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Phosphorylation of Hepatitis B Virus Precore and Core Proteins

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Hepatitis B virus precore and core proteins are related. The precore protein contains the entire sequence of the core protein plus an amino-terminal extension of 29 amino acids. The amino-terminal extension of the precore protein contains a signal sequence for the secretion of the precore protein. This signal sequence is removed after the translocation of the precore protein across the endoplasmic reticulum membrane to produce the precore protein derivative named P22. We demonstrate that both P22 and the core protein can be phosphorylated in cells. Microsomal fractionation and trypsin digestion experiments demonstrate that a fraction of phosphorylated P22 is located in the endoplasmic reticulum lumen. Phosphorylation of P22 likely occurs in the carboxy terminus, since the P22 derivative P16, which lacks the carboxy terminus of P22, is not phosphorylated. Linking the carboxy terminus of the precore-core protein to heterologous secretory and cytosolic proteins led to the phosphorylation of the resulting chimeric proteins. These results indicate that phosphorylation of P22 and the core protein is likely mediated by cellular kinases.

The hepatitis B virus (HBV) C gene encodes two related proteins named the core and precore proteins. Both proteins are translated from the same reading frame but from different initiation codons. Thus, the precore protein contains the entire sequence of the core protein plus an amino-terminal extension of 29 amino acid residues (precore region). The core protein is the major constituent of the nucleocapsid (core particle) of HBV, and the precore protein is the precursor of the serum e antigen detected in HBV-infected patients (for reviews, see references 6 and 24).

The first 19 amino acids of the precore region constitute a signal sequence (18, 25). This signal sequence directs the precore protein to the endoplasmic reticulum, where it is cleaved off, resulting in the translocation of the precore protein derivative P22 into the lumen of endoplasmic reticulum (ER) (3, 7, 13). After an initial event of forming a high-molecular-weight complex, possibly through self-aggregation (17), P22 is further processed by a protease(s) at its carboxy terminus and secreted as smaller-molecular-weight species known as e antigen (18, 20, 28).

After cleavage of its signal sequence, the majority of P22 is translocated into the ER lumen for secretion. However, a small but significant portion of P22 is released back into the cytosol and subsequently transported into the nucleus (7, 21). This observation has led us to the discovery of a nuclear localization signal in the carboxy terminus of the precore-core protein sequence (31).

Core particles purified from the mature HBV particles contain a protein kinase activity (1, 8). This kinase activity can phosphorylate the core protein in the core particles. This kinase activity is also found in the core particles of related hepadnaviruses, including duck HBV (22), ground squirrel HBV (5), and woodchuck HBV (12). The nature of this kinase activity is unclear. It could be of host or viral origin; alternatively, because of its tight association with the core particle, it could be an intrinsic activity of the core protein (12). Because the core protein isolated from duck HBV-infected hepatocytes is phosphorylated and the core protein isolated from mature duck HBV virion is not, it has been speculated that phosphorylation of the core protein regulates the maturation of the virion (22).

Although it has been very well demonstrated that the core protein can be phosphorylated, the phosphorylation of precore protein has not been studied. Lanford and Notvall (14) showed that the precore protein expressed in insect cells with a baculovirus vector is phosphorylated. In their report, however, the precore protein accumulated in the cytosol and was not proteolytically processed and secreted.

In this report, we describe our studies on the phosphorylation of precore and core proteins in human hepatoma cells. Our results demonstrate that the precore protein derivative, P22, can be phosphorylated. Some of the phosphorylated P22 is located in the ER lumen, and the majority of it is at least partially exposed in the cytosol. Furthermore, our results indicate that the phosphorylation of precore and core proteins is likely mediated by a cellular kinase(s).

