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Treatment of hepatitis B virus-infected cells with α-glucosidase inhibitors results in production of virions with altered molecular composition and infectivity

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Abstract

Trimming of the *N*-glycans attached to the envelope proteins of hepatitis B virus (HBV) is required in different steps of the viral life cycle. Inhibition of the host enzymes α-glucosidases, involved in the endoplasmic reticulum (ER)-associated processing of the N-linked glycans, results in misfolding of the HBV envelope proteins, prevention of HBV secretion and accumulation of viral DNA within infected cells. However, the impact of these effects on HBV morphogenesis and infectivity of the viral particles that are still released from cells with inhibited α-glucosidase has not been addressed so far. Using *N*-butyldeoxynojirimycin (NB-DNJ), a competitive inhibitor of the ER α-glucosidases, we analyzed the role of these enzymes on HBV assembly and infectivity of the virions released from HepG2.2.2.15 cells. HBV secreted from drug-treated cells contained an envelope with altered composition of the disulfide-linked oligomers and no detectable middle (M) protein. These molecular changes had a significant effect on HBV infectivity, reducing it to 20% compared to controls, for the highest concentrations of NB-DNJ used. Our data show for the first time that an active α-glucosidase activity is crucial for production of infectious HBV and provide new insights into the controversial role of the M protein in this process.

Keywords: HBV; Infectivity: HepaRG cells; Alpha-glucosidase inhibition; NB-DNJ

1. Introduction

Hepatitis B virus (HBV) is an important human pathogen that causes chronic liver disease leading to cirrhosis, hepatocellular carcinoma and death (Beasley, 1988; Hollinger, 1995). HBV is a small virus, member of the Hepadnaviridae family, which contains a partially double stranded DNA genome of approximately 3.5 kb (Leenders et al., 1992). The HBV envelope consists of a lipid membrane derived from the infected host cell in which multiple copies of the viral surface proteins are inserted. These proteins, designated large (L), middle (M) and small (S) are translated from alternative start codons of the same open reading frame (ORF) on the HBV genome and share the same S domain at the C-terminal region (Nassal, 1996). In addition to the S domain, the M protein contains the pre-S2 region, also present in L. The N-terminal region of the L protein further contains the pre-S1 domain, which is unique to this protein (Fig. 1). The virus-encoded envelope proteins are synthesized as glycosylated and nonglycosylated entities, depending on the occupancy of the *N*-glycosylation site at Asn-146 of the S domain. A second *N*-glycosylation site is located at Asn-4 of the pre-S2 domain; however, it is only utilized by the M protein, which is also *O*-glycosylated at Thr-37 in its pre-S2 domain (Heermann and Gerlich, 1992; Werr and Prange, 1998).

The *N*-glycosylation sites are strictly conserved among all HBV genotypes, suggesting an important role of the glycans attached to the envelope proteins in the HBV life cycle (Norder et al., 1994). Indeed, inhibition of *N*-glycosylation or *N*-glycan

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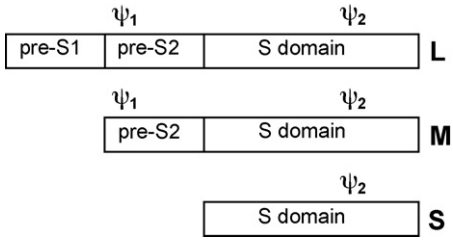

Fig. 1. Schematic representation of HBV envelope glycoproteins. ψ_1 and ψ_2 are potential *N*-glycosylation sites. ψ_1 is only occupied in M glycoprotein. ψ_2 is partially occupied in all HBV envelope glycoproteins.

trimming prevents HBV secretion, implying a role for carbohydrates in the assembly and trafficking of viral glycoproteins (Lu et al., 1997; Mehta et al., 1997). Also, the *N*-glycan-dependent association of the M and L glycoproteins with calnexin, an endoplasmic reticulum (ER)-resident chaperone involved in protein folding (Bergeron et al., 1994) was suggested to play a crucial role in the proper assembly of HBV (Prange et al., 1999; Xu et al., 1997). Calnexin recognizes monoglucosylated glycans attached to newly synthesized proteins, which are produced in the ER by the sequential action of the α-glucosidases I and II (Hebert et al., 1995). Inhibition of glucosidase activity results in proteasomal degradation of L and M proteins, possibly as a consequence of their misfolding when the interaction with calnexin is prevented (Simsek et al., 2005), suggesting a role for the *N*-glycans in the acquirement of the native conformation of the HBV envelope proteins. However, another report showed that the N-linked glycans on the HBV envelope proteins were not important for the infectivity of hepatitis delta virus (HDV) (Sureau et al., 2003), a defective virus which depends on the envelope proteins supplied by HBV for its own assembly (Bonino et al., 1986). Thus, further studies are still needed for understanding the precise role of *N*-glycosylation in the HBV life cycle.

