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Virology 342 (2005) 150 – 158

VIROLOGY

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Suppression of hepatitis B virus replication by SRPK1 and SRPK2 via a pathway independent of the phosphorylation of the viral core protein

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Received 18 May 2005; returned to author for revision 29 June 2005; accepted 25 July 2005
Available online 24 August 2005

Abstract

The SR-domain protein kinase (SRPK) 1 and 2 are two important kinases involved in cellular RNA splicing. Recently, it was suggested that these two kinases, which could bind to the hepatitis B virus (HBV) core protein, might be the major cellular kinases that phosphorylate the core protein to regulate HBV replication. In this report, we tested the role of SRPK1 and SRPK2 in HBV replication and found that both of them could suppress HBV replication by reducing the packaging efficiency of the pgRNA without affecting the formation of the viral core particles. This suppressive effect of SRPK1 and SRPK2 on HBV replication cannot be explained by their phosphorylation activities on the HBV core protein as the over-expression of these two kinases had no detectable effects on HBV core protein phosphorylation in vivo and their mutants that lacked the kinase activity could still suppress HBV DNA replication. Thus, these findings demonstrate a negative role of SRPK1 and SRPK2 in the regulation of HBV replication through a mechanism not involving the phosphorylation of the core protein.

Keywords: Hepatitis B virus; HBV core protein phosphorylation; HBV replication; SRPK1; SRPK2

Introduction

Hepatitis B virus (HBV) is an enveloped virus with a DNA genome of 3.2 kb. This virus is a liver-tropic virus. Upon infection of hepatocytes, the viral DNA genome is transported into the nucleus where it serves as the template to direct the synthesis of viral RNAs. One of the viral RNAs is larger than the genome size and codes for the core protein and the viral DNA polymerase, which is also a reverse transcriptase. During the maturation of HBV, the core protein packages its own mRNA and the viral DNA polymerase to form the core particle. The viral DNA polymerase subsequently converts the core protein RNA, which is also known as the pregenomic RNA (pgRNA), into the viral DNA genome. The DNA containing core particles then interact with viral envelope proteins, commonly referred to as the surface antigens, on the membrane of the endoplasmic reticulum (ER). The virion is then released into the ER lumen and secreted from infected cells (for reviews, see Ganem and Schneider, 2001; Yen, 2001).

In addition to the viral genome and the DNA polymerase, the HBV core particle also contains a kinase activity. This kinase activity can phosphorylate the core protein that forms the core particle (Albin and Robinson, 1980; Gerlich et al., 1982). The core protein is hyperphosphorylated in cells whether it is expressed by itself or from the HBV genome (Roossinck and Siddiqui, 1987; Yeh and Ou, 1991). Three serine residues, serine-157, serine-164 and serine-172, have previously been identified as the major phosphorylation sites in the core protein sequence (Liao and Ou, 1995). These serine residues are located in the carboxy-terminal arginine-rich sequence. The conversion of these three serine residues to glutamic acid or aspartic acid to mimic phosphoserine did not prevent the core protein from packaging the HBV pgRNA. However, it suppresses the viral genomic DNA replication (Gazina et al., 2000; Kock et al., 2004; Lan et al., 1999). The conversion of these serine residues to alanine to mimic nonphosphorylated serine suppresses the packaging of the pgRNA. Therefore, it has been suggested that the

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doi:10.1016/j.virol.2005.07.030

regulation of the core protein phosphorylation is important for viral pgRNA packaging and DNA replication (Lan et al., 1999). Daub et al. (2002) identified two additional serine residues, serine-178 and serine-180, as the phosphorylation sites in the core protein sequence.

The nature of the endogenous kinase activity associated with the core particle is controversial. It has been suggested to be protein kinase C (PKC) (Kann and Gerlich, 1994) and a 46 kDa protein kinase associated with ribosomes (Kau and Ting, 1998). These two kinases as well as the cdc2 kinase can phosphorylate the core protein in vitro (Yeh et al., 1993). In an attempt to identify the cellular kinase that may be responsible for the phosphorylation of the HBV core protein, Daub et al. (2002) fused the core protein sequence to the glutathione-*S*-transferase (GST) and used this fusion protein to isolate cellular proteins that might bind to the core protein. They identified SRPK1 and SRPK2 as such proteins. SRPK1 and SRPK2 are approximately 95 kDa and 115 kDa in size, respectively. These two kinases can phosphorylate serine/arginine-rich domain (SR-domain)-containing proteins to affect their biological functions (Gui et al., 1994a, 1994b; Wang et al., 1998). SR-domain proteins are RNA binding proteins involved in the formation of spliceasomes (Zahler et al., 1992). These two kinases can also phosphorylate the core protein in vitro. Daub et al. (2002) suggest that these two kinases are most likely the cellular kinases that mediate the core protein phosphorylation during HBV infection. In this report, we have tested the possible effects of SRPK1 and SRPK2 on HBV replication. Our results indicate that both SRPK1 and SRPK2 could suppress HBV DNA replication by inhibiting the packaging of the HBV pregenomic RNA without significantly affecting the formation of viral core particles. Further studies indicate that this suppressive effect of SRPK1 and SRPK2 on HBV replication is independent of their kinase activities.

