Hepatitis B Virus Pre-S\textsubscript{2} Mutant Surface Antigen Induces Degradation of Cyclin-Dependent Kinase Inhibitor p27\textsuperscript{Kip1} through c-Jun Activation Domain-Binding Protein 1

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Abstract

The hepatitis B virus (HBV) large surface antigen (LHBS) mutant with deletion at the pre-S\textsubscript{2} region accumulates in endoplasmic reticulum (ER) and is associated with HBV-induced hepatocellular carcinogenesis. In this study, we found that the pre-S\textsubscript{2} LHBS mutant directly interacts with the Jun activation domain–binding protein 1 (JAB1). Association of pre-S\textsubscript{2} LHBS with JAB1 dissociated JAB1 from the JAB1/IRE1 complex in ER. The free (active) JAB1 then translocated into cell nuclei and rendered the Cdk inhibitor p27\textsuperscript{Kip1} to cytosolic proteasome for degradation. The pre-S\textsubscript{2} LHBS mutant induced hyperphosphorylation of tumor suppressor retinoblastoma (RB) via cyclin-dependent kinase 2 (Cdk2), a downstream molecule regulated by p27\textsuperscript{Kip1}. This effect is independent of the ER stress signaling pathway. The transgenic mice carrying the pre-S\textsubscript{2} mutant LHBS gene also exhibited Cdk2 activation, p27\textsuperscript{Kip1} degradation, as well as RB hyperphosphorylation. The mouse hepatocytes exhibited morphologic abnormalities such as chromatin condensation, multinucleation, and dysplasia of hepatocytes. In summary, the pre-S\textsubscript{2} LHBS mutant with deletion at the pre-S\textsubscript{2} region accumulates in endoplasmic reticulum (ER) and is associated with HBV-induced hepatocellular carcinogenesis. In this study, we found that the pre-S\textsubscript{2} LHBS mutant directly interacts with the Jun activation domain–binding protein 1 (JAB1). Association of pre-S\textsubscript{2} LHBS with JAB1 dissociated JAB1 from the JAB1/IRE1 complex in ER. The free (active) JAB1 then translocated into cell nuclei and rendered the Cdk inhibitor p27\textsuperscript{Kip1} to cytosolic proteasome for degradation. The pre-S\textsubscript{2} LHBS mutant induced hyperphosphorylation of tumor suppressor retinoblastoma (RB) via cyclin-dependent kinase 2 (Cdk2), a downstream molecule regulated by p27\textsuperscript{Kip1}. This effect is independent of the ER stress signaling pathway. The transgenic mice carrying the pre-S\textsubscript{2} mutant LHBS gene also exhibited Cdk2 activation, p27\textsuperscript{Kip1} degradation, as well as RB hyperphosphorylation. The mouse hepatocytes exhibited morphologic abnormalities such as chromatin condensation, multinucleation, and dysplasia of hepatocytes. In summary, the pre-S\textsubscript{2} LHBS mutant causes p27\textsuperscript{Kip1} degradation through direct interaction with JAB1. The pre-S\textsubscript{2} mutant LHBS is suggested to be a potential oncoprotein for HBV-related hepatocellular carcinoma. (Mol Cancer Res 2007;5(10):1063–72)

Introduction

Chronic viral hepatitis is the major cause for hepatocellular carcinoma, the most frequent visceral neoplasm worldwide. The main causative agents for hepatocellular carcinoma are HBV and HCV, which together are responsible for the majority of hepatocellular carcinomas in humans. These viruses cause necroinflammatory liver disease of variable duration and severity. A major portion of the viral hepatitis progresses into liver cirrhosis and dysplasia and, ultimately, hepatocellular carcinoma. In this process, the viral proteins are believed to be important players, which cross-talk with various host proteins, affecting the host signaling pathways. A number of HBV gene products have been identified as viral tumor proteins. The X protein (pX) is oncogenic because it activates the Ras/Raf-1 signal transduction pathway and inhibits DNA repair (1, 2).

HBV surface protein (HBsAg) expression in the chronic phase of HBV infection is also associated with hepatocellular carcinoma incidence. HBsAg causes sustained hepatic inflammation and injury, an important marker identifying chronic HBV carriers (3).

