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Inhibition of Host ER Glucosidase Activity Prevents Golgi Processing of Virion-Associated Bovine Viral Diarrhea Virus E2 Glycoproteins and Reduces Infectivity of Secreted Virions

Robert Jordan,*[,1] Olga V. Nikolaeva,* Lijuan Wang,* Bertha Conyers,* Anand Mehta,* Raymond A. Dwek,† and Timothy M. Block*

*Department of Biochemistry and Molecular Pharmacology, The Jefferson Center for Biomedical Research, Thomas Jefferson University, Doylestown, Pennsylvania 18901; and †Department of Biochemistry, Glycobiology Institute, University of Oxford, Oxford, United Kingdom

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Recently, it was shown that replication of bovine viral diarrhea virus (BVDV) is sensitive to inhibitors of host ER glucosidases. Consistent with these findings, we report that incubation of BVDV-infected MDBK cells with the glucosidase inhibitor *n*-butyl-deoxynojirimycin (nB-DNJ) reduced BVDV yields by 70- to 100-fold ($n = 27$), while having no effect on MDBK cell viability. However, the 70- to 100-fold reduction in infectious virus was associated with only a 2-fold reduction in genomic RNA synthesis and secretion of enveloped virus particles. Analysis of secreted virions showed that in the absence of glucosidase inhibitor, approximately 50% of the virion-associated BVDV E2 glycoprotein was resistant to endoglycosidase H (endo H) digestion, whereas intracellular E2 was completely sensitive to endo H digestion. In the presence of glucosidase inhibitor, virion-associated E2 and intracellular E2 were completely sensitive to endo H digestion. Taken together, these results suggest that BVDV is secreted through a Golgi-mediated pathway and that host ER glucosidase activity is required for production of infectious virions and Golgi processing of envelope E2 protein during virus egress. © 2002 Elsevier Science (USA)

Key Words: flavivirus; pestivirus; BVDV; glycoprocessing; glucosidase inhibitor; Golgi processing; E2; gp53.

INTRODUCTION

Many viruses require host ER glucosidase activity for efficient replication and production of infectious particles (Fisher *et al.,* 1996a,b; Courageot *et al.,* 2000; Lu *et al.,* 1997; Bolt *et al.,* 1999; Zitzmann *et al.,* 1999; Block *et al.,* 1998a). ER glucosidases I and II catalyze the sequential removal of terminal glucose residues on nascent glycoproteins in the lumen of the ER (Cannon *et al.,* 1996; Cannon and Helenius, 1999). Trimming of the terminal glucose residues yields monoglucosylated glycoproteins which serve as substrates for calnexin and calreticulin, host ER chaperone proteins that facilitate glycoprotein folding (Cannon *et al.,* 1996). Glucosidase inhibitors, such as *n*-butyl-deoxynojirimycin (nB-DNJ), *n*-nonyl deoxynojirimycin (nN-DNJ), or castanospermine, inhibit glucose trimming and block glycoprotein interaction with calnexin and calreticulin, thereby greatly retarding the rate of glycoprotein folding (Ellgaard *et al.,* 1999; Cannon *et al.,* 1996). Glucosidase inhibitors do not dramatically alter the function of many host proteins, since cell viability is maintained at concentrations of compound that inhibit viral glycoprotein function and prevent virion morphogenesis or other critical steps in the virus life cycle (Fisher *et al.,* 1996a,b; Courageot *et al.,* 2000; Lu *et al.,* 1997; Bolt *et al.,* 1999; Zitzmann *et al.,* 1999; Block *et al.,* 1998a). Thus, glucosidases play an essential role in viral glycoprotein folding and viral envelope function.

Bovine viral diarrhea virus (BVDV) is a pestivirus which belongs to the *Flaviviridae,* a virus family that also comprises important human pathogens such as dengue virus, yellow fever virus, and hepatitis C virus. BVDV replication is sensitive to glucosidase inhibitors, suggesting that viral glycoprotein processing is essential for virus replication (Zitzmann *et al.,* 1999). BVDV strain NADL contains a total of 14 putative N-linked glycosylation sites distributed among three glycoproteins termed Erns, E1, and E2 (Donis, 1995). These glycoproteins along with core protein comprise the major structural elements of the virion and are likely required for receptor-mediated entry into permissive cells (Donis *et al.,* 1988; Donis and Dubovi, 1987). Inhibition of N-linked glycosylation by addition of tunicamycin inhibits BVDV replication, suggesting that cellular glycoprocessing is necessary for the production of infectious virus (Donis and Dubovi, 1987). The precise role of N-linked glycosylation in maturation of BVDV is unknown.

In this report, initial studies were performed toward understanding the role of ER glucosidases in the BVDV life cycle. Using specific inhibitors of ER glucosidases, the effects of these compounds on virus replication and infectivity were examined. While glucosidase inhibitors slightly reduced both BVDV genomic replication and virion secretion, the major impact upon virus replication was the reduction of infectivity of newly synthesized

[1] To whom correspondence and reprint requests should be addressed. Fax: (215) 489-4920. E-mail: robert.jordan@mail.tju.edu.

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virions. Moreover, glucosidase inhibitors prevented Golgi processing of virion-associated E2 glycoprotein. These results suggest that BVDV virions are secreted through a Golgi-mediated pathway and that ER glucosidase activity is required for production of infectious virions and Golgi processing of E2 protein. Given the similarities of BVDV replication with other members of the *Flaviviridae,* ER glucosidases may serve as targets for development of therapies for the treatment of flavivirus infection.

