Biological significance of amino acid substitutions in hepatitis B surface antigen (HBsAg) for glycosylation, secretion, antigenicity and immunogenicity of HBsAg and hepatitis B virus replication

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Amino acid substitutions of hepatitis B surface antigen (HBsAg) may affect the antigenicity and immunogenicity of HBsAg, leading to immune escape and diagnostic failure. The amino acid positions 122 and 160 are known as determinants for HBsAg subtypes d/y and w/r, respectively. The substitution K122I has been shown to strongly affect HBsAg antigenicity. In this study, we investigated the significance of naturally occurring amino acid substitutions K122I, T123N, A159G and K160N. Both T123N and K160N substitutions resulted in additional N-glycosylated forms of HBsAg, while the other mutations produced more glycosylated HBsAg compared with the wild type (wt). Detection of HBsAg by ELISA and immunofluorescence staining indicated that variant HBsAg (vtHBsAg) with K122I was not recognized by HBsAg immunoassays, while vtHBsAg with T123N, A159G, K160N and A159G/K160N had reduced antigenicity. DNA immunization in BALB/c mice revealed that wtHBsAg and vtHBsAg with T123N and K160N are able to induce antibodies to HBsAg (anti-HBs), whereas K122I and A159G greatly impair the ability of HBsAg to trigger anti-HBs responses. The cellular immune response to the HBsAg aa 29–38 epitope was enhanced by the K160N substitution. Using replication competent clones of hepatitis B virus (HBV), T123N and A159G substitutions were shown to strongly reduce virion assembly. The amino acid substitution K160N appeared to compensate for the negative effect of A159G on virion production. These results reveal complex effects of amino acid substitutions on biochemical properties of HBsAg, on antigenicity and immunogenicity, and on the replication of HBV.

INTRODUCTION

The three surface antigens of the hepatitis B virus (HBV) (L-, M- and S-HBsAg) share 226 carboxyl-terminal amino acid residues. The aa 99–169 central region of S-HBsAg has been designated as a major hydrophilic region (MHR) and harbours many B-cell epitopes. In particular, the immunodominant aa 124–147 ‘a’ determinant in HBsAg represents a cluster of epitopes targeted by neutralizing antibodies. A number of variant HBsAgs with amino acid substitutions within or around the ‘a’ determinant have been found in patients. Some have shown reduced affinity for monoclonal antibodies (mAbs) to HBsAg (anti-HBs) or they may escape detection by commercial HBsAg ELISA assays (Carman, 1997; Carman et al., 1990, 1999; Cooreman et al., 2001; Gunther et al., 1999; Lu & Lorentz, 2003; Seddigh-Tonekaboni et al., 2000).

The amino acid residues at positions 122 and 160 of HBsAg are known as determinants for HBsAg serotypes d/y and w/r, respectively. HBsAg serotypes d/y and w/r are defined by amino acid residues Arg/Lys at position 122 and Arg/Lys at...
position 160 (Kay & Zoulim, 2007; Okamoto et al., 1987). Substitutions at positions 122 and 160 have been identified in different cases and were found to be associated with immune escape or diagnostic failure. Substitutions at the position 122 occurred in chronically HBV-infected patients that were tested negative for HBsAg (Alexopoulou et al., 2004; Grethe et al., 1998; Hou et al., 2001; Weinerberger et al., 2000). Tian et al. (2007) further confirmed that the K122I substitution had a major influence on HBsAg antigenicity. HBV isolates with a Lys to Asn substitution at amino acid position 160 (K160N) were identified in a renal transplant recipient who developed de novo HBV infection despite pre-existing protective anti-HBs (Lu & Lorentz, 2003). The K160N substitution was accompanied by an Ala to Gly substitution at position 159 (A159G). The variant HBsAg isolated from this patient had reduced antigenicity and immunogenicity. However, the roles of amino acid substitutions K160N and A159G in antigenicity and immunogenicity are not clearly defined. Substitutions to Asn were frequently found in HBsAg, for example, at the position 123 in a liver transplant patient during the treatment with anti-HBs immunoglobulin (Carman et al., 1999) or in patients with atypical serological profiles for HBV infection (Hou et al., 2001). The recognition of such variants by anti-HBs appeared to be reduced (Ireland et al., 2000; Tian et al., 2007). These published data indicate that such amino acid substitutions may occur in HBV isolates of different genotypes.

