Identification of a glycosylation site in the woodchuck hepatitis virus preS2 protein and its role in protein trafficking

O. Schildgen,† M. Roggendorf and M. Lu

Institut für Virologie, Universitätsklinikum Essen, Hufelandstraße 55, 45122 Essen, Germany

The middle surface antigen (M-sAg) of hepadnaviruses is one of three envelope proteins that share a common C-terminal S domain. M-sAg contains the preS2 domain in addition to the S region. The preS2 region of woodchuck hepatitis virus (WHV) contains a potential glycosylation site Asn-Gln-Thr at amino acid (aa) positions 3–5. In this study, we mutated this site by site-directed mutagenesis and confirmed that glycosylation occurs here. In in vitro translation assays, the mutation Thr to Asn at aa 5 significantly impaired glycosylation of M-sAg. The mutated M-sAg formed abnormal clustered structures in transfected cells as determined by immunofluorescent staining. Confocal microscopic analysis showed that an enrichment of this glycosylation-deficient protein in the Golgi apparatus occurred, which is not typical for the wild-type protein. These results are consistent with earlier findings that incorrect glycosylation of viral proteins may interfere with virus assembly.

INTRODUCTION

The three surface antigens of mammalian hepadnaviruses are encoded by a region on the viral genome with three different translational starts. The corresponding products, the small, middle and large surface antigens (S-, M- and L-sAg), share a domain of 222–226 aa in length at the C terminus. The major part of the viral envelope of mammalian hepadnaviruses consists of S-sAg. The M- and L-sAg contain the preS2 and preS1–preS2 sequences, respectively, at the N terminus in addition to the S domain. The functions of the L- and M-sAg are not fully understood. It is assumed that the preS1 region on the L-sAg of duck hepatitis B virus contains a region that is important for recognition of a cellular receptor (Urban et al., 1998; Breiner et al., 2001). The N-terminal region of the large hepatitis B virus (HBV) surface antigen (L-HBsAg) is required for infectivity of virions and harbours a determinant of host specificity (Le Seyec et al., 1999; Chouteau et al., 2001). The function of M-HBsAg is unknown. HBV mutants without a functional M-HBsAg have been found in chronically infected patients, indicating that M-HBsAg may be dispensable for the virus life cycle. However, the presence of HBV mutants lacking M-HBsAg is often found to be associated with fulminant hepatitis (Pollicino et al., 1997).

The short N-terminal preS2 region on the M-sAg of mammalian hepadnaviruses has various glycosylation sites.

†Present address: Institute for Medical Microbiology and Immunology, University of Bonn, Sigmund-Freud-Strasse 25, 53105 Bonn, Germany.

Indeed, M-HBsAg and M-sAg of woodchuck hepatitis virus (WHV) possess N- and O-glycans (Tolle et al., 1998; Schmitt et al., 1999). Although the precise function of the glycosylation of the M-sAg of WHV (M-WHsAg) has not been studied, it appears that impaired glycosylation of M-WHsAg may inhibit virus assembly. Block et al. (1998) showed that an inhibitor of α-glucosidase was able to block M-WHsAg processing and intracellular trafficking, thus leading to inhibition of virus assembly. Treatment with an α-glucosidase inhibitor suppressed the production of enveloped virions in woodchucks chronically infected with WHV (Block et al., 1998). Therefore, the glycosylation of viral proteins may represent a target for antiviral therapies.

The sites of glycosylation on the preS2 region of M-WHsAg (WHVpreS2 region) have not been precisely identified. The asparagine (Asn) residue at amino acid (aa) 3 is followed by glutamine-threonine (Gln-Thr) and therefore represents a potential site for N-glycosylation (Tolle et al., 1998). In this work, a substitution of Thr to Asn at aa 5 of the WHVpreS2 region on the M-WHsAg by site-directed mutagenesis eliminated this potential N-glycosylation site. It could be shown that this amino acid substitution impaired glycosylation of M-WHsAg and led to an aberrant accumulation of the mutated protein in the cellular compartment. These results confirmed the assumption that the sequence Asn-Gln-Thr at aa 3–5 of the WHVpreS2 region represents an N-glycosylation site. Furthermore, correct N-glycosylation of M-WHsAg appears to be essential for its intracellular trafficking.
METHODS

