Processing of hepatitis B virus surface antigen expressed by recombinant Oka varicella vaccine virus

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We have constructed a recombinant Oka varicella vaccine virus expressing hepatitis B virus (HBV) surface antigen (HBsAg). HBsAg was synthesized as 26K and 30K proteins in infected cells and secreted into the culture supernatant as 30K and 35K proteins. Inhibitors and glycosidase treatments, and pulse–chase labelling experiments, revealed the glycosylation process of HBsAg. The latter was synthesized as a non-glycosylated 26K protein and subjected to N-linked glycosylation to form a 30K protein with high mannose glycans. Three species of dimers composed of 26K and 30K subunits were then formed with disulphide bonds. Both subunits of the dimers were further subjected to O-linked glycosylation and conversion from high mannose glycans to complex glycans followed by sialylation. Three species of dimers composed of 30K and 35K subunits were secreted into the culture supernatant as HBsAg particles. HBsAg was synthesized, glycosylated with both N- and O-linked glycans, sialylated, and then secreted into the culture supernatant within 1 h. These modifications of HBsAg by glycans might stabilize its structure and enhance its immunogenicity as a live HBV vaccine.

Introduction

Hepatitis B virus (HBV) surface antigen (HBsAg) has been genetically expressed in bacteria (Pasek et al., 1979), yeast (Valenzuela et al., 1982), and mammalian cells (Dubois et al., 1980; Marion et al., 1979; Patzer et al., 1984, 1986). Expression of HBsAg has been studied in Xenopus oocytes (Persing et al., 1986; Standring et al., 1986), and regulation and pathological effects of HBsAg expression in the tissues have been analysed in transgenic mice (Babinet et al., 1985). HBsAg has been expressed and produced in insect cells by a baculovirus vector (Kang et al., 1987). Recombinant animal viruses expressing HBsAg have been constructed by using vaccinia virus (Smith et al., 1983), adenovirus (Davis et al., 1985; Molnar-Kimber et al., 1988), herpes simplex virus (Shih et al., 1984), simian virus 40 (Laub et al., 1983; Moriarty et al., 1981) and papillomavirus (Hsiung et al. 1984).

We have constructed a recombinant Oka varicella vaccine virus expressing HBsAg which has been shown to be immunogenic as a live varicella and HBV vaccine (Shiraki et al., 1991). The expected HBsAg should initiate at the ATG of the varicella-zoster virus (VZV) thymidine kinase gene followed by a serine codon created at the insertion site, 25 amino acids of preS2, and the entire S peptide. HBsAg was synthesized in the form of 26K and 30K proteins in the cells and secreted as HBsAg particles composed of 30K and 35K proteins into the culture medium. These changes in Mr, may be mainly due to glycosylation which has been recognized as an important feature of HBsAg in maintaining its structure and enhancing its immunogenicity (Burrell et al., 1973, 1976; Mishiro et al., 1980; Neurath et al., 1975; Peterson et al., 1982; Shiraishi et al., 1977). We have clarified the glycosylation process of HBsAg by using inhibitors and glycosidases, and the unique features of glycosylation by using VZV as an HBsAg expression vector in this study.

Methods

Cells and virus. Human embryonic lung (HEL) cells were grown and maintained in MEM supplemented with 10% and 3% foetal bovine serum, respectively. Recombinant Oka varicella vaccine virus expressing HBsAg (Shiraki et al., 1991) was used.

