Phosphorylation and Nuclear Localization of the Hepatitis B Virus Core Protein: Significance of Serine in the Three Repeated SPRRR Motifs

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Received 23 September 1994/Accepted 9 November 1994

Hepatitis B virus core protein (antigen) is an important serologic marker of hepatitis B virus infection. This protein is found in the cytoplasm or the nuclei, or both, of infected hepatocytes. A nuclear localization signal has previously been identified in the core protein sequence. This signal overlaps three repeated SPRRR motifs. In this report, we demonstrate that substitution of all of the serine residues in these three SPRRR motifs with alanine can prevent almost entirely the phosphorylation of the core protein in Huh-7 hepatoma cells, enhance nuclear localization of the core protein in both Huh-7 and nonhepatic cells, and abolish cell cycle regulation of nuclear localization of the core protein. Since the three core protein mutants which retained only one serine residue of each of the three SPRRR motifs could be phosphorylated to similar degrees, these three serine residues likely could serve as the acceptor sites for phosphorylation with equal efficiency. These results, together with the observation that the three SPRRR motifs overlap the nuclear localization signal of the core protein, raise the possibility that nuclear localization of the core protein is negatively regulated by phosphorylation of the serine residues in the SPRRR motifs.

Hepatitis B virus (HBV) core protein is a 21-kDa protein. This protein is the major capsid protein of HBV and, by immunofluorescence staining analysis, was found localized to the nuclei or the cytoplasm, or both, of infected hepatocytes (13). The subcellular localization of the core protein appears to be affected by the disease states of the patients (2, 7). Its subcellular localization in hepatocytes is more heterogeneous if the patients have acute or severe hepatitis symptoms. However, its subcellular localization is more homogeneous with a predominant nuclear localization pattern if the patients have mild or no hepatitis symptoms. Predominant nuclear staining of the HBV core protein was also observed in transgenic mice carrying either the core protein gene alone (6) or the entire genomic sequence of HBV (4), although in the latter model, some cytoplasmic staining of the core protein was also detected. The core protein was found localized to either the cytoplasm or the nuclei of hepatocytes in transgenic mice carrying the core protein gene after partial hepatectomy (6). Thus, the observations with both HBV patients and animal models indicate a role of liver injury and regeneration in the regulation of subcellular localization of the core protein.

Our recent studies have suggested a role of the cell cycle in the nuclear localization of the core protein (24). We found that nuclear localization of the core protein was enhanced during the G₁ phase and the quiescent G₀/G₁ phase of the cell cycle and suppressed during the S phase of the cell cycle. Thus, the heterogeneous subcellular localization of the core protein during aggressive hepatitis could be attributed to liver injury and regeneration which cause originally quiescent hepatocytes to enter cell cycles. On the basis of the transgenic mice model, it has also been suggested that the relocalization of the core protein to the cytoplasm is due to the dissolution of the nuclear membrane during cell division (6). This process allowed the core protein which had formed capsid-like particles in the

nucleus to be released back into the cytoplasm. Besides the cell cycle, the large envelope protein of HBV has also been shown to regulate the subcellular localization of the core protein (23).

The core protein is a phosphoprotein (1, 22). Phosphoamino acid analysis has revealed that most, if not all, of the acceptor amino acid residues for phosphorylation in the core protein sequence are the serine residues (18). These phosphorylation sites apparently reside in the carboxy-terminal arginine-rich domain of the core protein, since truncation of this domain reduced the phosphorylation of the core protein to an undetectable level (22). This carboxy-terminal arginine-rich domain contains three repeated SPRRR motifs, the consensus sites for p34^{cdc2} phosphorylation (Fig. 1A) (18). In this report, we have investigated whether the serine residues in these three repeated SPRRR motifs serve as the phosphorylation acceptor sites. Our results revealed that substitution of these serine residues with alanine reduced the phosphorylation of the core protein to an almost undetectable level, indicating that these serine residues are the major phosphorylation acceptor sites in the core protein sequence. In addition, these serine-to-alanine substitutions enhanced nuclear localization of the core protein and abolished cell cycle regulation of nuclear localization of the core protein. These results, together with the previous finding that these three repeated SPRRR motifs overlap the nuclear localization signal of the core protein (3, 21), raise the possibility that phosphorylation of these SPRRR motifs negatively regulates nuclear localization of the core protein.