Plasmid construction. pMCS3, a chimeric construct of the coding regions for M-WHSAg (nt 107–650 according to Galibert et al., 1982) and HBsAg (nt 509–860 according to Stoll-Becker et al., 1997) was used (Zheng et al., 2002; Fig. 1). This plasmid expresses a middle chimeric surface antigen (MCSAg3) with the WHVpreS2 and the S region including the ‘a’ determinant of HBsAg. The chimeric construct pMCS3 provides two advantages: (i) MCSAg3 can be detected by a polyclonal antibody to the WHVpreS2 region and by any monoclonal antibody (mAb) to the HBsAg ‘a’ determinant, allowing detection and differentiation of both domains separately; (ii) HBsAg is known to contain one glycosylation site at Asn 146. A single mutation at the putative glycosylation site within the WHVpreS2 region was introduced by PCR using the primers WpreS2Ngl (sense: 5′-AAGCTTATGAAAAATCAGAATTTTCA-3′, nt 116–135, according to Galibert et al., 1982, plus a HindIII restriction site, underlined) and HBStop (antisense: 5′-GAATTCCCATCTTTTGTGTTAGG-3′, nt 860–838, according to Stoll-Becker et al., 1997, plus an EcoRI site), resulting in the construct pMCS3Ngl. Another construct, pMCS3PreS2, with a mutation in the WHVpreS2 start codon, was generated using primers WPreS2 (sense: 5′-AAGCTTACGAAAAATCAGACTTATTTCA-3′, nt 116–135, according to Galibert et al., 1982, plus a HindIII restriction site) and HBStop.

PCR was performed in an Eppendorf Cycler Master gradient in a 50 μl volume containing 3.5 mM MgCl2, 2.5 mM KCl, 10 mM Tris/HCl, pH 8.8–8.5, 5 pmol primer each, 2.5 mM dNTP mix and 1.5 U Expand High Fidelity enzyme mix (Roche). PCR was carried out for 35 cycles of 95°C denaturation for 1 min, 48°C annealing for 45 s and 72°C elongation for 1 min, with a final elongation step for 5 min at 72°C. PCR products were cloned with the TOPO-TA cloning kit following the manufacturer’s instructions. The translations were performed with and without the microsomal fraction (Promega) to study post-translational modifications of the in vitro-translated products. Further controls were performed in the presence of 2 μg tunica-mycnax ml−1 for inhibition of N-glycosylation (Sigma) or 2 mM p-nitrophenyl N-acetyl x-D-galactosaminide (Sigma) for inhibition of O-glycosylation.

Transfection of cell lines, indirect immunofluorescence (IF) staining and laser-scanning microscopy. HepG2 or HeLa cells were cultivated in chamber slides (Nunc) and transinfected with the recombinant DNA constructs pMCS3, pMCS3Ngl and pMCS3PreS2 with the Effectene Transfection Reagent (Qiagen) following the manufacturer’s instructions. For each well of a twowell chamber slide, 2 µg plasmid DNA was used for transfection. Transfected cells were incubated for 24–48 h in an incubator at 37°C and 5% CO2. Cells were then washed once with PBS, fixed with 50% methanol (v/v) for 30 min at 4°C, washed twice with PBS and air-dried. Antibodies against HBsAg and markers of the Golgi apparatus (Golgi-Zone; BioTrend) and the endoplasmic reticulum (ERAB, ER antibody binding protein; BioTrend) were used for IF staining at a dilution of 1:100, as recommended by the manufacturer. Cells were incubated with primary antibodies at 37°C for 1 h, washed twice with PBS and further incubated with appropriate secondary antibodies. Secondary antibodies against rabbit, goat or mouse IgGs labelled with Cy2 or Cy3 (Dianova) were used at a 1:200 dilution in PBS containing 0.1% Evan’s blue. Additional incubation for 1 h at 37°C, cells were washed twice and covered with mounting medium. HBsAg stained red and the Golgi stained green (see Fig. 4). Additionally, the ER was stained blue with 100 nM of the ER blue/white tracker (Molecular Probes) 30 min before fixation, according to the manufacturer’s protocol. Stained cells were analysed by confocal laser-scanning microscopy (Carl Zeiss).

BHK cells were transfected and fixed as described above and stained with a monoclonal mouse serum (diluted 1:100) to the HBsAg ‘a’ determinant (Biotrend) or with a polyclonal rabbit serum (Zheng et al., 2002) to the WHVpreS2 domain (diluted 1:20). The secondary antibodies against mouse and rabbit used in this assay (Dianova) were coupled to FITC and used at a dilution of 1:500.