RESULTS

The effects of α-glucosidase inhibitors on BVDV replication

Single-cycle BVDV replication. A previous report has demonstrated that BVDV replication is sensitive to inhibitors of ER glucosidases in virus yield and plaque reduction assays (Zitzmann *et al.,* 1999). In those experiments, the effects of glucosidase inhibitors on BVDV replication were studied at very low multiplicities of infection (0.001 PFU/cell), and virus yields were measured after multiple rounds of infection. To measure the effects of glucosidase inhibitors at low and high multiplicities of infection in a single round of replication, MDBK cells were infected with 0.05 or 5.0 PFU/cell of BVDV strain NADL in the presence and in the absence of an amount of nB-DNJ that was sufficient to inhibit 99% of the ER glucosidase activity (4.5 mM). Virus yields were measured every 4 h after infection for a 24-h period and the results are shown in Fig. 1.

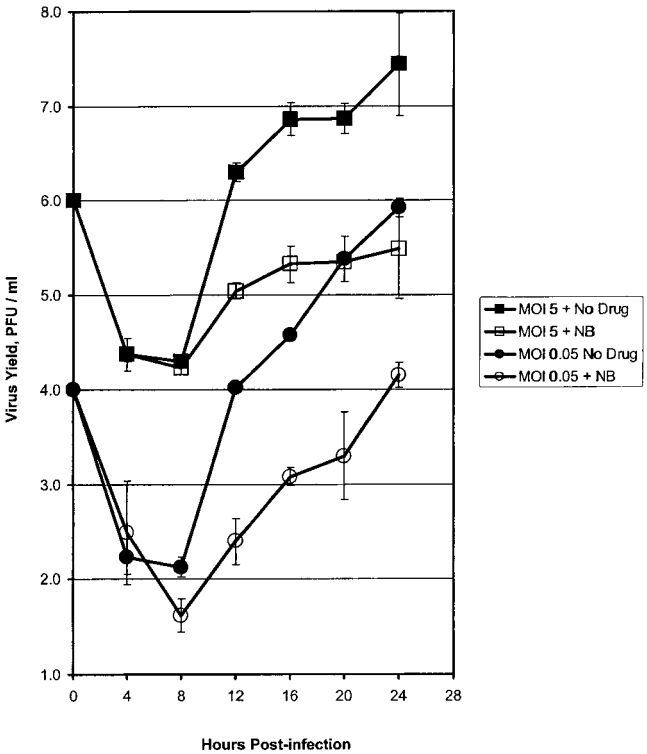

FIG. 1. The effects of glucosidase inhibitors on single-cycle BVDV replication. MDBK cells were infected in triplicate at a multiplicity of infection of 0.05 PFU/cell (circles) or 5 PFU/cell (squares). At 1 h postinfection the virus inoculum was removed and MDBK medium alone (filled symbols) or containing 4.5 mM NB-DNJ (open symbols) was added to the cultures. At 0, 4, 8, 12, 16, 20, and 24 hpi the cultures were harvested and virus yields were measured by plaque assay. Error bars represent the standard deviation from three infections.

In this experiment, treatment of infected MDBK cells with nB-DNJ reduced BVDV yields at 24 h postinfection by 60-fold at a low (0.05 PFU/cell) multiplicity of infection and 81-fold at a high (5.0 PFU/cell) multiplicity of infection relative to untreated controls (Fig. 1). On average, treatment of infected MDBK cells with 4.5 mM nB-DNJ reduced 24-h virus yields by 70-fold ($n = 27$) relative to untreated controls. Increasing the multiplicity of infection from 0.05 to 5.0 PFU/cell had little effect on the antiviral activity of nB-DNJ, suggesting that increasing the multiplicity of infection could not overcome the antiviral effects of the glucosidase inhibitor. Similar results were obtained in 24-h yield assays with castanospermine, a non-imino sugar glucosidase inhibitor, and *n*-nonyl-deoxynojirimycin, a derivative of nB-DNJ (data not shown). The results of these tests demonstrated that BVDV replication is sensitive to inhibitors of host ER α-glucosidases in a single round of infection (Zitzmann *et al.,* 1999).

Previous studies have shown that glucosidase inhibitors have a greater effect upon virus replication than upon cell viability (Block *et al.,* 1994b; Lu *et al.,* 1997). Indeed, the concentration of nB-DNJ needed to kill 50% or the cells (CC_{50}) was not reached and is therefore greater than 38 mM, which was the highest concentration tested (data not shown). This concentration is well above the concentration of inhibitor required to block virus replication. Moreover, there was little observable effect on cellular morphology at inhibitor concentrations that reduced virus yield by 70-fold and inhibited glycoprocessing of secreted glycoproteins by 95% (data not shown). Taken together, these results suggest that the activity of viral glycoproteins with respect to production of infectious virus is more sensitive to glucosidase inhibition than the activity of many cellular proteins required for host cell viability and protein secretion.

Viral RNA synthesis. To begin a determination of the role of glucosidases in BVDV replication, the effects of glucosidase inhibitors on BVDV genomic RNA synthesis were examined. MDBK cells were either mock-infected or infected with BVDV at >1 PFU/cell in the presence or absence of nB-DNJ. At 20 h postinfection, newly replicating BVDV RNA was radiolabeled by the addition of [^{3}H]-uridine to the culture medium in the presence of actinomycin D (act D) (Purchio *et al.,* 1983; Myers *et al.,* 2001). Act D was added to the cultures to reduce the background due to incorporation of radiolabel into cellular mRNA. The BVDV RNA-dependent RNA polymerase is resistant to act D at the concentrations used in these

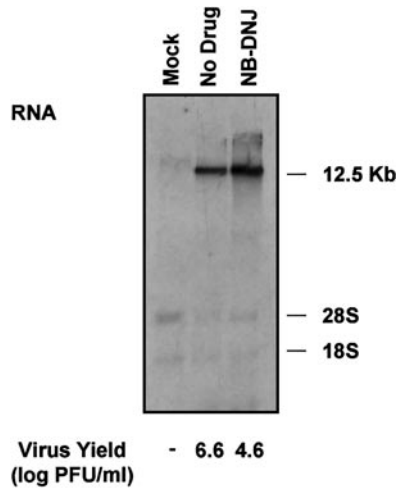

FIG. 2. The effects of glucosidase inhibitors on BVDV RNA synthesis. MDBK monolayers were infected with 5 PFU/cell BVDV and at 20 h postinfection BVDV RNA was radiolabeled with [^{3}H]uridine as described under Materials and Methods. The radiolabeled RNA was separated by denaturing agarose gel electrophoresis and visualized by autoradiography. BVDV genomic RNA migrated as a single band of 12.5 kb. The two lower molecular weight species comigrate with 28S and 18S ribosomal RNA.

experiments and had no observable effect on BVDV replication (Purchio *et al.,* 1983). Total intracellular RNA was isolated at 5 h postlabeling and separated by denaturing agarose gel electrophoresis (Fig. 2). The radiolabeled RNA was visualized following autoradiography.

