Hepatitis B Virus DNA-negative Dane Particles Lack Core Protein but Contain a 22-kDa Precore Protein without C-terminal Arginine-rich Domain*

Tatsuki Kimura‡§, Nobuhiko Ohno‡, Nobuo Terada‡, Akinori Rokuhara¶, Akihiro Matsumoto¶, Shintaro Yagi‡, Eiji Tanaka¶, Kendo Kiyosawa‡, Shinichi Ohno‡, and Noboru Makit†

From the ‡Research and Development Division, Advanced Life Science Institute, Inc., Wako, Saitama 351-0112, Japan, the §Department of Anatomy, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, Tamakochō, Yamanashi 409-3898, Japan, and the ¶Department of Internal Medicine, Shinshu University School of Medicine, Matsumoto, Nagano 390-8621, Japan

DNA-negative Dane particles have been observed in hepatitis B virus (HBV)-infected sera. The capsids of the empty particles are thought to be composed of core protein but have not been studied in detail. In the present study, the protein composition of the particles was examined using new enzyme immunoassays for the HBV core antigen (HBcAg) and for the HBV precore/core proteins (core-related antigens, HBcrAg). HBcrAg were abundant in fractions slightly less dense than HBcAg and HBV DNA. Three times more Dane-like particles were observed in the HBcrAg-rich fraction than in the HBV DNA-rich fraction by electron microscopy. Western blots and mass spectrometry identified the HBcrAg as a 22-kDa precore protein (p22cr) containing the uncleaved signal peptide and lacking the arginine-rich domain that is involved in binding the RNA pregenome or the DNA genome. In sera from 30 HBV-infected patients, HBcAg represented only a median 10.5% of the precore/core proteins in enveloped particles. These data suggest that most of the Dane particles lack viral DNA and core capsid but contain p22cr. This study provides a model for the formation of the DNA-negative Dane particles. The precore proteins, which lack the arginine-rich nucleotide-binding domain, form viral RNA/DNA-negative capsid-like particles and are enveloped and released as empty particles.

Hepatitis B virus (HBV)1 infects more than 300 million people and is a major cause of liver diseases. The HBV belongs to the Hepadnavirus family and is a small (42 nm) enveloped DNA virus, which possesses a 27-nmicosahedral nucleocapsid composed of core protein and a 3.2-kb partially double-stranded, circular genome (1). Although the term “Dane particles” refers to the 42-nm HBV particles (2) and is often used in reference to the complete HBV particles, electron microscopic studies have suggested that the DNA-negative “empty” Dane particles are predominant in sera (3–6). The capsids of the empty particles are thought to be composed of core protein but have not been studied in detail.

The HBV genome encodes two core-related open reading frames, precore and core genes (Fig. 1). These are expressed because of two in-frame ATG initiation codons located at the 5′ end of the genes. The first ATG encodes a 25-kDa protein (p25) containing the 29-amino acid (aa) precore sequence fused to the N terminus of the HBV core antigen (HBcAg). The p25 is directed toward the secretory pathway by a 19-aa signal sequence that is cleaved during translocation into the lumen of the endoplasmic reticulum (ER), producing a 22-kDa protein. Subsequent proteolytic cleavages within the arginine-rich C-terminal region (94 aa) generate a 17-kDa protein that is secreted as hepatitis B e antigen (HBeAg) (7–10). A heterogeneous population of these precore derivatives has been observed in the sera of patients and is serologically defined as HBeAg (9, 11, 12). Conversely, the second ATG specifies the 21.5-kDa HBcAg, which assembles into dimers that form the virus capsid (7, 9, 13–15). HBcAg is a 183-residue protein with two domains, the assembly domain that forms the capsid and the C-terminal arginine-rich domain that is responsible for RNA packaging (Fig. 1). The assembly domain, lacking the C-terminal domain, is sufficient for self-assembly into capsid particles. The arginine-rich C-terminal domain is involved in binding to the HBV RNA pregenome or the HBV DNA genome but is dispensable for HBV capsid assembly in Escherichia coli (16–19) and insect cells (20). The capsid is enclosed within an envelope containing the viral glycoprotein surface antigen (HBsAg) and released to the circulation as Dane particles.