In the chronic phase of HBV infection, the HBV genome often integrates into the host chromosome (4). In this status, the large form of HBsAg (LHBS) becomes largely expressed (5). Compared with small-form HBsAg, the large form includes an additional pre-S region, which is the upstream promoter region for the small form (5). LHBS is pro-oncogenic: it induced hepatocellular carcinoma in a transgenic mouse model (6). These findings indicate that LHBS interacts with host factors to regulate the mechanism of hepatocellular carcinogenesis.

Ground glass hepatocytes are the histologic hallmarks of chronic HBV infection (7). The type II ground glass hepatocytes display marginal staining pattern of HBsAg and has been found to carry specific mutations in the pre-S\textsubscript{2} region (8). This mutant HBS gene is deleted in approximately nucleotides 4 to 57 of the pre-S\textsubscript{2} region and often contains a point mutation in the start codon of the region, which leads to a dramatic decrease in the synthesis of small-sized and middle-sized surface antigens. Type II ground glass hepatocytes often appear in hepatic nodules and proliferate in clusters, strongly suggesting that they are involved in HBV-related hepatocarcinogenesis. This mutant form of the HBS gene, designated pre-S\textsubscript{2} HBS mutant, emerges only in the late or nonreplicative phase of chronic HBV infection and eventually becomes a dominant HBV gene product in hepatocytes (9, 10).

The pre-S\textsubscript{2} type of LHBS mutant is predominant in hepatocellular carcinoma patients with HBV infection (10-13). Based on epidemiologic studies, HBV carriers who presented with the pre-S\textsubscript{2} LHBS mutant in serum had worse disease
outcomes than those who did not (13). Although the correlation between the prevalence of pre-S2 LHBS mutant and hepatocellular carcinoma has been clearly shown, the molecular mechanism of hepatocellular carcinogenesis induced by the pre-S2 LHBS mutant is not yet clear. In a recent study (14), we found that pre-S2 mutant LHBS accumulated in ER and induced strong ER stress, which induced oxidative stress, DNA damage, and mutagenesis, all of which resulted in genomic instability in hepatocytes. The pre-S2 LHBS mutant also induced overexpression of cyclin A, which is associated with the G1-S cyclin-dependent kinases (Cdk), leading to cell cycle progression in the presence of DNA lesions (15). In the present study, we searched for the molecule directly targeted by the pre-S2 LHBS mutant and the mechanism for pre-S2 LHBS mutant–induced hepatocellular carcinogenesis.

Results
Pre-S2 LHBS Mutant Is Associated with Jun Activation Domain–Binding Protein 1

We used yeast two-hybrid assays to identify the human protein directly targeted by the pre-S2 LHBS mutant. We found that Jun activation domain–binding protein 1 (JAB1) was associated with the pre-S2 mutant LHBS (Fig. 1A; ref. 16). In the human hepatoma HuH-7 cells transfected with the wild-type (WT) or pre-S2 mutant LHBS, we found that the JAB1 protein was associated with the pre-S2 LHBS mutant, but not with the WT LHBS, shown by immunoprecipitation experiments (Fig. 1B and C). One study (17) reported that JAB1 is associated with the ER transmembrane kinase/RNase IRE1 in ER lumen and dissociated from it on ER stress. We found that the association was nearly completely abolished in cells expressing pre-S2 LHBS mutant (Fig. 1D-F). In addition, such association between JAB1 and IRE1 could not be recovered even in the presence of ER stress inhibitor vomitoxin, indicating that the pre-S2 LHBS mutant disrupted JAB1-IRE1 binding by specifically interacting with JAB1 (Fig. 1F).

Macrophage migration inhibitory factor (MIF) protein has also been associated with JAB1 in cytosol (18). Immunoprecipitation studies showed that pre-S2 LHBS mutant also disrupted the association between MIF and JAB1 (Fig. 1G). Therefore, the pre-S2 LHBS mutant seems to competitively bind with JAB1 and dissociate JAB1/IRE1 and JAB1/MIF complexes.