Radiolabeled BVDV genomic RNA from infected MDBK cells migrated as a single species of approximately 12.5 kb, which is consistent with the reported size of 12.5 kb for the full-length BVDV genome (Fig. 2, center and right most lanes) (Collett *et al.,* 1988). In mock-infected samples, no radiolabeled RNA was detectable at this molecular weight (Fig. 2, left). The low-molecular-weight RNA species (below the BVDV genomic RNA bands) comigrates with 28S and 18S rRNA. Virus yields were measured from duplicate cultures to determine the antiviral efficacy of each compound (Fig. 2).

In the presence of nB-DNJ, the amount of radiolabel incorporated into virus-specific RNA was 1.5-fold higher in nB-DNJ-treated samples compared to untreated samples. In contrast, in the presence of nB-DNJ, the 24-h yields of infectious BVDV were reduced by 2 logs relative to untreated samples. Thus, while nB-DNJ had little effect on uridine incorporation into BVDV RNA, there was a more dramatic loss of infectious virus (~100-fold). These data indicate that glucosidase inhibitors block a step(s) in the virus life cycle that occurs after RNA synthesis.

Virion secretion. It has been demonstrated that glucosidase activity is required for assembly and secretion of Dengue virus and Sinbis virus (McDowell *et al.,* 1987; Courageot *et al.,* 2000). To determine whether glucosidase inhibitors interfere with BVDV assembly and secretion, the level of BVDV RNA in the medium was measured by Northern blot analysis. MDBK cells were infected with BVDV at >1 PFU/cell and treated with medium alone or with medium containing 4.5 mM nB-DNJ. The medium was collected at 24 h postinfection and virus yields were measured by plaque assay and the level of BVDV RNA was measured by Northern blot analysis (Fig. 3) (Sullivan and Akkina, 1995). The Northern blots were probed with radiolabel probes specific for the BVDV E2 gene.

Northern blot analysis detected a single RNA species that migrated at the approximate molecular weight of full-length BVDV RNA (12.5 kb) from BVDV-infected samples (Fig. 3, center and rightmost lanes). In mock-infected samples, no RNA species were detected at this molecular weight (Fig. 3, left lane). No appreciable differences were observed in the level of secreted RNA in the presence or in the absence of nB-DNJ (Fig. 3, center and rightmost lanes). Similar results were obtained using RT-PCR to measure the amounts of secreted BVDV RNA (data not shown). In contrast, the 24-h virus yields were reduced by 63-fold (Fig. 3, center and rightmost lanes). These results suggest that glucosidase inhibitors had a greater effect upon the production of infectious virus than upon secretion of viral particles. Bovine actin mRNA was not detected by RT-PCR in these RNA preparations, while bovine actin mRNA was readily detectable in preparations of MDBK cell RNA, suggesting that these virus RNA preparations used for Northern blot and RT-PCR analyses did not contain detectable cellular mRNA contamination (data not shown). Taken together, these results suggest that glucosidase activity is required for the production of infectious virus.

Virion infectivity. Equilibrium sedimentation was used to test the role of glucosidases in the production of infectious virus and to provide a more quantitative analysis of the effects of nB-DNJ on virus particle secretion. MDBK cells were infected with >1 PFU/cell of BVDV in the presence and absence of nB-DNJ and viral RNA was

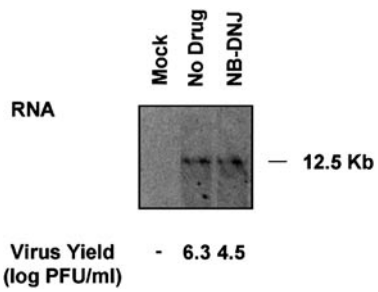

FIG. 3. Northern blot analysis of secreted BVDV particles in the presence and absence of nB-DNJ. Northern blot analysis of secreted BVDV mRNA at 24 h postinfection from MDBK cells infected with BVDV (NADL) at >1 PFU/cell and incubated in the presence and absence of 4.5 mM. Viral RNA from the supernatants was purified and transferred to membranes by Northern blotting. The Northern blots were probed with BVDV cDNA specific for E2. Virus yields in culture supernatants were measured by plaque assay. The average reduction in virus yield in the presence of 4.5 mM nB-DNJ is 65-fold.