We previously developed enzyme immunoassays (EIAs) for HBcAg (21) and HBV core-related antigens (HBcrAg) (22, 23). Serum specimens were pretreated with SDS to release and denature antigens and to inactivate antibodies. The HBcAg assay specifically measures core protein (21), and the HBcrAg assay measures precore/core proteins, including core protein and HBeAg (22, 23).

The present study investigated precore/core proteins in HBV-infected human sera using the new assays. The results suggest that most of the Dane particles were DNA-negative and were composed of a 22-kDa precore protein containing the uncleaved signal peptide and lacking the C-terminal arginine-rich domain. We present a new model for the formation of HBV DNA-negative particles.
Empty HBV Particles Contain a 22-kDa Precore Protein

EXPERIMENTAL PROCEDURES

Serum/Plasma Samples—Hepatitis B plasma panels were purchased from Boston Biomedica, Inc. (BBI, West Bridgewater, MA), or ProMedDx (Norton, MA). Clinical sera were collected between 1997 and 2001 at the Shinshu University Hospital (Matsumoto, Japan) from patients with persistent HBV infection. Seventeen of these serum samples containing ≥0.05 ng/ml HBcAg were immunoprecipitated to examine HBcAg/HBcrAg ratios. Of the 30 total serum samples (from 23 males and 7 females), 22 were HBcAg-positive, and 7 were HBeAb-positive. The remaining sample was positive for both HBcAg and HBeAb. None of the 30 patients was treated with anti-viral agents such as interferon or lamivudine. All sera were stored at −30 °C or below until testing. The study design conformed to the 1975 Declaration of Helsinki and was approved by the Ethics Committees of the institutions involved in this study. A written informed consent was obtained from each patient.

Recombinant HBV Core-related Antigens—Recombinant HBcAg (rHBcAg, aa 1–183) and HBcAg (rHBcAg, aa 10–149) were expressed and purified as described (21, 22). The concentration of these antigens was determined using the BCA protein assay kit (Fierce) and bovine serum albumin standards according to the manufacturer’s instructions.

Monoclonal Antibodies and EIAs for HBcAg or HBcrAg—Anti-HBcAg and anti-HBcrAg monoclonal antibodies were established as reported previously (21, 22). The HBcAg-specific monoclonal antibody, HB50, recognizes SPRRR repeats in the arginine-rich domain of HBcAg (21), whereas the anti-HBcrAg monoclonal antibody, HB91, recognizes aa 1–19 of HBcAg and thus reacts to denatured HBcAg, HBcAg, and other precore/core proteins (22).

HBcAg and HBcrAg were measured by EIA as described previously (21–23). The assays contain a sample pretreatment step that inactivates antibodies and dissociates antigens in samples. The assays can thus detect antigens within the viral envelope or complexed with antibodies in addition to free antigens.

HBV DNA Measurement—HBcAg and HBsAg were measured by radioimmunoassay or by chemiluminescent immunoassay (Abbott, Tokyo, respectively). HBV DNA was detected by PCR using the Amplipcr HBV monitor test (Roche Applied Science). Samples showing values over the detection range were remeasured after dilution to obtain quantitative results.

Sucrose Density Gradient Ultracentrifugation—Aliquots (1.7 ml) of a reaction buffer from the kit and 50 μl of a magnetic bead suspension. The mixture was incubated for 30 min at room temperature with gentle agitation and then magnetically separated. HBcAg and HBcrAg in supernatant and precipitate were measured by EIA.

Electron Microscopy—A 500–μl aliquot of HBV-positive plasma was subjected to ultracentrifugation on linear 10–50% (w/w) sucrose density gradients. The high density HBcAg peak fractions (corresponding to Fig. 3A, fractions 23 and 24) and HBcAg peak fractions (corresponding to Fig. 3A, fractions 25 and 26) were separated by the second ultracentrifugation through linear 35–50% (w/w) sucrose density gradients. The fractions were fixed by adding paraformaldehyde solution to a final concentration of 4%. A 4-μl aliquot of each fraction was diluted in 90 μl of distilled water in 5-mm diameter polyallomer centrifugation tubes (Beckman Instruments), and copper grids films with Formvar membranes and treated additionally with poly-L-lysine were placed on the bottom of the tubes in the solution. Ultracentrifugation (200,000 × g, 4 °C, 2 h) was performed in a Beckman TLS-55 swinging bucket rotor to concentrate the virus particles and allow them to attach to the Formvar membranes on the copper grids. Afterward, the attached virus particles were negatively stained with 4% uranyl acetate and observed at an accelerating voltage of 80 kV in an electron microscope (H-7500, Hitachi, Tokyo). Fifteen electron micrographs of the virus particles from each fraction were taken randomly at a magnification of ×80,000. The number of virus particles in the 3.76 μm² area was then counted on each electron micrograph. The diameters of the virus particles in each fraction were also measured.