MATERIALS AND METHODS

Construction of DNA plasmids. Plasmids pRSV-PC and pRSV-C express the precore and the core proteins, respectively. To construct pRSV-PC, site-directed mutagenesis was used to generate a *Hin*dIII site at nucleotide 1757 (sequence TAGGTT to AAGCTT) of the adw HBV genome (29). The *Hin*dIII-*Bgl*II HBV DNA fragment containing the entire genomic information was then inserted into the *Hin*dIII-*Bam*HI polylinker site of pSP64 (Promega Biotec) to generate pSP64-PC. The 300-bp *Nae*I-*Hin*dIII fragment in pSP-PC was then replaced with the 400-bp *Nru*I-*Hin*dIII fragment isolated from pRSV-CAT (10). Thus, in this plasmid construct, the precore sequence is located immediately adjacent to the Rous sarcoma virus long terminal repeat. The construction of pRSV-C was essentially identical to that of pRSV-PC, except that the *Hin*dIII site was generated at nucleotide 1821 (sequence AACTTT to AAGCTT), 1 bp downstream from the precore ATG.

Plasmids pECE-GH and pECE-G express human growth hormone and human alpha-globin sequences, respectively. In these two plasmids, expression is regulated by the simian virus 40 early promoter. The construction of these two plasmids has been described before (21, 31).

Plasmid pECE-GHC expresses the fusion protein contain-

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ing the growth hormone and the carboxy-terminal sequence of the precore-core protein. To construct this plasmid, the ~500-bp *Hpa*II-*Bst*EII DNA fragment was isolated from the HBV genome, made blunt ended, and inserted into the *Sal*I site (blunt ended) of pECE-ATG, a plasmid previously constructed in our laboratory (31). This ligation resulted in the restoration of a *Sal*I site. A ~500-bp *Sal*I (blunt ended)-*Xba*I fragment was isolated from the resulting plasmid and ligated to the 4.3-kb *Bgl*II (blunt ended)-*Xba*I fragment of pECE-GH. This results in the creation of the plasmid pECE-GHC.

Plasmid pECE-GlobC expresses the fusion protein containing the alpha-globin sequence and the carboxy-terminal sequence of precore-core protein. The construction of this plasmid has been described before (31). This plasmid was originally named pECE-HpaG.

Cell lines and DNA transfection. COS-7 cells and Alexander hepatoma cells (PLC/PRF/5) were maintained in Dulbecco modified Eagle medium containing 5% fetal bovine serum. DNA transfection was carried out by the $CaPO_4$ precipitation method (11). The Alex-PC stable transfectant was established by cotransfecting Alexander cells with pRSV-PC and p007LTR, a DNA plasmid containing the neomycin gene sequence (19), followed by selection with G418, a neomycin analog. The Alex-C stable transfectant was established by cotransfecting Alexander cells with pRSV-C and p007LTR.

Cell labeling for radioimmunoprecipitation. Cells starved for methionine (or phosphate) for 2 h were labeled with [^{35}S]methionine (or $^{32}P_i$) for 1 h and then lysed in TBS (10 mM Tris-HCl [pH 7.2], 150 mM NaCl) containing 0.5% Nonidet P-40 (NP40). After a brief centrifugation to remove nuclei and NP40-insoluble cell debris, the cell lysate was mixed with an equal volume of RIPA (10 mM Tris-HCl [pH 7.0], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate for radioimmunoprecipitation.

Microsomal fractionation and trypsin digestion. The procedures for microsomal fractionation and trypsin digestion experiments have been described before (17). Briefly, cells labeled with either [^{35}S]methionine or $^{32}P_i$ as described above were lysed in 0.1× TBS. After a brief centrifugation to remove the nuclei, the cell lysate was divided into three aliquots: to the first aliquot, 100 μl of phosphate-buffered saline was added; to the second aliquot, 100 μl (50 μg) of trypsin (GIBCO) was added; and to the third aliquot, 100 μl of trypsin plus NP40 (final concentration, 0.5%) was added. The samples were incubated at 37°C for 1 h, and the reactions were stopped by the addition of 100 U of aprotinin (Boehringer Mannheim) and phenylmethylsulfonyl fluoride to a final concentration of 2 mM. After the termination of the reactions, the microsomes were pelleted through 4 ml of 10% sucrose cushion in a Beckman SW55 rotor at 45,000 rpm for 3 h. The supernatant was combined with an equal volume of RIPA, and the pellet was suspended in 0.5 ml of RIPA for radioimmunoprecipitation experiments.

