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The HBSP gene is expressed during HBV replication, and its coded BH3-containing spliced viral protein induces apoptosis in HepG2 cells ☆

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Abstract

The mechanisms of liver injury in hepatitis B virus (HBV) infection are defined to be due not to the direct cytopathic effects of viruses, but to the host immune response to viral proteins expressed by infected hepatocytes. We showed here that transfection of mammalian cells with a replicative HBV genome causes extensive cytopathic effects, leading to the death of infected cells. While either necrosis or apoptosis or both may contribute to the death of infected cells, results from flow cytometry suggest that apoptosis plays a major role in HBV-induced cell death. Data mining of the four HBV protein sequences reveals the presence of a Bcl-2 homology domain 3 (BH3) in HBSP, a spliced viral protein previously shown to be able to induce apoptosis and associated with HBV pathogenesis. HBSP is expressed at early stage of our cell-based HBV replication. When transfected into HepG2 cells, HBSP causes apoptosis in a caspase dependent manner. Taken together, our results suggested a direct involvement of HBV viral proteins in cellular apoptosis, which may contribute to liver pathogenesis.

Keywords: HBV replication; Apoptosis; HBSP; BH3

Hepatitis B virus (HBV) infection leads to a wide range of liver diseases, including viral hepatitis and hepatocellular carcinoma (HCC). Viral hepatitis is characterized by an inflammatory reaction in the infected liver cell, and is associated with cell damage and death [1]. One of the typical processes of cell death is apoptosis which is generally considered to be a mechanism of host defense against viral infections [2,3]. Apoptosis, or programmed cell death, is a highly conserved, tightly controlled self-destruction process to ablate damaged and neoplastic cells in multicellular organisms. On the other hand, viruses have evolved strategies to counteract and regulate apoptosis in order to maximize the production of virus progeny and promote the spread of virus progeny to neighboring cells. During the course of persistent HBV infection, continuous intrahepatic inflammation maintains a cycle of liver cell destruction and regeneration that often terminates in HCC. While the expression and retention of viral proteins in hepatocytes may influence the severity and progression of liver disease, the mechanisms of liver injury in viral hepatitis are defined to be due not to the direct cytopathic effects of viruses, but to the host immune response to viral proteins expressed by infected hepatocytes [4].

Recent reports have pointed to the involvement of the mitochondria-dependent apoptotic pathway which is governed by Bcl-2 family of proteins in the development of liver diseases [5,6]. However, little is known on the involvement of HBV viral proteins in this pathway at molecular level. The interest of characterizing the Bcl-2 mediated apoptosis in HBV related hepatitis lies in the fact that these proteins are able to either delay or induce apoptosis. While the former would ensure prolonged viral

☆ *Abbreviations:* HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HBSP, HBV spliced variant protein; BH3, Bcl-2 homology domain 3; GST, glutathione- *S*-transferase.

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replication, it is now known that some viruses are able to induce apoptosis at late stages of infection [7]. This process may result in the dissemination of viral particles with minimum host immune response as well as protecting progeny viral particles from host neutralizing antibodies.

The Bcl-2 family of proteins consists of both suppressors and promoters of apoptosis. Four conserved domains within the Bcl-2 family of proteins have been identified through sequence comparisons, these are named as Bcl-2 homology (BH) domains 1 to 4, with BH3 only proteins being pro-apoptotic [8].

We report in this study evidence of direct involvement of HBV viral protein in apoptosis. HBV replicating HepG2 cells showed characteristics of cellular apoptosis such as detachment from substrate and rounding up, followed by cell death. This observation was supported by FACS analysis and caspase-3 activation assay. Our study further revealed the presence of BH3 domain in HBSP, a spliced HBV protein, which has been reported to induce cellular apoptosis. While its apoptotic effect was confirmed in our study, mutation of the well-conserved residue in the BH3 domain (L25A) significantly reduced the apoptotic effect. The significance of our findings was discussed.