Western Blot Analysis—Samples were subjected to SDS-PAGE through a 15–25% polyacrylamide gel under reducing conditions. Proteins in the gel were electroblotted onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore) at 15 V for 45 min. The membrane was blocked and probed using alkaline phosphatase-conjugated HB50 (for HBcAg) or HB91 (for HBcrAg) monoclonal antibody at room temperature for 1 h. The membrane was washed and incubated with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate solution (KPL, Gaithersburg, MD) for 15 min (for HBcAg) or 90 min (for HBcrAg), respectively.

N-Terminal Amino Acid Sequence Analysis—A 4-ml aliquot of HBV-positive plasma was subjected to ultracentrifugation on linear 10–60% (w/w) sucrose density gradients, and subsequently the high density HBcAg peak fractions (Fig. 3A, fractions 23 and 24) were separated by gel filtration through Superose 6 HR (Amersham Biosciences). Void fractions were collected and ultracentrifuged at 200,000 × g for 15 h at 4 °C using a Beckman SW 50.1 rotor. The precipitate was separated by SDS-PAGE and electroblotted onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore) at 15 V for 45 min. Proteins on the membrane were stained using Coomasie Brilliant Blue-R250. The N-terminal amino acid sequence of the 22-kDa band was analyzed using the Procise 494 cLC protein sequencing system at the ProSci Life Science Institute, Inc. (Tokushima, Japan).

Mass Spectrometry Analysis—The 22-kDa protein was purified as described above. The 22-kDa band was cut from the SDS-polyacryl-
FIG. 2. Reactivity of the HBcAg assay and the HBcrAg assay. Recombinant HBcAg (▲) and HBcrAg (□) were diluted and tested for the HBcAg assay (A) and the HBcrAg assay (B). The assay reactivity is shown as log relative luminescence intensity (RLI).

RESULTS

Specificity of HBcAg and HBcrAg EIAs—The specificity of the HBcAg and HBcrAg assays was confirmed by using rHBcAg and rHBeAg. The HBcAg assay specifically reacted to rHBcAg but not to rHBeAg (Fig. 2A). The HBcrAg assay reacted equally to rHBcAg and rHBeAg (Fig. 2B).

Density Distribution of HBV Precore/Core Proteins—HBV DNA-positive plasma (ProMedDx 9990776, HBsAg-positive, HBcAg-positive, HBV DNA 9.1 log copies/ml) was subjected to ultracentrifugation through a 10–60% (w/w) sucrose density gradient. Fractions were tested for HBcAg, HBcrAg, HBsAg, HBeAg, and HBV DNA (Fig. 3A). HBcAg appeared in the high density fractions and peaked in the same fraction (fraction 25) as HBV DNA. HBsAg was distributed in fractions of lower density, and HBeAg was dispersed widely in fractions of much lower density. HBcrAg peaked in fraction 24, slightly lower in density than the HBV DNA and HBeAg peaks in addition to a peak corresponding to HBeAg at much lower density. The concentration of HBcrAg in fraction 24 was 13-fold higher than the concentration of HBcAg in fraction 25. The high density HBcrAg peak was therefore predominantly composed of precore proteins other than core protein.

High density HBcrAg fractions (Fig. 3A, fractions 23–26) were reanalyzed under gentler (30–50%) sucrose density gradient sedimentation (Fig. 3B). HBcrAg concentration peaked in lower density fractions than HBcAg and HBV DNA, indicating that high density HBcrAg clearly differs from HBcAg. HBsAg concentration exhibited a shoulder at the HBcrAg peak fraction.