MATERIALS AND METHODS

Cell lines and cell cycle synchronization. Vero cells and NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% calf serum. Huh-7 human hepatoma cells were maintained in a medium containing 1:1 ratio of DMEM and F-12 medium supplemented with 10% fetal bovine serum. For cell cycle synchronization, the cells were grown to 80% confluence and further incubated in the serum-free DMEM for 30 h. The cell cycle was then initiated with DMEM containing 20% calf serum.

Construction of DNA plasmids. Mutagenesis of the HBV core protein coding sequence was conducted by using PCR. The sequence of the 5'-end PCR primer

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FIG. 1. Localization of the major phosphorylation sites in the core protein sequence. (A) The sequence highly conserved among mammalian hepatitis B viruses is boxed (21). This sequence contains the nuclear localization signal of the core protein and the three SPRRR repeats (underlined). Asterisks marked the locations of the serine residues in the SPRRR repeats. (B) Phosphorylation analysis of the core protein. (C) Western blot analysis of the core protein. The M1 mutant sequence was created as described in Materials and Methods. Huh-7 cells were transfected with DNA, using the CaPO_4 precipitation method. On day 2 after transfection, the cells were split into two plates; one was used for ^{32}P labeling and radioimmunoprecipitation experiments, and the other was used for Western blot analysis using rabbit anticore (16). Lanes: 1, cells transfected with the wild-type core protein sequence; 2, cells transfected with the M1 core protein mutant sequence; 3, mock-transfected cells; 4, the core protein marker from an NIH 3T3 stable transformant carrying the core protein sequence (11). Arrowheads mark the locations of the core protein bands.

(the sense primer) was located between nucleotides 1889 and 1907 of the HBV sequence (adw subtype). The sequence of the 3'-end PCR primer (the antisense primer) was 5' CCG AGA TTG AGA TCT TCT GCG ACG CGG CGC CTG AGA CCT GCG TCT GCG AGG CGC GGG AGT TCT TCT TCT AGG GGC CCT GCC TCG GTC 3'. This sequence was located between nucleotides 2437 and 2359 of the HBV sequence (adw subtype) and contained the antisense coding sequence of the three repeated SPRRR motifs. In this antisense sequence, the serine anticodons in the three SPRRR motifs were mutated to the alanine anticodons. The anticodons of these three alanine residues are underlined. In this particular sequence, the *Bgl*II site located at nucleotide 2404 was also removed by a silent mutation. PCR was conducted with the wild-type HBV core protein coding sequence as the template. The PCR product was further digested with *Bgl*II, and the 445-bp *Bgl*II fragment was then used to replace the same sequence in the HBV core protein coding sequence. These procedures created the M1 mutant core protein sequence. Similar procedures were used to construct other HBV core protein mutants except that, depending on the mutants, the serine in only one or two of the three SPRRR repeats was mutated to alanine. All mutated sequences were verified by direct sequencing.

The mutated core protein sequences were inserted into the vector pCMV/Rc (Invitrogen) under the expression control of the immediate-early promoter of cytomegalovirus.