Fig. 1. Construction of plasmids encoding chimeric hepadnaviral surface proteins. The plasmid pMCS3 encodes MCSAg3, a chimeric hepadnaviral surface protein consisting of the WHVpreS2 domain aa 1–60, the N-terminal part of the WHV S domain aa 1–116 and the C-terminus of HBsAg aa 121–226 including the ‘a’ determinant (Zheng et al., 2002). The putative glycosylation site Asn-Gln-Thr at aa 3–5 within the WHVpreS2 region is indicated by an asterisk. The construct pMCS3PreS2 has undergone an aa substitution of Thr to Asn at aa 5 by site-directed mutagenesis, which eliminates the putative glycosylation site. As in the viral genome, the constructs pMCS3 and pMCS3Ngl have a second start codon for the small surface antigen. A control plasmid pMCS3PreS2 was constructed by elimination of the preS2 start codon by site-directed mutagenesis. The construct pMCS3PreS2 is able to express the small surface antigen only.
Fractionation of cellular compartments. HepG2 cells were transfected as described above and harvested 48–72 h post-transfection. The cells were pelleted at 3000 r.p.m. for 10 min in an Eppendorf 5416 centrifuge at room temperature and the pellet was washed twice with ice-cold PBS. The cells were lysed under native conditions by dounce homogenization and sonication and mounted on to a sucrose gradient (20–60 %, w/v) following the recommendations of Rickwood (1992). The cellular compartments were separated by ultracentrifugation at 100,000 g for 4 h. The fractions were collected after centrifugation. For each fraction, both the refractive index and the total amount of protein were determined. According to their density, the fractions were assigned to the cellular compartments ER, Golgi or plasma membrane and spotted on to a nitrocellulose membrane to detect the chimeric proteins by Western blot analysis. The membranes were saturated with fat-free milk powder (10 % in PBS), washed twice with PBS and incubated with primary antibodies against HBsAg, the ER and the Golgi for 1 h at 37 °C. The membranes were washed twice with PBS-T (0.05 % Tween-20 in PBS) and incubated for 1 h with a secondary antibody coupled to alkaline phosphatase. Detection of bound antibodies was carried out using NBT/BCIP.

RESULTS

Construction of chimeric genes of WHV and HBV

Plasmids encoding chimeric proteins containing the WHVpreS2 region and the HBsAg ‘a’ determinant were constructed. The plasmid pMCS3 has been described previously and encodes a chimeric protein consisting of the WHVpreS2 region (aa 1–60), the N-terminal part of WHsAg (aa 1–116) and the C-terminal region of HBsAg (aa 121–226) including the ‘a’ determinant (Fig. 1). Two different monoclonal antibodies to the WHVpreS2 region or to the HBsAg ‘a’ determinant were used to detect the chimeric protein. This approach overcomes the problem that no defined mAb to the C-terminal part of WHsAg is available. The coding region was cloned into pcDNA3 and placed under the control of a bacterial promoter (T7) and a eukaryotic promoter (CMV), allowing both in vitro translation of the chimeric protein and expression in mammalian cells by transient transfection (Fig. 1).

The construct pMCS3 encoded the wild-type WHVpreS2 sequence containing the potential glycosylation site Asn-Gln-Thr at aa 3–5 (Fig. 1). In the construct pMCS3Ngl, the codon for Thr at aa 5 was changed to Asn by site-directed mutagenesis, thus eliminating the potential Asn-Gln-Thr glycosylation site. As an additional control, the preS2 start codon was mutated by site-directed mutagenesis in pMCS3ApreS2, which thus encoded a chimeric protein comprised of aa 1–116 of WHsAg and aa 121–226 of HBsAg.

Mutation of the WHVpreS2 region affects the glycosylation of M-sAg

The chimeric proteins encoded by plasmids pMCS3, pMCS3Ngl and pMCS3ApreS2 were in vitro translated using the TNT-coupled reticulocyte lysate system (Promega). Microsomal fractions were added to the reactions to study post-translational modification of translation products. In addition, translation reactions were carried out in the presence of the glycosylation inhibitors tunicamycin and p-nitrophenyl-N-acetyl-α-D-galactosaminide, which block N- and O-glycosylation, respectively. The in vitro translation products were subjected to SDS-PAGE and visualized by autoradiography (Fig. 2).