Analysis of HBsAg induced in recombinant virus-infected cultures. The recombinant varicella virus-infected cells were treated with 5 &micro;g/ml of tunicamycin or 1 &micro;m-monomensin for 2 h. The cultures were then labelled with 50 &micro;Ci/ml of [35S]methionine and cysteine in medium without methionine for 4 h in the presence of each inhibitor. HBsAg was also labelled with 50 &micro;Ci/ml of [3H]glucosamine in medium without glucose in the absence or presence of tunicamycin or monensin. The labelled...
cells were sonically lysed with RIPA buffer (20 mM-Tris-HCl pH 8.0, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM-NaCl and 1 mM-PMSF) followed by centrifugation at 35000 r.p.m. for 1 h. Culture supernatants of labelled cells were centrifuged at 3000 r.p.m. for 20 min. Both supernatants were mixed with anti-HBs rabbit serum and the immune complexes were separated on Protein A-Sepharose CL-4B and solubilized in the sample buffer (50 mM-Tris-HCl pH 8.2, 1% SDS, 10% glycerol and 0.01% phenol red) with or without 5% 2-mercaptoethanol by boiling for 3 min (Shiraki et al., 1991; Shiraki & Hyman, 1987). The immune complexes were analysed by SDS-PAGE under non-reducing and reducing conditions.

Digestion with glycosidases. HBsAg was prepared from the infected culture labelled with [35S]methionine and cysteine for 6 h. The immunoprecipitates with anti-HBs serum were digested with N-glycosidase F (N-glycosidase), endo-β-N-acetylgalactosaminidase (O-glycosidase), neuraminidase and endoglycosidase H (endo H). Glycosidases used were purchased from Boehringer Mannheim.

Pulse-chase labelling experiment. Infected cells were labelled with [35S]methionine and cysteine for 5 min and the HBsAg synthesized was analysed by SDS-PAGE. Infected cells were labelled with [35S]methionine and cysteine for 10 min and incubated with unlabelled medium for 4 h after washing three times with unlabelled medium. HBsAg in the cells and their supernatant was analysed by SDS-PAGE at the indicated times shown in Fig. 6.

Results

Effects of tunicamycin and monensin on the formation and secretion of HBsAg

Fig. 1 (a) shows the HBsAg synthesized in the cells and secreted into the supernatants in the presence of tunicamycin and monensin. HBsAg was synthesized as 26K and 30K proteins and secreted as 30K and 35K proteins in the absence of inhibitors. The supernatant 30K and 35K proteins were glycosylated but cellular proteins labelled with [3H]glucosamine were barely detected in repeated experiments. Tunicamycin inhibited the synthesis of 30K in the cells and 35K in the culture supernatant. The supernatant 30K and 35K were glycosylated, suggesting the occurrence of tunicamycin-resistant glycosylation. Monensin inhibited the Mr shift of HBsAg (26K and 30K to 30K and 35K, respectively) during secretion. Glycosylated HBsAg was similarly detected in both the cells and the supernatant. Detection of cellular glycosylated HBsAg was due to the accumulation of HBsAg by the inhibition of transport. The detected glycoprotein corresponding to 26K showed a slightly slower electrophoretic mobility than the 35S-labelled 26K. This suggested the presence of a monensin-resistant glycosylation process modifying HBsAg.

HBsAgs with an Mr of 50K to 65K were detected under the non-reducing conditions (Fig. 1 b). This suggested that HBsAg was present in the cells and supernatant as dimers formed via disulphide bonds. Some HBsAg was also present as monomeric forms which had the same mobility as that detected under the reducing conditions. The Mr of the dimers were proportional to those of the monomers under either of the conditions used.

Fig. 2 shows the compositions of dimers formed in...
cells and supernatants. The dimer fractions with the slower, medium and faster mobilities were composed mainly of the 26K–26K, 26K–30K and 30K–30K dimers in infected cells, respectively (Fig. 2a). Similarly, the dimer fractions with the slower, medium and faster mobilities were composed mainly of the 30K–30K, 30K–35K and 35K–35K dimers in the supernatants, respectively (Fig. 2b). Thus three species of HBsAg dimers were formed in infected cells and secreted into the supernatant.