Results

Suppression of HBV DNA replication by SRPK1 and SRPK2

To investigate the possible effect of SRPK1 and SRPK2 on the replication of HBV, we performed the co-transfection experiment. The HBV genomic dimer was co-transfected with the Flag-tagged SRPK1 or SRPK2 expression plasmid into Huh7 hepatoma cells. Forty-eight hours after transfection, cells were lysed and treated with DNaseI and micrococcal nuclease to remove the transfected HBV DNA. The replicated HBV DNA was then released from the core particles by proteinase K digestion and analyzed by Southern blot. As shown in Fig. 1A, the co-expression with SRPK1 and SRPK2 led to the reduction of the HBV DNA level by approximately two-fold and eight-fold, respectively. The suppressive effect of SRPK2 was reproducibly more prominent than SRPK1 in different experiments (also see below). To investigate how SRPK1 and SRPK2 might have suppressed HBV DNA replication, we performed the Northern blot experiment to analyze HBV RNAs. As shown in Fig. 1B, the co-expression with SRPK1 and SRPK2 did not significantly affect the HBV RNA levels, indicating that the effect of SRPK1 and SRPK2 on the replication of HBV was most likely a post-transcriptional event.

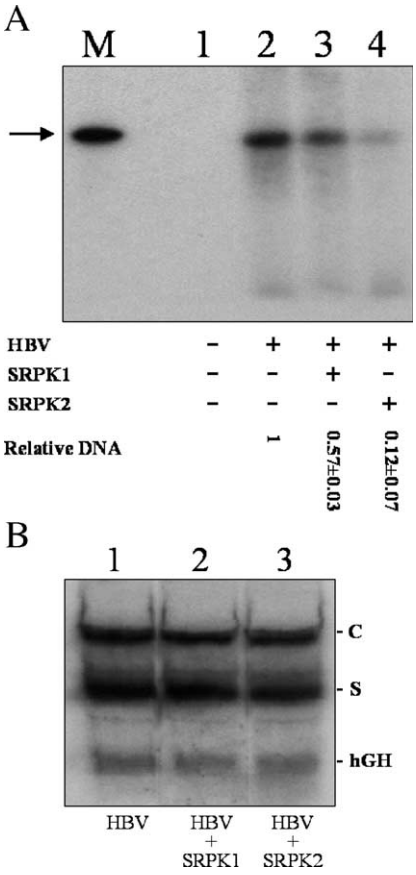

Fig. 1. Suppression of HBV DNA replication by SRPK1 and SRPK2. (A) Southern blot analysis. Huh7 cells were co-transfected with the HBV genomic dimer and a control vector (lane 2) or a plasmid that expresses SRPK1 (lane 3) or SRPK2 (lane 4). A plate of cells was also transfected with pUC19 and the control vector to serve as a negative control (lane 1). Cells were lysed 48 h after transfection and treated with DNaseI and micrococcal nuclease. The HBV DNA packaged in the core particles was then isolated by proteinase K digestion followed by phenol extraction. The linearized HBV DNA genome was shown in lane M to serve as a marker. The arrow denotes the 3.2 kb HBV DNA band. The relative DNA levels were measured using SigmaScan Pro 5. The numbers shown represent the average of at least three independent experiments. (B) Northern blot analysis. Huh7 cells were co-transfected with the HBV genomic dimer and a control vector (lane 1) or an expression vector that expresses SRPK1 (lane 2) or SRPK2 (lane 3). A plasmid that expresses the human growth hormone (hGH) was also used for co-transfection to monitor the transfection efficiency. C, S and hGH mark the locations of the C gene transcripts, the S gene transcripts and the hGH RNA.

Suppression of pgRNA packaging by SRPK1 and SRPK2

To further investigate how SRPK1 and SRPK2 may suppress HBV DNA replication, we had studied their effects on the encapsidation of the HBV pgRNA. The HBV core

particles were isolated by ultracentrifugation, and the pgRNA packaged in the core particles were extracted and quantified by the primer-extension analysis. Interestingly, as shown in Fig. 2, the co-expression of Flag-tagged SRPK1 and SRPK2 reduced the amount of the pgRNA packaged in the core particles by approximately two-fold and four-fold, respectively. As a control, we had also quantified the pgRNA level in the total cell lysates. As shown in the same figure, SRPK1 and SRPK2 did not affect the overall expression level of the pgRNA and its related precore protein RNA in Huh7 cells (Fig. 2). This result indicated that the effect of SRPK1 and SRPK2 on HBV replication most likely occurred at the encapsidation step of the pgRNA, which in turn led to the reduction of the HBV DNA level in the core particles.

Suppression of pgRNA packaging but not core particle formation by SRPK2

To further investigate how SRPK affected the encapsidation of the HBV pgRNA, we also preformed the isopycnic centrifugation to analyze the HBV core particles. To simplify our experiments, we decided to focus our studies on SRPK2, which had a higher suppressive effect on HBV replication. Huh7 cells were co-transfected with the HBV genomic DNA and the SRPK2 expression plasmid or its control vector. Cells were lysed 48 h after transfection and analyzed on a density gradient. The density gradient was fractionated, and the core particle peaks were analyzed by ELISA. As shown in Fig. 3, in the absence of SRPK2, two major core particle peaks with densities of 1.35 g/cm^3 (Peak I) and 1.30 g/cm^3 (Peak II) were identified. When these two peaks were analyzed by electron microscopy, they were found to contain core particles with a similar size and morphology (Fig. 3, insets). However, when individual fractions of the gradient were analyzed for HBV DNA by Southern blot, only the high-density peak fraction was found to contain the HBV DNA (Fig. 3, bottom panels).