Materials and methods

Cell culture and transfection. HepG2 cells (ATCC, USA) were cultured in Gibco Dulbecco's minimal essential medium (Invitrogen Inc., USA), supplemented with 10% fetal bovine serum (Invitrogen Inc., USA), 1% anti-mycotic (Invitrogen, USA) under 37 °C and 5% CO_2. Effectene transfection reagent (Qiagen, Germany) was the expression system for HBV in HepG2 cells. After adherent HepG2 cells reach 70% confluency, the cells were transfected with 2 μg replicative HBV genome constructed as described previously [9]. Transfected cells were maintained at 37 °C and 5% CO_2 for various times (6, 12, 24, 36, 48, and 60 h) and examined under the microscope.

Cloning of HBSP in pXJ-40 and site-directed mutagenesis. The coding region of HBSP [10] was amplified by PCR, joined in-frame with glutathione- *S*-transferase (GST) gene, and cloned in pXJ-40 containing a HA tag. This resulted in a fusion gene consisting of HA-GST-HBSP on pXJ-40 vector. This construct was used as template for generating mutation (L25A) in HBSP coding region by site-directed mutagenesis (Stratagene, USA). Separately, the coding region of GST alone was also cloned in-frame with the HA tag into pXJ40, resulting in a HA-GST fusion protein.

Reverse transcriptase-PCR. Two microgram of pcDNA3.1 containing the replicative HBV genome was transiently transfected into 3×10^5 HepG2 cells in each well of a 6-well plate. Transfected cells were collected at different time points after transfection (6, 12, 24, 36, 48, and 60 h). mRNA at each time point was extracted using miniRNAse kit (Qiagen, Germany), and served as template for reverse-transcriptase PCR using HBSP specific primers (5′-AT GAATTC ATG CCC CTA TCT TAT CAA-3′ and 5′-TT GCGGCCGC AAG CCC AGG ACG ATG GGA AT-3′). The PCR product using these primers encompassed the coding region of HBSP with an expected size of 279 bp [10]. Positive PCR products were sequenced to confirm their identity as HBSP.

Apoptotic effect of HBV and HBSP in HepG2 cells by flow cytometry analysis. pcDNA3.1 containing the replicative HBV genome and pXJ-40-HA-GST-HBSP were transiently transfected separately into 3×10^5 HepG2 cells. HepG2 cells transfected with empty pXJ-40 and cells treated by 50 μM cisplatin for 16 h were used as negative and positive controls, respectively. Transfected cells were incubated at different time points (6, 12, 24, and 48 h) before being harvested and analyzed by ApoAlert™ Annexin-V kit (BD Biosciences, USA). Cells were rinsed in binding buffer and resuspended in the same buffer. Five microliters of Annexin-V-FITC and 10 μl prodium iodide was added into each sample. Following 30 min incubation in dark, samples was analyzed on FACS station (BD Bioscience, USA).

Apoptotic effect of HBSP in HepG2 cells by caspase-3 assay. Increasing amount of pXJ-40-HA-GST-HBSP (2, 4, 6, and 8 μg) was transiently transfected into 3×10^5 HepG2 cells in each well of a 6-well plate. HepG2 cells transfected with empty pXJ40 and cells treated by 100 μM of cisplatin for 16 h were used as negative and positive controls, respectively. Samples were collected 48 h after transfection and analyzed by using ApoAlert™ Caspase-3 Fluorescent Assay Kit (BD Bioscience, USA). Cells were lysed in 50 μl of lysis buffer for 30 min on ice. After centrifugation, 50 μl

