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Inhibition of Hepatitis B virus replication by Phospholipid scramblase 1 in vitro and in vivo

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ABSTRACT

Human Phospholipid scramblase 1 (PLSCR1) is an α/β interferon-inducible protein that mediates antiviral activity against RNA viruses including vesicular stomatitis virus (VSV) and encephalomyocarditis virus (EMCV). In the present study, we investigated the antiviral activity of PLSCR1 protein against HBV (Hepatitis B virus). Firstly, PLSCR1 mRNA and protein expression was found to be downregulated in HepG2 cells after HBV infection. Then by performing co-transient-transfection experiments in cells and hydrodynamics-based transfection experiments in mice using a HBV expression plasmid and a PLSCR1 expression plasmid, we found that PLSCR1 inhibited HBV replication in vitro and in vivo through a significant reduction in the synthesis of viral proteins, DNA replicative intermediates and HBV RNAs. We also demonstrated that the antiviral action of PLSCR1 against HBV occurs, partly at least, by activating the Jak/Stat pathway. In conclusion, our results suggest that the expression of PLSCR1 is involved in HBV replication and that PLSCR1 has antiviral activity against HBV.

1. Introduction

Hepatitis B virus (HBV) infection is a significant global health problem. Despite the success of universal hepatitis B vaccination in many countries, more than 350 million individuals worldwide are chronically infected and 15–40% of those will develop cirrhosis and/or hepatocellular carcinoma if left untreated (Dienstag, 2008). The principal treatment for chronic hepatitis B involves the use of alpha interferon (IFN-α) or nucleoside analogs (Papatheodoridis et al., 2008). IFN-α belongs to the IFN-α/β system, which mediates antiviral, antiproliferative, immune, and other cellular effects. It engages the IFN-α receptor complex to activate the Jak/Stat pathway and induces the transcription of IFN-stimulated genes (ISGs) (Li et al., 2010). IFN-α inhibits HBV replication through a variety of mechanisms. It has been reported that IFN-α can suppress viral gene expression, prevent the formation of viral RNA-containing core particles, and reduce the accumulation of viral replicative intermediates (Gordien et al., 2001; Rang et al., 1999; Romero and Lavine, 1996; Uprichard et al., 2003; Wieland et al., 2000, 2005). However, the precise antiviral mechanisms of IFN-α and the biological functions of many ISGs have not been fully elucidated.

PLSCR1 is among the most potently activated of the interferon (IFN)-stimulated genes (Der et al., 1998; Zhou et al., 2000, 2005). Reports showed that PLSCR1 expression correlates with acute myelogenous leukemia (Yokoyama et al., 2004), systemic lupus erythematosus (Suzuki et al., 2010), colorectal cancer (Kuo et al., 2011) and acute phase response (Lu et al., 2007), and that PLSCR1 had a tumor suppressor function against leukemia and ovarian carcinoma (Huang et al., 2006; Silverman et al., 2002). As reported by Dong et al., PLSCR1 enhanced the IFN response and suppressed the replication of vesicular stomatitis virus (VSV) and encephalomyocarditis virus (EMCV) in vitro partly through increased expression of a select subset of potential antiviral genes (Dong et al., 2004). However, the relationship between PLSCR1 and HBV and the possible antiviral activity of PLSCR1 against HBV are not clear.

The aim of the present study was to determine whether PLSCR1 is involved in HBV replication and whether PLSCR1 plays a role in the antiviral activity against HBV. Therefore, we firstly demonstrated that the expression of PLSCR1 was involved in HBV replication by RT-PCR and Western blotting analysis. Then, we performed co-transient-transfection experiments in cells and hydrodynamics-based transfection experiments in mice using a HBV expression plasmid and a PLSCR1 expression plasmid. We found that PLSCR1 inhibited HBV replication through a significant reduction in the synthesis of viral proteins, DNA replicative intermediates and

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HBV RNAs. We also demonstrate that the antiviral activity of PLSCR1 against HBV occurs, partly at least, by activating the Jak/Stat pathway.

2. Materials and methods

2.1. Plasmids

To construct the HA-tagged PLSCR1 (pCMV/HA-PLSCR1), the complete cDNAs of human PLSCR1 carried on pPC86 were inserted into the EcoRI and XhoI sites of pCMV/HA vectors. The construct was confirmed by DNA sequencing. The HBV1.3 expressing plasmids (HBV1.3) contains a 1.3-copy of the HBV genome (kindly provided by Prof. Tang H, Chongqing Medical University, China).

2.2. Cell culture

The HepG2.2.15 cells (clonal cells derived from HepG2 cells that were transfected with a plasmid containing HBV DNA), which secrete hepatitis B virions (Sells et al., 1987, 1988) were kindly provided by the Beijing Medical University and was originally obtained from the Mount Sinai Medical Center (New York, NY). HepG2.2.15, HepG2 and Huh7 cells were cultured in Dulbecco's modified Eagle's medium (Hyclone) supplemented with 10% (v/v) fetal bovine serum (Invitrogen), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO_2. Three hundred and eighty micrograms per microliter G418 was needed in HepG2.2.15 cells.

2.3. Transfection and treatment of IFN

Transient transfection was performed using FuGENE HD (Roche) according to the manufacturer's instructions. Confluent cell monolayers (80–90%) were transfected with a 5:2 ratio of HD reagent to DNA. All transfection experiments were performed using equal total amounts of plasmid DNA normalized with empty vector. Equal amount of plasmid cytomegalovirus (pCMV)-β-galactosidase (β-gal) were cotransfected for normalizing the transfection efficiency. As a reference drug, 1000 units of IFN-α-2a (Roche) were added to the cell culture transfected only with pHBV1.3, empty vector and cytomegalovirus (pCMV)-β-galactosidase (β-gal). Cells and cell supernatants were harvested at 48 h post-transfection for the analysis of HBV DNA, HBV RNA, HBsAg, HBeAg and HBcAg. Transfection efficiency was normalized by detecting the activity of β-gal in cell lysates using the β-Galactosidase Enzyme Assay System (Promega).