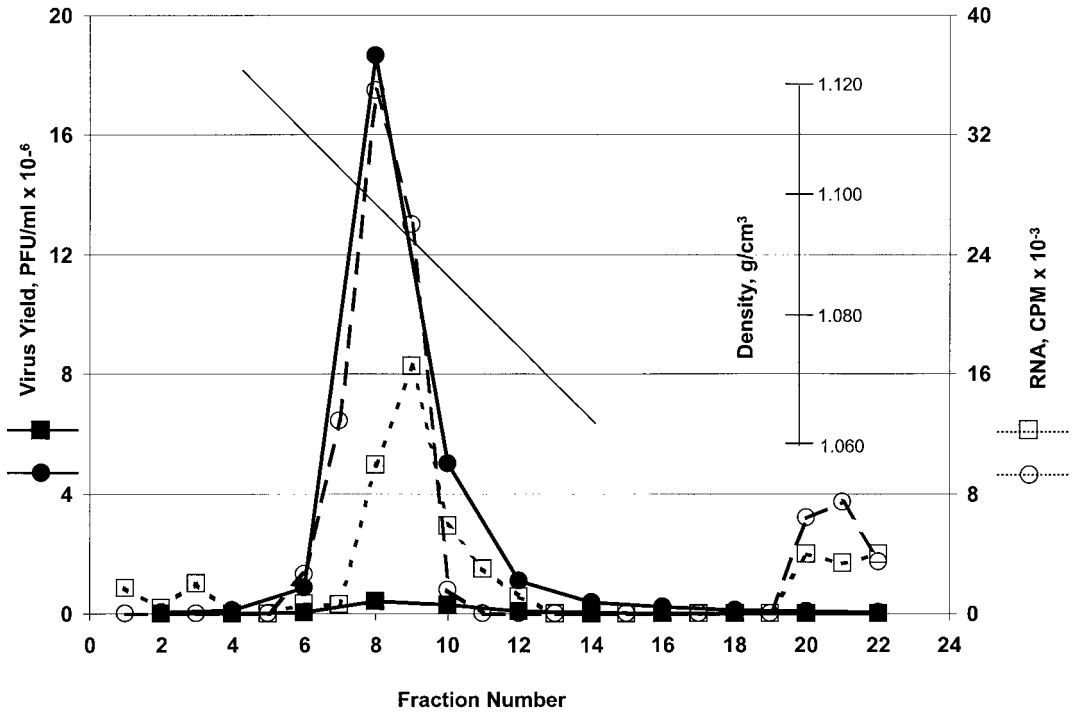

FIG. 4. Equilibrium sedimentation of radiolabeled BVDV virions. MBDK monolayers were infected with >1 PFU/cell of BVDV. At 7 h postinfection [^{3}H]uridine was added to the cultures in the presence of actinomycin D and in the presence (squares) and absence (circles) of nB-DNJ. At 24 h postinfection secreted virus was concentrated by PEG precipitation and sedimented to equilibrium on a 20 to 40% sucrose gradient. The amount of radiolabeled material in each fraction was determined by TCA precipitation and liquid scintillation counting (open symbols and dashed lines). The amount of infectious virus in every second fraction was measured by plaque assay (filled symbols and solid lines). The refractive index was used to measure the density of each sample.

radiolabeled by incorporation of radiolabeled uridine. At 24 h postinfection, secreted radiolabeled virions were purified by PEG precipitation and sedimented to equilibrium on a 20 to 40% sucrose gradient. The amount of radiolabeled material in each gradient fraction was determined by TCA precipitation and liquid scintillation counting. In addition, the amount of infectious virus in every second fraction was measured by plaque assay (Fig. 4).

Consistent with previously published reports, infectious BVDV and radiolabeled RNA cosedimented at a density of 1.10 g/cm^3 (Parks *et al.,* 1972). In control experiments, removal of viral envelope by treatment of virion preparations with 0.5% NP-40 prior to equilibrium sedimentation eliminated the peak of infectivity and radiolabeled material, suggesting that unenveloped virus particles sediment at a different density than enveloped virus under these conditions (data not shown). In the presence of nB-DNJ, the migration of virus particles in the gradient, as determined by the amount of radiolabeled RNA in each gradient fraction, was shifted by one fraction toward higher densities. This shift in particle density was not consistently observed in replicative experiments. These results suggest that there were no gross alterations in virion structure in the presence of glucosidase inhibitor that could be resolved by equilibrium sedimentation.

In the presence of nB-DNJ, the amount of infectious virus in the peak fractions from the gradient was reduced 45-fold relative to the untreated controls. However, the amount of radiolabeled RNA was reduced approximately 2-fold, suggesting that nB-DNJ had a greater effect on virus infectivity rather than particle secretion. Since inhibition of glucosidase activity did not dramatically affect the incorporation of radiolabeled uridine into viral genomic RNA (Fig. 2), the level of secreted radiolabeled BVDV RNA was used as a surrogate marker for BVDV particle number. Thus, comparing the level of BVDV RNA to the level of infectious virus in peak fractions gave an estimate of the relative infectivity of BVDV particles (particle to PFU ratio). The ratio of viral RNA to infectivity in peak fractions from virus produced in the presence and absence of nB-DNJ was 9.9×10^{-3} and 0.46×10^{-3} cpm/PFU, respectively. Thus, virus produced in the presence of nB-DNJ had a higher relative particle to PFU ratio than virus produced in the absence of compound. The results of these tests suggest that virions produced in the presence of nB-DNJ are less infectious than virions produced in the absence of glucosidase inhibitor.

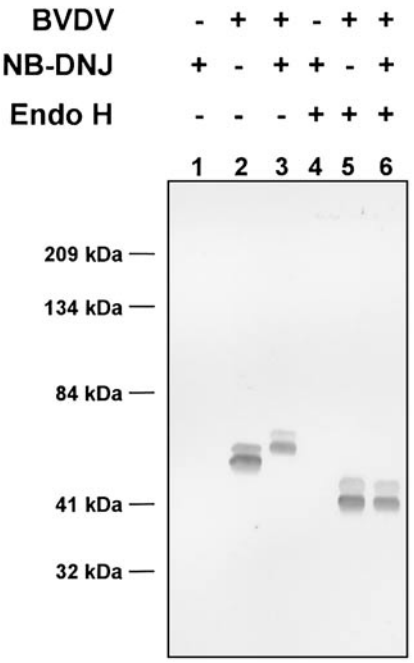

FIG. 5. The effects of nB-DNJ on posttranslational processing of intracellular forms of BVDV E2. MDBK monolayers were infected with BVDV at >1 PFU/cell in the presence and absence of 4.5 mM nB-DNJ. Protein lysates were prepared at 18 h postinfection and analyzed by Western blotting. The Western blot was probed with monoclonal antibody WB166 specific for BVDV E2 protein and developed using alkaline phophatase-conjugated goat anti-mouse antibodies and BCIP/NBT colorimetric substrates. The Western blot was scanned to produce a digital image of the blot.