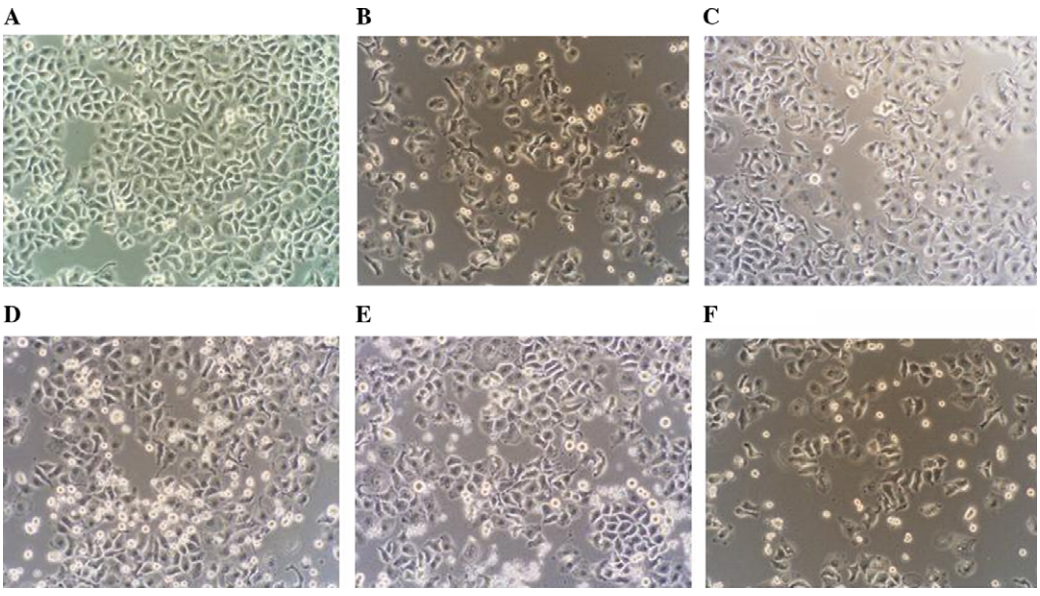

Fig. 1. Analysis of morphology of HBV-replicating HepG2 cells. A series of phase contrast images of HepG2 cell transfected with replicative HBV genome and incubated at 6 h (C), 12 h (D), 24 h (E), and 48 h (F). HepG2 cells transfected with empty vector pXJ40 vector (after 48 h) are shown in (A). Cells treated with cisplatin, an agent inducing apoptosis, are shown in (B). Increasing detachment of cells and their rounding up were observed in (B), and progressively from (C–F). Images were taken using Inverted Microscope (Olympus IX71).

supernatant was mixed with 50 μl of reaction buffer. Five microlitres of caspase-3 substrate DEVE-AFC was added into the mixture and incubated in 37 °C, 5% CO_2 for another 3 h. This mixture was then pipetted into 96-well plate and analyzed by using microplate fluorescence reader (FL600, Bio-TEK, USA). At an excitation wavelength of 400 nm the cleaved AFC gave out an emission at 505 nm and absorbance was automatically recorded. The caspase-3 activity for each time point was measured in cells transfected with pXJ40-HA-GST-HBSP in three independent experiments.

Western blot analysis. HA-GST-HBSP and HA-GST proteins expressed in HepG2 cells were detected by primary anti-HA antibody (Santaclause, USA) in 1:5000 dilution and secondary anti-mouse antibody conjugated with horseradish peroxidase (Pierce, USA) in 1:5000 dilution. Anti-actin antibody (Sigma, USA) was used to detect actin protein as an internal control.

Results and discussion

HBV transfected HepG2 cells show evidence of apoptosis

To generate a cell-based system for HBV replication, a linearized form of HBV genome has been constructed in the mammalian expression vector pcDNA3.1 [9] which has been shown to set viral replication in HepG2 by producing HBV particles in the culture medium [11]. This system was chosen to analyze effects of HBV replication on the cells.