2.4. Hydrodynamics-based transfection in mice

Specific pathogen-free male Balb/c mice were purchased from Experimental Animals Center of Beijing Institute of Medical Sciences (Beijing, China). A total of 10 μg of HBV1.3 and 20 μg of pCMV/HA or pCMV/HA-PLSCR1 was diluted in 2 ml of saline and injected into the tail vein of 6- to 8-week-old mice within 5–8 s. One, 3, 5 and 7 days after the injection, HBsAg and HBeAg in the serum of mice were determined by ELISA. Eight days after the injection, animals were sacrificed and the liver tissues were obtained. Formalin-fixed liver sections were processed for immunohistochemical HBcAg detection. The animal experiments were carried out according to the Guide for the Care and Use of Medical Laboratory Animals (Ministry of Health, PR China, 1998).

2.5. RT-PCR analysis

cDNA templates were synthesized from 2 μg total RNA from HepG2 cells, HBV1.3-transfected cells and HepG2.2.15 cells, respectively, using SuperScript II reverse transcriptase (Invitrogen) and oligo (dT) primer. PCR was performed in a single reaction of 20 μl volume. The schedule consisted of incubation for 5 min at 94 °C followed by 20–30 cycles of 94 °C for 20 s, 55 °C for 20 s and 72 °C for 20 s, then incubation for 5 min at 72 °C. The PCR products were analyzed by running the reaction products on 1% agarose/ethidium bromide gels. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as inner standard.

2.6. Real time RT-PCR analysis

Total RNAs from HepG2 cells transfected with pHBV1.3 for 24, 48 and 72 h were isolated and subjected to detecting PLSCR1 mRNA expression levels using one-step real time RT-PCR detection kit (SYBR green) according to the manufacturer's instructions (Tiangen Co., Beijing, China). The forward primer of PLSCR1 were 5′-cagcctccattaaactgtcc-3′, the reverse primer is 5′-tcttagtggtctctccagag-3′. GAPDH gene was used as inner standard. The forward primer of GAPDH were 5′-gtcaagctcatttcctggtatg-3′, the reverse primer is 5′-gtcaagctcatttcctggtatg-3′.

2.7. Western blot analysis

One hundred micrograms of total protein was separated by 10% SDS–polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene fluoride membranes (Millipore). The membranes were then blocked with 5% nonfat milk in Tris-buffered saline–Tween buffer (25 mmol/L Tris, 190 mmol/L NaCl, and 0.5% [v/v] Tween 20, pH 7.5) and then incubated with primary antibody (Santa Cruz Biotechnology) against HBcAg, PLSCR1, HA, p-Stat1, p-Stat2 or β-actin at 4 °C overnight. After being washed, the membranes were incubated at 25 °C for 1 h with HRP-labeled secondary antibody (1:2000 dilution; Santa Cruz Biotechnology). After being washed, the membranes were developed with an enhanced chemiluminescence reagent (GE healthcare) and exposed to X-Omat BT Film (KodakRochester). Bands were quantified by densitometry using an ATTO Densitograph (ATTO Corporation), normalized relative to beta-actin as internal control.

2.8. Enzyme-linked immunosorbent assay (ELISA)

HBsAg and HBeAg concentrations were determined using diagnostic kit for HBsAg and diagnostic kit for HBeAg (Kewei Biotechnology Co., Beijing, China) with the method described in the manufacturer's manual and quantified relative to a standard curve of serial dilutions of recombinant HBsAg or HBeAg.

2.9. Core-associated HBV DNA purification

The method for purification of cytoplasmic core-associated HBV DNA was adapted from Pugh et al. (1988) and Turelli et al. (2004). Briefly, cells were disrupted in lysis buffer (100 mM Tris–HCl pH 8.0, 0.2% NP-40). The cell lysate was clarified by centrifugation at 13,000 rpm for 1 min to pellet nuclei and insoluble material. The supernatant was adjusted to 6 mM $MgOAc_2$ and incubated for 2 h at 37 °C with 60 U of DNase I (Takara) and 100 μg/ml of RNase A (QIAGEN). Following digestion, the lysate was centrifuged for 1 min at 13,000 rpm. HBV DNA from the supernatant was further purified with an QIAamp DNA mini kit (QIAGEN) as recommended by the manufacturer. Purified DNA was subjected to Southern blot or HBV DNA quantification using real-time PCR.

HBV DNA from the cell culture supernatant was purified as follows. 400 μl of cell culture supernatant was collected and centrifuged at 14,000*g* for 10 s to remove cell debris (Turelli et al., 2004). The supernatant was then digested with 60 U of DNase I

for 30 min at 37 °C, and purified with an QIAamp DNA mini kit (QIAGEN).

2.10. Southern blot analysis

Purified HBV DNA was separated on a 1.2% agarose gel. The gels were treated as described (Chou et al., 2005), and the DNA samples were transferred onto nylon membranes (Amersham). After ultraviolet crosslinking and prehybridization, the membranes were hybridized with digoxigenin-labeled DNA probe generated using full-length HBV DNA and Dig High Primer DNA Labeling Kit according to the manufacturer's instructions (Roche).

2.11. Real-time PCR analysis

HBV DNA levels were detected by real-time PCR kit (DA AN GENE CO., Guangzhou, China) following the instruction manual provided. Amplification and detection were performed with an iCycler (Bio-Rad) Detection System. The program was optimized with denaturation at 94 °C for 2 min followed by 40 cycles of amplification (at 94 °C for 20 s, 55 °C for 20 s, 72 °C for 20 s).

2.12. Northern blot analysis

Total RNA was isolated using TRIzol solution (Invitrogen), which was followed by phenol/chloroform extraction and isopropanol precipitation. Fifteen micrograms of total RNA was separated using a 1.2% formaldehyde-agarose gel and transferred onto nylon membranes. The membranes were then hybridized with digoxigenin-labeled DNA probes as already described.

2.13. Immunohistochemical analysis

Paraffin-embedded liver sections were treated with 3% hydrogen peroxide and blocked with 5% bovine serum albumin. The sections were then incubated sequentially with anti-HBcAg antibodies (Dako), biotin-labeled secondary antibody, and avidin–biotin complex (ABC). Peroxidase stain was developed with 3,3′-diaminobenzidine (DAB) solution and counterstained with hematoxylin.

