Phosphorylation of the hepatitis B virus core protein by glyceraldehyde-3-phosphate dehydrogenase protein kinase activity

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In order to characterize the hepatitis B virus (HBV) hepatocellular receptor, several proteins have previously been identified in HepG2 hepatoma cells and in primary cultured normal human hepatocytes (PCHs) that reacted with an anti-idiotypic antibody against a preS1(21–47)-specific MAb (F35.25). Here, we report the identification of one of these preS1-binding proteins, a 35 kDa protein (preS1-BP35), as glyceraldehyde-3-phosphate dehydrogenase (GAPD). GAPD is well-known as a key enzyme involved in glycolysis and gluconeogenesis. Nevertheless, GAPD has also been shown to have many other functions such as protein kinase activity (GAPD-PK). HBV core particles derived from infected hepatocytes possess an associated kinase activity that phosphorylates HBcAg, and the nucleocapsid may acquire sequential functions through selective phosphorylation. Therefore, we have investigated the potential role of GAPD-PK in HBV replication. In this study, we found that the endogenous PK associated with human liver-derived HBV core particles (hL-HBcAg) and GAPD-PK were sensitive to the same types of inhibitors. Interestingly, capsid protein phosphorylation decreased in a concentration-dependent manner (at concentrations of 5–30 mM) in the presence of specific inhibitors for GAPD-PK (NADH and GAP). Furthermore, we demonstrated in vitro that GAPD-PK could phosphorylate the major core protein P22 in hL-HBcAg particles. The data suggest that GAPD is an additional cellular kinase which might interfere in the life-cycle of HBV.

Introduction

The initial event in the life-cycle of a virus is attachment to specific receptors on a host cell. This event is often a major determinant of virus tropism or pathogenesis and a potential target for antiviral agents. So far, the cell structure of the hepatitis B virus (HBV) receptor remains unknown despite intensified efforts by several groups during the last few years. The envelope antigen (envAg) of HBV consists of three related proteins designated the small (S), the middle (M) and the large (L) hepatitis B surface (HBs) proteins, which share a common 226 amino acid residue S-sequence that represents the C-terminal portion of MHBs and LHBs proteins. The MHBs protein has an additional 55 aa preS2 sequence located at the N terminus. The LHBs protein differs from the MHBs protein by an additional N-terminal 119 aa (or 108 aa, depending on the HBV subtype) preS1 sequence. Many cellular proteins have been found to bind to HBV surface antigens through the S (Hertogs et al., 1993; Mehdi et al., 1994), the preS2 (Machida et al., 1984; Pontisso et al., 1989; Krone et al., 1990; Franco et al., 1992; Budkowska et al., 1995) or the preS1 regions (Neurath & Strick, 1990; Neurath et al., 1992; Pontisso et al., 1992; Budkowska et al., 1993; Treichel et al., 1994). The preS1-specific domain of the LHBs proteins is preferentially expressed on the surface of infectious complete virions, and possesses an HBV hepatocyte receptor (Hep-R) binding site between amino acid residues 21 and 47 (Neurath et al., 1986), which is involved in the direct interaction between cell membranes and virions (Pontisso et al., 1989, 1992; Petit et al., 1991a).

Using an anti-idiotypic experimental approach, several preS1-binding proteins with apparent molecular masses of 35, 40, 43 and 50 kDa have previously been identified in HepG2 cells (Petit et al., 1992) and in primary cultured normal human hepatocytes (PCHs) (Mabit et al., 1996). The antiserum used

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was raised against MAAb F35.25 that recognized the preS1 domain (aa 21–47) corresponding to the HBV Hep-R binding site (Petit et al., 1989, 1991a, b).

In this report, the N-terminal part of the 35 kDa preS1-binding protein (preS1-BP35) was found to display homology to glyceraldehyde-3-phosphate dehydrogenase (GAPD; EC 1.2.1.12), a key enzyme in glycolysis. GAPD was originally described as a soluble enzyme that is responsible for the oxidative phosphorylation of glyceraldehyde 3-phosphate (GAP) by NAD+ and inorganic phosphate to give 3-phosphoglycerate with the generation of 2 mol ATP. However, in addition to its key role in glycolysis, there is now increasing evidence that this enzyme participates in many other functions. Among others, the enzyme appears capable of being phosphorylated with MgATP in a Ca2+-independent manner in skeletal muscle and then transferring the phosphate to membrane proteins (Kawamoto & Caswell, 1986). The phosphoenzyme complex formed is a high-energy acyl phosphate, which behaves as a protein kinase (PK). Dephosphorylation of acyl phosphate enzymes is normally associated with energy transduction either in active transport or in transfer of phosphate to a substrate. Biological activities of various proteins may be modulated by phosphorylation and dephosphorylation (Johnson & Barford, 1993), and the effects may be diversified to various processes or dedicated to a specific response. In duck HBV replication, the state of phosphorylation of the capsid protein has been shown to influence multiple functions of the core particle (Yu & Summers, 1994). Therefore, we speculated that GAPD might be an attractive candidate host factor involved in the HBV life-cycle through its PK activity by phosphorylating the viral nucleocapsid protein. Indeed, our results demonstrated that (i) the HBV core-related PK and GAPD-PK share the same properties, and (ii) GAPD-PK is able to phosphorylate the major core protein P22 in human liver-derived HBV core particles (hL-HBcAg).

