N-Linked Glycosylation of West Nile Virus Envelope Proteins Influences Particle Assembly and Infectivity

Sheri L. Hanna, Theodore C. Pierson, Melissa D. Sanchez, Asim A. Ahmed, Mariam M. Murtadha, and Robert W. Doms*

Department of Microbiology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

Received 1 April 2005/Accepted 25 July 2005

West Nile virus (WNV) encodes two envelope proteins, premembrane (prM) and envelope (E). While the prM protein of all WNV strains contains a single N-linked glycosylation site, not all strains contain an N-linked site in the E protein. The presence of N-linked glycosylation on flavivirus E proteins has been linked to virus production, pH sensitivity, and neuroinvasiveness. Therefore, we examined the impact of prM and E glycosylation on WNV assembly and infectivity. Similar to other flaviviruses, expression of WNV prM and E resulted in the release of subviral particles (SVPs). Removing the prM glycosylation site in a lineage I or II strain decreased SVP release, as did removal of the glycosylation site in a lineage I E protein. Addition of the E protein glycosylation site in a lineage II strain that lacked this site increased SVP production. Similar results were obtained in the context of either reporter virus particles (RVPs) or infectious lineage II WNV. RVPs or virions bearing combinations of glycosylated and nonglycosylated forms of prM and E could infect mammalian, avian, and mosquito cells (BHK-21, QT6, and C6/36, respectively). Those particles lacking glycosylation on the E protein were modestly more infectious per genome copy on BHK-21 and QT6 cells, while this absence greatly enhanced the infection of C6/36 cells. Thus, glycosylation of WNV prM and E proteins can affect the efficiency of virus release and infection in a manner that is cell type and perhaps species dependent. This suggests a multifaceted role for envelope N-linked glycosylation in WNV biology and tropism.

* Corresponding author. Mailing address: Department of Microbiology, University of Pennsylvania School of Medicine, 225 Johnson Pavilion, 3610 Hamilton Walk, Philadelphia, PA 19104. Phone: (215) 898-0890. Fax: (215) 898-9557. E-mail: doms@mail.med.upenn.edu.
flaviviruses (2, 28, 30, 31). Removal of the N-linked glycosylation site in prM or E significantly reduced the level of SVP and virus release from transfected or infected cells. However, reporter particles capable of a single round of infection and recombiant virus particles containing glycosylation-deficient prM or E were still infectious, albeit slightly less than the wild type. The effects of E protein glycosylation on viral infectivity were more interesting. While the absence of N-linked glycosylation on lineage I or II E proteins was associated with a modest increase in the infectivity of mammalian or avian cells, there was a markedly enhanced infection of C6/36 mosquito cells by these E glycosylation-null viruses. Thus, N-linked carbohydrate structures can significantly impact particle assembly and viral infectivity in a manner that is cell type and perhaps even species dependent.

MATERIALS AND METHODS

Plasmids. (i) prM-E expression vectors. WNV lineage I envelope proteins were expressed as a polypeptide using plasmid pcBNW (13) and as individual envelope proteins, prM and E protein, from pcDNA 6.2 (Invitrogen, Carlsbad, CA). WNV lineage II envelope proteins, subcloned from molecular clone SpWNV/Xba (68), were expressed individually and also as a polypeptide from pcDNA6.2. Glycosylation mutants of lineage I envelope proteins and lineage II prM were introduced using QuikChange site-directed mutagenesis (Invitrogen, Carlsbad, CA) by creating two nucelotide substitutions resulting in an amino acid change (N to Q), thereby removing the glycosylation site while introducing a chemically conservative mutation. Using overlap extension PCR methodology, the consensus WNV glycosylation site (NYST) was added to the lineage II E protein starting at amino acid 134.

(ii) WNV lineage II. Infectious molecular clones of WNV encoding point mutations that add or delete N-linked glycosylation sites were constructed by modification of a DNA-launched molecular clone of a lineage II strain of WNV that encodes GFP (WNII-IF) (48). WNII-IF was modified to introduce silent restriction sites that flank the sequences encoding prM and E, simplifying the introduction of different envelope protein gene cassettes. This was accomplished with two sequential overlap extension PCR procedures. First, a silent AvIII site (underlined below) was introduced downstream of E by engineering five mutations into an oligonucleotide primer (ACCCTCTCG to ACCTTAAGT) for use in overlap extension PCR. Flanking primers were used to generate a fragment containing this silent mutation into novel BglII and NsiI sites in WNII-IF. In addition to insertion of the silent AvIII site, this cloning step removed the majority of sequence encoding prM and E proteins. Next, a silent AgeI site was introduced using a similar approach (ACAGGGC to ACCGGG), generating pWNII(ΔME)-IF. A variant of this prM-E recipient vector lacking the IRES- GFP reporter gene cassette was also constructed by restricting the plasmid with NotI followed by religation [pWNII(ΔME)-Not]. Introduction of prM-E genes from WNV lineage II was accomplished by generating PCR fragments flanked by AgeI and AvIII sites and introducing them into either pWNII(ΔME)-IF or pWNII(ΔME)-Not using standard techniques. Sequences of primers and infectious clones are available upon request.