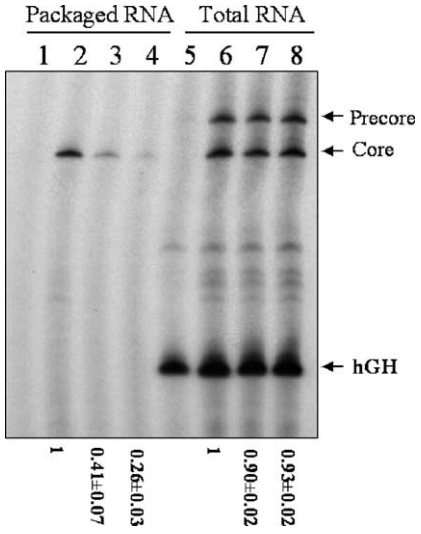

Fig. 2. Primer-extension analysis for the HBV pgRNA. Huh7 cells co-transfected with the HBV genome and a control vector (lanes 2 and 6) or a plasmid that expresses SRPK1 (lanes 3 and 7) or SRPK2 (lanes 4 and 8) were lysed for the isolation of the pgRNA packaged in the core particles (lanes 1–4) or the total cellular RNA (lanes 5–8). Lanes 1 and 5 are the cells that were transfected by pUC19 and the control expression vector. The plasmid that expresses hGH was also used for co-transfection to monitor the transfection efficiency. Details for the isolation of the viral RNA and the primer-extension are described in Materials and methods. Precore, core and hGH indicate the locations of the precore RNA, the core RNA and the hGH RNA. The numbers shown at the bottom of the gel represent the relative core RNA (pgRNA) levels. The core RNA signals were measured using SigmaScan Pro 5. The results represent the average of at least three independent experiments.

In the presence of SRPK2, the high-density DNA-containing core particle peak was greatly diminished (Fig. 3). This result is consistent with the results shown in Figs. 1 and 2, which indicated that SRPK2 could suppress the encapsidation of the HBV pgRNA and hence the subsequent viral DNA replication. Interestingly, SRPK2 had no apparent effect on the low-density core particle peak, indicating that SRPK2 did not affect the formation of the core particles.

Suppression of HBV replication by SRPK1 and SRPK2 via a mechanism independent of core protein phosphorylation

As SRPK1 and SRPK2 have been suggested to be the major kinases that phosphorylate the core protein during HBV replication, we decided to investigate whether these two kinases suppressed the encapsidation of the HBV pgRNA by enhancing the phosphorylation of the core protein. Huh7 cells were co-transfected with the HBV genome and a control vector or a plasmid that expressed Flag-tagged SRPK1 or SRPK2. The cells were then split into two plates a day after transfection. One of the plates was labeled with ^{32}P-orthophosphate followed by immunoprecipitation with the anti-core antibody, and the other plate was lysed and used for the Western blot analysis using the same antibody. As shown in Fig. 4A, the co-expression of SRPK1 or SRPK2 with the HBV genome did not significantly affect the phosphorylation or the expression level of the core protein (Fig. 4A). Similar results were obtained when the HBV core protein was expressed by itself in the absence of other HBV gene products (Fig. 4B). These results raised the possibility that SRPK1 and SRPK2 did not suppress the encapsidation of the HBV pgRNA by enhancing the phosphorylation of the core protein.

To further test whether the kinase activities of SRPK1 and SRPK2 are important for the suppression of HBV replication, we used the kinase-deficient forms of SRPK1 (SRPK1M) and SRPK2 (SRPK2M) for our studies. We first tested the kinase activities of SRPK1M and SRPK2M to ensure that they indeed lacked the kinase activity. Huh7 cells were transfected with a control vector, the expression plasmid for the Flag-tagged SRPK1 or SRPK2 or the expression plasmid for the Flag-tagged SRPK1M or

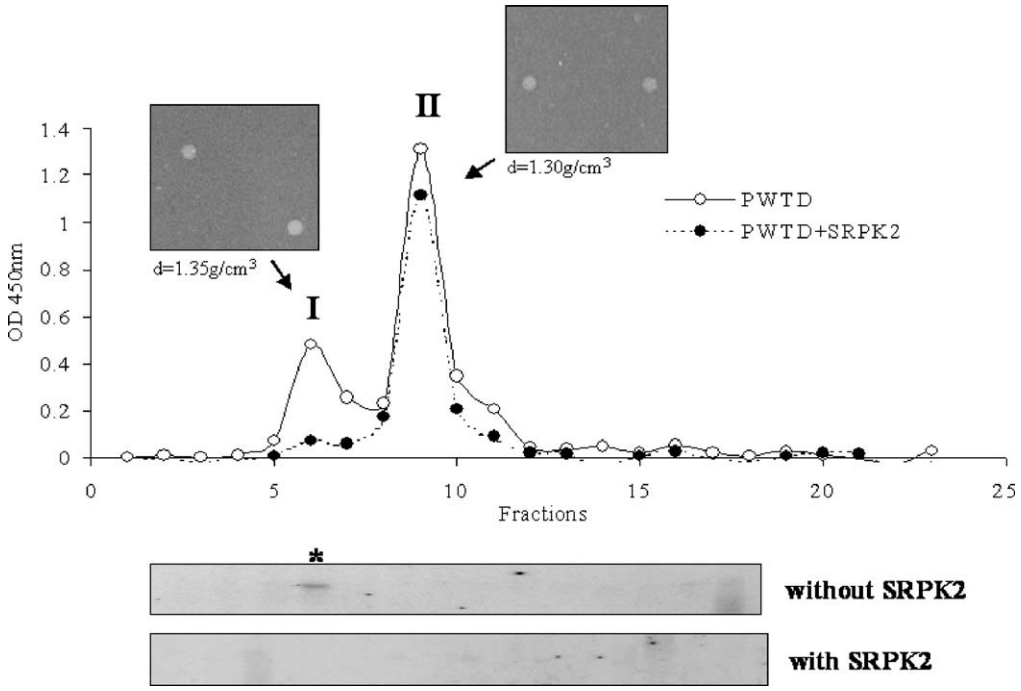

Fig. 3. Density gradient analysis of the HBV core particles. Huh7 cells transfected with the HBV genome with a control vector (solid line) or a plasmid that expresses SRPK2 (dashed line) were lysed and centrifuged on a 20–50% CsCl density gradient. The gradient was fractionated from the bottom and analyzed by ELISA (Materials and methods). The high-density (1.35 g/cm^3) and the low-density (1.30 g/cm^3) core particle peaks are indicated by I and II, respectively. An aliquot of fractions 3–17 was used for the isolation of the HBV DNA, which was then analyzed by Southern blot (bottom panels). The asterisk marks Fraction #6, which is the peak fraction of the high-density core particle. The core particle peak fractions were also negatively stained for electron microscopy for the analysis of the core particles (insets).

