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Differential Formation of Disulfide Linkages in the Core Antigen of Extracellular and Intracellular Hepatitis B Virus Core Particles

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Our understanding of the assembly of hepatitis B virus is still very limited. We present evidence to demonstrate that the HBc antigen formed oligomers through disulfide linkages in the extracellular hepatitis B virus core (HBc) particles. However, the intracellular HBV core particles did not contain disulfide-linked HBc antigens. Furthermore, the extracellular particles which had disulfide bonds were more stable than intracellular particles at pH 7.5 and 10 and in 3 M NaCl and 4 M urea. These data suggest that the formation of disulfide bonds in the HBc antigen is important for the integrity of the viral core particles.

The nucleocapsid core of hepatitis B virus (HBV) is composed of the core (HBc) antigen (2), the viral genome (24), a 5′-end-binding protein (11), a polymerase (15), and a core particle-associated protein kinase (1). Very little is known about the assembly of these components during the formation of the nucleocapsid core of HBV. Apparently, the HBc antigen is the major structural constituent of the nucleocapsid core (12). The C terminus of the HBc antigen is extremely rich in arginine, serine, and proline residues, resembling protamine. The surface of the HBV core particle is an icosahedrally symmetrical structure with 180 HBc molecules (21). The HBc antigen and the HBe antigen, which is a derivative of HBc with the protaminelike domain deleted, are able to self-assemble into 27-nm particles morphologically similar to the nucleocapsid of HBV (10). The core antigen appears to play an important role in the assembly of the core nucleocapsid. Previous studies have also revealed that the HBc/HBe antigen sometimes appears as a high-molecular-weight species on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or Western immunoblot analysis (3, 23, 27). These results suggest that disulfide bonds may have been formed in the HBV core particles. However, direct evidence for disulfide linkage of the HBc antigen is lacking. Furthermore, the importance of disulfide linkage of the HBc antigen in the process of formation of the viral core is unknown.

To further understand these questions concerning the HBV core particles, we have analyzed the interaction of core antigens. Initially, the culture fluid from pMH3/3097-transfected HuH-7 cells (13, 19) was clarified by centrifugation at 20,000 × *g* for 30 min. The supernatant was then treated with 0.5% Nonidet P-40 overnight and centrifuged in a Beckman Ti55.2 rotor at 227,000 × *g* for 80 min. The resulting pellet was resuspended in TNE buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA). The partially purified HBV core particles were then immunoprecipitated (5) with rabbit antiserum against the HBc and HBe antigens and subsequently labeled with [γ-^{32}P]ATP in vitro. The protein kinase reaction was performed on the immunoprecipitates by addition of protein kinase reaction mixture, consisting of 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 0.4% Nonidet P-40, and 10 to 20 pmol of [γ-^{32}P]ATP (7,000 Ci/mmol; ICN, Irvine, Calif.). After incubation at 37°C for 30 to 60 min, the protein A-Sepharose was washed four times with NET buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) and subjected to SDS-PAGE (15% acrylamide) (17). As shown in Fig. 1B, only HBc antigen was specifically phosphorylated by the HBV core particle-associated protein kinase. This result further confirms that viral particles produced from HuH-7 cells are similar to naturally occuring HBV in containing virus-associated protein kinase (1). Recently, Wu et al. reported that the X protein of HBV was present in core particles and had kinase activity with a high level of autophosphorylation (30). However, owing to the relatively small amount of culture fluid (10 ml) from HBV DNA-transfected HuH-7 cells, we did not detect the presence of phosphorylated X protein in HBV core particles in these experiments. Since the specific activity of the [γ-^{32}P]ATP (7,000 Ci/mmol) is higher than that of [^{35}S]methionine (1,100 Ci/mmol), the immunoprecipitation test with in vitro-phosphorylated HBc antigen would be severalfold more sensitive than with the [^{35}S]methionine-labeled HBc antigen.

The disulfide linkage of the core proteins in viral core particles was investigated by SDS-PAGE analysis. The results revealed that under nonreducing conditions (Fig. 1A), several HBc antigens with molecular masses of 21, 40, and 58 kDa and larger species were present. When the immunoprecipitates from the same preparation were run under reducing conditions (Fig. 1B), all these forms of HBc antigens shifted to the 21-kDa monomer. This result suggests that the antigen in the core particles contained disulfide linkage.