^{32}P labeling and radioimmunoprecipitation of the core protein. Cells grown in a 60-mm-diameter dish were transfected with plasmid DNA. On day 2 after transfection, the cells were split into two 60-mm-diameter dishes and processed for either ^{32}P labeling or Western immunoblot analysis. For ^{32}P labeling, the cells were rinsed twice with a phosphate-free medium, starved for phosphate in the same medium for 2 h, and subsequently labeled with 1 mCi of $^{32}\text{P}_i$ (ICN) in 2 ml of phosphate-free medium for 3 h. After ^{32}P labeling, the cells were rinsed twice with ice-cold phosphate-buffered saline and lysed with radioimmunoprecipitation assay buffer (10 mM Tris-HCl [pH 7], 150 mM NaCl, 1% Triton X-100, 1% sodium dodecyl sulfate). The chromosomal DNA was sheared by pipetting, and the core protein was then immunoprecipitated with rabbit anticore (16), using our previous procedures. For Western blot analysis, the cells were lysed directly in 2× Laemmli sample buffer (9). The sample was then electrophoresed on a

12.5% polyacrylamide gel and subsequently transferred to a nitrocellulose membrane for Western blot analysis.

RESULTS

The serine residues in the SPRRR repeats are required for efficient phosphorylation of the core protein. We have investigated whether the serine residues in the three repeated SPRRR motifs are the phosphorylation acceptor sites in the core protein sequence. These three serine residues were mutated to alanine by site-directed mutagenesis. The resultant DNA plasmid, pCMV-CM1, which contained the mutated core protein sequence under the expression control of the cytomegalovirus immediate-early promoter, was then transfected into Huh-7 hepatoma cells. Two days after transfection, the cells were labeled with $^{32}\text{P}_i$ and the core protein was immunoprecipitated. As shown in Fig. 1B, although the ^{32}P -labeled core protein was clearly detectable in cells transfected with the wild-type core protein sequence, it was almost undetectable in cells transfected with the mutant core protein sequence. To ensure that the mutant core protein was faithfully expressed in Huh-7 cells, we performed Western blot analysis. As shown in Fig. 1C, the M1 mutant core protein was expressed in Huh-7 cells at a level comparable to that of the wild-type core protein. Thus, the lack of phosphorylation of the M1 mutant core protein was not due to its instability or a low level of expression in Huh-7 cells. These results indicate that the three serine residues in the SPRRR motifs shown in Fig. 1A are important for phosphorylation of the core protein and are likely the major acceptor sites for phosphorylation in the core protein sequence.

To further investigate whether all of the serine residues in the three SPRRR repeats are important for phosphorylation, we substituted the serine in only one or two of the three SPRRR repeats with alanine. These mutagenesis experiments resulted in the generation of six additional core protein mutants (Fig. 2). These mutated core protein sequences were again transfected into Huh-7 hepatoma cells for phosphorylation studies. As shown in Fig. 2A, all six core protein mutants could be phosphorylated by ^{32}P . Since the three core protein mutants, M3, M5, and M8, which retained only one serine of each of the three serine residues in the SPRRR motifs, could be phosphorylated to similar degrees, these three serine residues apparently could serve with equal efficiency as the acceptor sites for phosphorylation. This speculation is further supported by the observation that the degree of phosphorylation of the individual core protein mutants appeared to be positively correlated with the number of serine residues in the SPRRR motifs retained in their sequences (Fig. 2A). The results of the control Western blot experiment (Fig. 2B) show that all of the mutant core protein sequences were expressed to similar levels in Huh-7 cells, suggesting similar stabilities of these proteins in the cells.

In this particular experiment, a small amount of the ^{32}P signal of the M1 core protein mutant was also detectable after a prolonged exposure of the film. Since the M1 mutant lacked all of the serine residues in the three SPRRR motifs, this result indicated that there might exist other minor phosphorylation sites in the core protein sequence.