SRPK2M. Cells were lysed 48 h after transfection, and the SRPK proteins were immunoprecipitated from the cell lysates using the anti-Flag antibody. The kinase activities of various SRPK proteins were then assayed using a peptide substrate and γ-^{32}P-ATP. As shown in Fig. 5A, both Flag-tagged SRPK1 and SRPK2 had high kinase activities, whereas Flag-tagged SRPK1M and SRPK2M displayed low kinase activities similar to those of the background controls. To examine whether SRPK2M retains the ability to bind to the core protein, we had performed the co-immunoprecipitation experiment. As shown in Fig. 5B, SRPK2 or SRPK2M expressed in Huh7 cells could be immunoprecipitated by the anti-core antibody in the presence, but not in the absence, of the core protein. Note that the expression levels of SRPK2 and SRPK2M in Huh7 cells were similar prior to immunoprecipitation, indicating that the lack of kinase activity of SRPK2M observed in Fig. 5A was not due to its instability. We were not able to detect the binding of SRPK1 or SRPK1M to the core protein in the same co-immunoprecipitation experiment (data not shown). This is consistent with the previous report, which indicated that SRPK1 had a significantly lower affinity than SRPK2 to the core protein (Daub et al., 2002). Nevertheless, the expression levels of SRPK1 and SRPK1M in Huh7 cells prior to immunoprecipitation were the same, again indicating that the loss of the kinase activity did not affect the expression level of SRPK1M (data not shown).

Next, we examined the effect of SRPK1M and SRPK2M on the replication of HBV DNA. The HBV DNA genome was co-transfected with the expression plasmid for SRPK1, SRPK1M, SRPK2 or SRPK2M into Huh7 cells. As shown in Fig. 5C, the ability of SRPK1M and SRPK2M to suppress HBV DNA replication was only slightly affected when compared with their respective wild-type proteins. Thus, these results demonstrated that SRPK1 and SRPK2 could suppress HBV replication via a kinase-independent pathway.

Discussion

SRPK1 and SRPK2 are highly specific kinases for the SR-domain containing proteins. By conducting the GST-pulling down experiment followed by mass spectrometry, Daub et al. (2002) found that SRPK1 and SRPK2 could bind to and phosphorylate the core protein in vitro. In their co-expression experiment in Huh7 cells, they found that SRPK2 could be co-immunoprecipitated with the core protein, indicating that SRPK2 could also bind to the core protein in Huh7 cells. In this report, we have investigated how the interaction between the core protein and SRPK1/2 may affect HBV replication. Our results indicated that both SRPK1 and SRPK2 could suppress HBV DNA replication, with the latter having a greater suppressive effect (Fig. 1A). Neither SRPK1 nor SRPK2 affected the transcription of

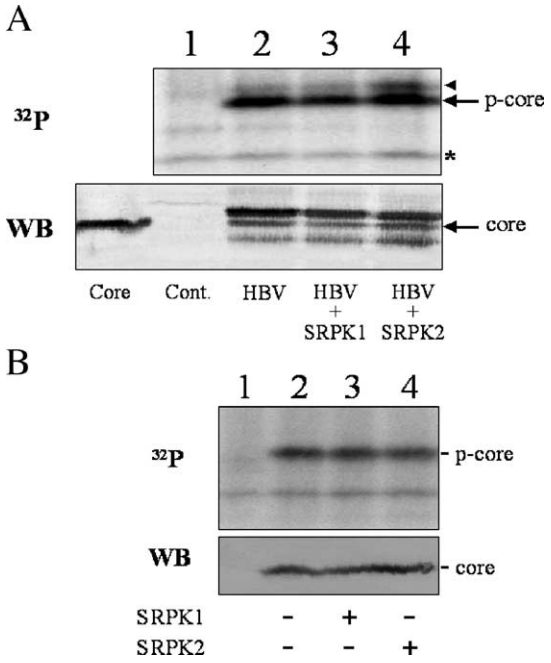

Fig. 4. Effects of SRPK kinases on the phosphorylation of the HBV core protein. (A) Phosphorylation analysis of the core protein expressed from the HBV DNA genome. Huh7 cells co-transfected with the HBV genome and a control vector (lane 2) or a plasmid that expresses SRPK1 (lane 3) or SRPK2 (lane 4) were split into two plates the second day. One plate of cells was labeled with ^{32}P-orthophosphate on the third day followed by immunoprecipitation using the anti-core antibody, and the other plate was lysed and used for the Western blot (WB) analysis. Lane 1 is the negative control (Cont.) sample. It was derived from cells transfected with pUC19 and the control expression vector. The arrow marks the locations of the core protein. The arrowhead denotes the precore protein, which is also a phosphoprotein (Ou et al., 1989; Yeh and Ou, 1991). The asterisk marks the location of a background band, which served as a loading control. In the lower WB panel, cells transfected with a plasmid expressing only the core protein were also shown to the left to serve as a marker to identify the core protein band. The additional protein bands in the lower panel are the precore protein derivatives that cross-reacted with the anti-core antibody (data not shown; Ou et al., 1989). (B) Phosphorylation analysis of the core protein expressed from pCMV-core, a plasmid that expressed the HBV core protein only. Huh7 cells were transfected with pUC19 and a control vector (lane 1), pCMV-core and the control vector (lane 2), pCMV-core and the SRPK1 expression plasmid (lane 3) or pCMV-core and the SRPK2 expression plasmid.