Since the core particle-associated protein kinase phosphorylates only HBc antigen, it is possible that the HBc antigen links with other proteins through disulfide bonds. To rule out this possibility, we performed diagonal gel electrophoresis (29) on [^{35}S]methionine-labeled HBV core particles precipitated by anti-HBc/HBe antiserum. As shown in Fig. 2, the electrophoretic pattern on a nonreducing gel was very similar to that for the [γ-^{32}P]ATP-labeled products and all the forms of the HBc antigen shifted to 21 kDa under reducing conditions. This result indicates that molecules larger than the HBc antigen monomer were oligomers of HBc antigen linked through disulfide bonds. Furthermore, the oligomers of phosphorylated HBc antigens did not arise from a selected population of core particles undergoing endogenous kinase

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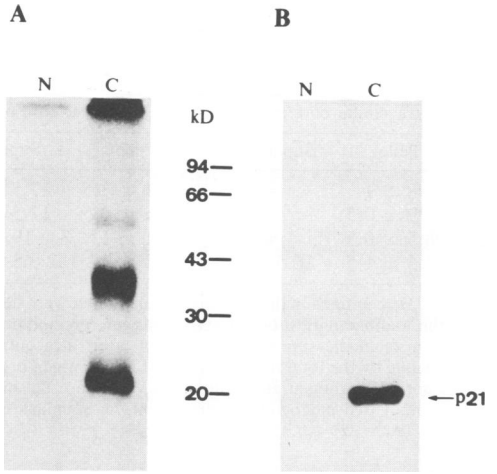

FIG. 1. Electrophoretic behavior of HBc antigen in nonreducing and reducing SDS-PAGE. The immunoprecipitates of anti-HBc/HBe antigen (lanes C) and normal serum control (lanes N) were analyzed by nonreducing SDS-PAGE (15% acrylamide) (A) or reducing SDS-PAGE (B) after in vitro phosphorylation with [γ-^{32}P]ATP. Molecular masses of the marker proteins (in kilodaltons) are given in the center.

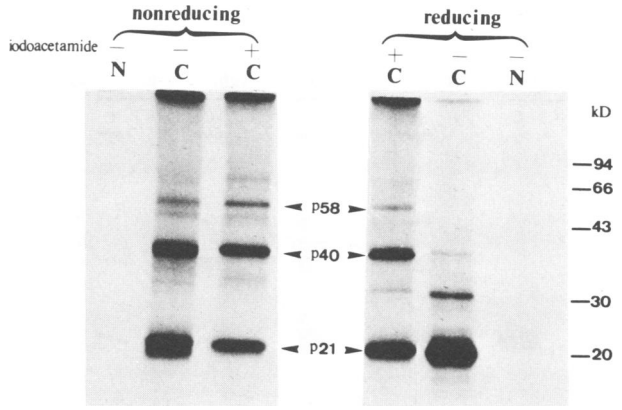

FIG. 3. Electrophoretic behavior of HBc antigen after iodoacetamide treatment. The iodoacetamide-treated core particles (see text) were immunoprecipitated with anti-HBc serum and phosphorylated as in Fig. 1. The immunoprecipitates of anti-HBc antigen were treated with or without 100 mM iodoacetamide after boiling. The resulting immunoprecipitates were separated by nonreducing SDS-PAGE (15% acrylamide) and detected by autoradiography of the dried gel (left panel). In control experiments, the reducing sample buffer (containing 1% 2-mercaptoethanol) was pretreated with or without 200 mM iodoacetamide for 1 h at room temperature (right panel). Molecular masses of the marker proteins (in kilodaltons) are given on the right. Lanes N and C are defined in the legend to Fig. 1.

reaction, since approximately 60% of the HBc antigens in the extracellular core particles formed oligomers as measured by densitometry and beta counts for [^{35}S]methionine-labeled core particles (Fig. 1 and 2).

The HBc antigen contains three cysteine residues. The oligomerization of the HBc antigen might have occurred during sample preparation. To rule out this possibility, we added iodoacetamide (final concentration, 10 mM) to the medium immediately after harvest and to the immunoprecipitates after boiling to block the free sulfhydryl groups. As shown in Fig. 3 (left panel), the electrophoretic patterns of the monomeric and oligomeric HBc antigens were the same with and without iodoacetamide treatment. As a control, the sample buffer (containing 2-mercaptoethanol) was pretreated with iodoacetamide. As shown in Fig. 3 (right panel), the migration pattern of the HBc antigen was similar to that under nonreducing conditions. This result demonstrates that iodoacetamide was able to block the free sulfhydryl group under our experimental conditions. Taken together, these data indicate that the HBc antigens form oligomers through disulfide bonds in the extracellular HBV core particles and that the disulfide linkages were formed before sample preparation.