Pre-S2 LHBS Mutant–Induced Degradation of Cdk Inhibitor p27Kip1

The nuclear JAB1 has been shown to target the Cdk inhibitor p27Kip1 and bring it to proteasome 26S for degradation (19). To confirm that the pre-S2 LHBS mutant triggered nuclear localization of JAB1, the nuclear and cytosolic fractions were analyzed for JAB1 protein levels. We found that, in the presence of pre-S2 LHBS mutant, nuclear JAB1 levels were consistently significantly higher than those induced by the WT (Fig. 2A and B). In addition, JAB1 also enhanced the activity of transcription activator protein 1 (AP-1), which was known to be regulated by JAB1 (Fig. 2C).

We also found that pre-S2 LHBS mutant down-regulated p27Kip1; however, after treatment with the 26S proteasome inhibitor lactacystin, the loss in p27Kip1 protein level was abrogated (Fig. 3A). By communoprecipitation studies, it was found that the amount of p27Kip1 associated with JAB1 was diminished in cells carrying the pre-S2 mutant LHBS but was fully recovered after lactacystin treatment (Fig. 3B). This revealed that pre-S2 LHBS mutant activated JAB1 and caused p27Kip1 to be degraded via proteolysis.

p27Kip1 is an inhibitor for the kinase activity of the cyclin-Cdk2 complex (20). To show whether the Cdk2 was indeed activated by the pre-S2 mutant LHBS, the activated Cdk2, indicated by its phosphorylation on the threonine residue at amino acid 160, was detected in the HuH-7 cells carrying the WT or the pre-S2 LHBS mutant (21). It was found that the phosphorylation on Thr160 of Cdk2 was significantly greater in cells carrying the pre-S2 LHBS mutant than in cells carrying the WT LHBS (Fig. 3C). On the contrary, the levels of cyclin D1, associated with Cdk4 in the G1-Cdk complex, were not changed in various transfected cells (Fig. 3C; ref. 22). These findings indicate that the pre-S2 LHBS mutant causes activation of the cyclin-Cdk2 complex through p27Kip1 degradation. In addition, these effects were dependent on JAB1 because the p27Kip1 degradation and Cdk2 activation were nearly abolished when cells were transfected with siRNA knockdown construct of JAB1 (Fig. 3D).

Pre-S2 LHBS Mutant–Induced Retinoblastoma Hyperphosphorylation

The cyclin A-Cdk2 complex plays a key role in the entry into S phase of the cell cycle by phosphorylating the tumor suppressor retinoblastoma protein (RB; refs. 23). We found that human hepatoma HuH-7 cells with the pre-S2 LHBS mutant, but not those carrying the WT LHBS, showed RB hyperphosphorylation (Fig. 4A). Such RB hyperphosphorylation was indeed caused primarily by Cdk2, which phosphorylates RB at the threonine residue at amino acid 821 (Fig. 4B; ref. 24). To show that such an effect induced by pre-S2 LHBS mutant was not cell type specific, the T24 human bladder cancer cell line was also used for the present study, and it showed RB hyperphosphorylation by Cdk2 when transfected with the pre-S2 LHBS mutant gene (Fig. 4C and D), indicating that RB hyperphosphorylation induced by the pre-S2 LHBS mutant is solely dependent on expression of the pre-S2 LHBS mutant gene. It was also found that the pre-S2 mutant LHBS rendered cells to progress through cell cycle after serum starvation, whereas the WT LHBS did not (Fig. 4E).