In this study we have used *N*-butyl-deoxynojirimycin (NB-DNJ), an inhibitor of the α-glucosidases, to investigate in more detail the importance of the ER-associated *N*-glycan trimming on HBV assembly and infectivity of the virions released from infected cells. Studies regarding HBV infection *in vitro* have been difficult to carry out so far, because of the lack of susceptible cell lines and also the low infectivity rates, which make the results more difficult to interpret.

Purification and quantification of viral DNA from cell medium confirmed the previously reported inhibitory effect of NB-DNJ on HBV secretion (Block et al., 1994). Western blotting analysis of virions and subviral particles secreted from drug-treated cells showed a decreased content of the L and S proteins, which correlated with the inhibition of HBV secretion, while the M proteins were undetectable at higher drug concentrations. Analysis of the HBV envelope proteins under non-reducing conditions showed an altered composition of the disulfide bond-linked oligomers in the presence of NB-DNJ, compared to controls. The implication of these modifications for the infectivity of HBV was investigated using the newly developed HepaRG cells, which are permissive for HBV infection *in vitro* (Gripon et al., 2002). Our data show for the first time that an active α-glucosidase activity is required for production of infectious HBV.

2. Materials and methods

2.1. Cells, inhibitors and enzymes

HepG2.2.2.15 cells stably transformed with two copies of the HBV genome (Sells et al., 1987) were grown in RPMI 1640 medium (Euroclone, UK) containing 10% fetal bovine serum (Euroclone, UK) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin and 200 μg/ml of G418 (GIBCO, UK). HepaRG cells were grown in William's E medium (GIBCO, UK) supplemented with 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml insulin, and 5×10^5 M hydrocortisone hemisuccinate, as described (Gripon et al., 2002). NB-DNJ and human lactoferrin were from Sigma. Endo-β-D-*N*-acetylglucosaminidase H (Endo H) and peptide *N*-glycanase F (PNGase F) were from New England Biolabs (UK).

2.2. Antibodies

Mouse monoclonal antibodies (Mabs) that recognize the pre-S2 region (aminoacids 131–139) of both L and M proteins were from Abcam (UK). Anti-S Mabs were purchased from Zymed (USA). The anti-mouse horseradish peroxidase-conjugated secondary antibodies were from Amersham (UK).

2.3. HBV preparation from cell supernatants

HepG2.2.2.15 cells were grown in 6-well plates in the absence (control) or presence of different concentrations of NB-DNJ. Supernatants were collected 3 days later and clarified from cell debris by a brief centrifugation at $10{,}000 \times g$. Virus and subviral particles were pelleted by ultracentrifugation through a 20% sucrose cushion in a SW 41 Ti Beckman rotor at 36,000 rpm, for 4 h. The pellet was resuspended in phosphate-buffered saline (PBS) resulting in 150-fold concentrated virus compared to that of the initial supernatant.

2.4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

Concentrated virus samples prepared as above, were lysed in a buffer containing 0.5% Triton X-100, 50 mM Tris–Cl (pH 7.5), 150 mM NaCl, and 2 mM EDTA (Triton TSE buffer) and a mixture of protease inhibitors (Sigma), for 1 h, on ice. Lysates were clarified by centrifugation at $10{,}000 \times g$ for 15 min. The proteins in the supernatant were either boiled under non-reducing conditions (NR) or boiled in the presence of 5 mM DTT (reducing conditions-R) before analysis by SDS-PAGE and western blotting. Where indicated in the figures, samples were digested with either Endo H or PNGase F, following the protocol supplied by the manufacturer. The proteins were transferred to nitrocellulose membranes using a semi-dry blotter (Millipore). The blots were incubated with

either anti-pre-S2 (dilution 1/1000) or anti-S (dilution 1/1000) Mabs, followed by anti-mouse Mabs (dilution, 1/2000) conjugated to horseradish peroxidase. The proteins were detected using an enhanced chemiluminiscence (ECL) detection system (Amersham, UK) according to the Manufacturer's instructions.

2.5. HBV purification and infection of HepaRG cells

HepG2.2.2.15 cells were grown in 75 cm^2 flasks in the absence (control) or presence of different concentrations of NB-DNJ for 3 days. The supernatants were clarified by centrifugation at 10,000 × *g*. The virus particles were concentrated 150-fold by ultracentrifugation through a 20% sucrose cushion, in a SW 41 Ti Beckman rotor at 36,000 rpm, for 4 h. The pellet was resuspended in phosphate-buffered saline (PBS) and the HBV concentration was determined by dot blot using serial dilutions of known amounts of a pTriEX-HBV vector for comparison. The HepaRG cells were seeded in 6-well plates and prepared as described (Gripon et al., 2002). Approximately 10^6 differentiated cells were used for infection with 50 μl of concentrated HBV virus containing 1.2×10^9 genome equivalents (GEq). One day post-infection the viral inoculum was removed, cells were washed three times with PBS and 3 ml of fresh medium was added. Human lactoferrin (Lf) was added at the same time with the virus for 24 h, as a control for the specificity of HBV infection (Hara et al., 2002). Infected HepaRG cells were harvested after 9 days and the HBV DNA was analyzed by southern blotting.