Methods

1. Preparative electrophoresis, amino acid analysis and protein sequencing. The preS1-BP35 was preparatively isolated from cell extracts (HepG2 or PCHs) by continuous elution electrophoresis with a Bio-Rad New Model 491 Prep Cell and then subjected to partial amino acid sequence analysis. For this preparation, 8 ml extract (made 4 days after plating from about 106 cells) was loaded directly onto a 10% polyacrylamide gel in the Prep Cell for purification. Fractions (3 ml) were eluted with buffer containing 20 mM Tris–HCl (pH 7.2) and 20 mM NaCl for 7.5 h. Ligand blotting experiments were run on fractions 170–210; results revealed that the preS1-BP35 was collected in fractions 186–207. This material was transferred to a PVDF membrane by centrifugation (Prospin cartridge; Applied Biosystems) and was sufficient to yield the major core protein P22 in human liver-derived HBV core particles (hL-HBcAg).

Results

PreS1-BP35 in human hepatocytes (HepG2 and PCHs): sequence homology to GAPD

Three determinations of the first 20 residues of the N-terminal sequence of the PVDF-bound preS1-BP35 (from HepG2 cells or PCHs) were performed. The following sequence was obtained: GKVVKVNGFXGRLVTR. A database search indicated that this 19 amino acid sequence displayed 100% homology with the N-terminal region of the liver form of GAPD, a glycolytic enzyme, the monomer of which has a molecular mass of about 35 kDa (Arcari et al., 1984). We noticed that this peptide sequence contained the motif KVVKVG (the 6 underlined amino acids in the above sequence), which was previously identified by C. R. Howard (personal communication) using epitope libraries as the presumed preS1-binding motif (KXXVGX). In solid-phase binding assays, we previously showed that the specific binding activity of GAPD (Sigma) for the HBV preS1 region was Ca2+-independent and inhibited by EDTA (Mabot et al., 1994).
GAPD and HBV core-related PK share the same properties

In an initial approach, we investigated the influence of different ions, activators or specific inhibitors on the phosphorylation of GAPD and of the HBV core protein. Purified GAPD (P35) purchased from Sigma was shown to be autophosphorylated with MgATP [as P35-GAPD; cf. Fig. 1a (i–iii)], giving evidence for its PK activity (GAPD-PK). The effect of metabolites of enzyme glycolytic function (NADH...
and GAP), which are known to be specific inhibitors of GAPD-PK activity (Kawamoto & Caswell, 1986), was studied. The phosphoenzyme complex (P35-GAPD) was reduced in a concentration-dependent manner (at concentrations of 5, 10, 20 and 30 mM) by both NADH [Fig. 1a (i), lanes 2–5] and GAP [Fig. 1a (ii), lanes 2–5], compared to controls (lane 1). hL-HBcAg particles, previously studied for their immunological and biochemical properties (Petit & Pillot, 1985), were also used (Fig. 1b). Electron microscopic examination of the hL-HBcAg preparation revealed the presence of spherical particles 27 nm in diameter, and Western blotting analysis under reducing conditions showed only the major 22 kDa full-length core protein (P22c). We found that P22c was phosphorylated in vitro by endogenous PK activity, copurified with hL-HBcAg particles, as illustrated in Fig. 1b (i–iii), lane 1. It was demonstrated that both NADH and GAP, inhibitors that are known to be specific for GAPD-PK, caused a concentration-dependent (5–30 mM) inhibition of the endogenous PK of HBV [Fig. 1b (i and ii, respectively), lanes 2–5]. In Fig. 1(iii), we showed that the phosphorylation of both GAPD (a) and HBV P22c (b) was not inhibited by the protein kinase C (PKC) inhibitor staurosporine (20 nM, lane 2), and the protein kinase M (PKM) inhibitor quercetin (5 µM, lane 3) after an incubation period of 1 h; the calcium chelating agent EGTA was also ineffective (1 mM, lane 6). In contrast, the addition of 0.5 mM Zn²⁺ (lane 4) or 20 mM EDTA (lane 5) led to a partial or complete loss of activity in both systems. The data indicate a clear coordination of the HBV core-related PK with GAPD-PK.