Enhancement of nuclear localization of the core protein after substitution of the serine residues in the SPRRR repeats with alanine. To investigate the possible relationship between phosphorylation and nuclear localization of the core protein, the subcellular localization of the wild-type core protein and the M1 mutant core protein was investigated. As shown in Fig. 3A, reminiscent of the clinical observation, subcellular local-

FIG. 2. Phosphorylation analysis of the core protein mutants expressed in Huh-7 hepatoma cells. The procedures described in the legend to Fig. 1 were used for the ^{32}P -labeling (A) and the Western blot (B) experiments. C., mock-transfected cells; Wt, cells transfected with the wild-type core protein sequence. The serine-to-alanine substitutions are shown under the name of each core protein mutant. Arrowheads mark the locations of the core protein bands.

ization of the wild-type core protein in Huh-7 hepatoma cells was heterogeneous, with roughly 60% of the transfected cells displaying predominantly cytoplasmic staining of the core protein, 30% of the cells displaying both nuclear and cytoplasmic staining of the core protein, and slightly less than 10% of the cells displaying predominantly nuclear staining of the core protein (Fig. 3B). This pattern of subcellular localization of the core protein was significantly altered if the cells were transfected with the M1 mutant core protein sequence. As shown in Fig. 3B, the proportion of cells displaying predominantly cytoplasmic staining of the M1 mutant core protein was reduced to approximately 25%. In the meantime, the proportion of cells displaying predominantly nuclear staining of the core protein was increased from less than 10% to slightly over 40%. This result indicates that nuclear localization of the M1 mutant core protein is more efficient than nuclear localization of the wild-type core protein and suggests a possible negative role of the serine residues of the three SPRRR motifs on the regulation of nuclear localization of the core protein.

Similar studies were conducted with Vero cells, an immortalized African green monkey kidney cell line. The cells were transfected with the core protein DNA and analyzed when the cells were still subconfluent. As shown in Fig. 3C, while the wild-type core protein was stained in the cytoplasm of roughly 70% of the positive Vero cells, in the nuclei and cytoplasm of about 30% of the cells, and in the nuclei of almost no cells, the M1 mutant core protein was found in the nuclei of roughly 80% of the cells, in both the cytoplasm and the nuclei of about 20% of the cells, and in the cytoplasm of almost no cells. Similar enhancement of nuclear localization of the M1 mutant core protein was also detected in NIH 3T3 cells, an immortalized mouse fibroblast cell line (data not shown; also see below). Thus, similar to the results obtained with Huh-7 hepatoma cells, nuclear localization of the M1 mutant core protein was also greatly enhanced in nonhepatic cells. The enhancement of

FIG. 3. Subcellular localization of the core protein in Huh-7 cells and Vero cells. (A) Heterogeneous immunofluorescence staining pattern of the core protein in Huh-7 cells. The core protein was found localized to the cytoplasm (C) or nuclei (N), or both, of transfected cells. (B) Histogram of the subcellular localization of the wild-type (Wt) core protein and the M1 mutant core protein in Huh-7 cells. (C) Histogram of the subcellular localization of the various core protein mutants in Vero cells. Approximately 50 to 100 positive cells were counted. N>C, predominantly nuclear staining; N=C, staining of the whole cell; N<C, predominantly cytoplasmic staining.

nuclear localization of the M1 mutant core protein was also observed in a subcellular fractionation assay using our previous procedures (24) (data not shown).

In comparing the results shown in Fig. 3B and C, the effect of the serine-to-alanine mutations in the SPRRR motifs on the nuclear localization of the core protein seemed to be more dramatic in Vero cells than in Huh-7 hepatoma cells. This result was reproducible, and the reason is unclear at this moment.

The role of the SPRRR motifs in the regulation of the subcellular localization of the core protein was further exam-

FIG. 4. Immunofluorescence analysis of the wild-type core protein (a and b) and the M1 mutant core protein (c and d) in S-phase Vero cells. Vero cells synchronized by serum deprivation for 30 h were allowed to grow in DMEM containing 20% calf serum for 12 h. At this time point, on the basis of the [3 H]thymidine incorporation assay, the cells were in the S phase of the cell cycle (reference 23 and data not shown). While predominantly cytoplasmic staining and whole-cell staining were observed for cells transfected with the wild-type core protein sequence, predominantly nuclear staining was observed for cells transfected with the M1 core protein sequence.

ined by using the six additional core protein mutants that retained either one or two serine residues of the three SPRRR motifs. As shown in Fig. 3C, mutation of only one of the serine residues (mutants M4, M6, and M7) was sufficient to result in a significant enhancement of nuclear localization of the core protein in Vero cells. This result indicates that all three serine residues in the SPRRR motifs are important for the regulation of subcellular localization of the core protein.