RESULTS

Phosphorylation of precore and core proteins. To study whether the precore protein is a phosphoprotein, we expressed the core and the precore proteins in Alexander cells, a human hepatoma cell line. Two different stable cell clones, Alex-PC and Alex-C, expressing the precore and the core proteins, respectively, were established by G418 (a neomycin analog) selection procedures (see Materials and Methods). The expression of the precore and core protein sequences in these two cell clones was regulated by the Rous sarcoma virus promoter.

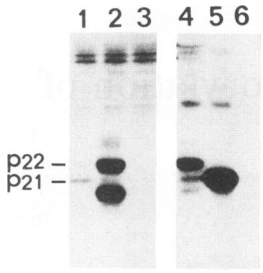

FIG. 1. Phosphorylation analysis of HBV precore and core proteins. Lanes: 1 through 3, cells labeled with [^{35}S]methionine; 4 through 6, cells labeled with $^{32}P_i$; 1 and 5, Alex-C cells; 2 and 4, Alex-PC cells; 3 and 6, control Alexander cells. Precore and core proteins were immunoprecipitated by rabbit anti-denatured core protein prepared by us (21). The relative amounts of P22 and the core protein expressed were measured by densitometer scanning.

The 21-kDa core protein (Fig. 1, lane 1) and the 22-kDa precore protein derivative P22 (lane 2) were produced in Alex-C and Alex-PC cells, respectively. For Alex-PC cells, an additional protein band of about 16 kDa (P16) was also detected. Pulse-chase labeling experiments indicated that P16 was derived from P22 (data not shown). This is consistent with previous reports showing that P22 was converted to P16 by proteolytic cleavage of its carboxy terminus before secretion (20, 27).

Both P22 (Fig. 1, lane 4) and the core protein (lane 5) can be labeled with ^{32}P. This demonstrates that both the core protein and P22 are phosphoproteins. P16 was not significantly labeled with ^{32}P; this indicates that the phosphorylation site(s) of P22 mostly likely resides in the carboxy terminus.

When [^{35}S]methionine was used as the label, the signal of the core protein band, as quantified by densitometer scanning, was approximately 10-fold weaker than that of P22. This indicates that the amount of the core protein expressed in Alex-C cells is approximately 10-fold lower than that of P22 expressed in Alex-PC cells. This observation was confirmed by RNA analysis (data not shown). Interestingly, when $^{32}P_i$ was used as the label, the signal of the core protein band was more than 40-fold higher than that of P22. This result, which is reproducible, indicates that the phosphorylation efficiency of the core protein is more than 400-fold higher than that of the precore protein. This difference of phosphorylation efficiency could be caused by different subcellular localization of the precore and core proteins and/or the involvement of different cellular kinases (see below). Alternatively, it could be caused by different tertiary structures of the precore and core proteins.

Subcellular localization of phosphorylated P22. Since P22 can be released into the cytosol and transported into the nucleus after cleavage of its signal sequence (7, 21), phosphorylated P22 may be located in the cytosol instead of the lumen of the ER. For this reason, we performed microsomal fractionation experiments to investigate the subcellular localization of phosphorylated P22.

Most P22 labeled with [^{35}S]methionine was associated with the microsomal (ER) fraction (Fig. 2, lanes 2 and 3); as determined by densitometer scanning, approximately 90% of it was resistant to trypsin digestion (lane 4). The resistance