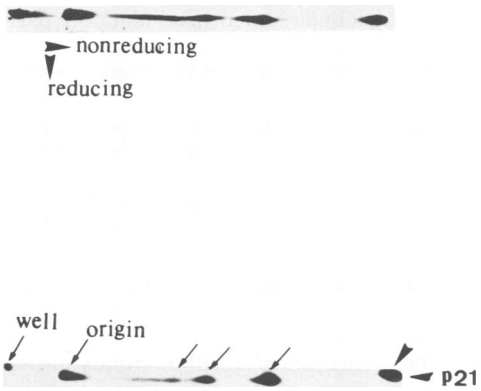

FIG. 2. Diagonal gel analyses of HBc antigen. [^{35}S]Methionine-labeled core particles were prepared and immunoprecipitated as in Fig. 1. The immunoprecipitates were analyzed by nonreducing (first dimension) and reducing (second dimension) SDS-PAGE. The monomer and oligomers of HBc antigens are indicated by the arrowhead and arrows, respectively. The well of the stacking gel and origin of the separation gel are also indicated.

The core particles released into the medium of transfected HuH-7 cells contain heavy cores, light cores, and nucleocapsids derived from the 42-nm Dane particles, whereas immature core particles exist in the cytoplasm (4, 18). The partially purified viral core particles from iodoacetamide-treated medium (without Nonidet P-40 treatment) was loaded onto a preformed discontinuous cesium chloride gradient containing 2 ml of 36%, 3 ml of 31%, 3 ml of 27%, and 2 ml of 22% cesium chloride and was centrifuged in an SW41 rotor (Beckman) at 220,000 $\times$ g for 24 h at 4°C. In Fig. 4B, extracellular heavy core particles, 42-nm Dane particles, and intracellular immature core particles contain HBV genome which can be repaired by the endogenous polymerase. As reported previously, the light core particles contain barely detectable levels of endogenous DNA polymerase activity (14). Interestingly, all the extracellular HBV core particles, including heavy cores, light cores, and the nucleocapsid of the Dane particles, contain disulfide linkages, whereas the intracellular immature cores do not have any detectable disulfide linkage (Fig. 4A).

To investigate whether the disulfide linkages play any role in maintenance of the viral core particles, we partially purified the extracellular and intracellular core particles from [^{35}S]methionine-labeled HuH-7 cells transfected with HBV DNA. Subsequently, these particles were treated for 5 h at 37°C with TNE buffer at pH 7.5, at pH 10, with 3 M NaCl in

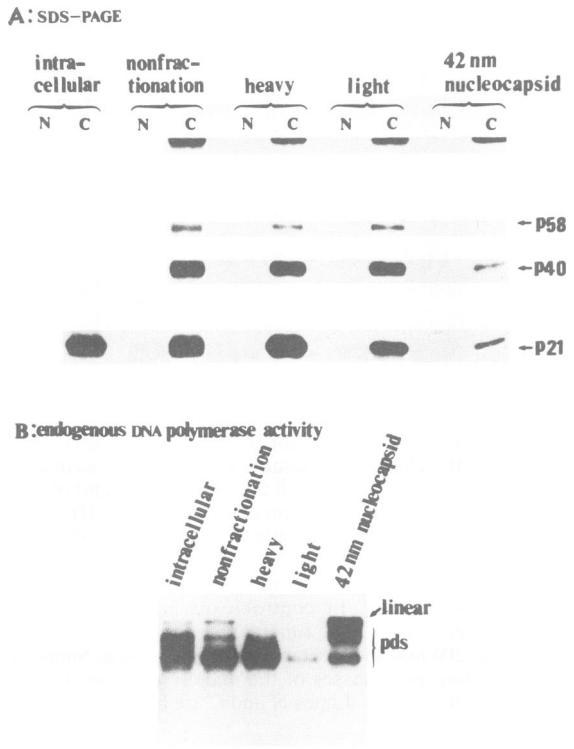

FIG. 4. Migration pattern of the extracellular and intracellular core particles on nonreducing SDS-PAGE. The CsCl gradient-separated extracellular core particles (containing heavy core, light core, and nucleocapsid of the Dane particle) and intracellular core particle (extracted with NET buffer containing 10 mM iodoacetamide and pelleted by ultracentrifugation) were phosphorylated as described in the legend to Fig. 1 and analyzed by nonreducing SDS-PAGE (A). Portions of the immunoprecipitates were removed before phosphorylation for the analysis of endogenous DNA polymerase activity (B). Similar amounts of radioactive DNA were loaded, except the light core, which contained barely detectable levels of endogenous DNA polymerase activities. Lanes N and C (panel A) are defined in the legend to Fig. 1.