In contrast to the widely accepted non-cytopathic nature of HBV, recent reports suggest a direct role of HBX (the smallest HBV viral proteins) in apoptosis [12,13]. Careful examination of infected cells by light microcopy showed characteristic signs of apoptosis during the infection process. As the incubation time after transfection prolonged, the number of dead cells which were detached from the culture dish increased (6, 12, 24, and 48 h, respectively) (Fig. 1C–F). The morphological anomalies observed in these cells were not detected in HepG2 cells 48 h after they were transfected by the empty plasmid pcDNA3.1 (Fig. 1A). Our data therefore suggested that the observed morphological changes were associated with HBV replication, but not due to transfection process or the empty plasmid. To our understanding, this is the first time that cell death associated with HBV replication has been reported.

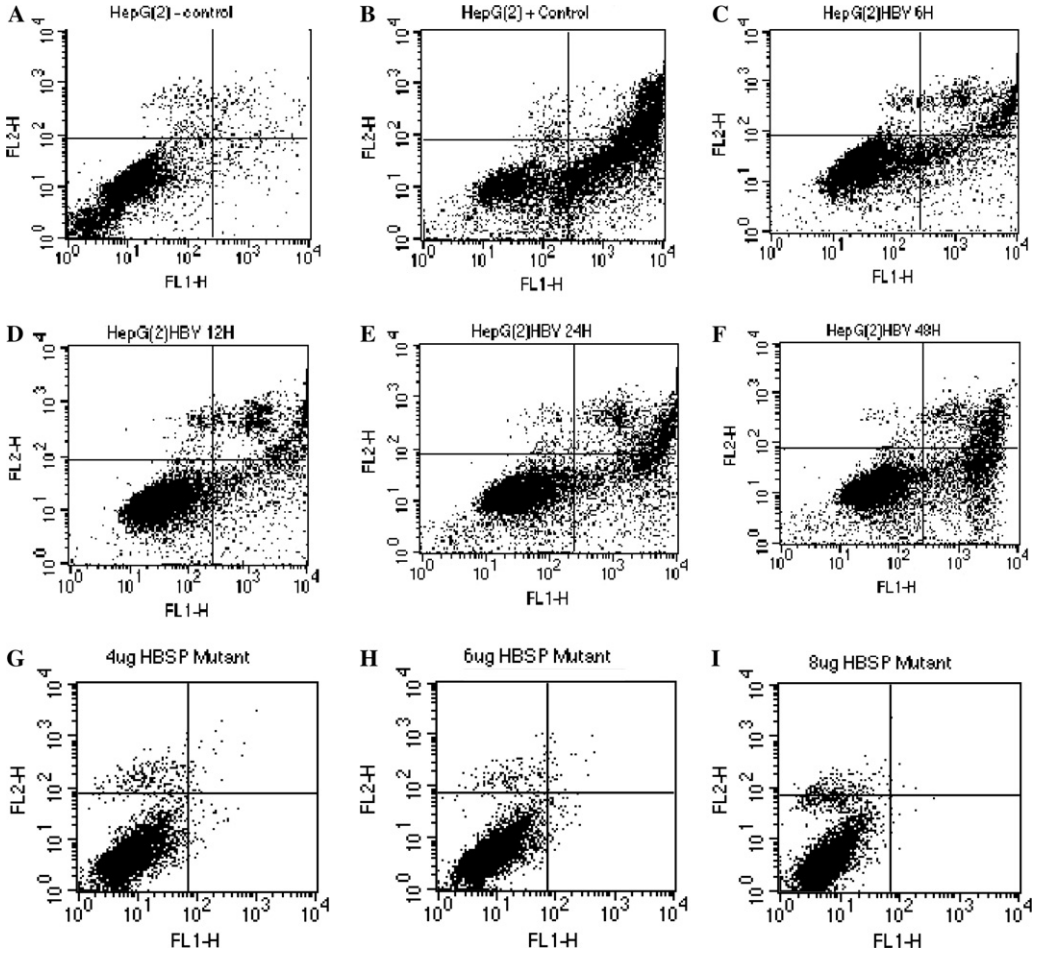

Fig. 2. Flow cytometry analysis of apoptotic effect of HBV replication in HepG2 cells. HepG2 cells were transfected with either pcDNA3.1 carrying the replicative HBV genome and incubated at different time points (12 h (C), 24 h (D), 36 h (E), and 48 h (F)) or empty pcDNA3.1 vector (A). Cells treated with cisplatin were used as positive control (B). The labeling of cells was by Annexin-V-FITC (FL1-H) and prodium iodide (FL2-H). In each panel, the bottom left square indicate the number of cells, the bottom right square the number of apoptotic cells, and top right square the number of necrotic cells. Increasing amount of apoptotic cells is observed from (C–F). (G–I) Represent FACS results on cells transfected with increasing amount (4 μg (G), 6 μg (H), and 8 μg (I)) of pXJ40-HA-GST-HBSP(L25A) mutant protein. There were fewer apoptotic cells which remained unchanged with the increased amount of mutant protein.

The morphological changes in HBV-infected cells at the 48 h time point (Fig. 1F) were similar to those in HepG2 cells treated with cisplatin, a known chemical that causes apoptosis (Fig. 1B).

To investigate if apoptosis was involved in the observed HBV-induced cell death process, flow cytometry (FACS) was used which is based on the observation that apoptotic cells would translocate the membrane phospholipids phosphatidylserine (PS) from the inner face of the plasma membrane to the cell surface which then becomes detectable by Annexin V [14]. Typically, the proportion of apoptotic cells is reflected in the bottom-right square of FACS profile (Fig. 2). In this study, HBV-replicating HepG2 cells were collected at the time points in Fig. 1 and were double-stained with