**GAPD-PK phosphorylates the HBV major core protein (P22c)**

In a second experimental approach, we tested the ability of GAPD-PK to catalyse the phosphorylation of the major core protein (P22c) in hL-HBcAg. Fig. 2a (i) shows that addition of exogenous purified GAPD (30 µg) significantly increased the phosphorylation of P22c from hL-HBcAg particles (0.5 µg protein) (lane 3 compared to lane 2). When increasing concentrations of exogenous GAPD (30, 40 and 50 µg) were added to hL-HBcAg particles (0.5 µg), a proportional en-

![Figure 2](image-url)
Enhancement in core phosphorylation was observed [Fig. 2a (ii)]. In the presence of GAPD-PK-specific inhibitors, NADH [Fig. 2b (i)] or GAP [Fig. 2b (ii)], used at concentrations of 5, 10, 20 and 30 mM (lanes 1, 2, 3 and 4, respectively), the phosphorylation of both P35-GAPD and the HBV core protein P22c was inhibited. These results demonstrate that the major core protein in hL-HBcAg particles can be a substrate for GAPD-PK in vitro.

Discussion

Initially, we compared the properties of GAPD-PK with those of the endogenous PK activity of hL-HBcAg particles (Petit & Pillot, 1985). Using inhibitors specific for different cellular PK, a clear correlation of the core-related PK to GAPD-PK was observed. Both kinases were strongly inhibited by EDTA and Zn++. More convincingly, the endogenous HBV PK activity was reduced in a concentration-dependent manner by selective inhibitors of GAPD-PK activity (NADH and GAP).

In contrast, no significant inhibitory effect of the PKC inhibitor staurosporine and the PKM inhibitor quercetin was observed under the conditions used. Thus, both the hL-HBcAg-associated PK and the GAPD-PK share the same properties, suggesting that both could belong to the same family of kinases. Secondly, we have demonstrated that exogenous GAPD led to an increased phosphorylation of the major core protein P22c in hL-HBcAg particles. The specificity of the phosphorylation reaction was proven using selective inhibitors of GAPD-PK activity, NADH and GAP. All these findings suggest that GAPD-PK could be encapsidated within HBV core particles. However, Western blotting experiments performed with purified liver-derived or virion-derived core particles did not clearly reveal the presence of GAPD-PK as a 35 kDa protein (data not shown). In a similar immunoblotting experiment, Kann & Gerlich (1994), as well as ourselves (data not shown), detected PKC within the liver-derived core particles, indicating that the PKC may be encapsidated. It is a fact that PKC is one major PK which is encapsidated by core particles during infection of the natural host, but it cannot be ruled out that minute amounts of GAPD-PK could be present within the lumen of all or a portion of the particles (direct immunoblotting was not sufficiently sensitive to detect this).

Our results, as well as those of Kann et al. (1993), on the inhibition of the endogenous PK activity of HBV by staurosporine and quercetin, were not very convincing, whereas inhibition by NADH and GAP is highly reproducible and reliable. Therefore, we cannot exclude the possibility that GAPD-PK may be encapsidated and play a role in the HBV life-cycle. Recently, M. Kann (personal communication) showed that the phosphorylation of the core protein was not only involved in the RNA encapsidation but was also essential for nuclear binding of the HBV core particles to the nucleus. Such a phosphorylation-dependent binding of the core particles required cytosolic factor(s), but was neither liver- nor species-specific. These data correlate well with the ubiquitous nature of GAPD. If PKC plays an important role during core particle reassembly (Kann & Gerlich, 1994), we postulate that GAPD-PK might phosphorylate the core subunits and thus increase nuclear transport of the viral genome. Hence, it could be expected that the phosphorylation of the nucleic acid binding site by PKC or PKA may be essential for genome encapsidation. However, this does not exclude the possibility of phosphorylation of core particles at another site by an additional endogenous and/or exogenous cellular PK. During the HBV infection process, GAPD might increase dissociation of the nucleocapsid from the envelope and transport it to the nucleus of the viral genome where it is converted into a viral covalently closed DNA (Mabit et al., 1994).

In conclusion, even though the role of GAPD in HBV entry to normal human hepatocytes through its preS1-binding activity may not be proven, we have provided evidence that GAPD-PK can phosphorylate the core subunits, and thus may be involved in the early steps of the replication cycle of HBV.

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