Immunoprecipitation by Anti-HBsAg—Sucrose density fractions (Fig. 3A) were immunoprecipitated by the anti-Envelope protein HBSAg. Most of the HBeAg (97.5, 97.8, 96.2, and 95.1% from fractions 23–26) was precipitated by anti-HBsAg. Although more than 94% (94.5, 94.1, and 94.3% from fractions 7, 10, 13) of low density HBcrAg was observed in the supernatant, more than 96% (96.2, 96.8, 96.9, and 96.5% from fractions 23–26) of high density HBcrAg was in the precipitate. These data suggest that similar to the core protein, the high density HBcrAg exists in enveloped particles.

Stability of HBcrAg Particles—The HBV core forms very stable capsid particles resistant to denaturing pH, temperature, or detergents (25). Particle fractions of HBV-positive plasma were treated with or without 3% Nonidet P-40 detergent at 37 °C for 30 min and then subjected to gel filtration through Superose 6 HR (exclusion limit = 4 × 10^7 Da). Fractions were tested for HBcAg and HBsAg. Regardless of Nonidet P-40 treatment, HBcAg and HBsAg appeared in the void fractions (Fig. 4), indicating that HBcAg formed high molecular mass (>10^7 Da) particles resistant to 3% Nonidet P-40
treatment, as did the HBcAg.

Electron Microscopy—HBcAg and HBcAg in plasma 9990776 were separated by sequential sucrose density ultracentrifugation. The resultant HBcAg-rich fraction (fraction A) contained 6.06-fold more HBcAg than the HBcAg-rich fraction (fraction B) but contained only 3 and 38% of the HBV DNA and HBcAg, respectively, found in fraction B (Table I). Virus particles in the two fractions were concentrated and attached to the copper grids by ultracentrifugation and then negatively stained and observed under the electron microscope. Although virus particles appearing similar to Dane particles were observed in fraction B, more such Dane-like particles were seen in fraction B. Fraction A contained 17.9/3.76 m²) /m²) in fraction A and 42.0/2.2 nm in fraction B (Table I). The Dane-like particles in fractions A and B were not morphologically distinguishable (Fig. 5) but were quite similar to those reported previously (2–4, 6). The mean diameters of the measured particles were 41.5 ± 2.2 nm in fraction A and 42.0 ± 2.2 nm in fraction B (Table I). The mean diameters were not significantly different from one another (n = 60, p = 0.27) and were similar to the sizes reported previously (2).

Identification of Particle HBcAg as a 22-kDa Precore Protein (p22cr) Lacking the C-terminal Domain—HBV DNA-positive plasma (BBI PHM95A-14) was subjected to a 10–60% sucrose density gradient and fractionated into 15 fractions. The fractions were then analyzed by Western blotting using monoclonal antibodies for HBcAg and HBcAg (Fig. 6A). HBcAg was detected only in fraction 8 and the original plasma. Conversely, four bands were detected by anti-HBcAg in plasma. HBcAg and two additional proteins, which were considered HBcAg precursors, were detected in low density fractions by anti-HBcAg. A 22-kDa protein, which was termed p22cr, was also detected in fraction 8. To confirm whether p22cr was identical to HBcAg, the p22cr band was compared with the neighboring HBcAg band (Fig. 6B). The p22cr protein exhibited slightly higher molecular weight than HBcAg. A fainter HBcAg band was also detected by anti-HBcAg. Because p22cr did not react with the HB50 anti-HBcAg antibody, SPRRR sequences (positioned at aa 155–174 as three repeats) were presumed absent. Furthermore, p22cr maintained its 22-kDa molecular mass without the N-glycosylation consensus site. These data suggest that p22cr contains a complete or nearly complete precore region, including the signal sequence.

The p22cr protein was purified, and the N-terminal amino acid sequence was analyzed. p22cr showed no significant amino acid signal (data not shown), suggesting that the N terminus of p22cr might be blocked.