Analysis of intracellular and virion-associated BVDV E2 glycoprotein. The reduced infectivity of virus particles grown in the presence of glucosidase inhibitors suggests that the loss of infectivity may be due to alterations in virion glycoprotein function. BVDV E2 glycoprotein contains a number of neutralizing epitopes, suggesting that E2 participates in receptor-mediated virus entry. To analyze the effects of glucosidase inhibitors on intracellular and virion-associated E2, a monoclonal antibody (WB166), specific for BVDV E2, was used to probe Western blots of intracellular protein lysates prepared from infected MDBK cell monolayers and from secreted and PEG precipitated virion preparations (Figs. 5 and 6).

BVDV E2 migrated as a doublet of 53 and 60 kDa (Fig. 5, lane 2) on Western blots of intracellular protein lysates. This doublet was due to incomplete proteolytic cleavage at the C-terminus of E2 (Harada *et al.,* 2000). Thus, two forms of E2 are present in infected cells, E2 and unprocessed E2-p7, which differ in molecular weight by 7 kDa (Harada *et al.,* 2000).

In the presence of nB-DNJ the mobility of both E2 and E2-p7 was retarded relative to untreated samples (Fig. 5, lane 3). The reduced mobility of E2 in the presence of nB-DNJ is likely caused by retention of terminal glucose residues on N-linked oligosaccharides (Branza-Nichita *et al.,* 2001). Indeed, removal of all N-linked glycans by endo F digestion caused both E2 and E2-p7 from untreated or nB-DNJ treated samples to migrate at approximately 41 and 48 kDa, respectively (data not shown). Thus, the increase in apparent molecular weight of E2 and E2-p7 in the presence of nB-DNJ was due to inhibition of glycoprocessing and not changes in proteolytic processing.

To determine whether intracellular forms of E2 were processed in the Golgi, protein lysates were treated with endo H and analyzed by Western blotting. Endo H digestion caused both E2 and E2-p7 to migrate at 41 and 48 kDa, respectively (Fig. 5, lanes 5 and 6), demonstrating that both E2 and E2-p7 are sensitive to endo H digestion. The apparent sensitivity to endo H digestion is consistent with ER retention of *Flaviviridae* glycoproteins (Cocquerel *et al.,* 1998, 1999; Flint and McKeating, 1999).

Western blots of protein extracts from secreted and PEG precipitated virus suspensions were used to measure the effects of glucosidase inhibitors on virion-associated E2. Western blots of protein extracts from untreated samples showed that virion-associated E2 migrated as a diffuse band of approximately 53 kDa (Fig. 6, lane 2). In the presence of NB-DNJ, the virion-associated E2 band was less diffuse and migrated at approximately 58 kDa (Fig. 6, lane 3). Removal of N-linked glycans by endo F digestion caused virion-associated E2 protein from both untreated and NB-DNJ-treated extracts to mi-

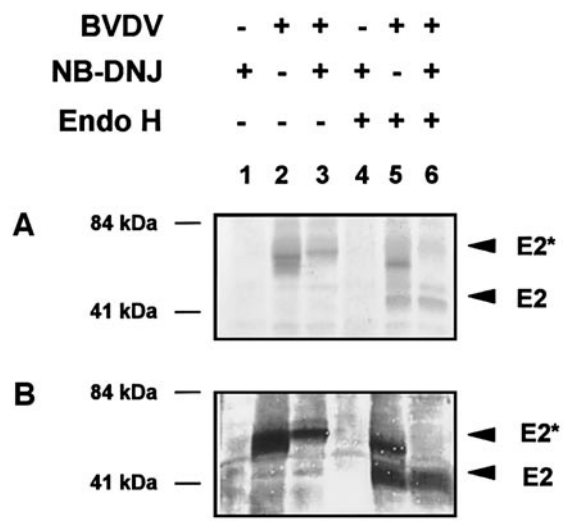

FIG. 6. The effects of nB-DNJ on posttranslational processing of secreted virion-associated forms of BVDV E2. MDBK monolayers were infected with BVDV at >1 PFU/cell in the presence and absence of 4.5 mM nB-DNJ. At 48 h postinfection the medium was collected and secreted virions in the supernatant fluids were PEG precipitated. Protein lysates were prepared and incubated in the presence (lanes 4–6) or absence (lanes 1–3) of endo H. The samples were analyzed by Western blotting using WB166 monoclonal antibody specific for BVDV E2 protein. The Western blots were developed as described in the legend to Fig. 5. The Western blots were scanned to produce digital images of each blot. (A) and (B) represent the results of two independent experiments. The triangles denote the mobilities of glycosylated (E2*) and deglycosylated E2.

grate with similar mobilities (data not shown). This result suggests that the altered mobility of virion-associated E2 in the presence of glucosidase inhibitor is due to changes in glycoprocessing.

To determine whether virion-associated E2 was processed in the Golgi, protein lysates were treated with endo H and analyzed by Western blotting. In the absence of nB-DNJ, densitometric analysis of scans of the Western blot indicated that approximately 50% of the virion-associated E2 glycoprotein was resistant to endo H digestion (Fig. 6, lane 5). Resistance to endo H digestion is associated with Golgi-mediated processing. Partial digestion by endo H was not due to loss of endo H activity since RNase B, which was used as a control, was digested to completion under the same reaction conditions. Furthermore, incubation of endo H reaction mixtures for 24 h did not increase the amount of cleavage of virion-associated BVDV E2 protein (data not shown). These results suggest that BVDV is secreted through a Golgi-mediated pathway and that a portion of the virion-associated E2 glycoprotein is processed in the Golgi during virus egress.

