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# Specificity and Localization of the Hepatitis B Virus-Associated Protein Kinase

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The nature of the protein kinase (PK) which phosphorylates the core protein of hepatitis B virus in vitro was studied. The PK copurified with the core particles during rate zonal centrifugation and gel chromatography. It showed the same size heterogeneity as the core particles, which consisted of a main fraction of 28-nm particles and a subfraction of 22- to 26-nm particles. DNA-containing heavy core particles with a density of 1.33 to 1.35 g/ml had less endogenous PK than did the light cores. The phosphorylation reaction had a rapid initial phase (several minutes) and a slow but long-lasting second phase (many hours). The PK had a high affinity for ATP ($K_M$ = 0.5 μmol/liter). Only few of the several hundred P21.9 subunits in one core particle were phosphorylated in vitro. The only amino acid which was phosphorylated in vitro was serine. The resistance of the introduced phospho group against alkaline phosphatase showed that the PK acceptor, and probably the enzyme itself, was located inside the core particle.

Hepatitis B virus (HBV) consists of a core particle, which encloses a small circular DNA (13), and an outer protein envelope (4). It has not yet been propagated in vitro, and it can be isolated only from the sera of infected persons or chimpanzees. Viral core particles and their major antigen (HBcAg) can be liberated from the protein envelope (2) by treatment with nonionic detergents. Nonenveloped core particles are also present in hepatocytes of certain chronically infected individuals (5), and a protein of approximately 20,000 molecular weight has been found to be their major component (8). The heavier subfraction of core particles (9) contains an endogenous DNA polymerase (DNA nucleotidyltransferase) (10) which fills up a single-stranded gap in the viral DNA in vitro (12). The HBV DNA has been cloned, and the primary structure of the major core protein has been derived from the DNA sequence (15).

Recently, a protein kinase (PK) was detected in purified preparations of core particles from virus or from liver; this enzyme phosphorylates the major core protein in vitro (1). It is not yet known whether this PK is an integral part of the core particles and which amino acid is phosphorylated. Cellular and viral PKs in general phosphorylate threonine or serine. The oncogenic products of certain tumor viruses, however, are PKs which phosphorylate tyrosine in vitro. In certain geographic regions, chronic infection with HBV is associated with the primary liver carcinoma (14), and its DNA has been found to be integrated into the DNA of the tumor cells (3).

This study presents evidence that the PK is indeed a part of the viral core particles. It phosphorylates one or several serine residues of the major core protein per particle at a site which is sterically protected against enzymatic hydrolysis.

(This publication contains parts of the M.D. thesis of W.W. [University of Göttingen, Göttingen, Federal Republic of Germany, 1982].)

## MATERIALS AND METHODS

**Purification of core particles.** Human liver containing HBcAg, as shown by immunofluorescence, came from an immunosuppressed patient. Twenty grams of frozen liver was minced and agitated with 200 ml of 0.13 M NaCl–0.01 M Tris-hydrochloride (pH 7.4)–0.001 M disodium-EDTA (TNE) for 10 min. The supernatant was discarded, and the washing cycle was repeated five times to remove adhering serum and antibody to HBcAg. The washed liver pieces were homogenized in 50 ml of TNE complemented with 0.5% Nonidet P-40 and 0.1 M dithiothreitol by using a rotating knife (VirTis S45) five times for 30 s each time at maximum speed. The homogenate was spun for 30 min at 12,000 rpm (23,000 × *g*) in an SW 27 rotor.

The supernatant was adjusted to a density of 1.16 g/ml with CsCl and layered on a CsCl gradient (density, 1.23 to 1.42 g/ml) in a B 14 zonal rotor. After 17 h at 38,000 rpm (100,000 × *g*) and 10°C, fractions with a density between 1.30 and 1.35 g/ml contained all HBcAg and no HBV surface antigen or soluble HBV

"e" antigen. The combined peak fractions were concentrated by ultrafiltration to 0.8 ml and passed through a column (90 by 1.5 cm) of Bio-Gel A 15M (200 to 400 mesh) with 2 ml of TNE per h. Protein concentration in the eluate was monitored by tryptophan-related fluorescence at 355 nm, using a spectrofluorometer with excitation at 290 nm.