2.14. Statistics

The results are reported as means ± standard deviation (SD). Statistical significance was determined using analysis of variance (ANOVA) and Tukey tests. The threshold of significance was set at $p = 0.05$.

3. Results

3.1. The expression of PLSCR1 is reduced by HBV replication

To examine whether PLSCR1 expression was involved in Hepatitis B virus replication, RT-PCR and Western blot analysis were carried out using HepG2, HepG2.2.15 and HBV1.3-tansfected HepG2 cells. As shown in Fig. 1a, PLSCR1 mRNA was reduced about threefold in HepG2.2.15 and eightfold in HBV1.3-transfected HepG2 cells compared to HepG2 cells. As shown in Fig. 1b, PLSCR1 protein was reduced about twofold in HepG2.2.15 and threefold in HBV1.3-transfected HepG2 cells compared to HepG2 cells.

To detect the kinetic change regarding the down regulation of mRNA and protein expression in the context of HBV1.3-transient-transfected experiment, the mRNA and proteins were isolated from cells 24, 48 and 72 h after pHBV1.3 vector had been transfected. Real time RT-PCR was used to detect PLSCR1 mRNA. Western blot analysis was used to detected PLSCR1 protein. As shown in Fig. 1c and d, PLSCR1 mRNA and protein were downregulated in a time-dependent manner when cells were transiently transfected with pHBV1.3 plasmid till 72 h.

The results above indicated that PLSCR1 expression is reduced by HBV replication.

3.2. PLSCR1 reduces the synthesis of HBV proteins in transfected HepG2 and Huh7 cells

Next, to evaluate the antiviral effects of PLSCR1 on the cell culture system, the HBV-producing plasmid HBV1.3 and PLSCR1-expressing or control vectors (pCMV/HA-PLSCR1 or pCMV/HA) were co-transfected into HepG2 and Huh7 cells. As a reference drug, 1000 units of IFN-α-2a (Roche) were added to the cell culture transfected with HBV1.3 and pCMV/HA vectors. At 48 h after transfection, the expression of PLSCR1 were detected by Western blotting with anti-HA antibody (Fig. 2a). HBsAg and HBeAg secretion were investigated using standard immunoassays. The secretion of HBsAg and HBeAg was profoundly and dose-dependently reduced by PLSCR1 in both HepG2 cells and Huh7 cells ($p < 0.05$, Fig. 2b and c). Cotransfecting HepG2 cells with HBV1.3 and pCMV/HA-PLSCR1 resulted in 79.8% reduction for HBsAg and 81.3% reduction for HBeAg, compared to cotransfecting HepG2 cells with HBV1.3 and pCMV/HA. Cotransfecting Huh7 cells with HBV1.3 and pCMV/HA-PLSCR1 resulted in 64.16% reduction for HBsAg and 50.17% reduction for HBeAg, compared to cotransfecting Huh7 cells with HBV1.3 and pCMV/HA. As a positive control, IFN treatment of the HBV1.3-transfected HepG2 cells and Huh7 cells led to a reduction of 17.12%, 36.38% for HBsAg and 13.24%, 28.83% for HBeAg. In order to establish whether this effect was due to intracellular accumulation of synthesized viral proteins, we analyzed the amounts of cytosolic HBsAg and HBeAg present at 2 days post-transfection, using the same ELISA tests. As Fig. 2d showed, the level of intracytoplasmic HBsAg and HBeAg were profoundly and dose-dependently reduced by PLSCR1 in HepG2 cells ($p < 0.05$). This result suggests that PLSCR1 inhibits the expression of HBsAg and HBeAg. In addition, we showed that PLSCR1 reduced the expression of HBcAg in HBV1.3-transfected HepG2 cells by Western blotting analysis (Fig. 2e). Taken together, our results are consistent with a marked reduction in HBV protein expression by PLSCR1.

3.3. PLSCR1 protein reduces the synthesis of HBV replicative intermediates in transfected HepG2 cells

In order to determine whether the decrease in viral protein expression was associated with a change to HBV DNA replicative capacity, the amount of encapsidated viral DNA was measured in HBV1.3 and pCMV/HA-PLSCR1–cotransfected HepG2 cells at 2 days post-transfection by real-time PCR and Southern blot analysis. As shown in Fig. 3a, there was a dose-dependent decrease in the levels of core-associated viral DNA either in PLSCR1-transfected cells or cell cultures ($p < 0.05$). Fig. 3b and c showed the results of Southern blot analysis, HBV DNA replicative intermediates were seen to have disappeared almost entirely in HepG2 cells cotransfected with HBV1.3 and pCMV/HA-PLSCR1. As a positive control, IFN treatment also led to a significant reduction in DNA replicative forms. Taken together, in addition to reduced viral protein synthesis, PLSCR1 protein reduced viral DNA replication in vitro.

3.4. HBV RNAs are reduced by PLSCR1

The reduction in HBV protein synthesis and DNA replicative forms by PLSCR1 could be due to a modulation of viral transcript synthesis. To investigate this possibility, Northern blot analysis was performed for the detection of HBV mRNA in transfected cells. As shown in Fig. 4, there was a significant reduction in the amount