2.6. Purification of HBV DNA and southern blotting

Encapsidated viral DNA was purified by phenol–chloroform extraction from both the supernatants of the HepG2.2.2.15 cells, treated or not with NB-DNJ, and the HepaRG cells infected with HBV, using a protocol adapted from Mehta et al., 2004. Briefly, 20 μl of the 150-fold concentrated virus or 10^6 infected HepaRG cells were lysed in 500 μl TLB buffer (50 mM Tris–HCl pH 8, 1 mM EDTA, 1% Nonidet P40), for 30 min, on ice. The samples were centrifuged for 5 min, at 10,000 × *g*. The resulting supernatant was supplemented with 5 μl of 1 mM Mg-acetate and 10 μl of 10 mg/ml DNase/RNase mixture (Sigma) before incubation for 2 h, at 37 °C. Samples were briefly centrifuged and 12.5 μl of 0.5 M EDTA, 10 μl of 5 M NaCl, 7.5 μl of 20 mg/ml proteinase K, and 11.5 μl of 20% SDS were added. Samples were incubated overnight, at 42 °C. Viral DNA was extracted in 0.5 ml phenol:chloroform:isoamyl alcohol mix and precipitated with isopropanol. The DNA pellet was resuspended in DNase/RNase-free water and analyzed in a 1.2% agarose gel. The DNA was transferred to a Hybond-N + membrane (Amersham, UK) using a vacuum transfer blotter (BioRad, UK). The HBV DNA fragments were hybridized with a fluorescein-labeled probe obtained by random priming using the HBV DNA genome as template. The DNA bands were detected using anti-fluorescein alkaline phosphatase (AP)-conjugated Mabs and the Gene Images CDP-Star detection kit (Amersham, UK).

3. Results

3.1. HBV released in the presence of NB-DNJ contains an envelope with altered protein composition

It was previously reported that NB-DNJ treatment of HBV-infected cells results in the proteasomal degradation of the L and M proteins (Simsek et al., 2005). However, the consequences of this process on HBV assembly in drug-treated cells as well as the infectivity of the released particles have not been investigated. To determine the molecular composition of the envelope of mature virions assembled in the presence of NB-DNJ, the HepG2.2.2.15 cells were treated with various drug concentrations for 3 days. High concentrations of NB-DNJ (4.5 mM) were found to prevent HBV secretion (Block et al., 1994); therefore, to obtain a partially inhibitory effect, the highest drug concentration used in this study was 2 mM. The virions purified from the medium were subjected to DNA and protein analysis by southern and western blotting, respectively.

As shown in Fig. 2A, virus-specific DNA bands migrating with electrophoretic mobility similar to those expected for the relaxed circular and linear forms (Sells et al., 1988) were detected by southern blotting in all samples. Clearly, the amount of HBV DNA recovered from NB-DNJ-treated samples decreased with increasing drug concentrations. The level of inhibition of HBV secretion was quantified by scan den-

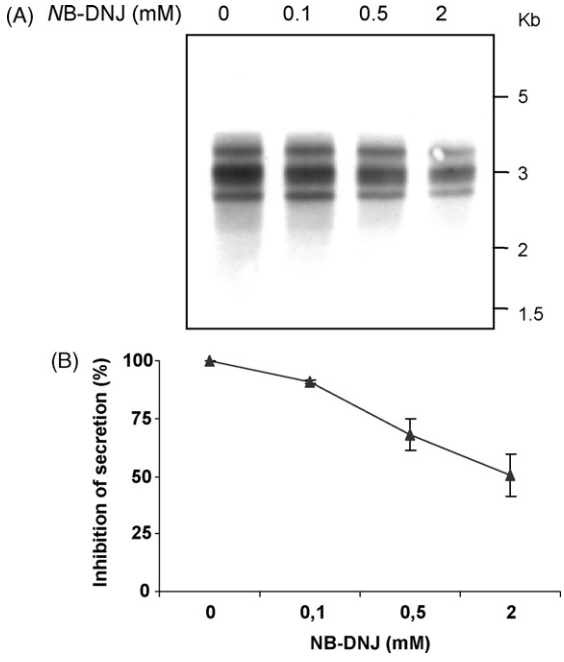

Fig. 2. Analysis of encapsidated HBV DNA secreted from α-glucosidase-inhibited HepG2.2.2.15 cells. HepG2.2.2.15 cells were grown in the absence (0 drug) or presence of different concentrations of NB-DNJ, for 3 days. The cell medium was collected and the HBV DNA was purified by phenol–chloroform extraction. Viral DNA was separated on a 1.2% agarose gel, followed by southern blotting and detection with a fluorescein-labeled HBV-specific DNA probe. The identification of viral DNA was performed using anti-fluorescein Mabs (A). The HBV DNA was quantified by scan densitometry analysis of the southern blots obtained in two separate experiments (B).