Annexin-V-FITC and propidium iodide (PI) separately. Results shown in Fig. 2 indicated there was an increase in the number of apoptotic cells as the infection time prolonged. Specifically, 10.07% of apoptotic cells were detected 6 h after transfection of HBV genome (Fig. 2C), while the proportion of such cells was at 13.8%, 14.85%, 20.75%, and 28.83% for 12 h (D), 24 h (E), and 48 h (F), respectively. This was significantly higher than HepG2 cells transfected with the empty pcDNA3.1 vector (1.37%, A), but lower than the positive control in which cells were treated by 50 μM cisplatin for 16 h (36.12%, B).

Our data therefore indicated that apoptosis was likely to be the main mechanism of HBV-induced cell death. This is consistent with other viruses capable of inducing cell death [15,16].

HBV spliced protein (HBSP) contains a BH3-like domain

To investigate whether HBV viral proteins were directly involved in the observed apoptosis, we screened amino acid sequences of HBV proteins for pro-apoptotic motif. Results revealed a BH3 domain in the N-terminus of the HBV DNA polymerase, spanning from residue 21 to 35, which included conserved and critical residues found in the BH3 domains of pro-apoptotic Bcl-2 family of proteins (Fig. 3A). The same BH3 domain was also found in HSBP, a spliced variant protein consisting of 46 amino acid residues of the N-terminal part of HBV DNA polymerase, and 47 new amino acid residues [10]. Interestingly, HBSP has been shown to induce apoptosis through a hitherto unknown mechanism [10,17]. The identification of a BH3 domain may therefore provide molecular insights into its apoptotic effect.

HBSP is expressed during HBV replication

Although HBSP has been found in HBV infected liver tissue and anti-HBSP antibodies can be detected in sera of HBV chronic carriers [10], its expression during HBV replication has not been characterized. Results from such an analysis would be particularly relevant to our observed apoptosis following HBV replication in HepG2 cells. To this end, HepG2 cells transfected with pcDNA3.1 carrying the replicative HBV genome were collected at various time points (6, 12, 24, 36, 48, and 60 h). mRNA was extracted from cells at each time point and used as template for reverse-transcriptase PCR. Positive PCR products were confirmed to be HBSP coding region by sequencing. Results shown in Fig. 3B indicated that HBSP gene was expressed in HBV transfected cells at 6, 12, and 24 h, but not thereafter. Our data therefore indicated that HBSP was expressed during HBV replication and may contribute directly to our observed apoptotic effect following HBV replication.