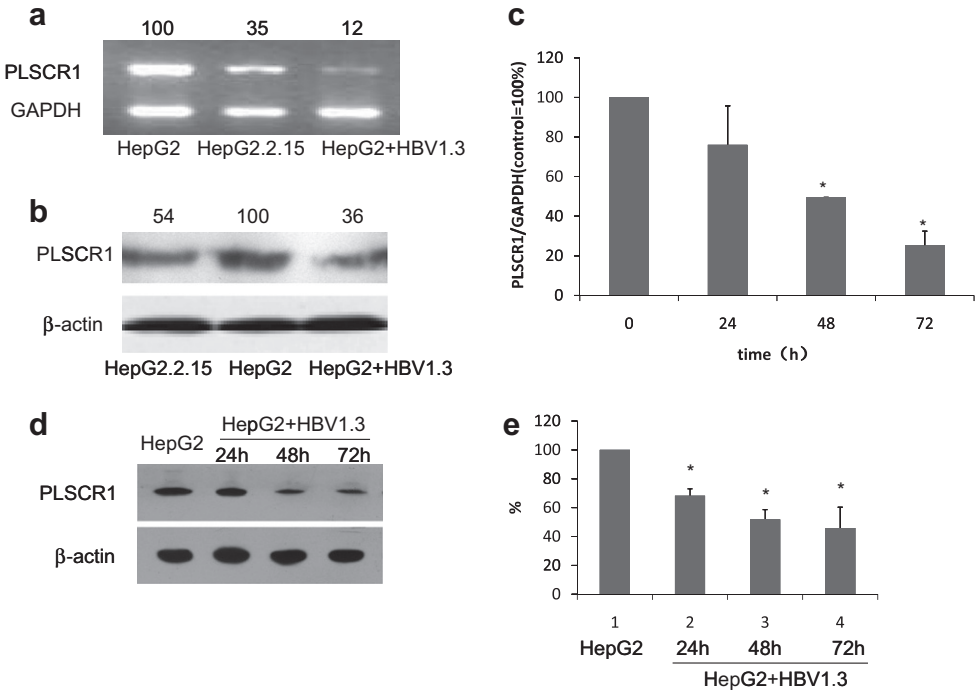

Fig. 1. HBV infection reduces the mRNA and protein levels of PLSCR1. (a) PLSCR1 mRNA expression in HepG2.2.15, HepG2 and pHBV1.3-transfected HepG2 cells. Bands from three independent experiments were quantified by densitometry and normalized to GAPDH bands. The average relative values of PLSCR1 mRNA expression were shown at the top, assuming that PLSCR1:GAPDH mRNA levels in HepG2 cells as 100. (b) The expression of PLSCR1 protein in HepG2.2.15, HepG2 and HBV1.3-transfected HepG2 cells. Blots from three independent experiments were quantified by densitometry and normalized to the corresponding β-actin bands. The average relative values of PLSCR1 protein expression were shown at the top, assuming that PLSCR1:GAPDH protein levels in HepG2 cells as 100. (c) The real-time RT-PCR analysis of PLSCR1 mRNA expression levels in HepG2 cells 24, 48 and 72 h after being transfected with pHBV1.3. Data are expressed as mean ± standard deviation (SD) from three independent experiments, assuming that the PLSCR1:GAPDH mRNA levels in HepG2 cells (0 h) as 100%. (d) The expression of PLSCR1 protein in HepG2 cells 24, 48 and 72 h after being transfected with pHBV1.3. (E) The expression levels of PLSCR1 protein from two independent experiments were quantified by densitometry and normalized against β-actin levels, assuming that the PLSCR1:β-actin protein levels in HepG2 cells (0 h) as 100%. Asterisks indicate a statistically significant difference as compared to the values in HepG2 controls (0 h) (*$p < 0.05$).

of HBV mRNA transcripts in PLSCR1-expressing cells compared to the control cells. The 3.5-kb mRNA most likely represented the HBV pregenomic template from which HBV replication starts.

3.5. PLSCR1 has no impact on the growth and apoptosis of HepG2 cells

To rule out the possibility that the inhibition of HBV replication is due to the impact of PLSCR1 on the growth or apoptosis of HepG2 cells, we examined the viability and apoptosis of PLSCR1-transfected HepG2 cells by MTS and Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining. The result showed that PLSCR1 had no impact on the growth and apoptosis of HepG2 cells in our experimental system ($p > 0.05$, Fig. 5a and b).

3.6. PLSCR1 has no impact on the release of LDH from HepG2 cells

To further rule out the possibility that the inhibition of HBV replication is due to the toxicity of PLSCR1 expression, the effects of PLSCR1 on host protein LDH secretion was detected by LDH ELISA analysis. The results showed that PLSCR1 had no impact on the secretion of host protein LDH ($p > 0.05$, Fig. 6).

3.7. PLSCR1 reduces the synthesis of HBV proteins and HBV replicative intermediates in a mouse model

Recently, a mouse model of acute HBV infection was established using hydrodynamic-based transfection (Yang et al., 2002). To examine the antiviral effect of PLSCR1 in vivo, Balb/c mice were hydrodynamically coinjected with plasmids expressing HBV (HBV1.3) and PLSCR1 (pCMV/HA-PLSCR1). One, 3, 5 and 7 days after the injection, HBsAg and HBeAg in the serum of mice were determined by ELISA. As shown in Fig. 7b and c, PLSCR1 significantly inhibited the synthesis of HBsAg and HBeAg in mouse serum ($p < 0.05$). Eight days after the injection, HBcAg in the mouse liver were subjected to immunohistochemistry. As shown in Fig. 7d, the expression of HBcAg was detected in the liver of HBV1.3-injected mice, while in the liver of HBV1.3 and PLSCR1 co-injected mice, the expression was almost not detected. These results indicated that PLSCR1 reduced the synthesis of HBV proteins in the hydrodynamic-based mouse model. Expression of PLSCR1 in transfected mouse liver was confirmed by Western blot analysis (Fig. 7a). Furthermore, possible toxicity of PLSCR1 to liver was assessed by determining the alanine aminotransferase (ALT) levels in the sera of mice. No difference in ALT levels was observed between mice injected with pCMV/HA and those injected with pCMV/HA-PLSCR1 (data not shown)..

3.8. PLSCR1 induces the phosphorylation of Stat1 and Stat2

To determine whether the expression of PLSCR1 affects the IFN inducing Jak/Stat signaling pathway, we detected the phosphorylation level of two important molecules Stat1 and Stat2 in IFN inducing Jak/Stat signaling pathway through Western blot analysis by using anti-p-Stat1 and anti-p-Stat2 antibodies. Results showed that the expression of phosphorylated Stat1 and Stat2 were induced by PLSCR1 (Fig. 8). This result indicates that PLSCR1 might activate the IFN inducing Jak/Stat signaling pathway.