RB Hyperphosphorylation in Transgenic Mice with the Pre-S2 LHBS Mutant

We established an FVB/N mouse model containing the pre-S2 LHBS mutant transgene (Fig. 5A; ref. 14). Liver tissue showed a large number of HBsAg+ hepatocytes (Fig. 5B) with abnormal morphologies (i.e., centrosome condensation, aneuploidy, and dysplasia; Fig. 5C). After examining the non-tumorous sections of liver in these mice, we found that the majority of transgenic mice with the pre-S2 LHBS mutant gene showed RB hyperphosphorylation, Cdk2 activation, and decrease in p27Kip1 protein levels, as compared with the mice carrying the WT LHBS gene (Fig. 5D). These findings were...
FIGURE 1. Association of the pre-S₂ mutant LHBS with JAB1. A. Yeast two-hybrid assays. The yeast colonies on growth plates and filter papers after the 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside reactions were shown as mirror images. B. Association of JAB1 with LHBS in the cells transfected with WT or pre-S₂ mutant (Δ2) LHBS genes in HuH-7 cells detected by coimmunoprecipitation assays with the antibody against hemagglutinin (HA) epitope fused to various types of LHBS, gp42, the 42-kDa glycosylated form of LHBS (39 kDa). C, control cells transfected with the plasmid vector pIRES-hrGFP-2α only. C. Reciprocal immunoprecipitation analysis to detect the association of pre-S₂ mutant LHBS with JAB1. D. Induction of ER stress by WT or pre-S₂ mutant LHBS in HuH-7 cells, detected by the alternative splicing patterns of XBP1 mRNA by multiplex reverse transcription-PCR analysis. Form I, total XBP1 (unspliced and spliced); Form II, spliced XBP1. TG, cells treated with thapsigargin (1 μM); TM, cells treated with tunicamycin (10 μg/mL). Levels of the form II and form I products were quantified and indicated below the gel electrophoresis. Columns, mean fold value (form II/I) from three independent experiments; bars, SD. E. Dissociation of IRE1 from JAB1, induced by the ER stress inducers tunicamycin and thapsigargin. F. Association of IRE1 with JAB1 in the cells transfected with WT or pre-S₂ mutant LHBS, shown by coimmunoprecipitation assays. VT, vomitoxin (150 ng/mL). G. Association of MIF with JAB1 in the cells transfected with WT or pre-S₂ mutant LHBS, shown by coimmunoprecipitation assays.
FIGURE 2. JAB1 subcellular localization and AP-1 activation by the pre-S2 mutant LHBS. A. JAB1 levels in the nuclear and cytosolic fractions in the HuH-7 cells transfected with WT or pre-S2 (Δ2) mutant LHBS, shown by Western blots. Cdk4 and α-actin were used as markers for proteins in nuclei and cytosol, respectively. V, cells transfected with pIRES-hrGFP-2a vector only. The relative levels of nuclear/cytosolic JAB1 were quantified and indicated below the Western blots.

B. Subcellular localization of JAB1 detected by immunofluorescence analysis. T24 cells transfected with WT, pre-S2 (Δ2) mutant LHBS, tagged with hemagglutinin epitope, or mock treated (C) were stained with mouse anti-JAB1 and rabbit antihemagglutinin antibodies. The antimouse antibody conjugated with Alexa488 (green) and antirabbit antibody conjugated with Alexa 594 (red) were then simultaneously incubated with the cells. Cell nuclei were stained with Hoechst 33258 (blue) fluorescent dye. Merge, the merged images of JAB1 and nuclear stainings. The relative levels of nuclear JAB1 in cells were quantified and indicated below the photographs. Columns, mean from 10 examined cells of each type; bars, SD.

C. AP-1 transcriptional activities in cells carrying the WT or pre-S2 mutant LHBS or mitogen-activated protein kinase kinase (MEKK). A, and C, columns, mean from three independent experiments; bars, SD.
consistent with ours in human HuH-7 in vitro cell cultures. This means that the pre-S2 LHBS mutant induces RB hyperphosphorylation through p27Kip1 degradation in in vivo mouse models.

Discussion

In this study, we found that the pre-S2 LHBS mutant directly interacts with the JAB1 protein and subsequently results in hyperphosphorylation of the RB tumor suppressor protein (summarized in Fig. 6). JAB1 is a multifunctional protein associated with the signaling pathway, cell cycle regulation, and development, and acts as a key subunit of the COP9 signalosome (CSN; ref. 16). Overexpression of CSN5/JAB1 promotes cell proliferation, increases AP-1 transcription, and stimulates or inhibits turnover of a number of proteins (16). CSN5/JAB1 binding has also been shown to stimulate protein degradation, whereas in other cases, such as hypoxia-inducible factor 1α, CSN5/JAB1 binding tends to promote stabilization (25). Given the multiple functionalities of JAB1, there might be other pathways of pre-S2 LHBS mutant–induced carcinogenesis in addition to p27Kip1 degradation. It was also reported that JAB1 participates in unfolded protein response through its association with and dissociation from ER factor IRE1 (17).