In this study, we investigated the significance of amino acid substitutions at positions 122, 123, 159, and 160 of HBsAg for the antigenicity and immunogenicity of HBsAg. We generated a series of expression constructs of variant HBsAg (vtHBsAg) with amino acid substitutions A159G, K160N, and A159G/K160N. The expression of vtHBsAg was examined by transient transfection in hepatoma cells and was detected by specific HBsAg immunoassays and immunofluorescence (IF) staining with anti-HBs antibodies. The ability of vtHBsAg to induce anti-HBs was determined by in vivo DNA immunization to assess the influence of single or combined mutations on the immunogenicity of HBsAg. Finally, replication competent HBV clones with corresponding amino acid substitutions were constructed to study the impact of the amino acid substitutions on HBV replication and viral assembly. Replication competent clones were transfected into hepatoma cells and intracellular HBV replication intermediates and virion DNA released in supernatants were detected.

**RESULTS**

**Expression of wild type (wt)- and vtHBsAg in transfected mammalian cells**

Western blot analysis was performed to detect wt- and vtHBsAg in culture supernatants and cell lysates of transiently transfected Huh7 cells using a specific antibody targeting the haemagglutinin (HA)-tag (Fig. 2a, b). The wtHBsAg and vtHBsAgs with K122I, A159G and A159G/K160N substitutions showed two bands with sizes 29 and 26 kDa that most likely represented HBsAg with and without glycosylation (Peterson, 1981; Tian et al., 2007). These proteins had slightly higher molecular masses than natural HBsAg due to the HA-tag. However, the 29 kDa band was stronger for vtHBsAgs with amino acid substitutions K122I, A159G and A159G/K160N than for wtHBsAg. An additional band with a size of about 32 kDa was detected for vtHBsAgs with amino acid substitutions T123N and K160N (Fig. 2a, b). Comparable results were obtained by using HeLa cells (data not shown).

The amino acid substitutions T123N and K160N may therefore have created new sites for N-glycosylation that led to the generation of the 32 kDa band. It has long been established that the classical N-glycosylation motif is Asn-X-Ser/Thr (with X not Pro, Gavel & von Heijne, 1990; Kaplan et al., 1987; Marshall, 1974; Mellquist et al., 1998). While the T123N substitution created the sequence Asn-Cys-Thr corresponding to the N-glycosylation motif, the K160N substitution resulted in the sequence Asn-Tyr-Leu that is atypical for a N-glycosylation site. To further investigate the glycosylation of vtHBsAg, Huh7 cells were treated with the glycosylation inhibitor tunicamycin after transfection with the expression vectors. As shown in Fig. 2(c), only the 26 kDa band was detected for wtHBsAg and all five vtHBsAgs. This indicates that the 29 and 32 kDa bands arose from glycosylation of 26 kDa HBsAg, as reported previously (Peterson, 1981; Tian et al., 2007). To identify the form of glycosylation, proteins obtained from transfected Huh7 cells were treated with N-glycosidase F (PNGase F) to remove N-linked glycans or with endoglycosidase H (EndoH) to specifically cleave high mannose and certain types of hybrid sugars, respectively. Fig. 2(d, e) shows that only the 26 kDa band was detected for wtHBsAg and all five vtHBsAgs in either PNGase F- or EndoH-treated samples. These results clearly demonstrate that the amino acid substitutions T123N and K160N create new sites for N-glycosylation. Consistently, glycosylated forms of HBsAg were predominant in culture supernatants (Fig. 2a).

**Reactivity of wt- and vtHBsAgs using commercial ELISA kit and ELISAs based on anti-HBs mAbs**

It was shown previously and in Fig. 3(a) that wtHBsAg with an HA-tag was recognized by anti-HBs antibodies and in HBsAg ELISAs (Tian et al., 2007). Here, wt- and vtHBsAg were expressed, vtHBsAg with amino acid substitution K122I was not detectable in culture supernatants and cell lysates. Although vtHBsAgs were expressed at a comparable level in cell lysates, vtHBsAg K122I was not reactive in several commercial ELISA kits used so far. A low amount of vtHBsAg K122I was detected in the cell supernatant by Western blot analysis (Fig. 2a).

The amino acid substitution K122I strongly impaired the reactivity of their respective vtHBsAgs in commercial HBsAg detection assays (Fig. 3a). Similar results were
obtained with culture supernatants and lysates of transfected cells. The wtHBsAg and vtHBsAgs with substitutions T123N, A159G, K160N and A159G/K160N were clearly recognized by the commercial ELISA kit, indicating that the protein was assembled properly and secreted into the culture medium (Fig. 3a, b).

The vtHBsAgs with substitutions of A159G, K160N and A159G/K160N had different reactivities to anti-HBs mAbs. Although reduced reactivity of these vtHBsAgs was observed in ELISAs based on mAbs S1, 2C8 and D12, vtHBsAgs with K160N and A159G/K160N were recognized well by an ELISA based on the 1C10 anti-HBs mAb and produced a higher S/N ratio when compared with wtHBsAg (Fig. 3b). Consistent with previous findings, no reactivity was found for vtHBsAgs with K122I and T123N substitutions with any mAbs when culture supernatants of transfected cells were tested (Fig. 3b).