4. Discussion

This report offers the first direct evidence for the involvement of PLSCR1 in HBV replication and the antiviral effects of PLSCR1

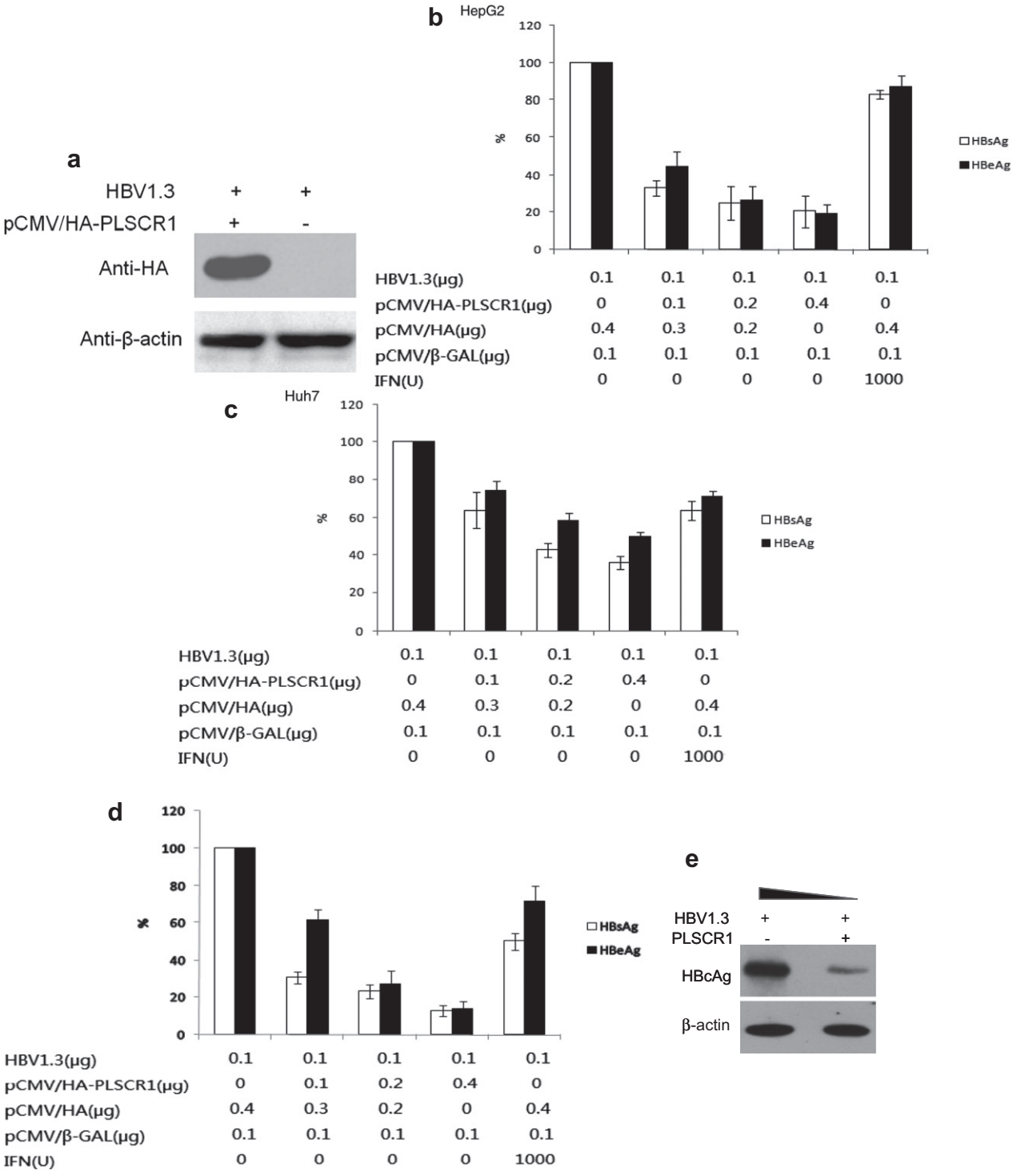

Fig. 2. PLSCR1 reduces the synthesis of HBV proteins in vitro. HepG2 cells or Huh7 cells were transfected with HBV1.3 plasmid in the presence or absence of pCMV/HA-PLSCR1. Forty-eight hours later, cell supernatants and cell lysates were collected. HBsAg/HBeAg in cell supernatants (b and c) and cell lysates (d) were assessed by ELISA. HBcAg (e) in cells were analyzed by Western blot using an anti-HBcAg antibody. β-Actin were detected for normalization. PLSCR1 expression in cells were confirmed by Western blot analysis using an anti-HA antibody (a). The values were calculated as percentages of the value for the HBV1.3, pCMV/HA and pCMV/β-gal cotransfected cells and shown as the mean ± standard error of three independent experiments performed in duplicate. The activity of β-gal in cell lysates was assessed to normalize transfection efficiency.

protein against HBV. Our results show that the expression of PLSCR1 is reduced by HBV replication. PLSCR1 protein induced a marked reduction in the synthesis of HBV proteins and HBV DNA replicative intermediates. This was associated with a marked reduction in the expression of total viral RNAs, suggesting that PLSCR1 inhibits HBV replication by suppressing expression of HBV RNA transcripts. In a mouse model, we also showed that PLSCR1 reduces the synthesis of HBV proteins.