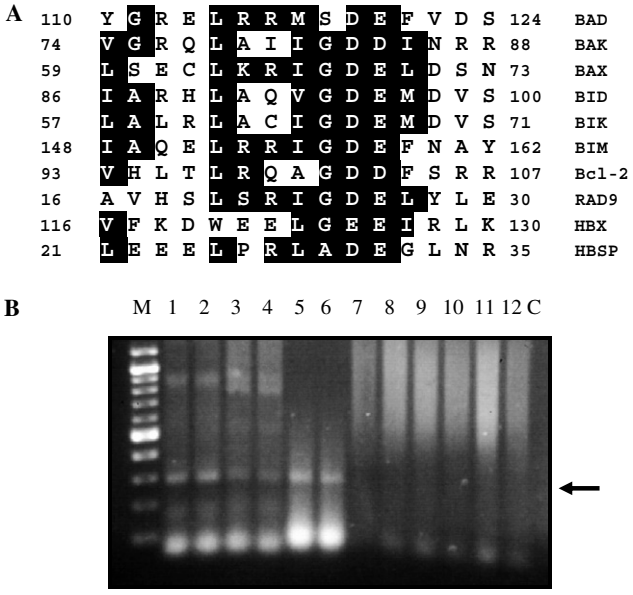

Fig. 3. (A) Alignment of BH3-homology regions of Bcl-2 family members and HBSP. Comparison of the BH3-like regions in HBVPolN and Bcl-2 family members. Conserved amino acids are shaded. (B) Expression of HBSP gene by reverse-transcriptase (RT) PCR analysis. mRNA was extracted from HepG2 cells transfected with replicative HBV genome, and used as template for RT-PCR analysis using primers encompassing the coding region of HBSP. Duplicate samples were analyzed for each of the following time points after transfection: 6 h (lanes 1 and 2), 12 h (lanes 3 and 4), 24 h (lanes 5 and 6), 36 h (lanes 7 and 8), 48 h (lanes 9 and 10), and 60 h (lanes 11 and 12). HBSP expression was detected up to 24 h after transfection (arrow head). Lane M represents 100 bp ladder. Lane C represents negative control for RT-PCR in which no mRNA was added.

HBSP induces apoptosis in mammalian cells and activates caspase-3 protease

To study the apoptotic effects on the cells conferred by either HBV DNA polymerase or HBSP, their respective coding region of 2526 and 279 bp was cloned in-frame with the GST and HA tag in mammalian expression vector pXJ40. HepG2 cells transfected by either HA-GST-HBV DNA Polymerase or HA-GST-HBSP construct were assessed for their viability. While cells transfected with HA-GST-HBSP showed decreased cell viability in a dose dependent manner as determined by MTT assay, no change of cell viability was observed for those transfected with HA-GST-HBV DNA Polymerase (data not shown).