We then applied mass spectrum analysis. Data from MALDI-TOF MS were analyzed by MS-Fit search using the NCBI non-redundant data base. The search selected 117 of 87,559 entries for the molecular mass range 15–30 kDa. The top 20 matches were all HBV core or precore proteins. Six of 50 input peptide masses matched five precore/core peptides (Table II) that spanned 40% (86 of 212 aa) of the sequence. The N-terminal precore tryptic peptide (peptide 1, aa -28 to aa -9) was found to be N-terminally acetylated and was, therefore, not directly accessible to Edman sequencing. p22cr lacked the first N-terminal methionine of the precore protein. Another peptide, peptide 5, was identified as a precore/core peptide comprising aa 128–150. LC-MS/MS analysis was also applied. Two peptide fractions corresponding to peptides 2 and 5 of Table II were recognized as HBV precore/core proteins. Thus, the p22cr protein was confirmed to be a precore protein from N-terminally
Empty HBV Particles Contain a 22-kDa Precore Protein

HBcAg and HBcrAg in plasma were separated by sequential sucrose density ultracentrifugation. The HBV-DNA, HBcAg, and HBcrAg concentrations in the resultant HBcrAg-rich fraction (fraction A) and HBcAg-rich fraction (fraction B) are shown. The numbers of virus particles in the 3.76 μm² area were counted on each of 15 electron micrographs (Fig. 5).

<table>
<thead>
<tr>
<th>HBV DNA</th>
<th>HBcAg</th>
<th>HBcrAg</th>
<th>Dane-like particles</th>
</tr>
</thead>
<tbody>
<tr>
<td>×10⁷ copies/ml</td>
<td>ng/ml</td>
<td>ng/ml</td>
<td>in 3.76 μm²</td>
</tr>
<tr>
<td>Fraction A</td>
<td>13</td>
<td>81</td>
<td>2,823</td>
</tr>
<tr>
<td>Fraction B</td>
<td>398</td>
<td>210</td>
<td>466</td>
</tr>
</tbody>
</table>

a Data are presented as mean ± S.D. n = 15; p < 0.001.
b Data are presented as mean ± S.D. n = 60; p = 0.27.

**DISCUSSION**

In the present study, we demonstrated that HBV DNA-negative Dane particles are dominant in serum and are composed of a precore protein p22cr, which contains an uncleaved signal sequence and lacks a C-terminal arginine-rich domain. Early electron microscopic and radiolabeling studies have suggested that less than 10% of Dane particles include full cores with viral DNA (3–6). However, the particle formation mechanisms have not been thoroughly examined. Core protein lacking the arginine-rich C-terminal domain can still assemble into capsid particles but fails to bind nucleic acids (16). Our findings present a new model for the formation of DNA-negative particles. The precore proteins, which lack the nucleotide-binding domain, form viral DNA-negative capsid-like particles, and the particles are enveloped and released to blood circulation.

Our new assays for HBcAg and HBcrAg enabled us to study precore/core proteins in HBV particles. The assays include sample pretreatment with SDS, which releases core protein from the particles, inactivates antibodies, and denatures antigens. Thus the HBcAg assay is able to detect the core protein in virion (21), and the HBcrAg assay is able to detect free HBcAg, HBcAg-antibody complex, and precore/core proteins in particles (22, 23). Unexpectedly, the HBcrAg assay detected abundant high density protein in addition to HBcAg and HBcrAg (Figs. 3 and 6). The protein formed Nonidet P-40-resistant particles (Fig. 4) that did not contain HBV DNA but were enveloped by HBcAg. The protein was detected together with HBV DNA-negative particles that were morphologically identical to the complete virion (Fig. 5). The unknown precore/core protein proved to be a 22-kDa precore protein species (p22cr) containing the uncleaved signal peptide (Table II) and lacking the arginine-rich domain (Fig. 6). The HBcrAg particles appear at a slightly lower density than HBcAg or HBV DNA (Fig. 3), which is also consistent with the observation that HBcrAg particles lack high density DNA components. Collectively, these data strongly suggest that p22cr forms the core of HBV DNA-negative Dane particles.

Our findings indicate that p22cr particles are more abundant than HBcAg capsid in sera (Figs. 3, 5–7, and Table I). In chronic hepatitis B sera, HBcAg comprised only 10.5% of HBcrAg (containing p22cr and HBcAg) in HBsAg-positive particles (Fig. 7). In addition, electron microscopic study indicated that Dane-like particles were more abundant in the HBcrAg-rich fraction than in the HBcAg/HBV DNA-rich fraction (Fig. 5 and Table I). This coincides with the previously reported abundance of empty particles (3–6). Empty and complete Dane particles were differently stained with uranyl acetate (3, 4, 6), but we could not distinguish Dane particles containing HBV DNA from those not containing HBV DNA. This might be due to differences in fixation and/or the negative staining procedure. We used paraformaldehyde for fixation to avoid biohazards.