sitometry analysis of the DNA bands. Approximately 50% of viral DNA was secreted in the presence of the highest NB-DNJ concentrations used, as compared to control (Fig. 2B).

The envelope proteins of the virions secreted from HepG2.2.2.15 cells, in the absence or presence of NB-DNJ, were detected under reducing conditions, using Mabs specific for the pre-S2 and the S domains. The anti-pre-S2 Mabs recognized three major bands in the control sample, with an electrophoretic mobility between 30 and 45 kDa, most likely representing the glycoforms of the L and M proteins (Fig. 3A). To precisely identify these species, the untreated sample was subjected to either Endo H or PNGase F digestion followed by western blotting (Fig. 3C). Following PNGase F digestion the apparent molecular weight of the proteins shifted to that corresponding to the fully deglycosylated L and M polypeptides (39 and 30 kDa, respectively). This demonstrates the presence of both glycosylated and unglycosylated L protein (the two upper bands) and that of mono- and diglycosylated M that co-migrate (the lower band). The complete resistance of the *N*-glycans attached to both L and M proteins, to Endo H digestion, indicated the acquirement of complex carbohydrate structures during trafficking of the virions through medial- and *trans*-golgi.

The NB-DNJ treatment resulted in a dose-dependent reduction of both L and M proteins detected in the virus samples. However, the M protein was clearly more affected by the drug as it became undetectable at the highest concentrations of NB-DNJ used (2 mM).

The amount of L and M proteins was further quantified by scan densitometry analysis of the western blot (as shown in Fig. 3A). Approximately 45% of L and only trace amounts of the M proteins were detected by the pre-S2 Mabs, in the 2 mM NB-DNJ-treated sample, compared to control (Fig. 3B). Since HBV secretion was inhibited by 50% in the presence of 2 mM NB-DNJ (Fig. 2B), it is plausible to conclude that the decreased amount of L protein detected in the virus sample is the consequence of this inhibition rather than that of secretion of virus with reduced content of L.

The virions purified from the medium of HepG2.2.2.15 cells, treated or not with NB-DNJ, were also analyzed for the presence of S, using the anti-S Mabs. Since the S domain is shared by all three envelope proteins, a complex mixture of L, M and S glycoforms were expected to be recognized by the anti-S Mabs in virus samples. The SDS-PAGE analysis of the heat-denatured and DTT-treated virus samples showed the complete lack of recognition of any of the HBV envelope proteins by the anti-S Mabs under reducing conditions (Fig. 4A, R samples). However, when the same samples were heat denatured in the absence of