Therefore, JAB1 also plays an active role in the ER stress signaling pathway, which activates the NFκB proto-oncogene (26). It has been documented that JAB1 is highly expressed in hepatocellular carcinoma and other common types of cancer (27, 28). In addition, the ER stress signaling pathways likely contribute to the JAB1-related carcinogenesis process.

Previous studies have shown that the pre-S2 LHBS mutant induced strong ER stress (10). In the present study, we found that association of pre-S2 LHBS mutant with JAB1 is independent of ER stress because the ER stress inhibitor did not block such association (data not shown), suggesting that the ER stress signaling pathway is not an essential mechanism for the pre-S2 LHBS mutant–induced JAB1 activation.

We previously showed that the pre-S2 mutant LHBS induced strong ER stress–dependent oxidative DNA lesions that activated the DNA repair mechanism and mutagenesis (14). The DNA damage potentially deactivates certain tumor suppressors or proto-oncogenes, leading to cellular transformation and hepatocellular carcinogenesis. The results of the present study have added another important mechanism for pre-S2 LHBS mutant–induced hepatocellular carcinogenesis: RB hyperphosphorylation and defects in G1-S cell cycle arrest. Taking these findings together, we hypothesize that in the presence of DNA damage, the failure of cell cycle arrest

![Graphical representation of the data]

**FIGURE 3.** p27Kip1 degradation affected by the pre-S2 mutant LHBS. A. Degradation of p27Kip1 in the HuH-7 cells carrying the WT or pre-S2 LHBS shown by Western blots. HA, Western blot with anti-hemagglutinin antibody to detect WT and pre-S2 mutant LHBS. LACTA, lactacystin (10 μmol/L), EtOH, the solvent for lactacystin. The levels of p27Kip1 in each experiment were quantified and indicated below the Western blots. Columns, mean from three independent experiments; bars, SD. B. Levels of p27Kip1 associated with JAB1 shown by coimmunoprecipitations. C. Activation of Cdk2 in the HuH-7 cells carrying the pre-S2 mutant LHBS by Western blot. D. Inhibition of p27Kip1 degradation and Cdk2 activation by siRNA against JAB1. V, HuH-7 cells transfected with pSUPER vector only.
evidently increases gene mutation rates and genomic instabilities and further enhances the carcinogenesis process (29). Our recent epidemiologic studies found that patients who presented with pre-S2 LHBS mutant in serum developed hepatocellular carcinoma at significantly higher rates (odds ratio, 3.2; data not shown) than those without. The results of the present study provide an explanation of the mechanism that yields such a high association of pre-S2 LHBS...
FIGURE 5. RB hyperphosphorylation in the transgenic mice carrying the pre-S2 mutant LHBS. A. Serum HBS gene levels were detected by PCR. M, DNA size marker. C, negative control; PCR reaction without addition of DNA template. pHBV3.6, plasmid containing the HBV genome DNA, used as a control for HBS gene (658 bp) in PCR reaction. GAPDH (293 bp), internal control. B. Expressions of the WT and pre-S2 mutant (Δ2) LHBS in hepatocytes of the transgenic mice. The brown-colored cells on the arrows are the LHBS-expressing cells. C, control, naïve mice. C. Morphologies of the liver sections in the transgenic mice, shown by H&E staining. Arrows, cells that display morphologic abnormalities. D, RB and Cdk2 phosphorylations, as well as p27<sup>Kip1</sup> levels, in the nontumor liver tissues of representative mice carrying the WT or pre-S2 mutant HBS transgenes. For each detected mouse, the serum HBsAg level (ng/mL), detected by the ELISA assays, was shown at the bottom of its PCR products. The relative levels of Cdk2 phosphorylation, p27<sup>Kip1</sup>, and hyperphosphorylated/hypophosphorylated forms of RB were quantified and shown at the bottom of Western blots.
mutant with hepatocellular carcinoma. Based on our studies, we propose that the pre-S 2 LHBS mutant is a potential oncoprotein and may represent an important predictive marker for the hepatocellular carcinoma caused by chronic HBV infection.