Glycosidase treatment of HBsAg

Fig. 3 shows the effects of glycosidases on the $M_r$ of cellular and supernatant HBsAg. Cellular HBsAg was sensitive to $N$-glycosidase but resistant to $O$-glycosidase and neuraminidase. Supernatant HBsAg was sensitive to $N$-glycosidase, $O$-glycosidase and neuraminidase. The shifts of the $M_r$ from 30K to 26K of cellular HBsAg and from 35K to 30K of the supernatant HBsAg indicated that both cellular 30K and supernatant 35K had $N$-linked glycosylation, which accounted for the only difference in the subunit structure between 26K and 30K in infected cells and between 30K and 35K in the supernatant. As supernatant HBsAg was sensitive to $O$-glycosidase and neuraminidase, it had $O$-linked glycosylation and sialylation in its structure. After $N$-glycosidase

Fig. 3. Intracellular (Cell) and culture supernatant (Supernatant) HBsAgs labelled with $[^{35}S]$methionine and cysteine. HBsAg (control) was treated with $N$-glycosidase ($N$-Gly), $O$-glycosidase ($O$-Gly) and neuraminidase (Neu), and analysed by SDS-PAGE.
treatment 30K was sensitive to neuraminidase (Fig. 4a). Supernatant HBsAg (35K) was sensitive to neuraminidase after O-glycosidase treatment (Fig. 4b). These results indicated that both N- and O-linked glycans had sialylation groups in their structures.

To characterize the N-linked glycosylation of HBsAg, cellular and supernatant HBsAgs were treated with endo H which cleaves high mannose glycans of glycoproteins but not complex glycans. Cellular HBsAg was sensitive to endo H but supernatant HBsAg was resistant to it (Fig. 5). Thus HBsAg was first modified by high mannose glycans in the infected cells and converted to complex glycans in the supernatant.

Pulse–chase labelling experiments

HBsAg synthesized during the 5 min pulse labelling period was analysed under non-reducing and reducing conditions (Fig 6a). Monomeric 26K and 30K were detected in greater amounts than dimeric forms in contrast to the results with a long labelling period (Fig 1b). This suggested that dimerization might follow the synthesis of 26K and glycosylation of 26K to 30K. HBsAg was synthesized as 26K and 30K in 10 min and secreted as 30K and 35K into the supernatant in 1 h (Fig. 6b); neither 30K nor 35K was detected in the cell fraction. This indicated that 26K and 30K were rapidly processed and secreted into the culture supernatant as 30K and 35K.

Discussion

HBsAgs are composed of large, middle and small antigens, which are encoded by the preS(1 + 2) and S genes, the preS2 and S genes, and the S gene, respectively.
Glycosylation of HBsAg expressed by VZV

Fig. 5. Endo H treatment (lanes 2 and 4) of cellular (lanes 1 and 2) and supernatant (lanes 3 and 4) HBsAg. Lanes 1 and 3, not treated with Endo H.

(Heermann et al., 1984; Laub et al., 1983; Neurath et al., 1985; Stibbe & Gerlich, 1983). Large surface antigens are synthesized but not efficiently secreted into the culture medium of cells expressing the large surface antigen or that of cells infected with recombinant vaccinia virus (Cheng et al., 1986; Cheng & Moss, 1987) or recombinant adenovirus (Molnar-Kimber et al., 1988). Middle and small surface antigens are expressed and secreted into the supernatant in expression systems using eukaryotic cells and recombinant viruses. Our recombinant virus was designed to express the partial preS2 (25 of 55 amino acids) and S gene in HEL cells. Therefore secretion of HBsAg into the supernatant would be expected in this system. However, HBsAgs produced in yeast or expressed in *Xenopus* oocytes are not efficiently secreted into the culture medium (Persing et al., 1986; Standring et al., 1986; Valenzuela et al., 1982). Thus secretion of HBsAg may depend on the construction of the HBsAg genome and expression systems.