The appropriate fractions were adjusted with CsCl to a density of 1.33 g/ml and spun for 3 days in tubes (2 by 0.5 in. [ca. 5.1 by 1.3 cm]) of a swinging rotor at 10°C and 34,000 rpm. The HBcAg-positive peak fractions were dialyzed and stored in aliquots at −80°C.

**HBcAg assay.** HBcAg was determined by solid-phase enzyme immunoassay, using a conjugate of immunoglobulin G containing antibody to HBcAg and horseradish peroxidase as described previously (6). The absorbance at 492 nm of the final test mixture was approximately proportional to core protein concentrations between 0.5 and 20 ng/ml.

**PK reaction and gel electrophoresis.** The PK reaction mixture contained 100 mM Tris-hydrochloride (pH 7.4), 10 mM $MgCl_2$, 0.4% Nonidet P40, and 5 to 20 pmol of [$\gamma$-$^{32}$P]ATP (1,000 to 3,000 Ci/mmol, New England Nuclear Corp.) in 10 μl. After 1 h at 37°C, 10 μl of sample buffer containing 6% sodium dodecyl sulfate and 10% dithiothreitol (11) was added, and the mixture was boiled for 4 min. The product was quantitatively transferred to electrophoresis wells and separated through a 15% polyacrylamide gel by using the Laemmli buffer system (11). After fixing, staining, and drying of the gel, the phosphorylated protein was localized by autoradiography, cut out, and counted in a liquid scintillation spectrometer.

Gels were stained with Coomassie blue for the localization of the molecular weight markers phosphorylase *b* (94,000), bovine serum albumin (68,000), ovalbumin (43,000), carbonate dehydratase (30,000), trypsin inhibitor (20,100), and lysozyme (14,300).

For preparative $^{32}$P labeling of core particles, the free [$\gamma$-$^{32}$P]ATP was removed by gel filtration through a column (0.4 by 5 cm) of Sephacryl S300 (Pharmacia Fine Chemicals) with TNE containing 0.1% albumin or by dialysis.

## RESULTS

**Copurification of PK and core particles.** Crude extracts of liver tissue contained very strong PK activities. At least 25 phosphorylated proteins were resolved by polyacrylamide gel electrophoresis after a PK reaction. Among these $^{32}$P-proteins the $^{32}$P-core protein was barely detectable, although the liver had a very high titer of HBcAg (1:2,048 in the enzyme immunoassay). Therefore we reinvestigated the question of whether the core particles remained PK positive after several purification steps.

Core particles were extracted from human liver, partially purified by rate zonal centrifugation, and then subjected to gel chromatography on 4% agarose beads. The fractions were tested for endogenous PK activity and HBcAg concentration. The $^{32}$P-labeled products of the PK reaction were separated from [$\gamma$-$^{32}$P]ATP and simultaneously analyzed by polyacrylamide gel electrophoresis and subsequent autoradiography (Fig. 1). At the void volume of gel chromatography (Fig. 1, fractions 33 to 37) a minor kinase