PLSCR1 is a calcium-dependent plasma membrane protein. It is known to partition into lipid rafts and implicated in regulating the organization of plasma membrane phospholipids (Sahu et al., 2007; Zhou et al., 2002). In the dermal epidermal junction zone

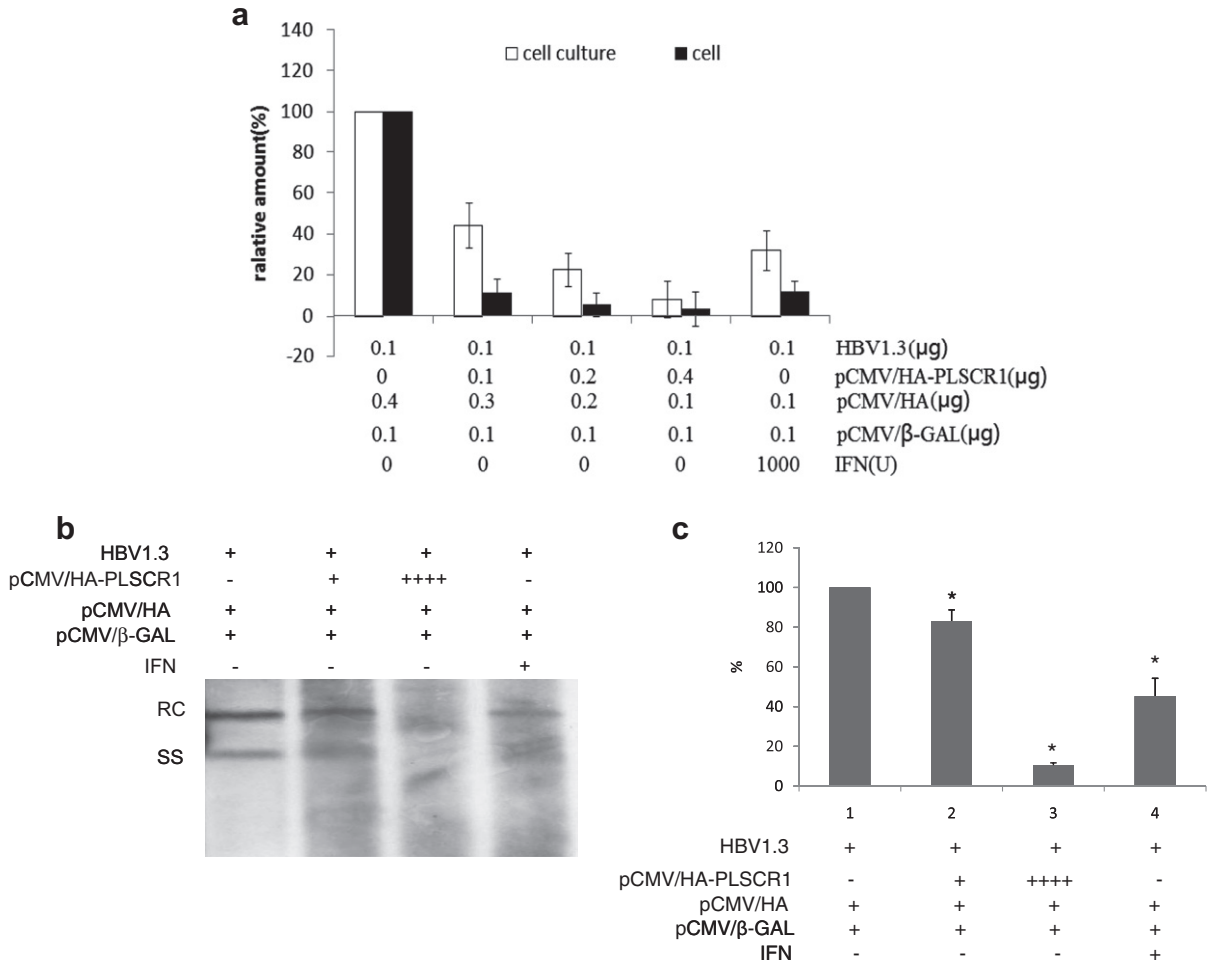

Fig. 3. PLSCR1 protein reduces the synthesis of HBV DNA replicative intermediates in vitro. HepG2 cells were transfected with HBV1.3 in the presence or absence of pCMV/HA-PLSCR1, with or without IFN; 48 h later, core-associated HBV DNA was isolated from core particles and analyzed by real-time PCR and Southern blot. (a) Real-time PCR analysis of core-associated HBV DNA in cell supernatants and cells. The values were calculated as percentages of the value for the HBV1.3 and pCMV/HA cotransfected cells and shown as the mean ± standard error of three independent experiments performed in duplicate (*$p < 0.05$). (b) Southern blot analysis of core-associated HBV DNA in HepG2 cells. The amount of DNA loaded on each lane is equivalent to that from a 60-mm diameter culture plate. RC, relaxed circular HBV-DNA; SS, single-stranded HBV-DNA. (c) Viral DNA levels by Southern blot analysis from two independent experiments were quantified. Asterisks indicate a statistically significant difference as compared to HBV DNA level in control cells co-transfected with pHBV1.3, pCMV/HA and pCMV/β-GAL ($p < 0.05$).