HBV RNAs (Fig. 1B). Further analysis indicated that these two kinases could suppress the encapsidation of the HBV pgRNA (Fig. 2). Thus, SRPK1 and SRPK2 apparently suppressed HBV DNA replication by suppressing the encapsidation of the pgRNA.

To further investigate how SRPKs may suppress HBV replication, we had also conducted the density gradient analysis. In our studies on SRPK2, we found that this kinase could reduce the level of only the high-density DNA containing core particles but not the low-density core particles devoid of viral DNA (Fig. 3). This result indicates that, although SRPK2 affects the encapsidation of the pgRNA, it does not significantly affect the core particle formation. Previous studies indicated that SRPK1 and SRPK2 could physically bind to the carboxy terminus of the core protein, with the former having a lower binding affinity (Daub et al., 2002). As the carboxy terminus of the core protein is important for the pgRNA packaging, it is likely that SRPK1 and SRPK2 suppress HBV replication by interfering with the interaction between the core protein and the pgRNA and/or the viral DNA polymerase. In this case, the lower suppressive activity of SRPK1 may be due to its lower binding affinity to the core protein. Our observation that the kinase-deficient mutant SRPK2M could bind to the core protein and suppress HBV replication with an efficiency similar to that of SRPK2 is also consistent with this hypothesis (Fig. 5B). The lack of effect of SRPK2 on the formation of the core particle may also be explained as the carboxy terminus of the core protein is not essential for the formation of the core particle (Kock et al., 2004).

As SRPK1 and SRPK2 can phosphorylate the core protein in vitro, we have also investigated whether these two kinases suppressed HBV replication via the phosphorylation of the core protein. As shown in Fig. 4, the co-expression of these two kinases did not increase the phosphorylation level of the core protein whether the core protein was expressed from the HBV genome or by itself using a heterologous promoter. This result raised the possibility that SRPK1 and SRPK2 did not suppress HBV replication by enhancing the phosphorylation of the core protein. This possibility is further supported by our studies using the kinase-deficient mutants. In our studies, we found that abolishing the kinase activities of SRPK1 and SRPK2 only slightly reduced their suppressive activities on the replication of HBV (Fig. 5C). Thus, these results together provide a compelling argument that SRPK1 and SRPK2 can suppress HBV replication via a phosphorylation-independent pathway. We cannot rule out the possibility that a kinase-dependent pathway may also be involved as the suppressive activities of SRPK1M and SRPK2M on HBV replication were slightly reduced.

SRPK1 and SRPK2 have been suggested to be the major cellular kinases involved in the phosphorylation of the core protein during HBV replication (Daub et al., 2002). In our studies, we were not able to observe a change in the phosphorylation level of the core protein whether the expression levels of these two kinases were increased by over-expression (Fig. 4) or decreased by the siRNA knockdown experiments (data not shown). One possible explanation to these results is that a very small amount of these two kinases was sufficient to fully phosphorylate the core protein. Hence, the increase or the decrease of their expression levels had no apparent effect on the phosphorylation of the core protein. Alternatively, it is also possible that these two kinases, which can bind to the core protein, are not the major enzymes involved in the phosphorylation of the core protein during HBV replication. Note that, if these two enzymes are indeed the kinase found associated with the core particles in the HBV virions (Albin and Robinson, 1980; Gerlich et