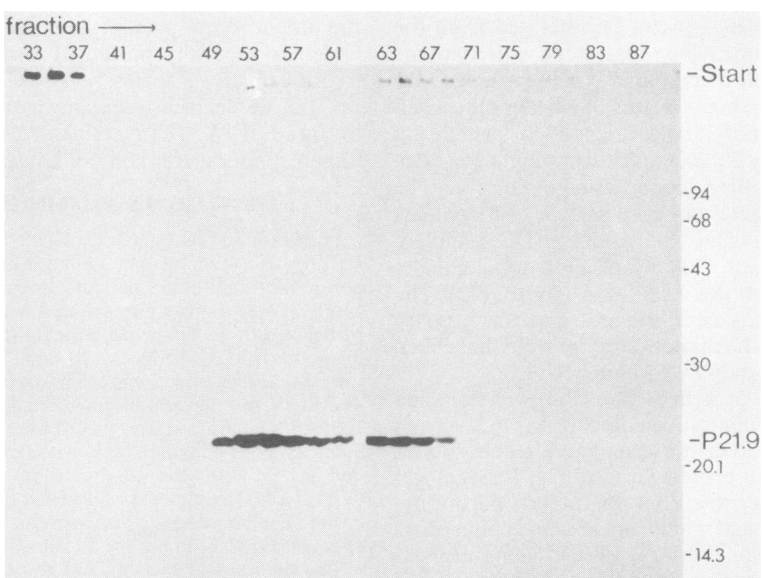


FIG. 1. Detection of [$^{32}$P]P21.9 in gel chromatography fractions after PK reaction. Semipurified core particles were passed through Bio-Gel A 15M, and 10-μl portions of the fractions were assayed by polyacrylamide gel electrophoresis as described in the text. The numbers at the right give the positions of the molecular weight markers. The correlation between the amount of [$^{32}$P]-P21.9 and core particles is shown in Fig. 2.

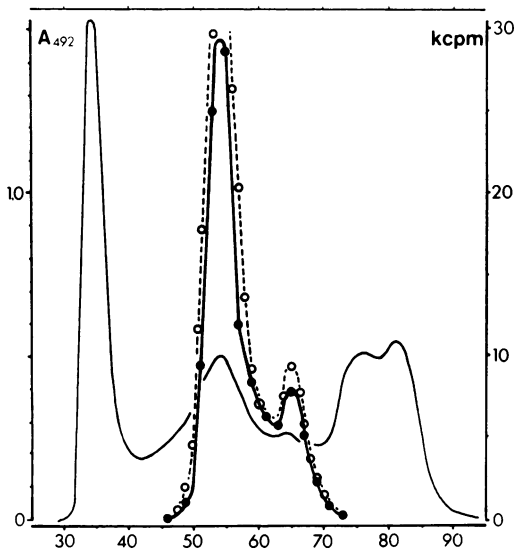


FIG. 2. Copurification of PK activity (●) and of HBcAg (○) in fractions 49 to 71 by gel chromatography. [$^{32}$P]P21.9 was cut out from the gel as shown in Fig. 1, counted, and taken as a parameter of endogenous PK activity. HBcAg was assayed by enzyme immunoassay (absorbance at 492 nm [$A_{492}$]) at 1:100 dilution. The thin line gives the protein profile, as determined by tryptophan-specific fluorescence, in arbitrary units.

activity was found. However, these fractions contained no HBcAg. The major substrate of phosphorylation by this kinase must have been very large, because it did not enter the polyacrylamide gel.

In fractions 49 to 71 a strong PK activity was found. A protein with an apparent molecular weight of 21,900 (P21.9) was the only phosphorylated product. No $^{32}$P-labeled product was found in fractions 73 to 89, where soluble proteins were eluted. For quantitation of endogenous PK activity, the bands of P21.9 were cut out from the polyacrylamide gel and their radioactivity was determined. The amount of $^{32}$P bound to P21.9 followed quantitatively the HBcAg concentration in the gel chromatography fractions (Fig. 2). Electron microscopy (fractions 51 to 57) showed that the major core peak consisted of numerous homogeneous 28-nm particles, whereas the minor peak (fractions 63 to 67) consisted of more heterogeneous (22- to 26-nm) particles.

The two core particle fractions were further purified by isopycnic banding in CsCl (Fig. 3). PK activity was only found in HBcAg-positive fractions. The light fraction of the core particles, with a density lower than 1.32 g/ml, was reproducibly found to have more endogenous PK activity than the heavy core particles, with a density of 1.33 to 1.35 g/ml. The heavier fraction was DNA polymerase positive, the lighter fraction was DNA polymerase negative (data not shown).