Different subcellular distributions of wt- and vtHBsAg

The vtHBsAg with the T123N substitution was shown to accumulate in the perinuclear region by using IF staining with an anti-HBs mAb (Tian et al., 2007). We refined the IF staining with the anti-HA mAb to detect all HBsAgs expressed in transfected cells and examined the subcellular distribution of wt- and vtHBsAgs (Fig. 4). The wtHBsAg and all vtHBsAgs including T123N, A159G, K160N and A159G/K160N were positively stained by anti-HA mAb with comparable intensities (Fig. 4b, d, f, h, j). This result was consistent with the previous finding that the amounts of wt- and vtHBsAgs produced in transfected cells were comparable (Fig. 2). The staining showed an even distribution through the cytoplasm of transfected cells for wt- and vtHBsAgs. The S1 anti-HBs mAb was able to recognize wtHBsAg and vtHBsAgs with amino acid substitutions T123N, A159G, K160N and A159G/K160N (Fig. 4a, c, e, g, i). IF staining of wtHBsAg with the anti-HBs mAb showed a diffuse intracellular distribution. It is notable that IF staining with the anti-HBs mAb showed a perinuclear distribution for wtHBsAg with T123N, consistent with the previous observation (Tian et al., 2007; Fig. 4c). The positive IF staining of wtHBsAg T123N with anti-HBs mAb was probably due to the high antibody concentration used in the assay, allowing sufficient binding of mAb to wtHBsAg. Although wtHBsAg with K160N was hyperglycosylated similar to T123N, it showed a diffuse distribution in cells similar to other wtHBsAgs (Fig. 4g). The wtHBsAg K122I was detected by using the anti-HA mAb but not the anti-HBs mAb (data not shown), which is consistent with a previous report (Tian et al., 2007). Taken together, IF staining with the anti-HA mAb showed that all HBsAgs variants were expressed in the cytoplasmic compartment. However, HBsAg variants differed in their detectability by the anti-HBs mAb recognizing HBsAg ‘a’ determinant. The fraction of HBsAg detected by the anti-HBs mAb appeared to be located differently from that detected by the anti-HA, as visible in the case of T123N. This observation may be explained by the association of folded HBsAg with the cellular ER/Golgi system. However, an optic effect of different staining intensities is not completely ruled out.

Induction of anti-HBs antibodies by wt- and vtHBsAgs

We next asked whether vtHBsAgs with amino acid substitutions could induce production of antibodies against HBsAg. Genetic immunizations in mice were performed to test the ability of wt- and vtHBsAg to induce anti-HBs antibody responses. Six groups of six mice were each immunized with plasmids pHbsAg-WT, pHbsAg-K122I, pHbsAg-T123N, pHbsAg-A159G, pHbsAg-K160N, and pHbsAg-A159G/K160N. All six plasmids were able to induce anti-HBs responses in mice but with different efficiencies as measured by using an anti-HBs commercial kit (Fig. 5). Notably, the commercial anti-HBs assay is primarily designed to detect antibodies specifically against wtHBsAg and may not recognize such antibodies only reactive with vtHBsAg. Mice immunized with plasmids expressing wtHBsAg and vtHBsAgs with the substitutions T123N and K160N showed an anti-HBs response with an average titre over 500 IU l\(^{-1}\), comparable to previous studies (Lu & Lorentz, 2003). The ability of vtHBsAgs with the substitutions K122I, A159G and A159G/K160N to induce anti-HBs antibody responses against wtHBsAg was greatly impaired. Although five of six mice developed anti-HBs titres after immunization with pHbsAg-K122I, these mice showed very low antibody titres compared with pHbsAg-WT (Fig. 5). Only one mouse from each group showed measurable anti-HBs titres after immunization with pHbsAg-A159G and pHbsAg-A159G/K160N (Fig. 5). These results indicate that changes in HBsAg glycosylation by T123N and K160N substitutions did not impair the immunogenicity of the corresponding vtHBsAgs. However, induction of anti-HBs antibodies was not efficient when K122I and A159G substitutions were present in HBsAg.