Two translation products, unglycosylated MCSAg3 and small CSAg3 were detected by SDS-PAGE using plasmid pMCS3 as a template (Fig. 2, lane 1). These products had molecular masses of 31 and 24 kDa, respectively. A shift towards higher molecular mass translation products was observed in the presence of microsomal fractions, as both MCSAg3 and small CSAg3 were glycosylated (Fig. 2, lane 2). The molecular mass of the glycosylated form of MCSAg3 was up to 41 kDa, consistent with previously published data (Tolle et al., 1998). Intermediate forms of glycosylated MCSAg3 were present, most probably due to the limited capacity of the post-translational modifications in vitro. However, the glycosylated small CSAg3 was not clearly visible in lane 2, since the glycosylated proteins were present at a low level and did not form a clear band on SDS-PAGE (Fig. 2, lanes 7 and 8). The glycosylation of in vitro-translated products was partially inhibited by both N- and O-glycosylation inhibitors (Fig. 2, lanes 3 and 4), consistent with the fact that MCSAg3 contains N- and O-glycosylation sites. These products, with reduced N- or O-glycosylation, had molecular masses between 31 and 41 kDa and therefore migrated to a similar position following SDS-PAGE. The in vitro translation of the plasmid pMCS3Ngl resulted in two unglycosylated proteins (lane 5), as for pMCS3, corresponding to MCSAg3 and small CSAg3. The product of the translation reaction for pMCS3Ngl in the presence of microsomal fractions clearly showed an impaired glycosylation (Fig. 2, lane 6). These results demonstrated that the mutation of Thr to Asn at aa 5 affected the glycosylation of MCSAg3, as predicted. As a control, translation with the control plasmid pMCS3ApreS2 produced a protein band that corresponded to small CSAg3 and shifted to a higher molecular mass in the presence of microsomal fractions (Fig. 2, lanes 7 and 8). Some products with high molecular mass were also present in the in vitro translation assay. The nature of these products is unknown but may be due to the ability of surface antigens to form dimers and multimers, as reported by Tolle et al. (1998).

The mutated M-sAg has an abnormal intracellular distribution

Glycosylation plays an important role in intracellular trafficking and localization of proteins. Therefore, we examined whether mutation of the glycosylation site in the WHVpreS2 domain had any influence on the intracellular localization of the hepadnaviral M-sAg. HepG2 or BHK cells were transfected with the plasmids pMCS3, pMCS3Ngl and pMCS3ApreS2. The cells were fixed and stained with an anti-HBs mAb or a polyclonal antiserum to the WHVpreS2 region (Fig. 3). IF staining of MCSAg3...
with antibodies to the ‘a’ determinant or to the WHVpreS region (Fig. 3, first row) showed a diffuse distribution of the chimeric proteins in the cytoplasm, which is regularly observed for wild-type middle surface antigens of hepadnaviruses (Zheng et al., 2002). Incubation of transfected cells with the glycosylation inhibitors tunicamycin and p-nitrophenyl N-acetyl α-D-galactosaminide led to an abnormal distribution of the wild-type protein MCSAg3 within cells, with the formation of granular structures (Fig. 3, second row). A similar cellular distribution of mutated MCSAg3Ngl expressed by pMCS3Ngl was found in transfected cells (Fig. 3, third row). These results strongly suggest that the absence of glycosylation on the WHVpreS region has a significant impact on the intracellular trafficking and localization of hepadnaviral surface proteins. The plasmid pMCS3Apres2 expressed only small surface antigens, which were positively stained by anti-HBs antibodies but not by antibodies to the WHVpreS2 region (Fig. 3, fourth row). Small CSAg3 showed a diffuse distribution within cells, similar to the wild-type MCSAg3.

**Co-localization of wild-type and mutated surface protein with cellular compartments**

To define the intracellular distribution patterns of wild-type and mutated MCSAg3 proteins further, we performed a triple staining (Fig. 4) of HBsAg (red, HBs), ER (blue) and the Golgi apparatus (green), analysed by confocal laser-scanning microscopy. In the merged staining (optical overlay of the three colours) the co-localization of the ER and HBsAg was characterized by a pink colour, co-localized Golgi and ER components by turquoise, co-localized Golgi and HBsAg by yellow and co-localization of all three components by bright yellow to white.

Fig. 4 shows the results of the confocal microscopic analysis. In the first row, staining of wild-type MCSAg3, ER and Golgi is shown. MCSAg3 was evenly distributed throughout the ER and Golgi. Small CSAg3 showed a similar distribution (Fig. 4, pMCS3Apres2). Again, an inhibition of glycosylation, either by inhibitors or by mutation, led to clustering of MCSAg3 in cells (Fig. 4, pMCS3 + inhibitor and pMCS3Ngl). The prominent white colour in the merged staining indicated that the majority of the MCSAg3 was localized in the ER-cis-Golgi region.

The intracellular distribution of MCSAg3 and MCSAg3Ngl was further examined by fractionation of cellular compartments by isopycnic centrifugation on a sucrose gradient. Transient transfected cells expressing wild-type and mutated MCSAg3 were harvested after 48 or 72 h, lysed and loaded on to a 20–60 % sucrose gradient. After centrifugation,
fractions containing particular cellular compartments – the ER, Golgi and plasma membranes – were selected and analysed for the presence of wild-type and mutated MCSAg3 (Table 1). While wild-type MCSAg3 was detected in association with all three fractions, mutated MCSAg3Ngl was enriched in the Golgi fraction.