In summary, we found that the pre-S 2 LHBS mutant directly interacts with the ER factor JAB1 and triggers p27Kip1 degradation and RB hyperphosphorylation. This is a novel mechanism for hepatocellular carcinogenesis caused by HBV. Based on the results of the present study, we conclude that the pre-S 2 LHBS mutant is an important prognostic marker for chronic HBV infection and should be widely applied in clinical medicine.

Materials and Methods

Cell Lines and Mice

Human hepatoma HuH-7 and bladder cancer T24 cell lines were used for in vitro cell culture studies. These cells were maintained and grown as previously described (14). The transgenic mice carrying WT or pre-S 2 LHBS mutant genes were constructed in our previous study (14).

Yeast Two-Hybrid Assays

The protocols for yeast two-hybrid assays followed those described elsewhere (30). Briefly, the pre-S 2 LHBS/pGBK7 (BD Clontech), which contains the Gal4 DNA-binding domain, was transformed into AH109 yeast strain, and then screened for
the pre-S$_2$ mutant LHBS–interacting proteins by using the human yeast two-hybrid testis cDNA library (BD Clontech). The positive genes were sequenced and identified by National Center for Biotechnology Information Blast search.

**Western Blotting**

The protocols for Western blotting basically followed those described elsewhere (14). The protein products of the large HBsAg were detected with monoclonal antibody raised against the sequence in the pre-S$_1$ region (IgMedica Biotechnology) or with the antihemagglutinin antibody because the WT or pre-S$_2$ mutant LHBS genes were cloned into the pRES-hrGFP-2a plasmid (Stratagene), which is tagged with hemagglutinin epitope. RB protein was detected with mouse monoclonal antibodies 11D7 and G3-245 (generously supplied by Dr. W-H. Lee, University of California, Irvine, CA), which recognize total RB protein (31, 32). To detect phosphorylated RB$^{T160}$ and Cdk2$^{T160}$, antibodies that specifically recognized the phosphorylated residues were used (AbCam plc and Novus Biologicals, Inc.). The other primary antibodies used in this study were mouse monoclonal antibodies against human JAB1 (Becton Dickinson Biosciences), MIF (Novus), cyclin D1, Cdk2, cyclin A, Cdk4, IRE1, p27 Kip1, and hemagglutinin epitope (Santa Cruz Biotechnology).

**Coimmunoprecipitation**

Protocols for coimmunoprecipitation experiments basically followed those previously described (14). To pull down HBV large HBsAg, mouse antihemagglutinin epitope antibody and protein A/G agarose beads (Santa Cruz Biotechnology) were mixed with cell-free extracts of HuH-7 cells transiently transfected with WT or pre-S$_2$ LHBS mutant genes cloned in pRES-hrGFP-2a plasmid. Immunoprecipitants were repeatedly washed with radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 10 mmol/L Tris (pH 7.2), 0.1% SDS, 1.0% Triton X-100, 1% deoxycholate, and 5 mmol/L EDTA] supplemented with protease inhibitor cocktail (Sigma-Aldrich), and then analyzed by Western blotting.

**Isolation of Nuclear and Cytosolic Fractions**

Human HuH-7 cells were washed with ice-cold PBS, collected with a cell scraper, and harvested by centrifugation. The cell pellets were lysed in solution A [50 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 1 mmol/L EDTA (pH 8.0), 1 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride, and protease inhibitors]. After cell lysis, 10% Triton X was gradually added to a final concentration of 0.5% to extract the cell nuclei. The cell pellets were lysed in solution B [50 mmol/L HEPES (pH 7.9), 0.4 mol/L NaCl, 1 mmol/L EDTA (pH 8.0), 1 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride, and protease inhibitors] with vigorous vortexing for 15 min at 4°C, and then centrifuged. The supernatant, which contained the nuclear extracts, was collected.