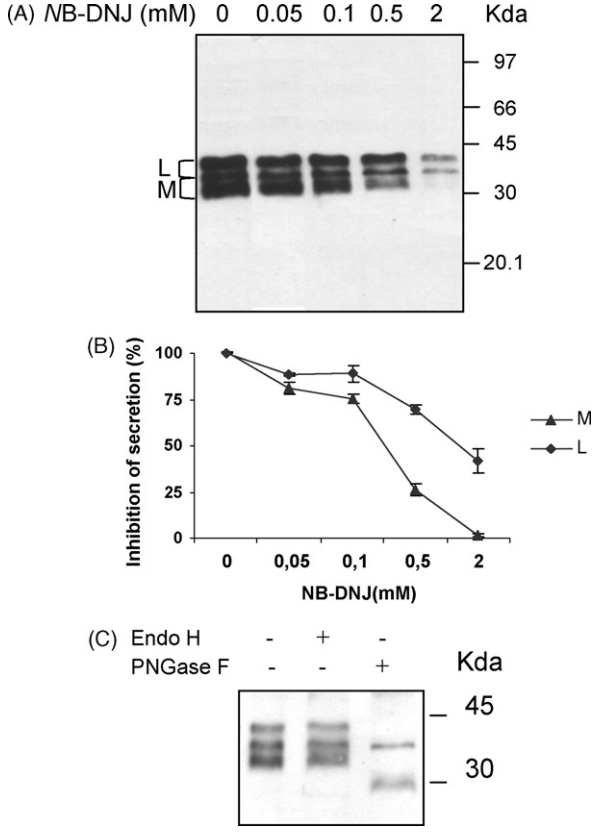

Fig. 3. Analysis of the L and M proteins in secreted virions. HepG2.2.2.15 cells were grown in the absence (0) or presence of different concentrations of NB-DNJ, for 3 days. The supernatants were collected and virion particles were purified and concentrated by ultracentrifugation through a 20% sucrose cushion. The proteins were separated by SDS-10% PAGE under reducing. The HBV proteins were identified by western blotting using the anti-pre-S2 Mabs specific for the L and M proteins (A). The virus-specific bands were quantified by scan densitometry analysis of the western blots resulted from two separate experiments (B). The untreated sample was also digested with either Endo H or PNGase F at 37 °C, overnight, before electrophoresis and western blotting with anti-pre-S2 Mabs (C).

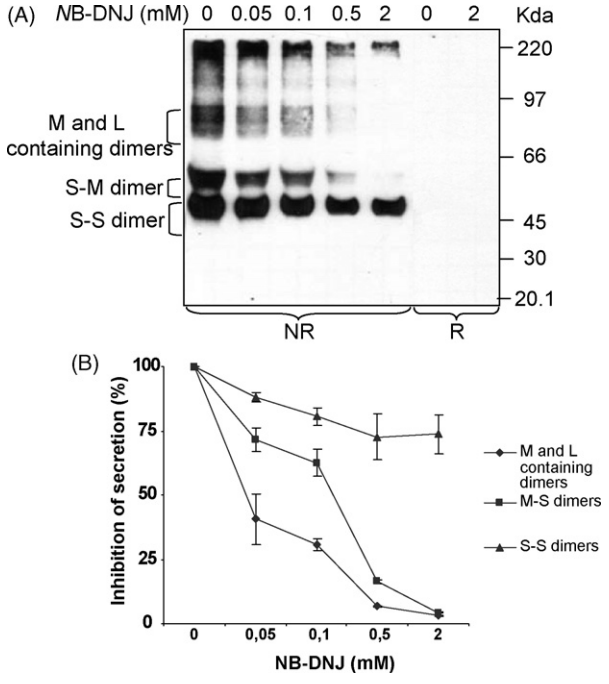

Fig. 4. Analysis of the S proteins and oligomers in secreted virions. HepG2.2.2.15 cells were grown in the absence (0) or presence of different concentrations of NB-DNJ, for 3 days. The supernatants were collected and virion particles were purified and concentrated by ultracentrifugation through a 20% sucrose cushion. The proteins were separated by SDS-10% PAGE under non-reducing (NR) or reducing (R) conditions and identified by western blotting using the anti-S Mabs (A). The virus-specific oligomers were quantified by scan densitometry analysis of the western blots from two separate experiments (B).

DTT, the envelope proteins were readily detected as disulfide-linked homo and heterodimers in controls, as well as NB-DNJ treated samples (Fig. 4A, NR samples). This result suggests that the anti-S Mabs recognize a disulfide bond-dependent epitope and can be used to monitor the oligomer-assembled enveloped proteins. Based on: (a) the apparent molecular weight of each of the S, M and L glycoforms, (b) their recognition by the anti-S Mabs and (c) the comparison with the electrophoretic mobility of the L and M monomers previously identified using the anti-pre-S2 Mabs (Fig. 3A), the bands migrating between the protein markers at 45–66 kDa were identified as the S–S and S–M homo and heterodimers (Fig. 4A). The remaining M- and L-containing dimers migrate as a broader band between 66 and 97 kDa. Attempts to improve the resolution of the upper part of the gel by running the samples on lower polyacrylamide concentration or gradient gels did not result in a better separation of this broad band into its components.

The intensity of the envelope protein oligomers decreased with increasing concentrations of NB-DNJ, however, the drug had different effects on the amount of the proteins detected by the anti-S Mabs. Thus, the bands corresponding to the L- and M-containing oligomers gradually decreased until disappearance, while the band corresponding to the S–S homodimers was only moderately affected. Quantification of these effects by scan densitometry analysis showed that, in contrast to the L and M dimers which were hardly detectable, the amount of the S–S dimers present in the virus sample was reduced by 25% (Fig. 4B). The S protein has the peculiar feature of assembling into empty (subviral) particles, which are secreted in large excess compared to mature virions, in infected patients (Heermann and Gerlich, 1992). The HepG2.2.2.15 cells are also able to secrete subviral particles (Sells et al., 1987), and their secretion is not affected by NB-DNJ, as shown previously (Mehta et al., 1997). Thus, a possible contamination of the virus samples with these proteins during HBV purification may account for the difference between the overall inhibition of HBV secretion and the reduction of the S homodimers in the virus sample.