The analysis shown in Fig. 3C was conducted when Vero cells were subconfluent. At this cell density, the contrast of the subcellular localization patterns of the wild-type core protein and the M1 mutant core protein was very dramatic. However, in confluent cells, there was an increase of nuclear localization of the wild-type core protein, and thus the difference between the subcellular localization patterns of the wild-type core protein and the M1 mutant core protein was reduced. The observation that the cell density affected the subcellular localization of the wild-type core protein is consistent with our previous results which indicated that nuclear localization of the wild-type core protein was enhanced in the confluent, quiescent G_0/G_1 -phase cells (24).

Loss of cell cycle regulation of nuclear localization of the mutant core protein. The effect of the cell cycle on the subcellular localization of the M1 mutant core protein was further investigated. Vero cells transfected with either the wild-type or the M1 core protein coding sequence were synchronized at the G_0 phase of the cell cycle with a serum-free medium for 30 h. After synchronization, the cell cycle was initiated with a medium containing 20% calf serum and progressed to the S phase. As shown in Fig. 4, consistent with our previous finding (24), the wild-type core protein was localized predominantly in the cytoplasm or both the cytoplasm and the nuclei of all transfected cells by immunofluorescence staining. Nuclear staining of the core protein was rare but not absent at this stage of the cell cycle. On the other hand, the M1 core protein lacking the major phosphorylation sites was stained predominantly in the nuclei of the great majority of the positive cells.

The same nuclear staining of the M1 mutant core protein was observed in all phases of the cell cycle (also see Fig. 3C), indicating a lack of cell cycle regulation of nuclear localization of the M1 mutant core protein. This result indicates that the serine residues in the SPRRR motifs are important for cell cycle regulation of the nuclear localization of the core protein.

DISCUSSION

The results shown in this report indicated that the three serine residues in the SPRRR repeats were important for the phosphorylation of the core protein, since substitution of these serine residues with alanine resulted in the loss of most of the phosphorylation signal of the core protein (Fig. 1B and 2A). These three serine residues likely serve as the major acceptor site for phosphorylation with equal efficiency, since the core protein mutants, M3, M5, and M8, that contained only each of the three serine residues were phosphorylated to similar degrees in Huh-7 hepatoma cells (Fig. 2A). Recently, three serine residues and one threonine residue located in the carboxy terminus of the core protein of the related duck hepatitis B virus (DHBV) have been identified as the major phosphorylation acceptor sites (25). Similar to our results, these four phosphorylation acceptor sites of the DHBV core protein were all juxtaposed to the amino-terminal end of proline. Thus, despite the divergence of the core protein sequences of HBV and DHBV, the requirement for the phosphorylation acceptor sites to be juxtaposed next to proline has been conserved during evolution.

Since the three SPRRR repeats overlap the nuclear localization signal of the core protein, the serine residues in these repeats likely are important for the activity of this signal. This apparently was the case, since substitution of all of the serine residues in the SPRRR motifs with alanine enhanced nuclear localization of the core protein in Huh-7 hepatoma cells and the nonhepatic Vero and NIH 3T3 cells (Fig. 3). Efficient suppression of nuclear localization of the core protein appeared to require the presence of all of the serine residues in the three SPRRR repeats, since substitution of any one of the three serine residues in these motifs with alanine would lead to a significant enhancement of nuclear localization of the core protein when the cell density favored cytoplasmic localization of the wild-type core protein (Fig. 3C). It has been reported that substitution of the phosphorylation acceptor sites in the DHBV core protein sequence had no effect on the subcellular localization of the DHBV core protein, which is predominantly cytoplasmic (26). Thus, DHBV may have a different mechanism to regulate the subcellular localization of its core protein. In this regard, it is important that nuclear localization of the DHBV core protein has not been positively demonstrated before.

