A modified hepatitis B virus surface antigen with the receptor-binding site for hepatocytes at its C terminus: expression, antigenicity and immunogenicity

Xie Xu, Guang-di Li,* Yu-ying Kong, Hai-ling Yang, Zu-chuan Zhang, Hui-Ting Cao and Yuan Wang

Shanghai Institute of Biochemistry, Academia Sinica, Shanghai, 200031, People's Republic of China

A modified hepatitis B virus (HBV) surface antigen, the SA-28 protein, was constructed and expressed by recombinant vaccinia virus in mammalian cells. This protein was composed of a PreS1 region-derived peptide (amino acids 21 to 47) that contained the hepatocyte receptor-binding site, joined to the C terminus of the major S protein at amino acid position 223. This modified surface antigen could be efficiently assembled into particles with a density of 1.23 g/ml and could be secreted from several mammalian cell lines. The results of immunoprecipitation revealed that the SA-28 protein was recognized by both the anti-S protein antibody and the anti-PreS1 antibody. A strong antibody response, against both the S protein and PreS1 epitopes, was induced in BALB/c mice immunized by the SA-28 particles, indicating good immunogenicity. These results suggested that the HBV surface antigen consisting of the SA-28 protein could be a promising candidate as a new HBV vaccine with higher efficacy.

The envelope of human hepatitis B virus (HBV) contains three coterminal proteins, designated small (S), middle (M), and large (L). The S protein has 226 amino acid residues and is the most abundant of the three. The M protein is composed of the S protein sequence and 55 additional amino acid residues at the N terminus, designated the PreS2 sequence. L protein is composed of the S protein and PreS2 sequence and, depending on the HBV subtype, an additional 108 or 119 N-terminal amino acids named PreS1. During an HBV infection, a large quantity of non-infectious 22 nm spherical particles are produced by the infected hepatocytes in addition to the infectious 42 nm HBV virions. Because the L protein is localized preferentially in virions, and the PreS region of the L protein represents the most peripheral structure of the virus particle, a role for the PreS region in morphogenesis and infectivity of HBV has been suggested (for review see Ganem & Varmus, 1987). Furthermore, Neurath et al. (1986) demonstrated that the HBV-binding site for the hepatocyte receptor is located within the PreS1 sequence, between residues 21 and 47. The interaction between HBV and HepG2 hepatoma cells is strongly inhibited by a PreS1-specific antiserum against this region. These data indicate that the PreS1 sequence between amino acids 21 and 47 [PreS1(21-47)] is indeed essential for the recognition of hepatocyte receptors and that the corresponding antibodies can efficiently block virus attachment. Thus it is expected that antibodies specific for such a recognition site on HBV virions would contribute to the neutralization of infectivity. A neutralizing effect by an anti-PreS1 peptide serum (amino acids 32 to 47) has been found by Neurath et al. (1988). Direct immunization of chimpanzees by a PreS1 peptide (amino acids 12 to 47) also induced protection against HBV challenge (Neurath et al., 1989). Therefore, the inclusion of the PreS1(21-47), the hepatocyte receptor binding site of HBV, into the present HBV vaccine seems to be a logical consideration.

Since it is difficult to express the native L protein in mammalian cells with high yield and efficient secretion, we tried to include PreS1(21-47) in the HBV surface antigen (HBsAg) by fusing it directly to the C terminus of the major S protein. The DNA fragment coding for PreS1(21-47) was amplified by PCR and fused to the 3' end of the truncated S gene at the position of amino acid 223. The sequences of PreS1 and its flanking region were checked by sequencing analysis (data not shown). The structure of the fusion protein SA-28 is presented in Fig. 1. The SA-28 coding sequence was then inserted into a vaccinia virus vector, pGIP-5 (Wu et al., 1988). This vector contained a thymidine kinase (TK) gene fragment within which a vaccinia virus promoter P7.5 was harboured. The coding sequence of the fusion protein was inserted so that it would be under the transcriptional control of this promoter. The detailed construction has been described elsewhere (Xu et al., 1995). When the
Short communication

Plasmid | Constructs | Recombinant vaccinia virus
---|---|---
pGJPS-3 | 1 | vTH-2
pGJPSA-28 | vwG ss | vSA-28

Fig. 1. Schematic diagram of the structures of the native S and modified SA-28 proteins expressed by the recombinant vaccinia viruses. The recombinant vaccinia viruses are indicated on the right. Corresponding plasmids are indicated on the left. The numbers below the S (■) and PreS1(21-47) (□) regions refer to the corresponding amino acids. The additional amino acids introduced into the construction are indicated. The amino acids between the S gene and PreS1(21-47) sequence (VWG) come from the Smal linker and the last two amino acids (SS) are from the cloning vector.