3.2. HBV envelope proteins secreted in the presence of NB-DNJ have similar N-glycosylation patterns

Inhibition of the ER α-glucosidases interferes with normal trimming of the *N*-glycans attached to the proteins resulting in retention of hyperglucosylated structures and prevention of the interaction with calnexin (Hebert et al., 1995). However, the α-glucosidase blockade can be bypassed in certain cell lines by a golgi-resident mannosidase pathway that allows for normal oligossaccharides processing to complex structures (Moore and Spiro, 1990).

To determine the glycosylation status of the HBV envelope proteins secreted from α-glucosidase inhibited HepG2.2.2.15 cells, the virus samples purified from the medium of both control (untreated) or 2 mM NB-DNJ-treated cells were subjected to Endo H and PNGase F digestions prior to western blotting analysis using the anti-pre-S2 Mabs. Analysis of the blots with the anti-S Mabs was not possible because of the lack of recognition of the S proteins under the reducing conditions required for Endo H optimal activity.

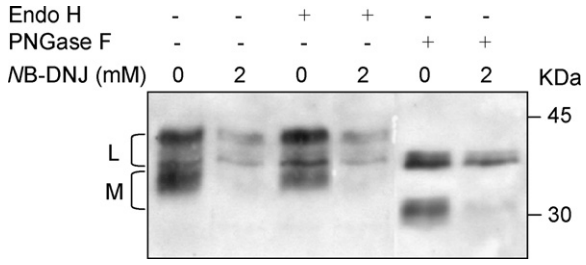

Fig. 5. Analysis of the glycosylation status of the L and M proteins in secreted virions. HepG2.2.2.15 cells were grown in the absence (0) or presence of 2 mM concentration of NB-DNJ, for 3 days. The supernatants were collected and virion particles were purified and concentrated by ultracentrifugation through a 20% sucrose cushion. Samples were digested with either Endo H or PNGase F overnight, at 37 °C, before electrophoresis on SDS-10% PAGE under reducing conditions and western blotting with anti-pre-S2 Mabs.

The L protein showed complete resistance to Endo H treatment in both control (confirming the results in Fig. 3C) and NB-DNJ-treated samples, indicating the processing of *N*-glycans to complex structures, despite the drug treatment (Fig. 5). This is in agreement with the normal phase HPLC results published by Mehta et al. (1997) showing that subviral particles secreted from α-glucosidase-inhibited HepG2.2.2.15 cells contain L and S proteins with complex glycan structures.

As shown in Fig. 5, less L and virtually no M proteins were detected in the virus sample secreted in the presence of 2 mM NB-DNJ, a result confirmed by the PNGase F digestion. The PNGase F treatment, which results in complete removal of the N-linked glycans from the protein backbone regardless to their structure, also rules out the possibility of the L and M proteins being less well recognized by the anti-pre-S2 Mabs when expressed in the presence of NB-DNJ, compared to their control counterparts.

3.3. NB-DNJ treatment of HepG2.2.2.15 cells results in secretion of HBV with impaired infectivity

The effects of α-glucosidase inhibitors on HBV replication and secretion have been reported before (Block et al., 1994). However, studies regarding the infectivity of the virions released from α-glucosidase-inhibited cells have not been carried out to date.

Analysis of HBV infectivity has been a difficult task due to the lack of cell lines susceptible to HBV infection *in vitro*. The recent development of the hepatoma-derived HepaRG cell line, permissive to HBV infection (Gripon et al., 2002), enables a re-evaluation of the antiviral activity of α-glucosidase inhibitors by determining their effects on the early steps of the HBV life cycle. Therefore, we used this system to investigate the infectivity of HBV particles assembled and secreted in the presence of NB-DNJ.

HBV was concentrated from medium of HepG2.2.2.15 cells grown in the absence or presence of NB-DNJ. The drug concentrations varied from 0.1 to 2 mM, which were shown to only partially inhibit HBV secretion (Fig. 2). Viral DNA was