Since substitution of serine with alanine would prevent phosphorylation, our results are in support of the hypothesis that phosphorylation of the serine residues in these SPRRR repeats could suppress nuclear localization of the HBV core protein. These results could also explain why the core protein in the nucleus was previously found to be hypophosphorylated (24). It has been shown that substitution of the serine with alanine would result in the conformational change of the DHBV core protein (25). Thus, it is also possible that enhancement of nuclear localization of the core protein mutants observed in this report was due to structural change of the core protein. Guidotti et al. (6) have suggested that because of its size, the HBV core particle cannot translocate across the nuclear membrane. Thus, the structural change or the reduction of phos-

phorylation of the core protein may affect the assembly process of the core particle and facilitate its nuclear transport.

We previously demonstrated that nuclear localization of the core protein was cell cycle regulated in Vero and NIH 3T3 cells. The nuclear localization of the core protein was facilitated during the G₁ phase and the confluent, quiescent G₀/G₁ phase and suppressed during the S phase. The experiments shown in Fig. 4 indicated that substitution of the serine residues in the SPRRR repeats with alanine would abolish this cell cycle regulation and lead to constitutive nuclear localization of the core protein. The SPRRR repeat is the recognition site of the cell cycle-regulated kinases p34^{cdc2} kinase (15, 20). This kinase is activated during the S phase and the M phase of the cell cycle. We have observed that p34^{cdc2} could phosphorylate the core protein in vitro (data not shown). Thus, if p34^{cdc2} and/or its related kinases could also phosphorylate the core protein in vivo, their phosphorylation on the core protein might lead to suppression of nuclear localization of the core protein during the S phase and immediately after the M phase when the nuclei of daughter cells were re-formed. A similar cell cycle regulation of nuclear localization has also been reported for the yeast transcription factor SWI5 (12, 14). For SWI5, it has been proposed that the cell cycle regulation of its nuclear localization is mediated by phosphorylation at the cdc2/CDC28 sites located in the nuclear localization signal (12).

The biological functions of this cell cycle regulation of nuclear localization of the HBV core protein remains elusive. It seems likely that during the early phase of infection, because of the quiescent state of the hepatocytes, nuclear entry of the core protein will be facilitated. This nuclear localization may be important for transporting the HBV genomic DNA into the nucleus for initiating viral mRNA transcription. It has been demonstrated that during DHBV infection, the number of viral DNA templates (i.e., the covalently closed circular DNA [cccDNA]) for RNA transcription is amplified in the nuclei of infected cells (19). Since the viral DNA replicates in the core protein particles in the cytoplasm, it is possible that nuclear localization of the core protein is also important for transporting the replicated viral DNA into the nucleus for cccDNA amplification. Although nuclear localization of the core protein of DHBV and other related mammalian hepatitis B viruses has not been positively demonstrated, localization of a small number of core particles to the nucleus during replication of these animal viruses will likely be sufficient to lead to cccDNA amplification. Lastly, the HBV core protein has been demonstrated to be a DNA-binding protein (5, 10). Thus, instead of playing a positive role for HBV replication, a high concentration of the core protein in the nucleus may lead to suppression of viral mRNA transcription as a result of the binding of the core protein to the cccDNA. This suppression may be important for the establishment of persistent infection in patients. In this regard, suppression of nuclear localization of the core protein during liver injury and regeneration may be important for enhancing viral gene expression from cccDNA to combat the immune response which is largely responsible for the liver injury.

ACKNOWLEDGMENTS

We thank T. S. Benedict for critical reading of the manuscript and members of J. Ou's laboratory for helpful discussions throughout this project.

This research was supported by research grant CA54533 from the National Institutes of Health and a research grant from the Council for Tobacco Research.

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