After isopycnic banding, the core particles appeared by gel electrophoresis to be free from contaminating protein. Only one component, P21.9, was found by protein staining (data not shown), and a peak of optical density at 280 nm appeared at the HBcAg peak (Fig. 3). Ferritin, a possible contaminant with a P22 subunit, was not seen in the electron micrographs of 28-nm

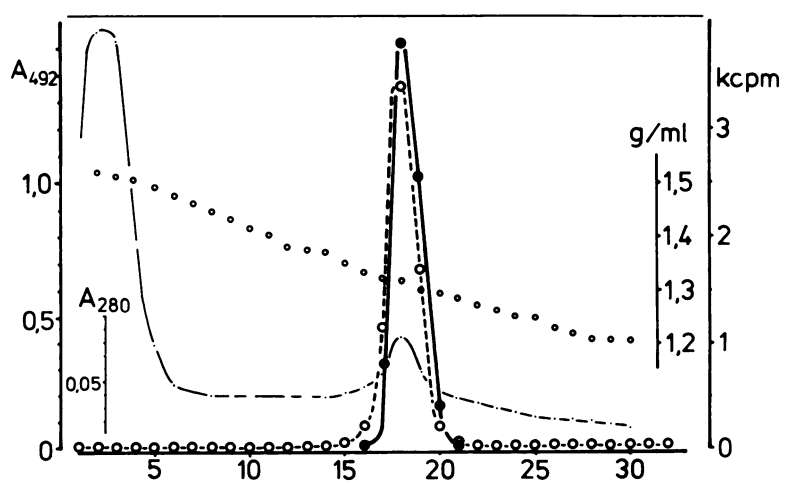


FIG. 3. Density difference (○○○) between PK activity (kilocounts per minute) (●) and HBcAg (absorbance at 492 nm [$A_{492}$]) (○). Purified 28-nm core particles (Fig. 2, fractions 51 to 57) were analyzed by isopycnic centrifugation in CsCl as described in the text. For PK activity, 1-μl samples were assayed at 1:10 dilution; HBcAg was tested at 1:1,000 dilution. The thin line (— · —) gives $A_{280}$.

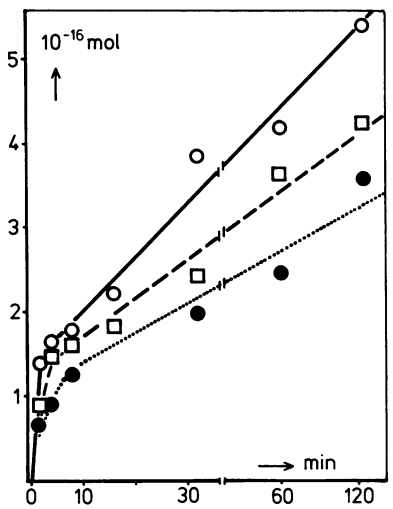


FIG. 4. Kinetics of $^{32}P$ incorporation into P21.9 of core particles. Three portions of 100 ± 50 ng of HBcAg were made 0.5 μM with [γ-$^{32}P$]ATP in 50 μl of PK reaction mixture. One portion was complemented with cold ATP to 1.5 μM (□), another was complemented to 5.5 μM (○), and the third remained 0.5 μM (●). At the indicated times 1-μl samples were removed in duplicate and analyzed by polyacrylamide gel electrophoresis as described in the text.

particles. Assuming 2 < optical density at 280 nm (0.1% protein) < 6 for HBcAg, the peak fraction of Fig. 3 contained between 5 and 15 μg of protein per ml. The uncertainty of this estimate was due mainly to the unknown DNA content of the core particles.

**Kinetics and extent of phosphorylation.** The incorporation of $^{32}P$ into HBcAg showed two different time phases (Fig. 4). Within a few minutes, approximately 0.15 fmol of $^{32}P$ were bound covalently to 2-ng portions of HBcAg particles. That amount corresponds to one phospho group per 800 ± 400 P21.9 molecules or one phospho group per one to three core particles. After this rapid incorporation the reaction rate decreased, but even after 3 h (data not shown) saturation was not reached. Depending on the ATP concentration, an uptake of approximately one phospho group per particle occurred in 30 to 120 min. From the relation between the ATP concentration and the incorporation rate of $^{32}P$, the binding constant of PK for ATP was estimated. If $K_M$ is calculated from the velocity of reaction between 10 and 30 min (Fig. 4), then $K_M$ = 0.5 ± 0.15 μmol/liter.