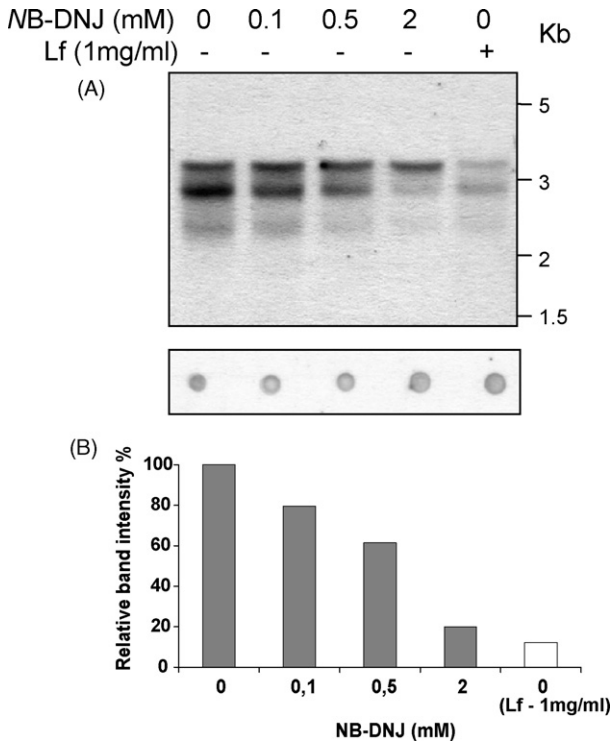

Fig. 6. Infectivity of HBV particles produced in α-glucosidase-inhibited cells. Differentiated HepaRG cells were infected with 10^3 GEq/cell of concentrated viral stock purified from supernatants of HepG2.2.2.15 cells grown in the absence (0 drug) or presence of different concentrations of NB-DNJ. Lactoferrin (Lf) was added during infection to one of the control samples (Lf+), at a final concentration of 1 mg/ml. The concentration of the HBV DNA used for infection of HepaRG cells was determined by dot blot and comparison to the hybridization signal obtained from serial dilutions of known amounts of double-stranded HBV DNA (A, lower panel). HepaRG cells were collected 9 days post-infection and lysed. The encapsidated HBV DNA was purified by phenol–chloroform extraction, followed by southern blotting. The identification of viral DNA was performed by hybridization using a fluorescein-labeled HBV-specific DNA probe followed by incubation with anti-fluorescein Mabs (A, upper pannel). The virus-specific bands were quantified by scan densitometry analysis (B).

extracted and quantified by dot blot analysis and comparison to the hybridization signal obtained from serial dilutions of known amounts of double-stranded HBV DNA. The HBV samples used to infect the HepaRG cells were adjusted so that each contained equal amounts of HBV DNA (approximately 1000 GEq/cell). The presence of the same amount of DNA used for infection was further confirmed by dot blot analysis of the viral genome extracted from the HBV samples (Fig. 6A, lower panel). To ascertain the specificity of the infection process, lactoferrin, a secretory protein known to protect hepatocytes from infection with HBV *in vitro* (Hara et al., 2002), was added during HBV infection of HepaRG cells to one of the untreated control samples.

The HepaRG cells were collected 9 days post infection and the intracellular HBV DNA was analyzed by southern blotting. Replicative forms of viral DNA were identified in HepaRG cells, as reported before (Gripon et al., 2002) (Fig. 6A, upper panel). The amount of viral DNA was reduced in cells infected with HBV assembled in the presence of NB-DNJ. This suggests that a lower number of HBV particles were able to enter or initiate replication cycles in HepaRG cells. Quantification of the total viral DNA by scan densitometry analysis revealed that NB-DNJ altered HBV infectivity in a dose-dependent manner (Fig. 6B). A significant inhibition of HepaRG infection (approximately 80%) was observed at the highest concentrations of NB-DNJ (2 mM). As expected, lactoferrin was able to inhibit HBV entry, confirming the specificity of the infectivity assay.

4. Discussion

The importance of *N*-glycosylation and *N*-glycan processing of the envelope proteins in the HBV life cycle has been investigated before and several roles have been suggested in folding, secretion and intracellular trafficking (Lu et al., 1995, 1997; Mehta et al., 1997; Simsek et al., 2005; Werr and Prange, 1998); however, important questions regarding the molecular mechanisms underlying these effects remained to be answered.

In this study we have used NB-DNJ to investigate the effect of α-glucosidase inhibition on the molecular composition and the infectivity of the released HBV particles. Our results show that HBV secretion was inhibited by NB-DNJ in a dose-dependent manner, up to 50% at the highest drug concentration used (2 mM). The amount of the envelope proteins detected in extracellular virus samples was reduced in the presence of NB-DNJ, compared to controls. The reduction of the total amount of L detected by the anti-pre-S2 Mabs correlates well with the inhibition of HBV secretion; however, the drug treatment resulted in release of virus containing no detectable M protein. This was confirmed by the analysis of the disulfide-linked oligomers using Mabs specific for the S domain, which showed that neither of the M containing oligomers was detectable in the HBV particles secreted from 2 mM NB-DNJ-treated cells. Under the same conditions, the S homodimer was readily detectable. Together, these results suggest that virion assembly and export from the cell are still possible in the absence of M. This is consistent with previously published data in which the M protein is shown to be dispensable for envelopment of the HBV nucleocapsid (Bruss and Ganem, 1991).

The absence of the M from the virus envelope can be a consequence of its retention and degradation in α-glucosidase-inhibited cells, as recently demonstrated by Simsek et al. (2005). In this work, treatment of HBV-infected cells with DNJ resulted in proteasomal degradation of up to 56% of L and 86% of M, while the S protein was not affected.