The expression plasmid was introduced into CV-1 cells (African green monkey kidney) infected with the vaccinia virus Tiantan line, homologous recombination can occur between the plasmid and the genome of the vaccinia virus through the TK gene sequence. Thus the recombinant vaccinia virus would then carry the SA-28 coding sequence with a mutated TK gene. The recombinant vaccinia virus, termed vSA-28, was selected for with 5-bromo-2′-deoxyuridine in human TK-143 cells (Mackett et al., 1984). The presence of the modified S protein gene in the recombinant vaccinia virus genome was confirmed by Southern blot hybridization with the S gene as the probe (data not shown).

The expression and secretion of the SA-28 protein produced by vSA-28 was first examined using the method of pulse labelling and immunoprecipitation. CV-1 cells were grown in 60 mm tissue culture dishes in Dulbecco’s modified Eagle’s medium (DMEM) with 5% fetal bovine serum (FBS, Gibco) and individual dishes of CV-1 cells (7 x 10⁵ cells per dish) were infected with vSA-28, vTH-2 (a recombinant vaccinia virus expressing S protein; Wang et al., 1990), or the vaccinia virus Tiantan line. After infection (20 h), cells were pulse-labelled (with 80 µCi of [³⁵S]methionine per dish) for 3 h and chased in 3 ml DMEM with 5% FBS for an additional 20 h. To prepare cytoplasmic lysates, cells were lysed in 1 ml of lysis buffer (0.01 M-sodium phosphate, 0.5% NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 4 mM-EDTA, 0.012 M-MgCl₂, 0.8% Trasylol). Samples (200 µl) of each cytoplasmic lysate (c) were used in parallel with 3 volumes of culture media (m) in immunoprecipitation with 2.5 µl of suitably diluted anti-S or anti-PreS antibody. Immunoprecipitation reactions followed the standard procedures (Yu, 1991). As shown in Fig. 2(a), with S protein as the control, the specific bands of 27K and 29K were detected upon SDS-PAGE both in the media and in the cytoplasmic lysates. The Mᵣ of the SA-28 protein was slightly larger than that of the major S protein, as calculated. The SA-28 protein could also be specifically immunoprecipitated when using a monoclonal antibody MA18/7 against the PreS(31-34) epitope (Heermann et al., 1984), whereas the S protein could not (Fig. 2b). The results indicated that the SA-28 protein

Fig. 2. Immunoprecipitation of the S and SA-28 proteins. (a) Precipitation with anti-S antibody. (b) Precipitation with anti-PreS1 antibody. The specifically immunoprecipitated bands indicate the glycosylated and unglycosylated S or SA-28 protein. Lane M, Mᵣ size markers; m, medium; c, cytoplasmic lysate.
could be recognized by both anti-S and anti-PreS1 antibodies and could be secreted efficiently from the cells. The expression and secretory behaviour of the SA-28 protein was compared in several mammalian cell lines. CV-1, Vero and primary chicken embryo cells (CEC) \((1 \times 10^6)\) were grown in 25 mm\(^2\) tissue culture flasks in 5 ml DMEM with 5% FBS and infected with the recombinant vaccinia virus vTH-2 or vSA-28 (m.o.i. of 0-1). When complete c.p.e. in each flask was observed, the cells were harvested, suspended in 5 ml PBS buffer and lysed by the freeze–thaw method and sonication. The cytoplasmic lysates and the corresponding supernatants were analysed by a radioimmunoprecipitation assay (RIA) (Fig. 3). Among the three cell lines, a higher expression and secretion level of the SA-28 protein was observed in the Vero cells and the CEC cells. About 50 % of the total synthesized SA-28 protein was secreted into the media, whereas the secretion level of the SA-28 protein in CV-1 cells was somewhat lower. The secretion level of the SA-28 protein was almost the same as that of the S protein (Fig. 3).

It has been confirmed previously that the S protein synthesized in mammalian cells could be assembled into particles with a diameter of 22 nm and density of 1-20 g/ml (reviewed by Ganem & Varmus, 1987). To examine the form of the SA-28 protein, CsCl gradient ultracentrifugation was performed. The media containing the SA-28 protein were added to 10 to 40 % (w/w; 1-08 to 1-43 g/ml) CsCl in 25 mm-sodium phosphate buffer pH 7-4 and centrifuged at 45000 r.p.m. for 40 h at 15 °C in an RSP65T rotor (Hitachi). The media containing the S protein, expressed by vTH-2, were used as a control. The gradient was fractioned, the density determined by refractive index and HBsAg antigenicity was measured by RIA. The SA-28 protein displayed an anti-S antibody-binding peak at a density of 1-23 g/ml (Fig. 4b), slightly higher than the density of the S protein, detected as 1-20 g/ml (Fig. 4a), in accordance with the results of a previous report (Wang et al., 1990). The anti-PreS1 antibody-binding peak was also found in the same position (data not shown). The particle form of the SA-28 protein was revealed by electron microscopy. After partial purification by filtration through hydrophobic chromatography gel, the sample containing SA-28 protein was further purified by equilibrium sedimentation as described above. The HBsAg peak fractions were pooled, concentrated and visualized by negative-staining with uranyl acetate. As shown in Fig. 5, the diameter of the SA-28 particles was 22 to 25 nm, similar to that of the S particles.