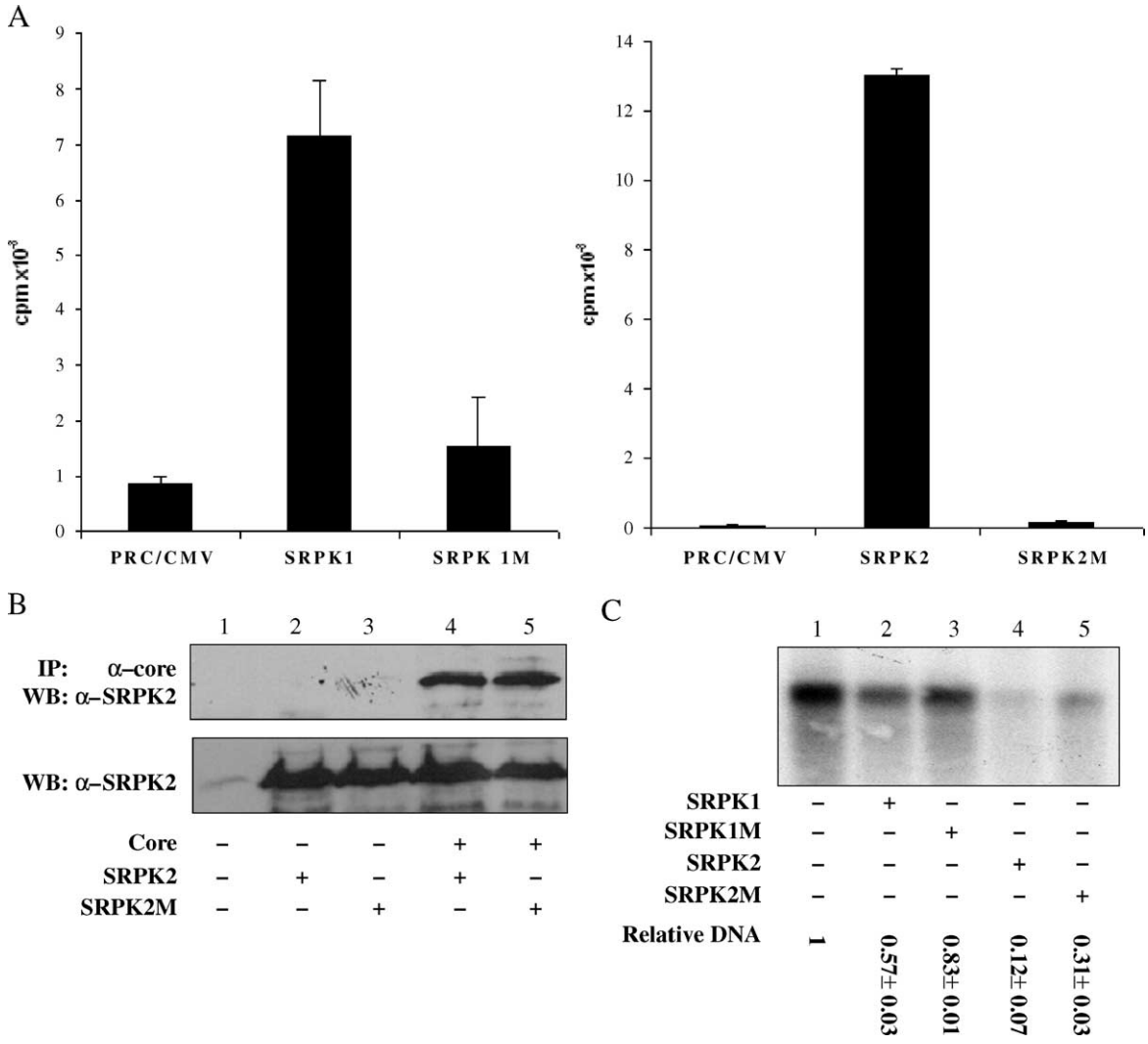

Fig. 5. Effects of the SRPK kinase-negative mutants on HBV DNA replication. (A) Analysis of the kinase activities of various SRPK proteins. Huh7 cells transfected by the control vector pRc/CMV or the expression plasmid for various Flag-tagged SRPK proteins were lysed 48 h after transfection. Cell lysates were immunoprecipitated with the anti-Flag antibody. The immunoprecipitates were then incubated with the SRPK substrate peptide for the analysis of the SRPK kinase activities. (B) Co-immunoprecipitation of SRPK2 and SRPK2M with the HBV core protein. Huh7 cells were co-transfected with control vectors, the HBV core protein expression plasmid, the SRPK2 plasmid and the SRPK2M plasmid as indicated under the gel. Forty-eight hours after transfection, cells were lysed. An aliquot of the cell lysates was used directly for Western blot using the anti-SRPK2 antibody (BD Transduction Lab.; lower panel). The rest of the cell lysates were immunoprecipitated using the rabbit anti-core antibody prior to Western blot analysis using the anti-SRPK2 antibody (upper panel). As shown in the figure, both SRPK2 and SRPK2M could be immunoprecipitated by the anti-core antibody only in the presence but not in the absence of the core protein. (C) Effects on HBV DNA replication. Huh7 cells were co-transfected with the HBV genome and the control vector (lane 1) or the expression plasmid for SRPK1 (lane 2), the SRPK1 mutant (SRPK1M) (lane 3), SRPK2 (lane 4) or the SRPK2 mutant (SRPK2M) (lane 5). The isolation of the HBV DNA and the subsequent Southern blot procedures were described in Materials and methods. The quantification of the relative DNA levels was determined by using SigmaScan Pro 5. The numbers shown represent the average of at least three independent experiments.

al., 1982), then the virus apparently packages these two kinases at the expense of its replication efficiency. Their packaging may be required for the proper initiation of HBV replication in the next infection cycle, such as facilitating the nuclear transport of the core particles as what has been proposed (Kann and Gerlich, 1994; Rabe et al., 2003).

In conclusion, in this report, we demonstrated that SRPK1 and SRPK2 could suppress HBV DNA replication via a pathway independent of their kinase activities. This suppressive effect of SRPK1 and SRPK2 affects the encapsidation of the pgRNA but not the formation of the core particle. Our studies thus implicate a negative role of these two kinases in the replication of HBV.

Materials and methods

DNA plasmids

The plasmid pWTD contains a head-to-tail dimer of the HBV (*adw2*) genome joined at the unique *Eco*RI site. This

plasmid has been described before (Zheng et al., 2004). pFlag-SRPK1 and pFlag-SRPK2 express human SRPK1 and SRPK2, respectively, using the immediate early promoter of cytomegalovirus (CMV). In both plasmids, the SRPK sequences were tagged with a Flag epitope at the N-terminus (Wang et al., 1998). pFlag-SRPK1M and pFlag-SRPK2M are the two kinase mutants which contain a lysine to methionine mutation in the ATP binding sites. The mutation is located at amino acid (aa.) 109 of the SRPK1 sequence and aa. 110 of the SRPK2 sequence. pCMV-core is a plasmid that expresses only the HBV core protein using the CMV promoter (Yeh and Ou, 1991).

DNA transfection and Southern blot analysis of replicated HBV DNA

Huh7 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum. Cells in a 60-mm dish were transfected by a total amount of 10 μg DNA plasmids using the calcium phosphate precipitation method when they were about 80% confluent. Cells were rinsed twice with phosphate-buffered saline (PBS) 48 h after transfection and lysed in Tris-buffered saline (TBS; 10 mM Tris–HCl, pH 7, 150 mM NaCl) containing 0.5% Nonidet P-40 (NP40) in a total volume of 500 μl. The nuclear pellet was removed by a brief centrifuge at 15,000 × *g* in a microfuge. The cytoplasmic supernatant was then incubated with 16 units of micrococcal nuclease (Amersham Pharmacia Biotech Inc.) and 5 μg of DNaseI in the presence of 4 mM $CaCl_2$ and 4 mM $MgCl_2$ at 37 °C for 1 h. This nuclease reaction, which removed the input DNA plasmid, was stopped by the addition of 40 μl 0.5 M EDTA. The replicated HBV DNA in the core particles was then isolated by proteinase K digestion followed by phenol extraction using our previous procedures (Zheng et al., 2003). The HBV DNA was subsequently analyzed by Southern blot using a ^{32}P-labeled HBV DNA probe.