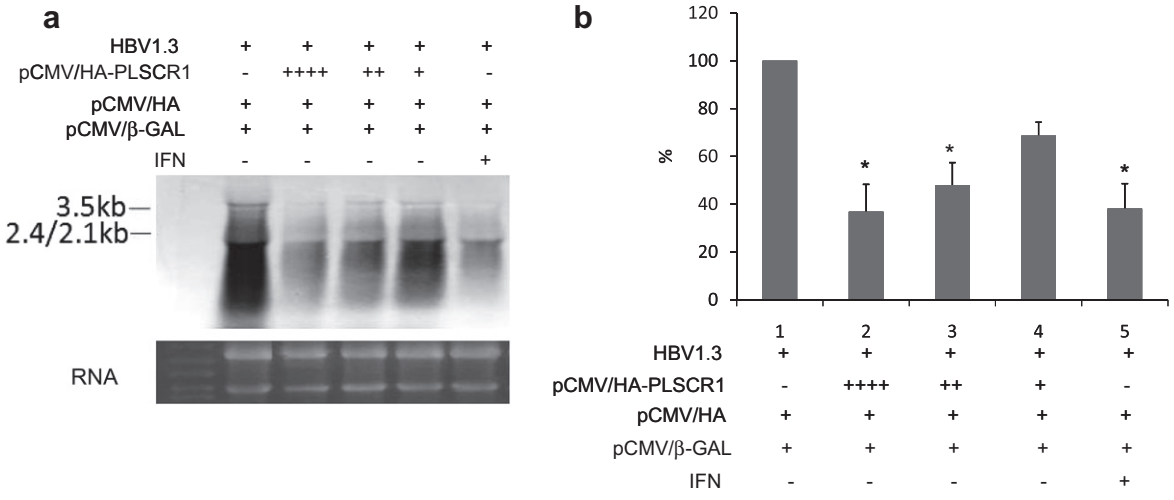

Fig. 4. PLSCR1 protein reduces the synthesis of HBV RNA in vitro. HepG2 cells were transfected with HBV1.3 in the presence or absence of pCMV/HA-PLSCR1, with or without IFN, 48 h later, total RNA were isolated and analyzed by Northern blot as described in Section 2. The 28s and 18s rRNAs were visualized under ultraviolet light for equal loading control (a). Viral RNA levels from two independent experiments were quantified and normalized by 28s and 18s rRNAs (b). Asterisks indicate a statistically significant difference as compared to HBV RNA level in control cells co-transfected with pHBV1.3, pCMV/HA and pCMV/β-GAL ($p < 0.05$).

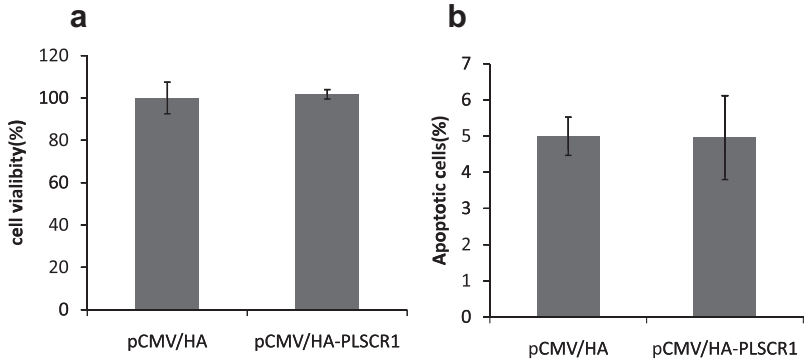

Fig. 5. The impacts of PLSCR1 on the growth and apoptosis of HepG2 cells. (a) Cell viability was determined by the MTS method. Results are presented as percent values in pCMV/HA-PLSCR1-transfected cells relative to that in pCMV/HA-transfected cells. (b) The apoptotic rates of transfected cells were determined by Annexin V-FITC/PI staining. Data are representative of three independent experiments.

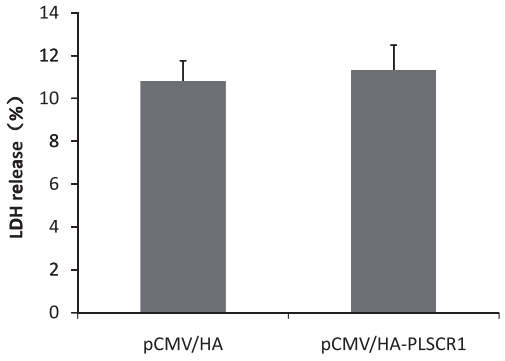

Fig. 6. The impacts of PLSCR1 on the releasing of LDH protein. HepG2 cells were transfected with or without pCMV/HA-PLSCR1. The release of LDH from cells was determined by LDH ELISA analysis. The results are presented as the ratios of LDH activity in cell culture against that in both cell culture and cells. Data are representative of three independent experiments in duplicates.

of human skin, it is secreted by a lipid raft-dependent pathway and interacts with the extracellular matrix protein 1 (Merregaert et al., 2010). PLSCR1 interacts with CD4 at the cell surface of T lymphocytes, inhibition of this interaction leads to the inhibition of HIV infection (Py et al., 2009). It is phosphorylated by select protein kinases, including Abl and Src, tyrosine kinases and PKCδ (Nanjundan et al., 2003; Pastorelli et al., 2001; Sun et al., 2001; Zhao et al., 2004). In addition to transcriptional induction by IFN, PLSCR1 expression is upregulated through each of the growth factor receptor pathways, and that PLSCR1 gene deletion has been shown to attenuate afferent receptor signaling and cellular response to a select group of cell surface growth factor receptors with specific effects on the activation of c-Src and potentially other protein kinases (Zhou et al., 2002). Under conditions of transcriptional induction, PLSCR1 has been shown to traffic to both the plasma membrane and the nucleus, events that appear to be regulated through its palmitoylation (Chen et al., 2005; Sahu et al., 2007), and once in the nucleus, PLSCR1, an acidic polypeptide, is found tightly bound to DNA (Ben-Efraim et al., 2004; Chen et al., 2005). Dong BH and his colleagues found that PLSCR1 enhances the expression of certain critical antiviral genes in Hey1B cells by using a custom viral response cDNA microarray. They suggested that the antiviral effect against VSV and EMCV mediated by PLSCR1 might be related to these effects (Dong et al., 2004). Taken together, the observed antiviral activity of PLSCR1 against HBV reflects activities of this protein at the plasma membrane that potentially influence afferent signaling through the Jak/Stat kinase pathway (or accessory signaling pathways recruited downstream of the activated receptor). This leads to an alteration of the repertoire of activated transcription factors, and/or affects the transcription of selected ISGs. The results of our study showed that PLSCR1 induced the expression of phosphorylated Stat1 and Stat2 in HBV1.3 transfected HepG2 cells, which indicates that PLSCR1 might activate the IFN inducing Jak/Stat kinase signaling pathway. Studies on whether PLSCR1 enhances the expression of antiviral genes in hepatocytes are ongoing. In addition, in contrast with our findings about the effects of PLSCR1, in IFN-treated HepG2 cell lines, no change or a moderate reduction in HBsAg and HBeAg secretion was evidenced (Caselmann et al., 1992; Hayashi and Koike, 1989). This suggests that, in addition to the IFN inducing pathway, PLSCR1 might exert its activity against HBV through other pathways, which needs further study.