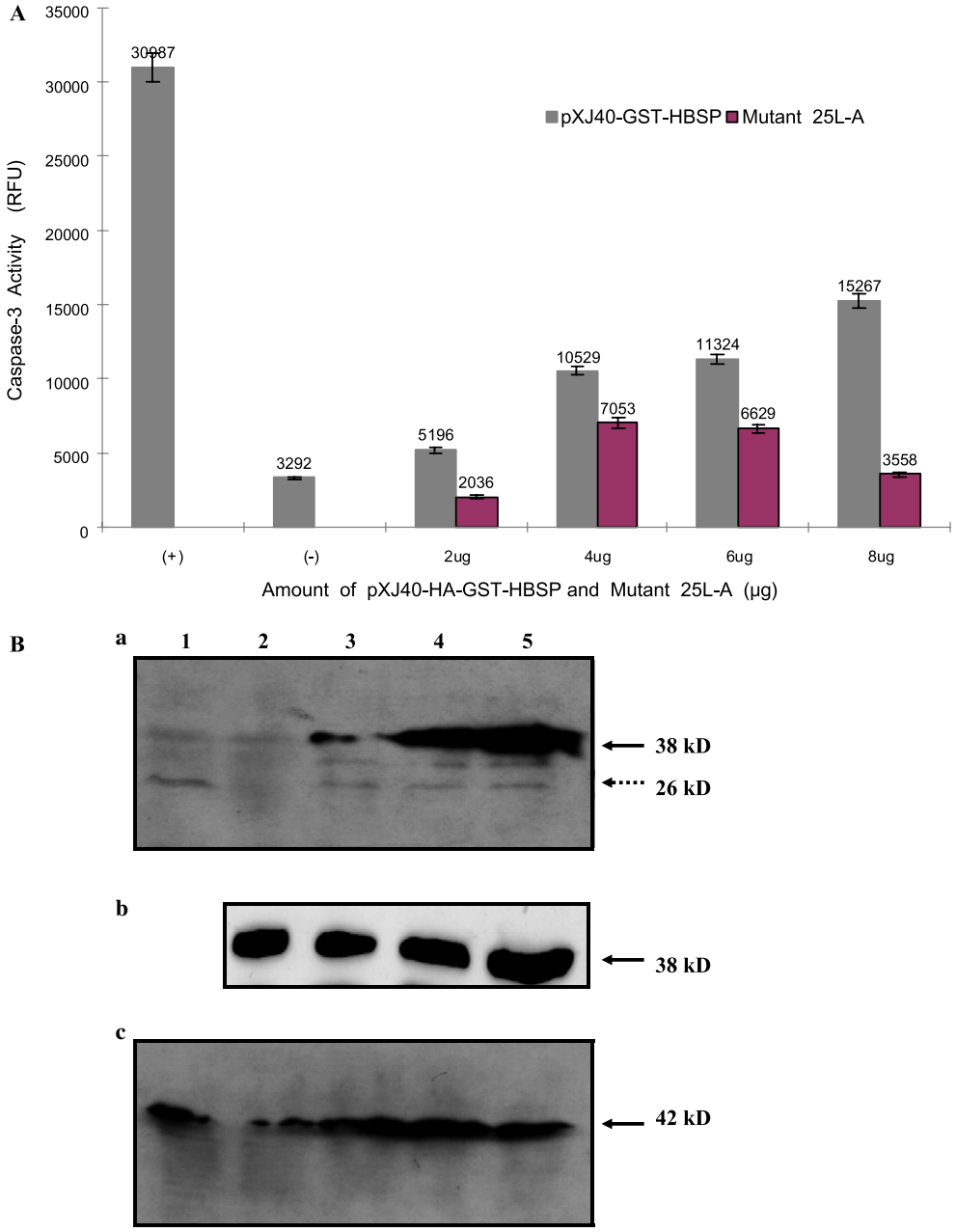

Fig. 4. (A) Apoptotic effects of HBSP in HepG2 cells by caspase-3 activity. HepG2 cells were transfected with increasing amounts of either wild type pXJ40-HA-GST-HBSP or mutant pXJ40-HA-GST-HBSP(L25A) and analyzed for caspase-3 activity (see Materials and methods). Similar analysis was carried out on cells treated with cisplatin (+) and those transfected with pXJ40-HA-GST (−). Caspase-3 activity was measured in three independent transfections for each amount of plasmid used in this study. (B) Western blot analysis of HA-GST-HBSP in HepG2 cells. Cells were transfected with the different amount of either the wild type pXJ40-HA-GST-HBSP (A) or mutant pXJ40-HA-GST-HBSP(L25A) (B) 2 μg (lane 2), 4 μg (lane 3), 6 μg (lane 4), and 8 μg (lane 5), respectively. HepG2 cells transfected with pXJ40-HA-GST were used as negative control (lane 1, a). Endogenous actin was used as an internal control. Antibodies used were anti-HA (a,b) and anti-actin (c), respectively. (a,b), Solid arrowhead indicates the fusion HA-GST-HBSP protein (either wild type or in panel a mutant in panel b) at 38 kD. The discontinued arrowhead indicates the 26 kD GST protein in cells transfected with pXJ40-HA-GST (lane 1, a). (c), Arrowhead indicates the endogenous action protein at 42 kD.