The present study demonstrated that p22cr is a precore protein from aa –28 to at least aa 150. HBcAg and HBcrAg Levels in HBsAg-positive Particles from Chronic Hepatitis B Sera—The levels of precore/core proteins were investigated in HBV particles of chronic hepatitis B sera. Sera were immunoprecipitated with anti-HBsAg, and then levels of HBcAg and HBcrAg in the supernatant and precipitate were measured. More than 91% of the HBcAg was detected in precipitate fractions. HBcAg in precipitate fractions included HBcAg and HBcrAg. In the precipitate fractions, HBcAg concentration ranged from 0.08 to 165 ng/ml, whereas HBcrAg ranged from 0.59 to 1,079 ng/ml (Fig. 7). In addition, electron microscopic study indicated that Dane-like particles were more abundant in the HBcrAg-rich fraction than in the HBcAg/HBV DNA-rich fraction (Fig. 5 and Table I). This coincides with the previously reported abundance of empty particles (3–6). Empty and complete Dane particles were differently stained with uranyl acetate (3, 4, 6), but we could not distinguish Dane particles containing HBV DNA from those not containing HBV DNA. This might be due to differences in fixation and/or the negative staining procedure. We used paraformaldehyde for fixation to avoid biohazards.

The present study demonstrated that p22cr is a precore protein from aa –28 to at least aa 150 (Table II). The assembly domains (residues 1 to 149) self-assemble into capsids (16–19). In addition, precore protein containing the assembly domain could form capsid-like particles (26–28), whereas precore proteins are secreted as soluble HBeAg (7–12, 29, 30). A precore protein similar to p22cr, but containing the first methionine, has been isolated as soluble HBeAg from pooled sera of HBV.
Empty HBV Particles Contain a 22-kDa Precore Protein

TABLE II
MALDI-TOF MS analysis of p22cr

The 22-kDa protein band was digested in-gel and analyzed by MALDI-TOF MS. The results were analyzed using the NCBI non-redundant database, taking into account probable post-translational modifications. Five precore/core peptides matched to six of 50 input peptide masses are shown.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>m/z observed</th>
<th>[M+H]+ matched</th>
<th>Δ</th>
<th>Peptide sequence</th>
<th>Modifications</th>
<th>Amine acids</th>
<th>Start</th>
<th>End</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2233.1438</td>
<td>2233.1183</td>
<td>11.4048</td>
<td>QLPHLCLILSCSCPTVQASK</td>
<td>N-terminally acetylated</td>
<td>−28</td>
<td>−9</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1237.6388</td>
<td>1237.6428</td>
<td>−3.2413</td>
<td>DLLDTASALYR</td>
<td></td>
<td>29</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1913.9167</td>
<td>1913.8928</td>
<td>12.4530</td>
<td>EALESEPHESGPHFLAR</td>
<td></td>
<td>40</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1552.7798</td>
<td>1552.8045</td>
<td>−15.8855</td>
<td>DLVSYVNTNNLKL</td>
<td>Acrylamide-modified Cys</td>
<td>83</td>
<td>96</td>
<td></td>
</tr>
</tbody>
</table>

Amino acids

\[
\begin{array}{c|c|c}
\text{Start} & \text{End} \\
10 & 1100 \\
25 & 39 \\
40 & 56 \\
83 & 96 \\
128 & 150 \\
\end{array}
\]

![Fig. 7. HBeAg and HBcAg levels in HBsAg-positive particles of chronic hepatitis B serum.](http://www.jbc.org/)

HBsAg-positive particles were immunoprecipitated by anti-HBsAg-coated magnetic beads from 30 samples of HBV-infected sera. Precipitated proteins were eluted by SDS solution. Levels of HBcAg and HBcAg in precipitate were measured by EIA. A, data are presented as HBcAg and HBeAg concentrations per ml of serum. O, HBeAg-positive; C, HBeAg-negative sample. B, HBcAg percentage per HBeAg in the precipitate. The box plots show the 10th, 25th, 50th, 75th, and 90th percentiles, and diamonds denote the outliers.

Carriers (30). This could represent the soluble form of p22cr, which was secreted without processing of the signal peptide. Our findings indicate that the majority of p22cr exists in enveloped particles (Figs. 6 and 7).