Characterization of cellular responses to HBsAg induced by wt- and vtHBsAgs

The question was raised as to whether the amino acid substitutions in HBsAg influence the induction of cell-mediated immune responses to HBsAg since vtHBsAgs are biochemically different to wtHBsAg. The cellular immunity elicited by the different variants was measured by using an ELISPOT assay 3 weeks after the last immunization. The major H-2L\(^{d}\) CTL epitope HBsAg aa 29–38 was used to stimulate antigen-specific gamma interferon (IFN-\(\gamma\)) secretion from splenocytes (Zheng et al., 2004). As shown in Fig. 6, all six different plasmids expressing wt- and vtHBsAg induced specific cellular responses to the H-2L\(^{d}\) CTL epitope of HBsAg. Notably, the most vigorous responses were observed in BALB/c mice from the A159G/K160N- and K160N-injected groups (in A159G/K160N, \(P<0.001\); in K160N, \(P<0.01\)) as compared with wt- and other vtHBsAgs.
Detection of HBV DNA in culture supernatants and transfected cells

The substitutions K122I, T123N and K160N in S-HBsAg in the present study led to the substitutions rtQ476H, rtN477K and rtI515L in HBV polymerase, K285I, T286N and K323N in L-HBsAg, and K178I, T179N and K215N in M-HBsAg, respectively. Thus, the impact of amino acid substitutions on viral replication and assembly was examined. Encapsidated HBV replicative intermediates were extracted from Huh7 cells transfected with replication competent clones and subjected to Southern blot analysis (Fig. 7a). No apparent differences were noted for HBV produced with wt- or vtHBsAg. We further quantified the amount of virion-associated HBV DNA in culture supernatants to analyse virion assembly and secretion. The amounts of virion-associated HBV DNA from cells transfected with pHBV1.3-T123N, -A159G, -K160N and -A159G/K160N were about 35, 17, 52 and 47 % of that from cells transfected with pHBV1.3-WT, respectively (Fig. 7b). Transfection with pHBV1.3-K122I produced the same amount of virion-associated HBV DNA as transfection with pHBV1.3-WT, despite the low antigenicity and immunogenicity of K122I VTBSAg. These observations indicate that the HBsAg T123N, A159G and K160N substitutions examined in this study may influence the assembly and secretion of the HBV virion, but not the formation of HBV replication intermediates.

It has been reported that transfection of replication competent clones into hepatoma cells may lead to the production and release of naked cores (Parekh et al., 2003). Therefore, virions and naked cores in culture supernatants of transfected Huh7 cells were separated by CsCl gradient ultracentrifugation and their relative proportions were determined by real-time PCR. The results indicated that the fractions of naked cores ranged between 1 and 13.1 % of the corresponding total HBV DNA amounts in supernatants (Supplementary Table S2, available in JGV Online).

DISCUSSION

In the present study, the significance of amino acid substitutions at positions 122, 123, 159 and 160 of HBsAg for glycosylation, secretion, antigenicity and immunogenicity of HBsAg and HBV replication was investigated. The results are summarized in Table 1.

We found that amino acid substitutions K122I, T123N, A159G and K160N led to altered glycosylation patterns for HBsAg. Both T123N and K160N substitutions led to the production of extra 32 kDa products that represent new N-glycosylated forms of HBsAg (Fig. 1). VTBSAgs with amino acid substitutions (pHBsAg-A159G, pHBsAg-K160N and pHBsAg-A159G/K160N) was constructed based on pHBsAg-WT. Mutations were introduced into the wtHBsAg sequence (subtype adw2) by PCR-based mutagenesis. Two previously constructed expression vectors pHBsAg-K122I and pHBsAg-T123N expressing VTBSAgs with the substitutions K122I and T123N were also used.

We found that amino acid substitutions K122I, T123N, A159G and K160N led to altered glycosylation patterns for HBsAg. Both T123N and K160N substitutions led to the production of extra 32 kDa products that represent new N-glycosylated forms of HBsAg (Fig. 1). VTBSAgs with amino acid substitutions K122I, A159G and A159G/K160N were detected in their unglycosylated 26 kDa form and also in an N-glycosylated 29 kDa form; these forms have been shown to natively exist for wtHBsAg (Peterson, 1981; Tian et al., 2007). However, the glycosylated 29 kDa form was present in greater proportions for VTBSAgs than for wtHBsAg, indicating that the amino acid substitutions altered the biochemical properties of HBsAg and facilitated glycosylation. Interestingly, the A159G substitution prevented the production of the 32 kDa form of HBsAg that was caused by the K160N substitution. Thus, the co-existence of A159G and K160N may be the result of

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**Table 1.** Summary of characteristics of wtHBsAg and VTBSAgs in the present study