Although IFN-α has been used for treatment of HBV infection for two decades, the antiviral mechanism of IFN-α and the biological function of many IFN-α response genes have not been fully elucidated. With different cell lines, it has been shown that the antiviral state induced by IFN-α is complex and can be established by induction of a wide pattern of genes and activation of a variety of cellular functional proteins (Gordien et al., 2001; Rang et al., 1999; Romero and Lavine, 1996; Uprichard et al., 2003; Wieland et al., 2000, 2005). Some interferon-inducible protein such as OAS, PKR, MxA (Gordien et al., 2001; Peltekian et al., 2005), members of the APOBEC3 family (Bonvin et al., 2006; Nguyen et al., 2007; Nguyen and Hu, 2008; Rosler et al., 2005; Turelli et al., 2004), TRIM22 (Gao et al., 2009) and MyD88 (Li et al., 2010; Lin et al., 2007; Xiong et al., 2004) which are involved in intracellular antiviral response, are well defined. However, it has also been shown that the inhibition of HBV replication by IFN-α is not limited to certain IFN-α-inducible proteins. For example, it has been reported that IFN-α induced suppression of HBV replication in MxA-deficient cells (Qin et al., 2009). It is supposed that multiple proteins interact each other to build up the IFN-α antiviral networks and determine the level of HBV replication and transcription. In this study, we showed that PLSCR1 inhibited HBV replication in HBV1.3-transfected cells and in a mouse model. Although the exact mechanism remains unresolved, from these data, we conclude that PLSCR1, an ISG induced by IFN, plays an antiviral role against HBV in our system. The previous studies demonstrated that IFN-α targets multiple steps of the HBV life cycle, including transcription, export and degradation of viral RNAs, as well as formation of the core particle and DNA replication (Gordien et al., 2001; Rang et al., 1999; Romero and Lavine, 1996; Uprichard et al., 2003; Wieland et al., 2000, 2005). Partly in line with the effect of IFN, in this study, PLSCR1 was shown to significantly inhibit the expression of viral proteins, DNA replication intermediates and viral pregenomic RNA. This indicates that IFN-α and PLSCR1 have comparable inhibitory effects on HBV. It is therefore likely

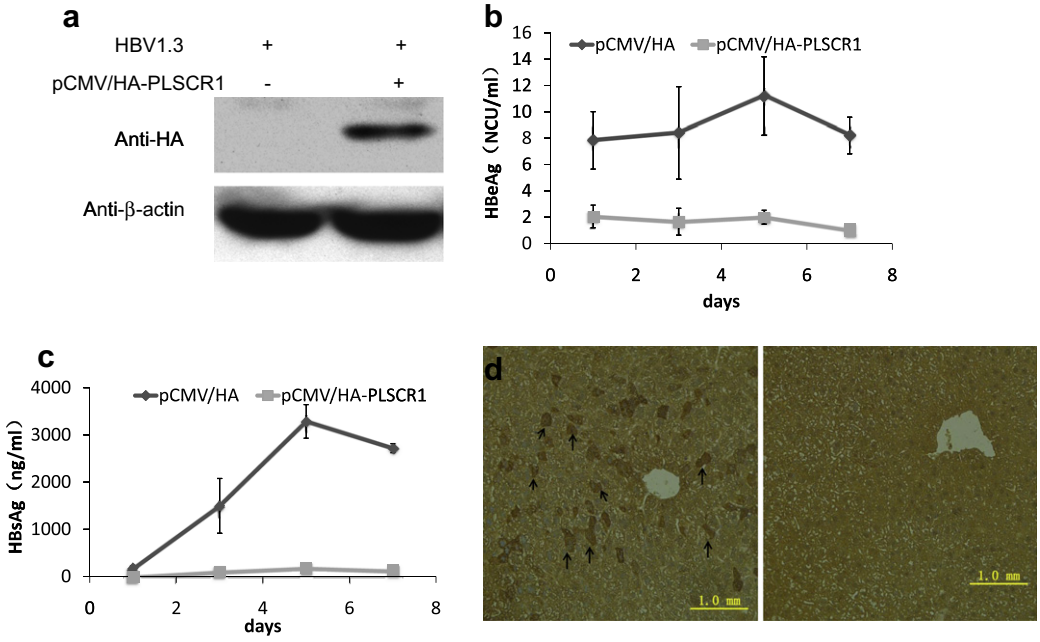

Fig. 7. PLSCR1 protein reduces the synthesis of HBV proteins in vivo. Balb/c mice were hydrodynamically coinjected with HBV1.3 and pCMV/HA or pCMV/HA-PLSCR1. At 1, 3, 5 and 7 days post-coinjection, HBsAg and HBeAg in mouse serum were determined by ELISA (b and c). Each value represents the average value of HBsAg and HBeAg detected from eight mice. At 8 days after coinjection, HBcAg in mouse liver was analyzed by immunohistochemistry using an anti-HBcAg antibody (d). The arrows indicate HBcAg. PLSCR1 expression in mouse liver was confirmed by Western blot analysis using an anti-HA antibody (a).

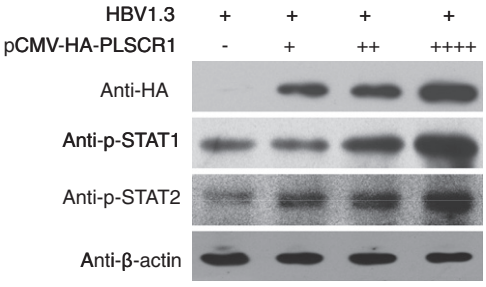

Fig. 8. PLSCR1 enhances the expression of phosphorylated Stat1 and Stat2 as determined by Western blot analysis. HepG2 cells were cotransfected with HBV1.3 and pCMV/HA-PLSCR1 for 48 h. Levels of proteins (indicated) were determined by probing Western blots of cell extracts with specific antibodies (see Section 2).

that the PLSCR1 protein plays a key role in the antiviral action of IFN against HBV.

Taken together, the present results demonstrate the involvement of PLSCR1 in HBV replication and provide evidence for a major antiviral role of the PLSCR1 protein against HBV. Additional studies are required to further define the precise mechanism(s) involved in the antiviral effect of PLSCR1 against HBV and how PLSCR1 might be used to develop new diagnostic and therapeutic approaches in the management of chronic HBV infection.

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