Mass spectrum study indicated that the protein band contained precore peptide (Table II). We believe that this peptide was not derived from minor precore protein contamination of the major core protein because: (a) core protein concentrations in the original plasma were much lower than concentrations of precore/core proteins; (b) the purified sample appeared as a single band on SDS-PAGE; (c) the 22-kDa protein band could not be sequenced by Edman degradation, indicating that the N-terminal end of the peptide was blocked; and (d) the antibody targeting the C-terminal SPRRR repeats did not react with the 22-kDa p22cr (Fig. 6), suggesting that p22cr possesses the nearly complete N-terminal precore sequence.

Although the median HBcAg to HBeAg (HBcAg + p22cr) ratio of HBsAg-positive particles was 10.5%, the actual ratios ranged widely from 3.1 to 37.4% (Fig. 7B). Because precore protein expression is abolished by precore nonsense mutation (31), the precore mutation must influence the HBeAg/HBcAg ratios. In addition, the particle HBeAg/HBeAg ratios would depend on the amount of precore proteins that are secreted as HBeAg or form p22cr particles. The ratios of particle-forming p22cr to soluble HBeAg in serum ranged from ~10:1 to 1:100.²

The manner in which precore protein containing the signal peptide forms particles remains unclear, but inefficient translocation of the precore protein might lead to particle formation in the cytosol. As with most secreted proteins, translocation of the precore protein across the ER membrane is mediated by signal recognition particles (8). However, translocation of the precore proteins is inefficient (8, 32, 33). In Xenopus oocytes, precore protein (p25, aa −29 to +183) was produced but not translocated into the ER lumen without processing (33). If translated precore proteins were to evade translocation to the ER, disulfide bridges would not form in the reducing environment of the cytosol. An intramolecular disulfide bridge between Cys-7 and Cys-61 determines the structure of the HBeAg (34, 35). HBe protein without Cys-7 also assembles into particles (29, 34–36). Conversely, Cys residues are not essential for the assembly of viral core particles (37). We therefore hypothesize that precore proteins remaining in the cytosol, which do not form disulfide bridges between Cys-7 and Cys-61, cannot assume the HBeAg conformation but can assemble into capsid-like particles.

The mechanisms for cleaving the C-terminal domain are unclear. Maassen et al. (38) reported that an N-terminal fusion core protein (with foreign sequences comprising 14 aa) assembles into capsid-like particles, but the fusion is sensitive to proteolytic attack within the arginine-rich C terminus. The uncleaved precore region (aa −28 to −1) might thus promote cleavage of the C-terminal domain.

Based on numerous in vitro or animal studies (14–19, 27, 29, 35, 38), the HBV capsid is believed to be a construct of core protein alone. However, nonsecreted precore protein and core protein can assemble to form hybrid nucleocapsids (28). The p22cr displayed a shoulder in virion fractions from density gradients (Fig. 3B, fraction 26–27), and the concentration of p22cr protein greatly exceeded that of HBeAg. The nucleocapsid of complete HBV particles could therefore contain p22cr.

Although the functions of the DNA-negative particles are largely unknown, the particles have been suggested to play a role in the persistence of HBV infection (3, 5, 6). p22cr in the particles may be a disturbing antigen for the host reactions. Overexpression of the precore gene results in inhibition of HBV replication in culture cells or transgenic mice (28, 39). The p22cr might be a molecule that inhibits HBV replication in carriers (30).
human hepatocytes during natural infection. Furthermore, the number of particles containing p22cr or the antibodies specific for p22cr could be clinical markers for hepatitis B.

REFERENCES

Protein Synthesis, Post-Translation Modification, and Degradation: Hepatitis B Virus DNA-negative Dane Particles Lack Core Protein but Contain a 22-kDa Precore Protein without C-terminal Arginine-rich Domain

Tatsuji Kimura, Nobuhiko Ohno, Nobuo Terada, Akinori Rokuhara, Akihiro Matsumoto, Shintaro Yagi, Eiji Tanaka, Kendo Kiyosawa, Shinichi Ohno and Noboru Maki

doi: 10.1074/jbc.M501564200 originally published online April 4, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M501564200

Find articles, minireviews, Reflections and Classics on similar topics on the JBC Affinity Sites.

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 39 references, 26 of which can be accessed free at http://www.jbc.org/content/280/23/21713.full.html#ref-list-1