<table>
<thead>
<tr>
<th>HBsAg</th>
<th>Glycosylation</th>
<th>Antibody recognition</th>
<th>Anti-HBs induction</th>
<th>Replication</th>
<th>Virion formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
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<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>K122I</td>
<td>Normal</td>
<td>Impaired</td>
<td>Impaired</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>T123N</td>
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<td>Impaired</td>
<td>Normal</td>
<td>Normal</td>
<td>Reduced</td>
</tr>
<tr>
<td>A159G</td>
<td>Normal</td>
<td>Reduced</td>
<td>Impaired</td>
<td>Normal</td>
<td>Reduced</td>
</tr>
<tr>
<td>K160N</td>
<td>Additional</td>
<td>Reduced</td>
<td>Normal</td>
<td>Normal</td>
<td>Slightly reduced</td>
</tr>
<tr>
<td>A159G/K160N</td>
<td>Normal</td>
<td>Reduced</td>
<td>Impaired</td>
<td>Normal</td>
<td>Slightly reduced</td>
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</tbody>
</table>
Amino acid substitutions in HBsAg

selective pressure for prevention of HBsAg glycosylation at locations other than aa 146.

The exact sites of additional glycosylation for T123N and K160N vHBsAg remain unknown, but the amino acid substitutions at the positions 123 and 160 definitely created two potential glycosylation sites. The consensus sequence Asn-X-Ser/Thr is generally required for N-linked oligosaccharides (Gavel & von Heijne, 1990; Kaplan et al., 1987; Marshall, 1974; Mellquist et al., 1998). The T123N substitution created the sequence Asn-Cys-Thr, which should allow glycosylation. However, the sequence Asn-Tyr-Leu that resulted from the K160N substitution does not represent a typical N-glycosylation motif, as no such site was described in the literature. HBV surface proteins may also be subjected to O-glycosylation based on the hydroxyl group of Ser/Thr (Schmitt et al., 1999). Thus, the possibility exists that the extra 32 kDa product, resulting from the T123N substitution, may represent new O-glycosylated forms of HBsAg. Nevertheless, results of endoglycosidase digestion supported that both vHBsAg

Fig. 2. Expression of HBsAg in Huh7 or HeLa cells transfected with different plasmids. Huh7 cell culture medium (a) and Huh7 cells without (b) or with 4 μg tunicamycin ml⁻¹ (c) were collected at 48 h after transfection with pHBsAg-WT (WT), pHBsAg-K122I (K122I), pHBsAg-T123N (T123N), pHBsAg-A159G (A159G), pHBsAg-K160N (K160N), pHBsAg-A159G/K160N (A159G/ K160N). Cell lysates were prepared and subjected to SDS-PAGE. A mouse mAb to the HA-tag was used for detection of expressed HBsAg. β-Actin was detected as a loading control. ck, Cells transfected with empty plasmid as a negative control. Another two aliquots of Huh7 cell lysates without tunicamycin collected at 48 h after transfection were incubated with 2 μl PNGase F (d) or 2 μl Endo H (e) at 37 °C overnight.

Fig. 3. Reactivity of expressed wt- and vHBsAg to a commercial kit or to mAbs. The wt- and vHBsAg were expressed by transient transfection. The culture supernatants and cell lysates were collected at 48 h after transfection and used for ELISA. The culture supernatants and cell lysates were detected with a commercial kit (Shiye Kehua) (a) and the culture supernatants were detected for ELISA with four mAbs S1, 2C8, D12 and 1C10 (b). The reactivity was expressed as the ratio of samples to the negative control (S/N ratio) with S/N ≥ 2.1 as the cut-off value. The average value of four replicates was calculated and given as the final reactivity of wt or vHBsAg. ck, Cells transfected with empty plasmid as a negative control.
possess additional N-glycosylation but not O-glycosylation, consistent with the creation of the corresponding sites. Further biochemical analysis is necessary to demonstrate that the new glycosylated 32 kDa form of vtHBsAg is indeed glycosylated at amino acid positions 123 and 160.

The S gene overlaps the gene for the viral DNA polymerase. In this study, the substitutions sK122I, sT123N and sK160N in HBsAg led to the substitutions rtQ476H, rtN477K and rtI515L in the polymerase. However, further studies demonstrated that the substitutions T123N and K160N significantly reduced HBV DNA levels in culture supernatants of transfected cells but did not change the intracellular HBV DNA levels, indicating that both substitutions mainly affect the virion assembly and secretion of HBV virions, but not the formation of replication intermediates. Apparently, the mutations on the amino acid sequence of the polymerase due to the substitutions in HBsAg had no phenotypic effect. Thus, the additional glycosylation due to substitutions T123N and K160N may be responsible for altered assembly. Unexpectedly, however, the A159G substitution also impaired virion secretion of HBV, although it did not influence the secretion of HBsAg. Thus, the A159G substitution likely altered the large or middle surface protein such that virions could not be properly assembled. The co-variation of K160N appeared to compensate for the effect of A159G. This may explain the necessity of the co-variation at aa 159 and 160.