In the presence of nB-DNJ, treatment of virion extracts with endo H showed that approximately 90% of the virion-associated E2 glycoprotein was sensitive to endo H digestion (Fig. 6, lane 6). This result suggests that glucosidase inhibitors block Golgi processing of E2 or that BVDV is secreted by a non-Golgi-mediated pathway.

DISCUSSION

In this article, we have shown that glucosidase inhibitors reduced the production of infectious virus in single-round replication assays at low and high multiplicities of infection. While these compounds reduced the production of infectious virus, they had little effect on intracellular BVDV RNA synthesis as measured by the incorporation of radiolabeled uridine into newly replicated genomes. Analysis of secreted BVDV RNA by Northern blot analysis (Fig. 3) and RT-PCR (data not shown) showed little effect on secretion of BVDV RNA in the presence of glucosidase inhibitor. A more quantitative analysis of the level of secreted virions as measured by equilibrium sedimentation of radiolabeled virus particles demonstrated that secretion of viral RNA was reduced 2-fold in the presence of glucosidase inhibitor. While glucosidase inhibitors reduced virion secretion by 2-fold, the reduction of infectious virus was much higher (~70-fold), suggesting that particles produced in the presence of glucosidase inhibitors were less infectious. Indeed, analysis of the infectivity of virus purified by equilibrium sedimentation showed that virus from peak fractions produced in the presence of glucosidase inhibitors was 45-fold less infectious relative to untreated samples. Thus, while there are modest effects on virion secretion, the predominant effect of glucosidase inhibitors on BVDV replication was a reduction in the infectivity of newly synthesized virus.

Zitzmann *et al.* (1999) have reported that glucosidase inhibitors block BVDV replication. These studies were performed in multiround replication assays at low multiplicity of infection and led them to conclude that glucosidase inhibitors prevented the secretion of virus particles. This conclusion was based on the amount of secreted viral RNA measured by RT-PCR at 3 days postinfection (Zitzmann *et al.,* 1999). While our results are consistent with a profound effect of glucosidase inhibitors upon the virus life cycle, there are several important differences. In contrast to Zitzmann and colleagues, we have demonstrated that glucosidase inhibitors have little effect on RNA synthesis and cause only slight (twofold) reductions in virion secretion in single-round replication assays. Moreover, we show that the predominant effect of glucosidase inhibitors is on the infectivity of virus particles. Zitzmann and colleagues concluded that glucosidase inhibitors blocked the secretion of virus particles based on RT-PCR analysis of viral RNA following low multiplicity of infection and multiple rounds of replication over a period of 3 days. Since glucosidase inhibitors reduced the infectivity of newly synthesized virus, multiple rounds of replication would amplify the antiviral effect and cause a larger difference in the amount of secreted viral RNA between treated and untreated samples. In our experiments, MDBK monolayers were infected at high multiplicities of infection and samples were harvested after a single round of replication. This method more accurately reflects the magnitude of the antiviral effect in a single round of infection and would show smaller differences in the amount of secreted viral RNA in the presence and in the absence of glucosidase inhibitor.

Secretion of BVDV is mediated through a Golgi-dependent pathway since approximately 50% of the virion-associated E2 glycoprotein on secreted virions is partially resistant to endo H digestion. Resistance to endo H digestion suggests that a portion of E2 was modified in the Golgi during virus egress to produce oligosaccharides containing high mannose and hybrid structures which are resistant to endo H digestion. In the presence of glucosidase inhibitor, virion-associated E2 was sensitive to endo H digestion, suggesting that glucosidase inhibitors prevented Golgi processing of virion-associated E2 or that virions were secreted by a non-Golgi-mediated pathway. Intracellular forms of E2 were completely sensitive to endo H cleavage in the presence and in the absence of glucosidase inhibitor, consistent with ER retention of *Flaviviridae* glycoproteins (Cocquerel *et al.,* 1998, 1999; Flint and McKeating, 1999). Taken together, these results suggest that glucosidase inhibitors reduced the infectivity of secreted virions and prevented Golgi processing of BVDV E2 glycoprotein.

Inhibition of E2 Golgi processing or retention of terminal glucose residues on viral glycoproteins caused by

inhibition of glucosidase activity in the ER is not expected to contribute to the reduced infectivity of secreted virions. BVDV replication is not inhibited by deoxymannojirimycin (DMJ), a Golgi processing inhibitor which blocks ER and Golgi mannosidases (Zitzmann *et al.,* 1999). Moreover, recent evidence examining the effects of glucosidase inhibitors on HIV glycoprotein function suggests that retention of terminal glucose residues on viral glycoproteins caused by the inhibition of glucosidase activity in the ER does not interfere with viral infectivity. Glucosidase inhibitors caused a loss of reactivity of monoclonal antibodies to surface epitopes on virion-associated HIV gp120 which is involved in receptor-mediated cell attachment. Removal of the terminal glucose residues on gp120 did not restore monoclonal antibody recognition to these epitopes (Fisher *et al.,* 1996a). These observations suggest that the effects of glucosidase inhibitors on glycoprotein function occurred prior to Golgi processing and virion secretion.

Inhibition of glucosidase activity affects viral glycoprotein function

The current evidence is consistent with the theory that inhibition of glucosidase activity reduces viral infectivity by causing misfolding of viral glycoproteins. Glucosidase inhibitors block glycoprotein interaction with host chaperone proteins and inhibit viral glycoprotein folding (Parodi, 2000; Ou *et al.,* 1993). Misfolded viral glycoproteins interfere with virion assembly, virion secretion, and/or infectivity of newly synthesized virus (Fisher *et al.,* 1996a,b; Courageot *et al.,* 2000; Schlesinger *et al.,* 1985, 2000). Thus, interaction with ER chaperones is an important component of viral glycoprotein biogenesis. Coprecipitation experiments have shown that both BVDV and HCV glycoproteins E1 and E2 interact with calnexin (Choukhi *et al.,* 1998; Branza-Nichita *et al.,* 2001). In addition, VSV G protein and influenza hemagglutinin directly bind to calnexin (Cannon *et al.,* 1996; Hebert *et al.,* 1997). Thus, the exquisite sensitivity of viral glycoproteins to glucosidase inhibitors may reflect the inability of viral glycoproteins to achieve their native conformations in the absence of interaction with ER chaperones.