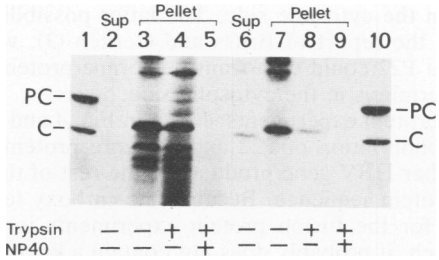

FIG. 2. Microsomal fractionation and trypsin digestion experiments of P22 expressed in Alex-PC cells. Details for the experiments are described in Materials and Methods. Lanes: 2 through 5, proteins labeled with [^{35}S]methionine; 6 through 9, proteins labeled with $^{32}P_i$; 1 and 10, full-length precore (25-kDa) and core (21-kDa) protein markers synthesized in vitro with synthetic RNA and rabbit reticulocyte lysates (21); 2 and 6, postmicrosomal supernatant; 3 and 7, microsomal pellet; 4 and 8, microsomes treated with trypsin; 5 and 9, microsomes treated with trypsin and NP40. A small amount of P22 but not the core protein was detected in lane 2 after a prolonged exposure of the gel on the film. Proteins were immunoprecipitated by rabbit anti-denatured core protein. Densitometer scanning was used to determine the relative amounts of P22 in lanes 3, 4, 7, and 8.

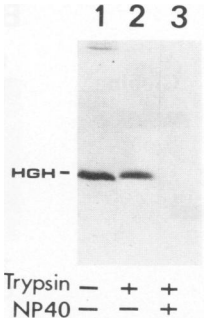

FIG. 3. Microsomal fractionation and trypsin digestion experiments of human growth hormone. Human growth hormone was expressed in COS-7 cells by plasmid pECE-GH (see Materials and Methods). Lanes: 1, microsomal pellet; 2, microsome treated with trypsin; 3, microsome treated with trypsin and NP40. Human growth hormone was immunoprecipitated with rabbit anti-human growth hormone. Densitometer scanning was used to measure the relative amounts of human growth hormone in lanes 1 and 2. Approximately 90% of human growth hormone, as revealed by densitometer scanning, is resistant to trypsin digestion.

to trypsin digestion is lost when the microsomal membrane is solubilized by the nonionic detergent NP40 (lane 5). These results indicate that most P22 associated with the microsomal fraction is located in the lumen of microsomes.

Most P22 labeled with $^{32}P_i$ was also associated with the microsomal fraction (Fig. 2, lane 7). Approximately 20% of P22 associated with the microsomal fraction was resistant to trypsin digestion in the absence of NP40 (lane 8), which is indicative of lumenal localization. The majority of phosphorylated P22, however, was trypsin sensitive in the absence of NP40 (lane 8), which is indicative of cytosolic localization. To make sure that this is not an artifact caused by the subcellular fractionation procedures, we performed a control microsomal fractionation experiment with human growth hormone, a known secretory protein. The results are similar to those with [^{35}S]methionine-labeled P22; approximately 90% of growth hormone is resistant to trypsin digestion, indicating lumenal localization (Fig. 3). These results demonstrate that the majority of phosphorylated P22 is at least partially exposed in the cytosolic side of the ER membrane.

[^{35}S]methionine labeling experiments (Fig. 2) demonstrate that most of P22 molecules are resistant to trypsin digestion and are located in the ER lumen. However, ^{32}P labeling experiments indicate that the majority of phosphorylated P22 molecules are sensitive to trypsin digestion and are at least partially exposed in the cytosolic side of the membrane. The discrepancy of these two experimental results would be resolved, if only a small fraction of P22 molecules were phosphorylated. This possibility is supported by the observation shown in Fig. 1, which indicates that the phosphorylation of P22, when compared with that of the core protein, is extremely inefficient. If this speculation is true, the result shown in Fig. 2 further suggests that phosphorylation increases the sensitivity of P22 to trypsin, possibly by impeding the process of membrane translocation.

A protein species with the same mobility on the gel as the core protein was detected in the cytosolic supernatant of Alex-PC cells (Fig. 2, lane 6). This protein is likely to be the core protein translated from the internal AUG codon of the precore mRNA. This protein band is not detected in the cytosolic supernatant when the protein is labeled with [^{35}S]methionine (lane 2). This could be explained by the high phosphorylation efficiency of the core protein, which probably substantially increased the sensitivity of detection.