To determine whether the SA-28 particles were immunogenic, the partially purified SA-28 protein sample was used to immunize BALB/c mice. One group of 6- or 7-week-old BALB/c mice were injected intra-peritoneally with SA-28 protein (1-9 µg per mouse) adsorbed to Alum adjuvant. The S protein (3-2 µg per mouse), prepared with the same procedure, was used in
Fig. 5. Electron micrograph of the SA-28 particles. Samples were negatively stained with 2% uranyl acetate prior to observation in an electron microscope (Hitachi). The bar represents 100 nm.

Table 1. Immunogenicity of SA-28 protein in BALB/c mice*

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>time (weeks)</th>
<th>Specific antibody titre (1/dilution)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA-28</td>
<td>3</td>
<td>S 70</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>S 390000</td>
</tr>
<tr>
<td>S</td>
<td>3</td>
<td>S 55</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>S 1200000</td>
</tr>
</tbody>
</table>

* Mice were immunized with 1.9 µg of SA-28 particles or 3.2 µg of S particles, and primary antisera (3 weeks) or secondary antisera (7 weeks) were analysed for reactivity with the indicated solid-phase antigens by ELISA.

Clinical use of the present HBV vaccine since 1976, which consists mainly of the S protein, has indicated that vaccine containing S protein alone is sufficient to confer protection to healthy recipients. But in the general population, there are still 10 to 20% of people who are low-responsive or non-responsive to immunization with the current vaccines. In addition, several HBV variants with mutations in the common group ‘a’ determinant region of the S protein have been found to be able to escape immune recognition, although the number of such variants remains small. Thus a more immunogenic vaccine with more extensive protective efficacy seems to be essential. In recent years considerable interest has been focused on the additional antigenic determinants located within the PreS region of HBV proteins. These PreS1 and PreS2 epitopes are present in a much higher proportion on the surface of the 42 nm HBV virions, and may be of importance in facilitating the uptake of the virus into hepatocytes as well as the clearance of viral particles during natural infection. Considering the low yield and retention of the L protein when expressed in mammalian cells, here we reported an alternative approach, the construction of a modified HBsAg, SA-28. This carries PreS1(21-47), the HBV-binding site for the hepatocytes receptor, joined to the S protein at amino acid 223. This altered surface antigen can be readily recognized by both anti-S and the anti-PreS1 antibody. It can also be assembled into similar particles as the S protein and secreted from cells efficiently. It seems that the fusion of the PreS1 sequence at the S protein C terminus has little effect on the assembly and secretion of HBsAg particles. Similar results were also observed by Machein et al. (1992). In their report, all or part of the PreS1 region was translocated from the N terminus to the C terminus of the S protein. When transiently expressed in HepG2 cells, such altered HBsAg proteins were secreted from HepG2 cells with high efficiency.

The study in mice showed that the PreS1(21-47) sequence inserted at the C terminus of the S protein could be presented in an immunogenic form. It has generally been suggested that the synthetic peptide PreS1(21-47) is not a good immunogen, especially in mice. Our results showed that the fusion of this sequence to the C terminus of the S protein greatly improved its immunogenicity. Similar to a report by Cheng et al. (1986) on the immunization of rabbits with live recombinant vaccinia virus that expressed the entire L protein, the antibody against the PreS1 epitope is produced at high titres earlier than the anti-S antibody. This opens the possibility of earlier protection for vaccinees. Furthermore, high titres of both anti-S and anti-PreS1 antibody were elicited and may provide more extensive protection against HBV infection. Because of its efficient yield and secretion, and its good antigenicity and immunogenicity, we conclude that the SA-28 protein, harbouring the hepatocyte receptor-binding site on the surface of HBsAg particles, could be a promising candidate as a new vaccine with higher efficacy.
The authors would like to thank W. H. Gerlich for providing us with monoclonal antibody MA18/7, Z. X. Gong & J. Y. Shen for help in electron microscopy, and Y. D. Zhang for critical reading of the manuscript. This research was supported by National High Technology Program.

References


(Received 25 April 1994; Accepted 29 July 1994)