Primer-extension analysis for the HBV pregenomic RNA

Cells transfected with the DNA plasmids were rinsed with PBS, lysed in TBS containing 0.5% NP40 and treated with micrococcal nuclease and DNaseI as described above. The core particles in the cytoplasmic lysates were pelleted at 50,000 rpm in a SW55 rotor for 1 h. The pregenomic RNA packaged in the core particles was then extracted with Trizol reagent (Invitrogen) following the manufacturer's protocol. The primer-extension analysis was conducted to quantify the amount of the pregenomic RNA packaged in the core particles using our previous procedures (Zheng et al., 2004). A ^{32}P-labeled oligonucleotide containing the antisense sequence (nucleotide 2052–2025) in the core protein coding region was used as the primer (Zheng et al., 2004). For the isolation of the total cellular RNA, cells were rinsed twice with PBS followed by immediate extraction with Trizol Reagent. A total of 10 μg total cellular RNA was also used for the primer-extension experiment to serve as a control.

Isopycnic centrifugation and enzyme-linked immunosorbent assay (ELISA)

A total of thirty million Huh7 cells that had been transfected with the DNA plasmids were lysed in 1.5 ml TBS containing 0.5% NP40 and 1 mM PMSF. The nuclei were removed by a brief centrifugation at 15,000 × *g* in a microcentrifuge. The supernatant was then loaded on a 20–50% CsCl density gradient and centrifuged at 36,000 rpm for 16 h using a SW40 rotor. The gradient was fractionated from the bottom of the tube, and 0.5-ml fractions were collected. The Bethyl ELISA kit (Bethyl Laboratories) was used to analyze the HBV core particles in the gradient. Briefly, each well of the ELISA plate was coated overnight at 4 °C with 100 μl rabbit polyclonal anti-core antibody (Ou et al., 1990) that had been diluted 1:100 in the Bethyl coating buffer. After the removal of the antibody, the wells were washed with the washing buffer and then blocked with 200 μl blocking buffer at the room temperature for 1 h followed by further washing. 50 μl of the samples from each gradient fraction was then mixed with 150 μl Bethyl sample dilution buffer and then added into each well. After the incubation at the room temperature for 1 h, the wells were washed and then incubated with the mouse monoclonal anti-HBV core antibody (Abcam; 1:1000 dilution) at the room temperature for another hour. After further washing, the wells were incubated with the horseradish-peroxidase-conjugated goat anti-mouse antibody (1:3000 dilution). The Bethyl TMB color reaction substrates were then added, and the color reaction was stopped by the addition of an equal volume of 2 N H_2SO_4. The absorbance was measured at 450 nm using an ELISA reader.

Negative staining of the core particles

Peak fractions of HBV core particles were concentrated using the Microcon (10 kDa molecular weight cutoff) ultrafiltration columns (Millipore). Samples were then stained with 2% uranyl acetate and analyzed with a Zeiss 10 transmission electron microscope.

Phosphorylation analysis of the HBV core protein

Huh7 cells in a 10-cm petri dish were transfected with the DNA plasmid and then split into two 60-mm dishes on the second day. One of the plates was used for the ^{32}P-labeling experiment, and the other plate was used for the Western blot analysis to monitor the expression level of the core protein. Forty-eight hours after transfection, cells in one of the 60-mm dishes were starved in the phosphate-free medium (Invitrogen) for 1 h and labeled with 0.5 mCi

^{32}P-orthophosphate (ICN) for 3 h. Cells were then lysed in the RIPA solution (Li et al., 2004). Chromosomal DNA were sheared with a 26-gauge needle and removed by two centrifugations in a microcentrifuge. The supernatant was then immunoprecipitated with the polyclonal rabbit anti-core antibody (Ou et al., 1990). For Western blot, cells in the other 60-mm dish were rinsed with PBS, scraped off the plate in TBS containing 0.5% NP40 and sonicated. Cell lysates were then subjected to the Western blot analysis using the rabbit anti-core antibody (Ou et al., 1990).

Analysis of the kinase activities of SRPKs and their mutants

Cells transfected with DNA plasmids were rinsed and collected in 1.5 ml PBS 48 h after transfection. After a brief centrifugation at 4000 rpm in a microfuge, cell pellets were sonicated in 300 μl TBS containing 0.5% NP40 in the presence of the protease inhibitor cocktail (Roche). Cell lysates were then incubated with 1 μl mouse monoclonal anti-Flag antibody (Stratagene) at 4 °C overnight. The immune complex was precipitated using Gamma-bind (Amersham Biosciences), washed three times in TBS containing 0.5% NP40 and finally resuspended in 20 μl 2× kinase buffer (100 mM HEPES, pH 7.0, 200 mM NaCl and 20 mM $MgCl_2$). The kinase reaction was then carried out by incubating 5 μl of the immunoprecipitates with 1 μCi γ-^{32}P-ATP (3000 Ci/mmol), 50 μM ATP and 10 μg SRPKtide (Upstate) at 37 °C for 30 min. Five-microliter reaction mixture from each reaction was spotted on a piece of 1 cm^2 3MM paper and air-dried. The 3MM paper was then washed at 4 °C once with 20% trichloroacetic acid (TCA) for 30 min, twice with 5% TCA for 10 min and then once with methanol for 10 min. The kinase activities were then determined by scintillation counting.

Co-immunoprecipitation experiments

Transfected cells in 60-mm dishes were collected and lysed using the same procedures as described above for the in vitro kinase assay. The rabbit anti-core antibody was used for the immunoprecipitation, which was carried out in TBS containing 0.5% NP40. The immunoprecipitates were Western-blotted using the mouse monoclonal anti-SRPK2 antibody (BD Transduction Lab).