Phosphorylation of core and precore proteins is probably mediated by cellular kinases. To investigate whether the phosphorylation of core and precore proteins requires other HBV gene products, we fused the putative phosphorylation sites (the carboxy terminus) of the precore-core protein to a secretory protein, the human growth hormone, to create the fusion protein GHC (Fig. 4A) and to a cytosolic protein, the human alpha-globin, to create the fusion protein GlobC (Fig. 5A). These two fusion proteins were then transiently expressed in COS-7 cells, a monkey kidney cell line.

Although growth hormone (Fig. 4B, lanes 2 and 5) and alpha-globin (Fig. 5B, lanes 2 and 5) were not phosphory-

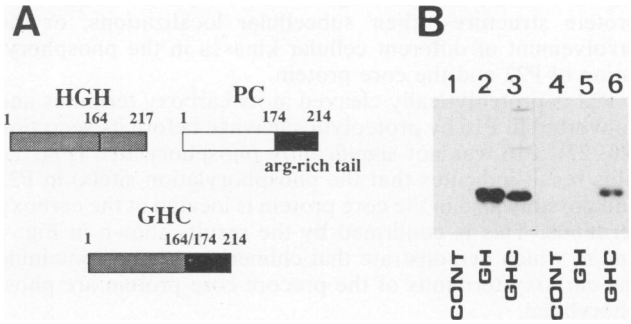

FIG. 4. (A) Schematic illustration for the construction of GHC fusion protein. The entire lengths of the full-length human growth hormone (HGH) and precore protein (PC) are 217 and 214 amino acids, respectively. GHC was created by fusing amino acids 1 through 164 of human growth hormone to amino acids 174 through 214 of the precore protein. This region of the precore protein sequence is arginine rich and contains the putative phosphorylation sites. Details for the construction of plasmid pECE-GHC for the expression of the fusion protein are described in Materials and Methods. (B) Phosphorylation study of the GHC fusion protein. Lanes: 1 through 3, proteins labeled with [^{35}S]methionine; 4 through 6, proteins labeled with $^{32}P_i$; CONT, control COS-7 cells; GH, COS-7 cells transiently transfected with pECE-GH; GHC, COS-7 cells transiently transfected with pECE-GHC.

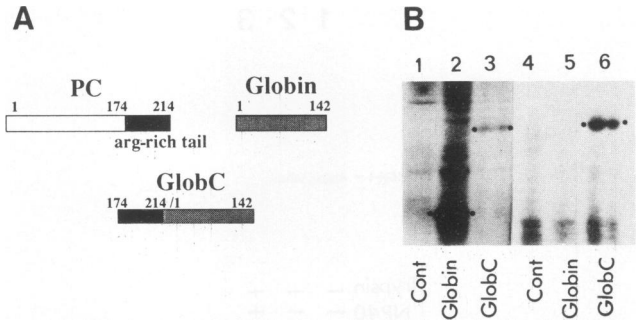

FIG. 5. (A) Schematic illustration for the construction of the GlobC fusion protein. Globin, Human alpha-globin; PC, precore protein. The length of the full-length globin is 142 amino acids. GlobC fusion protein was created by fusing amino acids 174 through 214 of the precore protein to the amino terminus of globin. The construction of plasmid pECE-GlobC for the expression of the GlobC fusion protein is discussed in Materials and Methods. (B) Phosphorylation study of the GlobC fusion protein. Lanes: 1 through 3, proteins labeled with [^{35}S]methionine; 4 through 6, proteins labeled with $^{32}P_i$; Cont, control COS-7 cells; Globin, COS-7 cells transfected with pECE-G; GlobC, COS-7 cells transfected with pECE-GlobC.

lated, the fusion proteins GHC (Fig. 4B, lanes 3 and 6) and GlobC (Fig. 5B, lanes 3 and 6) were. These results confirm that the carboxy terminus of the precore-core protein contains the phosphorylation site(s) and further demonstrate that phosphorylation of P22 and the core protein does not require HBV gene products and is probably mediated by cellular kinases.