Indeed, it has been demonstrated that glucosidase inhibitors cause aberrant intracellular BVDV E1–E2 heterodimer formation, suggesting that these proteins are incorrectly folded (Branza-Nichita *et al.,* 2001). The reduced infectivity of BVDV virions produced in the presence of glucosidase inhibitors may reflect incorporation of one or more misfolded glycoproteins that are required for binding or postbinding events during infection.

Several virus systems provide evidence in support of this hypothesis. Glucosidase inhibitors reduced infectivity of measles virus without causing major alterations in virion protein composition or viral protein processing (Bolt *et al.,* 1999). Measles virus produced in the presence of glucosidase inhibitors exhibited reduced reactivity to neutralizing monoclonal antibodies, suggesting that there are alterations in conformation-dependent epitopes on the virus surface required for infectivity (Bolt *et al.,* 1999). Likewise, glucosidase inhibitors caused changes in reactivity of HIV virions to neutralizing monoclonal antibodies and blocked HIV replication and HIV-induced syncytia formation by inhibiting postbinding events (Fisher *et al.,* 1996a,b). While the processing of HIV gp160 is altered in the presence of glucosidase inhibitors and some unprocessed gp160 is incorporated into the virion, the major effect of these compounds is alteration of the V1/V2 region of gp120, which is involved in HIV binding to cell receptors (Fisher *et al.,* 1996a,b). Taken together, these results suggest that glucosidase inhibitors induce conformational changes in surface epitopes of viral glycoproteins required for infectivity.

Potential therapeutic target for HCV and other flavivirus infections

BVDV and HCV are flaviviruses that have similar genomic structures and likely share similar replication strategies. Thus, BVDV is often used as a model for HCV infection (Zitzmann *et al.,* 1999; Ryan *et al.,* 1998). While glucosidase inhibitors reduced the infectivity of secreted BVDV virions, the effects of these compounds on HCV replication is unknown. Unlike BVDV, cell lines that support high-level replication of HCV are currently unavailable; therefore, infectivity of HCV virions produced in the presence of glucosidase inhibitors cannot be assessed. *In vitro* systems have been established to study the biogenesis and function of HCV glycoproteins (Choukhi *et al.,* 1998; Pileri *et al.,* 1998). In these systems, glucosidase inhibitors produced a 20% reduction in the amount of native HCV E2–E1 heterodimers (Choukhi *et al.,* 1998). Formation of native E2–E1 dimers is thought to be required for virion function (Choukhi *et al.,* 1998). However, glucosidase inhibitors had no observable effect on HCV E1–E2 interaction to the putative cellular receptor, CD81 (Chan-Fook *et al.,* 2000). While glucosidase inhibitors have no noticeable effect on glycoprotein biogenesis or function, it is possible that glucosidase inhibitors would block postbinding events in a manner similar to what has been observed for HIV infection (Fisher *et al.,* 1996a,b). Moreover, HCV envelope glycoproteins, like BVDV, may be postranslationally modified by Golgi enzymes during virus egress and thus viral glycoprotein activity would not be fully realized until virus particles are secreted. Without a reliable infectivity model for HCV replication, the effects of glucosidase inhibitors on HCV infection remain unknown. Thus, glucosidase inhibitors may serve as novel antiviral therapies for the treatment of HCV and other flavivirus infections.

MATERIALS AND METHODS

Cells, viruses, and glucosidase inhibitors

Madin–Darby bovine kidney cells (MDBK, ATCC-CCL22) were grown in DMEM/F12 (Gibco BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated horse serum (Gibco BRL). MDBK cells were tested for BVDV contamination by RT-PCR as described (Sullivan and Akkina, 1995). The cytopathic (cp) NADL strain of BVDV was kindly provided by Dr. Ruben Donis, University of Nebraska, and was prepared by transfection of infectious RNA transcribed *in vitro* from an infectious cDNA clone (Vassilev *et al.,* 1997). The resulting virus stock was plaque-purified three times on MDBK cell monolayers prior to large-scale virus stock preparation.

The glucosidase inhibitor nB-DNJ was provided by Synergy Pharmaceuticals (Sommerset, NJ).

BVDV single-cycle replication

MDBK cells (3×10^5 cells per 20-mm-diameter well) were infected in triplicate at a multiplicity of infection (m.o.i.) of 0.05 or 5 PFU/cell. At 1 h postinfection, the virus inoculum was removed, and the monolayers were washed twice with 1 ml of MDBK medium. MDBK medium, in the presence and in the absence of 4.5 mM nB-DNJ, was added to the cultures. At 0, 4, 8, 12, 16, 20, and 24 h postinfection the cultures were harvested and frozen at −70°C. The cultures were thawed at 37°C and immediately sonicated (Heat Systems ultrasonicator, Farmington, NY) at 40% power for 30 s at 0°C to release cell-associated virus. Cell debris was removed by centrifugation at 1000 *g* for 10 min at 4°C and virus yields in the supernatant fluids were measured by plaque assay.

Plaque assay for measuring virus yields

MDBK monolayers (3×10^5 cells per 20-mm-diameter well) were infected with 100 μl of 10-fold dilutions of virus suspensions. At 1 h postinfection the inoculum was removed and the monolayers were washed once with MDBK medium, and 1 ml of MDBK medium containing 1% methyl cellulose overlay was added to the monolayers. Since most plaques were counted at the 10^{-4} dilution of virus suspension, the concentrations of glucosidase inhibitors that might be present in the virus suspension were well below the concentrations needed to inhibit virus replication.