The amino acid substitutions K122I and A159G appear to have major impact on the antigenicity and immunogenicity of HBsAg. The residue at aa 122 may be especially critical for the HBsAg conformation and the induction of antibodies reactive to wtHBsAg with the natural conformation. Certainly, vtHBsAgs may induce antibodies reactive to themselves but not to wtHBsAg, as shown previously (Zheng et al., 2004). However, the anti-HBsAg assay used in this study would not allow us to detect antibodies that are reactive exclusively to vtHBsAgs but not wtHBsAg. Thus, the low anti-HBs responses seen for some variants may be due to a lack of expression/secretion of vtHBsAg, and/or a lack of recognition of specific antibodies to vtHBsAg in the anti-HBsAg assay. To solve this issue, a system allowing proper expression of the variant HBsAgs would be required. In contrast, vtHBsAgs with substitutions T123N and K160N retained the ability to induce antibody and cellular responses to wtHBsAg. This is particularly interesting since the T123N substitution reduced the antigenicity of HBsAg. Furthermore, the
A159G substitution also slightly impaired antigenicity and resulted in an anti-HBs antibody response with altered specificity. On the other hand, the double substitution of A159G/K160N more strongly impaired antigenicity and induced an antibody response similar to that observed with the A159G substitution. The results indicate that multiple substitutions may have cumulative effects on the conformation of HBsAg, and that the A159G substitution may have an important impact on the fine specificity of the antibody response.

The K160N substitution enhanced the cellular immune response. Previous studies reported that N-linked glycans of viral proteins play important roles in modulating the immune response (Tai et al., 2002; Wei et al., 2003; Zhang et al., 2004). Thus, alteration of wtHBsAg conformation due to the K160N substitution and its subsequent effect on glycosylation may contribute to cell-mediated immune responses.

The influence of amino acid substitutions on the properties of HBsAg is not explained at the basis of biochemistry of amino acid residues yet. However, no sufficient data about different amino acid substitutions at these particular sites are available for a reasonable analysis. We introduced a number of different amino acid substitutions at the position 145 of HBsAg (unpublished data). The preliminary results indicate that each amino acid substitution may have its specific effect on the conformation of HBsAg. Thus, more experimental data need to be accumulated for the understanding of the influence of each single amino acid substitution.

The double mutation was first identified from a renal transplant recipient who developed de novo HBV infection despite the presence of a pre-existing protective antibody (Lu & Lorentz, 2003). Thus, the concurrence of A159G and K160N in HBsAg may have some biological significance. Our experiments found that the A159G substitution reduced the antigenicity and immunogenicity of HBsAg. However, this substitution also prevented efficient virion production. The concurrence of K160N compensated for the negative effect of A159G on virion production and further reduced the antigenicity of HBsAg. Our results demonstrate the importance of investigating amino acid substitutions in HBsAg in the context of HBV replication. In addition, more attention must be paid to the mutual influences of concurrent amino acid substitutions in HBsAg.

**METHODS**

**Construction of plasmids encoding HBsAg with amino acid substitutions by site-directed mutagenesis.** A series of expression vectors of HBsAg variants with amino acid substitutions at positions 159 or 160 was constructed based on plasmid pHBSAg-WT, which...
was modified from pXF3H (a kind gift from Feng Xinghua, Baylor College of Medicine, Houston, TX, USA) with a wtHBsAg gene [nt 160–841 according to the HBV genome sequence (GenBank accession no. AF282918, adw)] fused with an amino-terminal HA-tag and driven by a cytomegalovirus immediate-early promoter as described previously (Tian et al., 2007). Mutations were introduced into the wtHBsAg sequence by PCR-based mutagenesis using the primer pairs SP1/A159GR or K160NR and SP2/A159GL or K160NL (listed in Supplementary Table S1, available in IGV Online) as described previously (Tian et al., 2007). Plasmids containing mutated sequences were identified by sequence analysis. Three new plasmids (pHBsAg-A159G, pHBsAg-K160N and pHBsAg-A159G/K160N) were produced (Fig. 1a). Two expression vectors pHBsAg-K122I and pHBsAg-T123N for vtHBsAg with K122I and T123N substitutions were isolated from cell culture supernatants at 72 h after transfection and probed with a 32P-labelled full-length HBV genome (a). Virion-associated HBV DNA was isolated from cell culture supernatants at 72 h after transfection for real-time PCR analysis to assess secreted HBV DNA in supernatants. Results are depicted as the per cent of wt control pHBV1.3-WT (b).