**Effect of AP on the $^{32}P$-core protein.** When HBcAg was phosphorylated in vitro with $^{32}P$ and treated thereafter with a large excess of alkaline phosphatase (AP), the [$^{32}P$]phospho group remained quantitatively bound to P21.9 (Fig. 5). Even in the presence of the nonionic detergent Nonidet P-40, AP did not exert any effect on the [$^{32}P$]phospho group. If the $^{32}P$-core particles were lysed with 2% sodium dodecyl sulfate at 100°C before enzyme digestion, AP rapidly removed the major part of the $^{32}P$ from [$^{32}P$]P21.9 (Fig. 5). However, part of the [$^{32}P$]phospho groups were still not susceptible to AP. Presumably, this was due to rapid refolding of the denatured core protein, because the digestion with AP was done in 0.1% sodium dodecyl sulfate.

**Site of phosphorylation.** The linkage of the [$^{32}P$]phospho group to P21.9 was completely stable in 0.1 N HCl at 60°C (Fig. 5). This finding suggests that the [$^{32}P$]phospho group is not bound to carboxy or amino groups (16).

In 0.1 N NaOH, lysis of the $^{32}P$-phospho group proceeded with a half-life of approximately 1 h (Fig. 5). Phosphothreonine and phosphoserine are both acid stable and alkali labile. In contrast, phosphotyrosine is stable in acid and alkali (16).

When [$^{32}P$]P21.9 was hydrolyzed in 6 N HCl for 2 to 6 h, the $^{32}P$ comigrated in high-voltage electrophoresis with free phosphate and with phosphoserine (Fig. 6). No [$^{32}P$]phosphothreonine and no [$^{32}P$]phosphotyrosine were detected. After 2 h of hydrolysis, partial hydrolysis products were seen, which disappeared at 4 h of

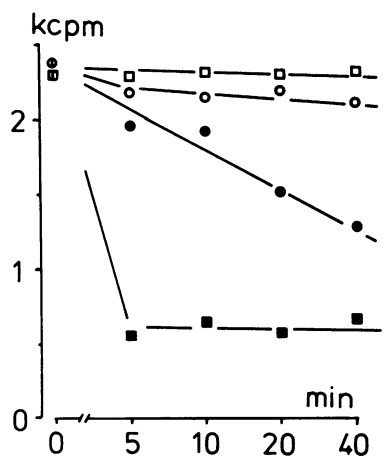


FIG. 5. Stability of the linkage between the [$^{32}P$]phospho group and the core protein. Aliquots were treated with 1,000 U of AP (calf intestine, Boehringer) per ml in 0.1 mM $ZnCl_2$–1 mM $MgCl_2$–100 mM glycine-sodium hydroxide (pH 10.5)–0.1% bovine serum albumin before (□) or after (■) lysis at 37°C, with 0.1 N NaOH (●) at 60°C, or with 0.1 N HCl (○) at 60°C. At the indicated times samples were spotted on Whatman no. 3 filter disks, precipitated and washed with 5% trichloroacetic acid, dried, and counted by liquid scintillation.