BVDV genomic RNA synthesis

BVDV genomic RNA synthesis was measured by the method of Purchio *et al.* (1983). MDBK monolayers (7×10^5 cells per 35-mm-diameter dish) were infected with >1 PFU/cell of BVDV. At 1 h postinfection the monolayers were washed twice with 1 ml of MDBK medium. MDBK medium alone or medium containing 4.5 mM nB-DNJ was added to the cultures. At 20 h postinfection, medium containing actinomycin D (5 μg/ml) in the presence and in the absence of glucosidase inhibitor was added to the cultures. Following a 2-h incubation in the presence of actinomycin D, 50 μCi/ml [5,6-^{3}H]uridine (43 Ci/mmol) was added to the culture medium and the cultures were incubated for 5 h. Total intracellular RNA was isolated using the Ultraspec RNA isolation system according to the manufacturer's specifications (Biotecx Laboratories, Inc., Houston, TX). The RNA was denatured by treatment with 1 M glyoxal in 50% DMSO for 1 h at 50°C and separated by electrophoresis on a 0.7% agarose gel. The gel was treated with 3% 2,5-diphenyloxazole in methanol–water and dried under vacuum. The radiolabeled BVDV RNA was visualized by autoradiography. The autoradiographic images following 12, 24, and 48 h of exposure were used to determine the pixel density in each band by densitometry.

Northern blot analysis

MDBK cell monolayers (7×10^5 cells per 35-mm-diameter dish) were infected with BVDV at >1 PFU/cell. At 1 h postinfection the cultures were washed twice with 1 ml MDBK medium and the cultures were incubated with medium alone or 4.5 mM nB-DNJ medium. At 24 h postinfection cell culture supernatants were prepared by centrifugation of medium at 1000 *g* for 10 min at 4°C to remove cell debris. Virus yields were determined from a portion of the supernatant fluids. The remaining supernatant fluids were incubated with 0.25% SDS and 200 μg/ml proteinase K for 20 min at 50°C. The samples were extracted twice with an equal volume of buffer-saturated phenol:chloroform:isoamyl alcohol (25:24:1, v/v) and once with an equal volume of chloroform:isoamyl alcohol (24:1, v/v). The RNA was precipitated in ethanol and collected by centrifugation at 14,000 *g* for 15 min at 4°C. The RNA was denatured in 1 M glyoxal for 1 h at 50°C and separated on a 0.7% agarose gel in sodium phosphate buffer (Ausubel *et al.*, 2001). The RNA was transferred to nylon membrane by Northern blotting and probed with ^{32}P-radiolabeled cDNA specific for the BVDV E2 gene. The Northern blot was visualized by phosphorimager analysis.

Equilibrium centrifugation

Equilibrium sedimentation of BVDV virions was performed as described by Pritchett *et al.* (1975) with the following modifications. MBDK monolayers (1.5×10^7 cells per 225-cm^2 flask) were infected with BVDV at >1 PFU/cell. At 6 h postinfection, 0.1 μg/ml actinomycin D in the presence and in the absence of 4.5 mM nB-DNJ was added to the cultures. At 7 h postinfection total RNA was labeled by addition of 100 μCi/ml [5,6-^{3}H]uridine (43 Ci/mmol) to the culture medium. At 24 h postinfection the cultures were harvested and cell debris was removed by centrifugation at 1000 *g* for 10 min at 4°C. Virus in the

supernatant fluids was precipitated in 10% polyethylene glycol (PEG, 8000 MW) and collected by centrifugation at 10,000 *g* for 60 min at 4°C. The PEG precipitate was resuspended in 1 ml of TEN buffer (10 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1 mM EDTA) and layered onto a 14-ml 10 to 40% continuous sucrose gradient prepared in TEN buffer. The samples were centrifuged to equilibrium at 90,000 *g* for 24 h at 4°C. The amount of radiolabeled material in each fraction from the gradient (0.4 ml) was measured by TCA precipitation and liquid scintillation counting. The amount of infectious BVDV in every second fraction was measured by plaque assay.

Western blotting

MDBK monolayers (1.5×10^7 cells per 225-cm^2 flask) were infected with BVDV at >1 PFU/cell in the presence and in the absence of 4.5 mM nB-DNJ. At 18 h postinfection the infected cell monolayers were scraped into 1× PBS and the cells were collected by centrifugation at 1000 *g* for 10 min at 4°C. The cell pellet was resuspended in 500 μl of lysis buffer (10 mM Tris–HCl, pH 7.6; 150 mM NaCl; 1 mM EDTA; 0.5% IGEPAL CA-630; 1× protease inhibitor cocktail; Sigma Inc., St. Louis, MO; Catalog No. P8340) and incubated for 5 min on ice. The insoluble debris was removed by centrifugation at 1000 *g* for 5 min at 4°C. An equal volume of 2× SDS–PAGE sample buffer was added to supernatant fluids which were stored at −80°C. Secreted virions were collected at 48 h postinfection and precipitated by the addition of PEG (8000 MW) to 10% in TEN buffer. The PEG precipitates were collected by centrifugation at 20,000 *g* for 60 min at 4°C and resuspended in 500 μl TEN buffer.

Virus samples from PEG precipitated material and intracellular protein lysates were analyzed by SDS–PAGE and Western blotting. Protein concentrations were determined by Bradford assay and equal amounts of protein were loaded in each lane of the gel. The Western blots were probed with a monoclonal antibody WB166 specific for BVDV E2 protein (Central Veterinary Laboratories, Surrey, UK). The Western blots were developed using alkaline phophatase-conjugated goat anti-mouse antibodies (Sigma Inc.) and BCIP/NBT colorimetric substrates. Densitometric analysis of scans of the Western blots was performed to quantify the relative amounts of each protein species.

Treatment of protein extracts with endo H (Boehinger Mannheim, Indianapolis, IN) was performed according to the manufacturer's recommendations. RNase B and Fetuin (Sigma Inc.) were used as positive and negative controls, respectively. RNase B was completely digested by endo H during the 1-h incubation period while Fetuin was resistant to endo H cleavage.

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