For construction of the replication-competent plasmid with amino acid substitutions, the wt HBV 1.3-fold over length plasmid pHBV1.3 (Lei et al., 2006) was used as a backbone. The region nt 252–689 containing amino acid substitutions was amplified from pHBsAg-WT, pHBsAg-K122I, pHBsAg-T123N, pHBsAg-A159G, pHBsAg-K160N and pHBsAg-A159G/K160N, respectively, using primer pairs X51/X52 (Supplementary Table S1) and inserted into the corresponding site in pHBV1.3. These procedures resulted in replication competent clones pHBV1.3-WT, K122I, T123N, A159G, K160N and A159G/K160N. Correct construction of all plasmids was verified by sequence analysis.

Transient transfection and detection of HBsAg by Western blot analysis and ELISA. Expression of HBsAg in Huh7 and HeLa cells by transient transfection and detection of HBsAg by Western blotting and ELISA were performed according to Tian et al. (2007). For Western blotting, a mouse anti-HA mAb (Cell Signaling Technology) was used as the primary antibody. Detection of the reactivity of wt- and vtHBsAg by a commercial HBsAg ELISA kit (Shiye Kehua) was performed according to the manufacturer’s instructions. In addition, four mAbs S1, 2C8, D12 and 1C10 generated to HBV particles were tested against vtHBsAgs. These mAbs were not reactive with denatured HBsAg and are therefore directed to conformational epitopes. Their fine specificities to the epitope on HBsAg are not known.

Tunicamycin treatment. The effects of amino acid substitutions on glycosylation were studied via transient transfection using plasmids expressing wt- and vtHBsAg. Transfected cells were treated with tunicamycin (Sigma), an inhibitor of N-linked glycosylation (Shi & Elliott, 2004). Tunicamycin was dissolved in DMSO at a concentration of 1 mg ml⁻¹ and then added to cells at 6 h after transfection at a final concentration of 4 μg ml⁻¹. An equivalent concentration of DMSO was included in control cultures. The cultures were maintained for a further 48 h until cells were assayed.

Endoglycosidase digestion. PNGase F and Endo H were purchased from New England Biolabs. These enzymes were used to remove N-linked glycans from glycoproteins according to the manufacturer’s protocols. Lysates of transfected cells were prepared by freeze–thaw cycles. After adding Glycoprotein Denaturing Buffer provided with the enzymes, cell lysates were heated to 100 °C for 10 min. Then, G7 Reaction Buffer, NP-40 and PNGase F were added and the reaction mix was incubated overnight at 37 °C followed by Western blot analysis using the anti-HA antibodies. Alternatively, G5 Reaction Buffer and Endo H were added and processed accordingly.

IF staining of transfected cells. IF staining of transfected cells was performed as described previously (Wang et al., 2008). Transfected cells were fixed with 3.7 % paraformaldehyde and permeabilized in 0.2 % Triton X-100. Then the cells were incubated with a mouse anti-HA mAb (Cell Signaling Technology) or anti-HBs mAb S1 as primary antibodies and fluorescein isothiocyanate-labelled secondary antibodies (Novagen) for laser confocal scanning microscopy after cell nuclei were stained with Hoechst.

DNA immunization. Adult female BALB/c mice (6–8-weeks-old) were kept under standard pathogen-free conditions in the Central Animal Laboratory of Wuhan Institute of Virology, Chinese Academy of Sciences and treated following the guidance of animal ethical standard. In vivo electroporation was performed according to a previously described protocol (Chen et al., 2005; Widera et al., 2000). Mice were immunized with 30 μg plasmid DNA dissolved in 30 μl PBS. Control mice were immunized with 30 μl PBS containing 30 μg vector DNA without inserts, or they received 30 μl PBS alone. A pair of electrode needles was inserted into the muscle 5 mm apart to cover the DNA injection site, and electric pulses were delivered using an electric pulse generator (Electro Square Porator T830M; BTX) following injection into the right quadriceps muscle. Three pulses of 100 V each, followed by three pulses of the opposite polarity, were delivered into each injection site at a rate of one pulse per second.

Fig. 7. Analysis of HBV DNA level in culture supernatants and cells. Huh7 cells were transiently transfected with HBV replication competent clones expressing wt- and vtHBsAg: pHBV1.3-WT (WT), pHBV1.3-K122I (K122I), pHBV1.3-T123N (T123N), pHBV1.3-A159G (A159G), pHBV1.3-K160N (K160N) and pHBV1.3-A159G/K160N (A159G/K160N), respectively. ck, Cells transfected with empty plasmid as negative control. Encapsidated HBV replicative intermediates were isolated from transfected cells at 72 h after transfection and probed with a 32P-labelled full-length HBV genome (a). Virion-associated HBV DNA was isolated from cell culture supernatants at 72 h after transfection for real-time PCR analysis to assess secreted HBV DNA in supernatants. Results are depicted as the per cent of wt control pHBV1.3-WT (b).
Each pulse lasted for 50 ms. DNA vaccination was repeated after 3 and 6 weeks using the same procedure.