Interestingly, HBsAgs were synthesized as 26K and 30K and secreted as 30K and 35K in the culture supernatant. HBsAg expressed by various other recombinant viruses is secreted into the supernatant without major $M_r$ shifts. HBsAg expressed in CHO cells transfected with the gene encoding HBsAg was synthesized as unglycosylated P20 and glycosylated G23, and secreted into the supernatant as P20 and G25. The $M_r$ shift was sensitive to monensin and mainly due to sialylation (Patzer et al., 1984). Therefore the $M_r$ shift of 26K and 30K to 30K and 35K by O-glycosylation and sialylation during secretion was unique to this expression system.

The process of glycosylation is summarized in Fig. 7. HBsAg was first synthesized as non-glycosylated 26K and then part of it was subjected to a high mannose type glycosylation to form 30K. Then the 26K and 30K subunits formed three species of dimers (26K–26K, 26K–30K and 30K–30K) linked by disulphide bonds (Burrell et al., 1976; Imai et al., 1974; Mishiro et al., 1980; Peterson et al., 1982; Sukeno et al., 1972). Both subunits were subjected to O-linked glycosylation and their high mannose glycans were converted to complex glycans. Thus the secreted HBsAg had sialylation in both N- and O-glycans, and disulphide bonds to form three species of dimers (30K–30K, 30K–35K and 35K–35K) in HBsAg particles. HBsAg purified from plasma also contains N-linked glycosylation with sialylation (Burrell et al., 1973, Neurath et al., 1975; Peterson et al., 1982; Shiraishi et al., 1977, Stibbe & Gerlich, 1983). These modifications of HBsAg by glycans might help maintain its structure and enhance its immunogenicity as a live HBV vaccine.

Monensin inhibits sialylation but allows O-linked glycosylation through the Golgi apparatus (Alonso & Compans, 1981; Johnson & Schlesinger, 1980; Grose, 1990; Patzer et al., 1984, 1986). O-linked glycosylation was confirmed with O-glycosidase treatment (Fig. 3, 4, 5) and a slight increase in the $M_r$ (26K and 30K) by labelling with $[^3H]$glucosamine was observed in the presence of monensin (Fig. 1a). Therefore this partial glycosylation of 26K and 30K in monensin-treated cells might be O-linked glycosylation. Both N- and O-glycans of supernatant HBsAg were sialylated, which coincided with the conversion of high mannose glycans to complex glycans.

The pathways for intracellular processing and transport to the cell surface have been studied and each glycoprotein matures at different rates or in different ways (Gumbiner & Kelly, 1982; Knipe et al., 1977; Patzer et al., 1984; Strous & Lodish, 1980). HBsAg expressed in CHO cells is secreted at its own rate with a relatively long half-time (approx. 5 h) (Patzer et al., 1984). In contrast, HBsAg was subjected to O-linked glycosylation and secreted within 1 h in the present expression system. On the other hand VZV glycoproteins gpI and gpII have both N- and O-linked glycans and
Fig. 6. Pulse labelling and pulse-chase labelling of HBsAg. (a) HBsAgs synthesized in 5 min were analysed under non-reducing (without 2-mercaptoethanol, lane 1) and reducing (with 2-mercaptoethanol, lane 2) (b) Infected cells labelled with [35S]methionine and cysteine for 10 min were incubated in unlabelled medium and HBsAg in the cells (C) and their culture supernatants (S) was analysed at the indicated times.

Fig. 7. Glycosylation processing of HBsAg expressed by the recombinant varicella virus. Disulphide bonds are indicated by s-s.

O-linked glycosylation is specific to this system and that HBsAg might be processed by a pathway unique to HBsAgs rather than that of VZV glycoproteins.

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References


sialic acid residues, but gpIII has N-linked glycans and not O-linked glycans (Grose, 1990; Namazue et al., 1985). Therefore the glycosylation of HBsAg was similar to that of gpI and gpII but not to that of gpIII which is secreted into the culture supernatant (Shiraki & Takahashi, 1982). These observations suggest that


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