The significantly different sensitivity of the HBV envelope proteins to α-glucosidase inhibition is intriguing since they are closely related and derive from the same ORF. An important difference between the three envelope proteins is the occupancy of the *N*-glycosylation site at Asn 4 residue in the pre-S2 domain, a feature unique to M. This glycan mediates the interaction of M with calnexin suggesting an essential role of the ER chaperone in the proper folding of M (Werr and Prange, 1998). Calnexin also interacts with L; however, in this case it has a role in the ER retention of the L protein, when it is expressed in the absence of S and M (Prange et al., 1999). Interestingly, L also associates with GRP78/BiP, another ER-resident chaperone, which specifically

binds to the pre-S1 domain of the L protein, implying a function in the folding process or assembly of L (Cho et al., 2003). This interaction may be sufficient for some L proteins to acquire an assembly competent conformation and further recruitment into the viral envelope, while the M protein has an absolute requirement for calnexin for proper folding. So far, there is no published evidence of an interaction of the S protein with the ER lectin-like chaperones, which may explain the lack of sensitivity of this protein to α-glucosidase inhibition.

The infectivity of the viral particles assembled in the presence of NB-DNJ was significantly impaired. The HepaRG cells infection by the HBV particles produced in the HepG2.2.2.15 cells was nearly completely inhibited by lactoferrin, a protein previously shown to prevent HBV infection in cultured human hepatocytes by blocking the viral receptors on cells (Hara et al., 2002). This confirms the specificty of the HBV infection on one hand and suggests similar virus entry pathways in the two cell culture systems used, on the other hand. The amount of viral DNA detected in HepaRG cells following infection with HBV formed in the presence of 2 mM NB-DNJ was reduced by 80% compared to controls. It is unlikely that a change in the glycan structure of the envelope proteins, as a consequence of α-glucosidase inhibition, would contribute to this effect, since at least the L protein contains fully processed *N*-glycans and M was undetectable in the presence of 2 mM NB-DNJ. These findings may provide new insights into the controversial role of the M protein in HBV infectivity. Studies published by Le Seyec et al. (1998) showed that most of the pre-S2 region is dispensable for HBV infection. However, Cho et al. (2001) reported the existence of a HBV receptor binding region located in the pre-S2 domain. More recently, the work of Saha et al. (2005) showed that amongst the vesicular stomatitis virus pseudotypes produced bearing HBV surface proteins in different combinations, the M pseudotype gave the highest infectious titer in HepG2 cells. In our hands, the reduced ability of HBV produced in the presence of NB-DNJ, to infect HepaRG cells, clearly correlated with the lack of M oligomers from the viral envelope. It may well be that in the absence of M, the remaining L and S, assembly competent monomers do not find the "right" oligomerization partner and are not able to pair up in the same combination as in untreated cells. These subtle changes in the molecular composition of the HBV envelope could provide an explanation for the reduced infectivity of the particles assembled in the presence of the inhibitor.

The infectivity of other enveloped viruses such as HIV (Fischer et al., 1995), BVDV (Branza-Nichita et al., 2001; Durantel et al., 2001) or that of virus-like particles coated with HCV envelope proteins (Chapel et al., 2006) has been shown previously to be affected by α-glucosidase inhibitors. The mechanism may in principle apply to a broad range of viruses bearing glycoproteins on the surface. However, the consequences of the α-glucosidase inhibition largely depend on the role played by the ER lectin chaperones in the folding of the viral envelope glycoproteins, which must be analyzed on a case by case basis.

Interestingly, it was recently shown that HDV particles coated with nonglycosylated HBV envelope proteins were infectious when assayed on primary cultures of human hepatocytes (Sureau et al., 2003). Assuming that HBV and HDV utilize the same receptor on target cells, these results seem to be contradicting our findings. However, it is important to note that in these experiments neither of the envelope proteins was degraded when expressed as nonglycosylated polypeptide, following removal of the *N*-glycosylation sites (Sureau et al., 2003). This suggests that other chaperones may substitute in this case for the role of calnexin, as opposed to the situation when the presence of an *N*-glycan is a recognition tag for a specific repertoire of folding chaperones and enzymes. This conclusion is sustained by the observation that nonglycosylated variants of S and L proteins are expressed normally in HBV-infected cells; moreover, they are recruited into the HBV envelope or secreted as components of subviral particles (Gerlich et al., 1992).

The strict conservation of the *N*-glycosylation sites among the HBV genotypes suggests that the attachment of the *N*-glycans to the HBV envelope polypeptides is not accidental. Here we show for the first time that their correct trimming within the ER of the host cell, is crucial, albeit indirectly, for the production of infectious HBV particles. This observation has also important implications in re-evaluating the antiviral effect of α-glucosidase inhibitors against HBV.

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