**DISCUSSION**

It was assumed that the sequence Asn-Gln-Thr at aa 3–5 of the WHVpreS2 region represented an N-glycosylation site. Sequencing analysis of the WHVpreS2 region has indicated that the Asn residue at this position may be post-translationally modified, as the nature of this amino acid residue could not be identified by sequencing (Tolle et al., 1998). Here we introduced an amino acid substitution of Thr to Asn at the aa 5, which eliminated the glycosylation site. It was clearly demonstrated that this amino acid substitution led to a significant impairment of glycosylation of MCSAg3. In addition, the mutated MCSAg3 appeared to be impaired in its intracellular trafficking. It formed unusual clusters around the ER in the transfected cell

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**Fig. 3.** IF staining of transiently transfected HepG2 cells for chimeric surface antigens. Cells were transfected with the plasmids pMCS3, pMCS3 in the presence of glycosylation inhibitors, pMCS3Ngl or pMCS3ΔpreS2 as indicated. After 48 h, cells were fixed with 50% methanol and subjected to IF staining with an anti-HBs mAb (HBs) or with a polyclonal serum to the WHVpreS2 region (WHpreS2). Arrows indicate the abnormal appearance of MCSAg3 with impaired glycosylation.
lines, while the wild-type MCSAg3 expressed after transient transfection showed a smooth and diffuse distribution over cellular compartments, except the nucleus. The appearance of clusters of mutated MCSAg3 was independent of the cell lines used for the transient expression, as the same results were obtained in HepG2, BHK and HeLa cell lines. Lu et al. (1997) has described similar observations for incorrectly glycosylated HBV proteins. These authors found that a disturbed glycosylation caused altered secretion of viral particles. Thus, misfolding or false processing can lead to an unusual distribution pattern of these proteins. In this study, we defined the intracellular site of clustering in more detail. The ER and the Golgi apparatus are cellular compartments where glycosylation takes place (reviewed

![Confocal microscopic analysis showing co-localization of MCSAg3 with ER and Golgi. HepG2 cells were transfected as described in Methods with the plasmids pMCS3, pMCS3 plus glycosylation inhibitors, pMCS3Ngl, pMCS3ΔpreS2 or pcDNA control as indicated. Triple staining of HBsAg, the ER and Golgi was performed with anti-HBs mAb (HBs), the ER blue/white tracker and antibodies to the Golgi. The final column (merge) shows the overlay of the three stains.](image)

**Table 1.** Isopycnic centrifugation of transient transfected cells expressing MCSAg3 and MCSAg3Ngl on sucrose gradient

*Weak (+), clear (+++) or strong (++++) signal in Western blot assay.

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<th>Sucrose density (g ml(^{-1}))</th>
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<th>Enrichment of MCSAg3Ngl(^*)</th>
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by Munro, 1998; Farquhar & Palade, 1998). The double or triple labelling of ER/Golgi and MCSAg3 by IF staining showed that mutated MCSAg3 clustered in the region between the ER and the Golgi. Interestingly, incubation of transfected cells with glycosylation inhibitors led to accumulation of wild-type MCSAg3 in the same region. It appears that even a partial block of N-glycosylation prevents the transport of MCSAg3 though the Golgi, as other glycosylation sites on the mutated protein were still available.

Based on these results, it appears that the mutant MCSAg3 did not simply misfold leading to aggregation within the cytoplasm, since the synthesized proteins were transported though the ER. In addition, the mutant MCSAg3Ngl was detected by anti-HBs mAbs. The binding of anti-HBs mAbs to the HBsAg ‘a’ determinant is sensitive for misfolding of surface antigens. Thus, this finding supports the conclusion that the folding of MCSAg3 was unimpaired by the introduced mutation.

We attempted to analyse the secretion of wild-type and mutant MCSAg3 from transient transfected cells. However, the production and secretion of MCSAg3 was at a relatively low level (Zheng et al., 2002). Though the secretion of the mutant MCSAg3 appeared to be reduced, we could not make an accurate quantitative determination of the amount of secreted protein.

Taken together our results suggest that hepadnavirus middle surface antigens contain glycosylation modifications that are essential for their biosynthesis and intracellular transport to their final destiny. Consistent with previously published data from other authors, inhibition of N-glycosylation leads to aggregation of proteins and therefore might inhibit virus assembly (Lu et al., 1997; Mehta et al., 1997). Thus, inhibition of glycosylation could serve as a novel therapeutic target, as demonstrated by Block et al. (1998).

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