To investigate the molecular basis of HBSP-induced apoptosis, HepG2 cells transfected with pXJ40-HA-GST-HBSP were analyzed by caspase-3 protease assay, a hallmark of apoptosis [18]. To this end, HepG2 cells were transfected with increasing amount of pXJ-HA-GST-HBSP (2, 4, 6, and 8 μg), incubated for 48 h, and analyzed for the caspase-3 protease activity. As shown in Fig. 4A, the caspase-3 activity (cleavage of DEVE-AFC substrate) of 3292 was found in cells transfected with pXJ40-HA-GST (negative control) whereas those cells treated with cisplatin (positive control) had the caspase-3 activity of 30987. In cells transfected with pXJ-HA-GST-HBSP

construct, the caspase-3 activity was at 5196 (2 μg), 10,529 (4 μg), 11,324 (6 μg), and 15,267 (8 μg), respectively. There was therefore a significant increase of caspase-3 activity in HA-GST-HBSP transfected cells, in a dose dependent manner. To correlate the expression level of HBSP in HepG2 cells with our observed caspase-3 activity, Western blot analysis was carried out in HepG2 cells transfected with HA-GST-HBSP construct using anti-HA antibody. Results shown in Fig. 4B indicated that an increase in intensity of the expected molecular weight of HA-GST-HBSP (38 kD, a) was detected, consistent with the increased amount of HA-GST-HBSP used in transfection. On the other hand, only the 26 kD GST protein was detected in cells transfected with pXJ-HA-GST. As an internal control, a constant intensity of the endogenous actin protein (42 kD, c) was observed in cells.

Mutant HBSP(L25A) induces reduced apoptosis

To determine the specificity of HBSP in inducing apoptosis, mutation was generated at the conserved residue (L25A) which has been reported to impact on the pro-apoptotic activity of BH3 containing proteins [19]. HepG2 cells were then transfected, in the same dose dependent manner as for the wild type HBSP, with pXJ40-HA-GST-HBSP(L25A) and the caspase-3 activity determined. Results shown in Fig. 4A indicated a significant reduction in caspase-3 activity for the mutant HBSP (L25 A), particularly in cells transfected with 2 and 8 μg of mutant construct. The moderate decrease in cells transfected with 4 and 6 μg of the same mutant construct suggested that other amino acid residues in the BH3 domain may also be involved in the pro-apoptotic activity. In addition, the HA-GST-HBSP(L25A) detected in each set of the transfected HepG2 cells (panel b, Fig. 4B) further suggested that the observed decrease in caspase-3 activity was due to the mutant HBSP, but not the absence of the mutant protein. Consistent with the analysis of apoptosis induced by HBV replication, HepG2 cells transfected with the increasing amount of pXJ40-HA-GST-HBSP(L25A) (4, 6, and 8 μg) were subjected to FACS analysis. Results shown in Fig. 2G–I, indicated that no significant number of apoptotic cells was detected (below 0.4% for all three panels), and remained unchanged even with the increased amount of plasmid used.

Taken together, our results indicated that HBSP induced apoptosis when overexpressed in HepG2 cells and may have a direct involvement in apoptosis associated with HBV replication. Our findings may also point to a direct involvement of HBV viral proteins in the viral pathogenesis unrelated to the host immune response. Despite a compact genome with overlapping coding regions, evidence of alternative spliced variants has been documented in HBV carriers [10,20,21]. One of these, HBSP which has been shown to induce apoptosis [10] and to be associated with HBV pathogenesis [17], contained a BH3 domain identified in this study. Our results suggest that HBV may possess an alternative mechanism of modulating its interaction with the infected host cells.

Acknowledgments

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