TNE, or with 4 M urea in TNE. The treated particles were then precipitated by ultracentrifugation at 280,000 × *g* for 30 min at 4°C. Under these conditions, the disintegrated core particles remained in the supernatant. The supernatants were then precipitated with an excess of rabbit anti-HBc antiserum coated on protein A-Sepharose. The pellets containing the core particles were redissolved in TNE buffer and immunoprecipitated with anti-HBc-coated protein A-Sepharose. The radioactivities of the HBc antigen in the supernatants and the pellets were calculated. The results indicated that the extracellular core particles were very stable to treatment in TNE buffer at pH 7.5, pH 10, and 3 M NaCl, whereas only about 82.5% of the intracellular core particles remained in the pellet under these conditions. After treatment with 4 M urea, 83.5% of the extracellular core particles and only 35% of the intracellular core particles were present in the pellet (Table 1). Similar results were obtained when an enzyme immunoassay was performed to measure the level of HBc antigen in the supernatants and in the pellets (data not shown). Thus, these data suggested that disulfide linkages in the extracellular core particles are important for the integrity of viral core particles.

We have presented evidence that the HBc antigen forms

TABLE 1. Differential stabilities of extracellular and intracellular core particles

Treatment	% HBc antigen in[a]:			
	Extracellular core particles		Intracellular core particles	
	Pellet	Supernatant	Pellet	Supernatant
pH 7.5	98 ± 1	<1	82.5 ± 3.5	17.5 ± 3.5*
pH 10	99 ± 0.5	<1	82.5 ± 0.5	17.5 ± 0.5**
3 M NaCl	98.5 ± 0.5	<1	81 ± 1	19 ± 1**
4 M urea	83.5 ± 1.5	16.5 ± 1.5	35 ± 4	65 ± 4**

[a] Percentages of HBc antigen in the supernatant and pellet were determined by measuring the radioactivities of the HBc antigen precipitated by an anti-HBc antiserum from the supernatant or pellet after ultracentrifugation and dividing this value by the total radioactivities of HBc antigen precipitated by an excess of anti-HBc antigen antiserum. The values are the means and standard deviations of two independent experiments ± standard deviation. Symbols: *, $P < 0.05$; **, $P < 0.01$.

oligomers through disulfide bonds in the extracellular HBV core particles. The authenticity of the disulfide linkage of the HBc antigen in the extracellular core particles is supported by the findings that (i) different forms of the HBc antigen were reduced to the monomeric 21-kDa HBc antigen in diagonal gel analysis (Fig. 2), (ii) different forms of HBc antigens were insensitive to iodoacetamide blockade (Fig. 3), and (iii) the intracellular immature cores similarly treated did not show the presence of any detectable disulfide linkage (Fig. 4). Gallina et al. reported that the HBc antigen expressed in bacteria was also able to form disulfide bonds (10). The amino acid sequence of HBc protein contains three cysteines (at positions 48, 61, and 107), all of which are strictly conserved among various HBV isolates and subtypes, as well as in other mammalian hepadnaviruses (7–9, 16, 20, 22, 26, 28). This observation suggests that disulfide bond formation may be important for the structural integrity of the viral core. Indeed, data in this report show that the extracellular core particles, which have disulfide linkages, were more stable than the intracellular core particles under conditions of high pH, high salt, and urea treatment. These results indicate that the disulfide bonds are important for the maintenance of the integrity of core particles. However, the data do not exclude the possibility that other differences between the extracellular and intracellular core particles also contribute to the stability of extracellular core particles. Disulfide linkages have also been reported to be involved in the assembly and stabilization of the viral nucleocapsid structure in some icosahedral viruses such as rubella virus (6), bovine rotavirus (25), and herpes simplex virus type 2 (31). The processes of assembly and secretion of the HBV core particles in the culture system are not clear. Our results indicate that all the extracellular viral core particles contain similar disulfide bonds, whereas intracellular core particles do not. The absence of disulfide bonds in the intracellular core particles may be a result of the presence of a high concentration of reducing agent, for example, glutathione, preventing the formation of disulfide bonds in the cytosol. However, the mechanism of the disulfide bond formation is unclear. Further work is needed to understand the mechanism of formation of the disulfide bonds of HBV core particles.

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