Acknowledgments

We wish to thank members of J. Ou's laboratory for helpful discussions during this project. We are also indebted to the Electron Microscopy Core facility of the USC Norris Cancer Center and the Molecular Biology Core facility of the USC Liver Center for their support. This work was supported by research grants from NIH.

References

Albin, C., Robinson, W.S., 1980. Protein kinase activity in hepatitis B virus. J. Virol. 34 (1), 297–302.

Daub, H., Blencke, S., Habenberger, P., Kurtenbach, A., Dennenmoser, J., Wissing, J., Ullrich, A., Cotten, M., 2002. Identification of SRPK1 and SRPK2 as the major cellular protein kinases phosphorylating hepatitis B virus core protein. J. Virol. 76 (16), 8124–8137.

Ganem, D., Schneider, R.J., 2001. Hepadnaviridae: the viruses and their replication. In: Knipe, D.M., Howley, P.M. (Eds.), Fields Virol., vol. 2. Lippincott Williams and Wilkins, Philadelphia, p. 3087. 2 vols.

Gazina, E.V., Fielding, J.E., Lin, B., Anderson, D.A., 2000. Core protein phosphorylation modulates pregenomic RNA encapsidation to different extents in human and duck hepatitis B viruses. J. Virol. 74 (10), 4721–4728.

Gerlich, W.H., Goldmann, U., Muller, R., Stibbe, W., Wolff, W., 1982. Specificity and localization of the hepatitis B virus-associated protein kinase. J. Virol. 42 (3), 761–766.

Gui, J.F., Lane, W.S., Fu, X.D., 1994a. A serine kinase regulates intracellular localization of splicing factors in the cell cycle. Nature 369 (6482), 678–682.

Gui, J.F., Tronchere, H., Chandler, S.D., Fu, X.D., 1994b. Purification and characterization of a kinase specific for the serine- and arginine-rich pre-mRNA splicing factors. Proc. Natl. Acad. Sci. U.S.A. 91 (23), 10824–10828.

Kann, M., Gerlich, W.H., 1994. Effect of core protein phosphorylation by protein kinase C on encapsidation of RNA within core particles of hepatitis B virus. J. Virol. 68 (12), 7993–8000.

Kau, J.H., Ting, L.P., 1998. Phosphorylation of the core protein of hepatitis B virus by a 46-kilodalton serine kinase. J. Virol. 72 (5), 3796–3803.

Kock, J., Nassal, M., Deres, K., Blum, H.E., von Weizsacker, F., 2004. Hepatitis B virus nucleocapsids formed by carboxy-terminally mutated core proteins contain spliced viral genomes but lack full-size DNA. J. Virol. 78 (24), 13812–13818.

Lan, Y.T., Li, J., Liao, W., Ou, J., 1999. Roles of the three major phosphorylation sites of hepatitis B virus core protein in viral replication. Virology 259 (2), 342–348.

Li, J., Zheng, Y., Choi, J., Ou, J.H., 2004. Phosphorylation analysis of hepatitis B virus core protein in mammalian cells. Methods Mol. Med. 95, 227–233.

Liao, W., Ou, J.H., 1995. Phosphorylation and nuclear localization of the hepatitis B virus core protein: significance of serine in the three repeated SPRRR motifs. J. Virol. 69 (2), 1025–1029.

Ou, J.H., Yeh, C.T., Yen, T.S., 1989. Transport of hepatitis B virus precore protein into the nucleus after cleavage of its signal peptide. J. Virol. 63 (12), 5238–5243.

Ou, J.H., Bao, H., Shih, C., Tahara, S.M., 1990. Preferred translation of human hepatitis B virus polymerase from core protein- but not from precore protein-specific transcript. J. Virol. 64 (9), 4578–4581.

Rabe, B., Vlachou, A., Pante, N., Helenius, A., Kann, M., 2003. Nuclear import of hepatitis B virus capsids and release of the viral genome. Proc. Natl. Acad. Sci. U.S.A. 100 (17), 9849–9854.

Roossinck, M.J., Siddiqui, A., 1987. In vivo phosphorylation and protein analysis of hepatitis B virus core antigen. J. Virol. 61 (4), 955–961.

Wang, H.Y., Lin, W., Dyck, J.A., Yeakley, J.M., Songyang, Z., Cantley, L.C., Fu, X.D., 1998. SRPK2: a differentially expressed SR protein-specific kinase involved in mediating the interaction and localization of pre-mRNA splicing factors in mammalian cells. J. Cell Biol. 140 (4), 737–750.

Yeh, C.T., Ou, J.H., 1991. Phosphorylation of hepatitis B virus precore and core proteins. J. Virol. 65 (5), 2327–2331.

Yeh, C.T., Wong, S.W., Fung, Y.K., Ou, J.H., 1993. Cell cycle regulation of nuclear localization of hepatitis B virus core protein. Proc. Natl. Acad. Sci. U.S.A. 90 (14), 6459–6463.

Yen, T.S.B., 2001. The molecular biology of hepatitis B virus. In: Ou, J.-H.J. (Ed.), Hepatitis Virus. Kluwer Academic Publishers, Boston, p. 304.
Zahler, A.M., Lane, W.S., Stolk, J.A., Roth, M.B., 1992. SR proteins: a conserved family of pre-mRNA splicing factors. Genes Dev. 6 (5), 837–847.
Zheng, Y., Li, J., Johnson, D.L., Ou, J.H., 2003. Regulation of hepatitis B virus replication by the ras-mitogen-activated protein kinase signaling pathway. J. Virol. 77 (14), 7707–7712.
Zheng, Y., Li, J., Ou, J.H., 2004. Regulation of hepatitis B virus core promoter by transcription factors HNF1 and HNF4 and the viral X protein. J. Virol. 78 (13), 6908–6914.