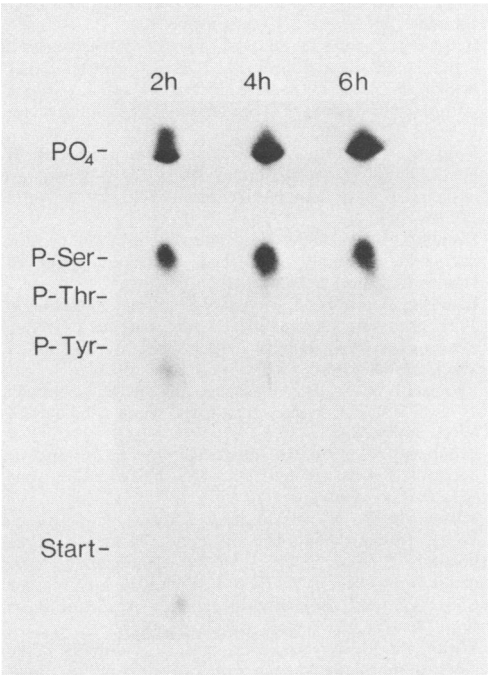


FIG. 6. Detection of [$^{32}$P]phosphoserine (P-Ser) in the PK reaction product. [$^{32}$P]P21.9 was cut out from a dried polyacrylamide gel, divided into three aliquots (5,000 cpm), mixed with 4 μl of phosphoamino acid mixture (5 mg of P-Ser, phosphothreonine [P-Thr], and phosphotyrosine [P-Tyr] per ml) and with 6 μl of 10 N HCl. After hydrolysis for 2, 4, or 6 h at 100°C, the samples were dried in vacuo and dissolved in 2 μl of electrophoresis buffer. The mixtures were separated by high-voltage electrophoresis (1,600 V, 40 min) on Whatman no. 3 paper in 50 mM pyridiniumacetate, pH 3.6. The carrier phosphoamino acids were stained with ninhydrin, and their positions are given at the left side. $^{32}$P was detected by autoradiography as shown above.

hydrolysis. After treatment of the hydrolysate with AP, the $^{32}$P migrated only with free phosphate and no longer with phosphoserine (data not shown).

## DISCUSSION

When the in vitro phosphorylation of the major core protein (here P21.9) was first described, it was suggested that PK, which is necessary for this process, would be a component of the hepatitis B virus, because it was found in preparations of purified core particles (1). The identical size distribution of the PK and the core particles found in this study confirms this conclusion, because it is highly improbable that the PK and the HBcAg would have the same size heterogeneity if they were not components of the same particle.

Between the density distributions of PK activity and HBcAg was a small but well-reproducible difference. The light core particles had a higher endogenous PK activity than did heavy core particles. It was suggested earlier that the light core particles do not contain DNA (9). If this is the case, there would be an inverse relationship between the DNA content and PK activity.

From the resistance of the [$^{32}$P]phospho group to AP it must be concluded that the acceptor site of the PK is not located at the surface of the core particle. Consequently, the active site of the PK, and most likely the whole enzyme itself, is located in the interior of the core particle.

The quantitative analysis showed that, on the average, only one or even less of the several hundred P21.9 subunits in a core particle were phosphorylated by the PK reaction in vitro. The low activity of the enzyme may be due to a lack of acceptor sites not yet phosphorylated in vivo or to steric hindrance. The kinetics of the PK reaction, with its rapid initial phase and its slow but steady continuation, is consistent with the latter possibility. Steric hindrance may also be the reason for the presumed inverse relationship between DNA content and PK activity. The high affinity of the enzyme for ATP, as seen by the low $K_M$, excludes the possibility that ATP is a weak substrate for the PK.

The finding that serine is the only amino acid which is phosphorylated in vitro could be explained in either of two ways: (i) the enzyme could have a narrow specificity and does not phosphorylate threonine or tyrosine, or (ii) the acceptor site has only serine residues available for phosphorylation. The second possibility is consistent with the amino acid sequence of P21.9 as it is derived from the HBV DNA sequence (15). The 38 COOH-terminal amino acids of the core protein contain 17 arginines and 7 serines, but only 1 threonine and no tyrosine. It is very likely that this portion of the molecule is the DNA-binding site of the core protein (15) and that it is, consequently, faced towards the interior of the particle.

The available experimental evidence does not allow any presumptions on the functional role of the PK and its relationship with the potential oncogenicity of HBV. Possibly, studies on the phosphorylation of cellular products by the soluble form of this PK could contribute to answering this question.

HBV contains three not yet identified proteins which interact with nucleotides: (i) the DNA polymerase (10), (ii) a protein which is covalently linked to the viral DNA and which may act as an endonuclease (7), and (iii) the PK. It is not known whether these enzymes are of viral or of host origin, but, in general, viral components are coded by the virus. The small genome of HBV

contains one open reading frame for a not yet identified 90,000-dalton protein (15) which may be a multifunctional precursor for these three activities.

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