**Immunofluorescence Studies**

T24 cells (5 × 10$^5$) transfected with WT or pre-S$_2$ mutant LHBS genes were seeded in a four-well Lab-Tek II chamber slide (Nalgene Nunc). After 48 h of transfection, the cells were fixed with 10% formalin and then washed with PBS (pH 7.4). The slides were incubated with mouse anti-JAB1 and rabbit antihemagglutinin antibodies for 2 h at room temperature. The secondary monoclonal antimouse antibody conjugated with Alexa 488 and antirabbit antibody conjugated with Alexa 594 fluorescent dye (Molecular Probes) were then simultaneously incubated with cells for 30 min at room temperature. Cell nuclei were stained with Hoechst 3324 fluorescent dye. Following the reactions, the slides were mounted and then observed by Leica TCS SP2 spectral confocal microscopy. The relative level of nuclear JAB1 in each cell was quantified by using the Metamorph 7 software (Molecular Devices).

**AP-1 Transactivation Assay**

The AP-1 activity was measured by using the PathDetect in vivo signal transduction pathway cis-reporting systems (Stratagene). Briefly, the cells were cotransfected with pAP-1-luc reporter plasmid and WT or pre-S$_2$ mutant LHBS in pRES-hrGFP-2a vector. The plasmid pFC-MEKK, which contains mitogen-activated protein kinase kinase kinase (MEKK), was used as the positive control for AP-1 activity. The cells were assayed for luciferase activities by using the luciferase assay kit (Applied Biosystems).

**RNA Interference**

JAB1 coding region, nucleotides 509 to 527 (5’-ttgtaccaacagagaata-3’), was cloned into pSUPER (33) to establish the RNAi construct. HuH-7 cells were transfected with JAB1-RNAi plasmid for 96 h. After transfections, the levels of JAB1, p27Kip1, and phosphorylated Cdk2$^{T160}$ were assessed by Western blot.

**ER Stress**

Human HuH-7 cells were treated with an ER stress inducer, tunicamycin (10 μg/mL) or thapsigargin (1 μmol/L), for 3 h and then returned to cell medium for 3 h for further incubation (34, 35). The cells were washed with PBS (pH 7.4), then lysed in radioimmunoprecipitation assay buffer supplemented with protease inhibitors. ER stress was detected by examining the alternative splicing patterns of the ER stress marker XBP1 using multiplex reverse transcription-PCR. The amounts of functionally active XBP1, which was unconventionaly spliced by IRE1 on ER stress, were measured by using PCR primers for total (unspliced and spliced) XBP1 transcripts and those specifically for spliced XBP1 transcripts (36). The PCR primers used for XBP-1 were forward (total), 5’-agcactcagactacgtgcac-3’; reverse (spliced form), 5’-acagagagaaggagcttg-3’; and reverse (total), 5’-acacaattagttgctec-3’.

Western blot analysis for RB was conducted with anti-pRB$^{S821}$ antibody. To detect whether pre-S$_2$ LHBS mutant–induced RB hyperphosphorylation is dependent on ER stress, the cells transiently transfected with WT or pre-S$_2$ mutant genes were treated with ER stress inhibitor vomitoxin (150 ng/mL; Sigma-Aldrich) or mock treated for 24 h (37). After the cells had been washed with ice-cold PBS (pH 7.4), they were lysed in radioimmunoprecipitation assay buffer and then analyzed by Western blotting.
Cell Cycle Analysis

T24 cells were transfected in serum-free medium with WT, pre-S2 mutant LHBS genes, or vector control for 48 h. After transfections, the cells were harvested by trypanosinization, washed twice with PBS (pH 7.4), fixed with ice-cold 70% ethanol overnight, and then stained with propidium iodide (40 μg/mL) supplemented with RNase A (0.1 mg/mL) for 10 min at room temperature. The cell cycle profiles were detected by FACScan flow cytometry (Becton Dickinson). The percentages of cells in the G1, S, and G2-M phases were measured by using the WinMDI software.

Pre-S2 HBs Transgenic Mouse Study

Control and pre-S2 HBs transgenic mice of ages 12 to 15 months were killed, and the genomic DNA of each was extracted and tested for the HBs gene by PCR. To detect the levels of p27Kip1 and the phosphorylation status of RB and Cdk2, mouse livers were homogenized with a mortar and pestle in ice-cold radioimmunoprecipitation assay buffer. The homogenates were transferred into microcentrifuge tubes and then centrifuged at 13,000 rpm for 15 min at 4°C. After they had been washed repeatedly, cell lysates were tested by Western blotting.

References
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