Measurement of anti-HBs antibody responses. Three weeks after the last immunization, blood samples were collected from anaesthetized animals and sera were recovered by centrifugation. Anti-HBs antibodies in sera were detected using a commercial enzyme immunoassay AUSAB kit (Abbott Laboratory). Tests were performed by a full automatic Architect i2000 (Abbott Laboratory) following the manufacturer’s instructions. The amount of anti-HBs antibody was expressed as IU 1−1.

ELISPot assay. The ELISPot assay was carried out using a Mouse IFN-γ Secreting Cell kit (U-Cytech) according to the manufacturer’s instructions. In brief, 1 × 105 mice splenocytes plus 5 µg ConA (Sigma) ml−1 and 0 or 10 µg HBsAg peptide (H-2Ld CTL epitope aa 29–38) ml−1 were added to each well of 96-well plates coated with anti-IFN-γ mAb and incubated for 18 h at 37 °C in 5 % CO2. Then biotinylated anti-IFN-γ antibody, GABA solution and activator I/II solution were added one by one. Finally, spots were counted using an automated ELISpot plate reader (BioReader 4000) and expressed as the number of spots per 1 × 105 cells.

Detection of virion-associated HBV DNA in culture supernatants. Replication competent HBV clones were transfected into HuH7 cells by using lipofectamine 2000. Virion-associated HBV DNA was isolated from cell culture supernatants at 72 h after transfection for real-time PCR analysis to assess secreted HBV DNA in supernatants. Virion-associated HBV DNA was extracted from cell culture supernatants as described previously (Karayiannis et al., 1995). In brief, the clarified supernatants obtained after low-speed centrifugation were treated with 100 µg DNase I (Promega) ml−1 to remove free DNA, then incubated for 10 min at 65 °C in the presence of stop solution (Promega) to heat-inactivate the enzyme. Then, an equal volume of 20 % PEG 8000 was added to supernatants to precipitate the virions, followed by digestion with 20 µg proteinase K ml−1. Finally, HBV DNA was extracted with phenol/chloroform followed by 2-propanol precipitation and resuspended in TE buffer at pH 8.0 (10 mM Tris/HCl pH 7.5, 1 mM EDTA).

A SYBR Green Mix kit (Toyobo) was used for quantitative PCR according to the manufacturer’s instructions with primer pairs F1/R1 (Supplementary Table S1). PCR parameters were as follows: 10 s at 95 °C, and then 38 cycles of 1 s at 95 °C, 15 s at 61 °C and 15 s at 72 °C. PCR specificity was tested by adding a 2 s data-acquisition step at 82 °C. Additionally, PCR products were loaded onto 1.5 % agarose gels for electrophoresis in order to further confirm the presence of specific bands of the correct size.

The relative proportions of HBV virions and naked cores in cell culture supernatants were determined. In brief, the clarified supernatants obtained after low-speed centrifugation were subjected to CsCl gradient ultracentrifugation to further separate Dane particles from naked core particles as previously described (Parekh et al., 1998). Supernatants obtained after low-speed centrifugation were treated with 100 µg DNase I (Promega) ml−1 to remove free DNA, the solution was subjected to extraction of HBV genome DNA by proteinase K digestion and phenol/chloroform extraction, as described above. The relative proportions of HBV DNA in separated fractions were determined by real-time PCR.

Detection of encapsidated HBV replicative intermediates. Replication competent HBV clones were transfected into HuH7 cells by using lipofectamine 2000. Encapsidated HBV replicative intermediates were isolated from transfected cells at 72 h after transfection and probed with a 32P-labelled full-length HBV genome. Analysis of HBV replicative intermediates was performed as described by Meng et al. (2008). Briefly, cells were lysed in lysis buffer (50 mM Tris/HCl, pH 7.4, 1 mM EDTA, 1 % NP-40). Nuclei were pelleted by centrifugation. The supernatant was adjusted to 10 mM MgCl2 and incubated for 2 h at 37 °C with 100 µg DNase I ml−1 and EDTA at a final concentration of 25 mM to stop the reaction. The supernatant after centrifugation was treated with 0.5 mg proteinase K ml−1 and 1 % SDS for 2 h at 55 °C. Finally, the sample was extracted with phenol/chloroform followed by 2-propanol precipitation. The purified DNA was subjected to Southern blot analysis. DNA samples were probed with a 32P-labelled full-length HBV genome.

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