DISCUSSION

We have demonstrated that both the core protein and the precore protein derivative P22 can be phosphorylated in cells. Furthermore, our results indicate that the phosphorylation efficiency of the core protein is approximately 400-fold higher than that of P22 (Fig. 1). This may be related to the protein structures, their subcellular localizations, or the involvement of different cellular kinases in the phosphorylation of P22 and the core protein.

P22 is proteolytically cleaved at its carboxy terminus and converted to P16 by proteolytic cleavage before its secretion (20, 27). P16 was not significantly phosphorylated (Fig. 1). This result indicates that the phosphorylation site(s) in P22 and possibly also in the core protein is located at the carboxy terminus. This is confirmed by the results shown in Fig. 4 and 5, which demonstrate that chimeric proteins containing the carboxy terminus of the precore-core protein are phosphorylated.

Microsomal fractionation experiments shown in Fig. 2 indicate that approximately 20% of phosphorylated P22 molecules are present in the lumen of the ER. However, the majority of phosphorylated P22 molecules are sensitive to trypsin digestion and thus are probably exposed in the cytosolic side of the membrane (Fig. 2). This result is reproducible and is unlikely to be an experimental artifact, because when human growth hormone, a secretory protein, was used as a control, it was found mostly (approximately 90%) in the ER lumen (Fig. 3).

It is unclear whether phosphorylated P22 is peripherally attached to the ER membrane or is as an integral membrane protein with the carboxy terminus (phosphorylation sites) exposed in the cytosolic side. The latter possibility is supported by the report of Bruss and Gerlich (3), which suggested that P22 could be a transmembrane protein with the carboxy terminus in the cytosolic side.

Fusing protein experiments shown in Fig. 4 and 5 indicate that phosphorylation of P22 and the core protein does not require other HBV gene products or the rest of the precore or core protein sequence. Because the carboxy terminus of P22 used for the fusion protein experiments is short and arginine rich, it probably does not contain a kinase activity. Thus, it is likely that phosphorylation is mediated by cellular kinases. Phosphorylation of P22 may occur in the ER and Golgi apparatus, since protein kinases and the phosphorylation of secretory proteins in these two subcellular compartments have been reported before (2, 4, 9, 16, 30). Alternatively, since the majority of phosphorylated P22 is at least partially exposed in the cytosol (Fig. 2), it is also likely that phosphorylation of P22 occurs in the cytosol before the complete translocation of P22 across the ER membrane.

Since the core protein is a cytosolic protein (21), its phosphorylation probably only occurs in the cytosol. This speculation is supported by the results shown in Fig. 2, which indicate that phospho-core protein is present only in the cytosolic supernatant (lane 6).

Phosphorylation of the core protein has been proposed to be important for regulating the maturation of HBV particles (22). The importance of P22 phosphorylation, on the other hand, is unclear. Since it has been reported that phosphorylation of casein, a secretory protein, can alter the sensitivity of casein to proteases (2, 15), it is conceivable that phosphorylation of P22 can also modulate proteolytic conversion of P22 to P16 for secretion.

The core proteins of HBV (5, 12, 26), woodchuck HBV (12), and ground squirrel HBV (5) are phosphorylated primarily on the serine residues. There are seven serine residues in the arginine-rich carboxy terminus of the precore-core protein, and six of them are in a highly conserved region (31). Interestingly, this region has been shown to contain a signal for nuclear transport (31). Since nuclear transport of the simian virus 40 T antigen can be facilitated by phosphorylation of the serine residues flanking the nuclear localization signal (23), phosphorylation of the serine residues in the carboxy terminus may also play an important role in regulating nuclear transport of P22 and the core protein.

In conclusion, our results demonstrate that both the precore protein derivative P22 and the core protein can be phosphorylated in cells. Furthermore, our results indicate that phosphorylation of P22 and the core protein is likely mediated by cellular kinases. The mechanism of P22 and core protein phosphorylation and its significance in the biology of HBV remain largely unresolved. Further research in this area will undoubtedly generate many more exciting results.

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