Virus production. WNV WNV lineage I strain 3000.00259 NY 2000 (NY2000) was propagated in C6/36 cells, collected 72 h postinfection (p.i.), and clarified through a 0.2 μm syringe filter (14). Lineage II viruses (strain 956 D117 3B) were produced by transfection of HEK-293T cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with pWNII-Not (48) or envelope glycosylation mutants as described above. Six hours after transfection, culture medium was removed and replaced with Dulbecco’s modified Eagle medium (DMEM)-low glucose containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Virus containing supernatant was collected 30 h postinfection, clarified through a 0.2 μm syringe filter, and used to infect BHK-21 or C6/36 cells. Virus was collected 48 h postinfection and clarified as described above. For storage, viral supernatants were mixed with a 30% volume of freezing medium (100% FBS, 30 mM HEPS, pH 8.0) and stored at −80°C.

Antibodies. Five flavivirus antibodies were used in this study: WNV hyperimmune ascites fluid (ATCC, Manassas, VA); dengue E monoclonal antibody (MAb) 4G2 (15); and WNV E MAbs 4E1 (53). E1 (kindly provided by Mike Diamond, Washington University, St. Louis), and 5H10 (BioReliance Corp., Rockville, MD). A polyclonal rabbit serum raised against calnexin (kindly provided by A. Helenius) was used for immunofluorescence detection of the endoplasmic reticulum. Secondary antibodies goat anti-mouse Alexafluor 594 (for WNV) and goat anti-rabbit Alexafluor 488 (for ER) (Molecular Probes, Eugene, OR) were used for all immunofluorescence studies. A sheep anti-mouse immunoglobulin G (IgG) horseshadish peroxidase (HRP) secondary antibody (Amersham Biosciences, Buckinghamshire, England) was used for all Western blots and plastic capture enzyme-linked immunosorbent assays (ELISAs). A streptavidin-HRP conjugate (Sigma) was used in the antigen capture ELISA. MAb 4E1 was directly conjugated to Alexafluor 647 for use in flow cytometry using an antibody conjugation kit (Molecular Probes, Eugene, OR).

Immunofluorescence microscopy. HeLa cells were plated at 7 × 10^5 cells per well in 24-well plates containing glass coverslips. The following day, cells were transfected with 1.7 μg of DNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Twenty-four hours posttransfection, all cells were fixed using 2% paraformaldehyde for 20 min at room temperature. Cells were then washed and permeabilized using 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 5 min at room temperature. Prior to incubation with antibody, coverslips were blocked with 5% FBS in PBS for 1 h (room temperature) to overnight (4°C). WNV prM and E proteins were detected using WNV hypermune ascites fluid (1:500 dilution; ATCC, Manassas, VA), and the endoplasmic reticulum was detected with a polyclonal rabbit antibody to the ER protein, calnexin (1:250 dilution). All antibodies were diluted in PBS containing 5% FBS. Secondary antibodies used at a 1:500 dilution were goat anti-mouse Alexafluor 594 for WNV ascites fluid and goat anti-rabbit Alexafluor 488 for the calnexin antibody (both from Molecular Probes, Eugene, OR). In addition, 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) was included with the secondary antibodies at a 1:500 dilution to stain the nucleus. Coverslips were mounted using ProLong (Molecular Probes, Eugene, OR) and imaged at 100× with a Nikon E600显微镜.

Electron microscopy. Vero cells were plated at 7 × 10^5 cells in a 10-cm^2 dish and infected with lineage II West Nile virus at a multiplicity of infection of 1.0. Twenty-four hours p.i., cells were washed and fixed with 4% glutaraldehyde and placed at 4°C. Cells were then dehydrated, placed in an Epon resin, thin sectioned, placed on a copper grid, poststained, and examined by transmission electron microscopy.

Particle isolation. HEK-293T cells were transfected with viral envelope protein expression plasmids as described above and the supernatant collected 48 h posttransfection. Supernatants were cleared with a low-speed spin at 500 x g for 10 min and then filtered through a 0.2-μm filter disk. Clarified supernatants were then layered over a 20% sucrose cushion (HEPES buffer, pH 8.0) and ultracentrifuged at 4°C in an SW55 rotor at 40,000 rpm for 1 h or in an SW41 Ti rotor at 38,000 rpm for 2 h to pellet the particles. Pelleted SVPs were resuspended overnight at 4°C in 60 μl resuspension buffer (150 mM NaCl, 50 mM HEPES [pH 8.0] with protease inhibitors [Roche Molecular Biochemicals, Indianapolis, IN]).

Equilibrium gradient centrifugation. Subviral and viral particles were separated based on density using a continuous 20 to 60% sucrose gradient. Resuspended SVPs were layered on the top of the gradient and centrifuged in an SW55 rotor for 38,000 rpm for 14 h at 4°C. Fractions were collected and the refractive index determined for each. Aliquots from each fraction were run on a 10 to 20% reducing sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gel and transferred to a polyvinylidene difluoride membrane. WNV prM and E proteins were detected using WNV HIAF (1:500; ATCC), visualized using a sheep anti-mouse IgG HRP secondary antibody, and imaged with SuperSignal West Femto maximum sensitivity substrate (Pierce Biotechnol.

Plastic capture ELISA. SLVs were bound to Immulon 2HB ELISA plates (Thermo Lab Systems Inc., Franklin, MA) in binding buffer (0.15 M Na carbonate, 0.35 M Na bicarbonate, pH 9.6) at 4°C overnight. Plates were washed with PBS three times and wells blocked with Biotto (1× PBS, 5% powdered milk, 0.1% Tween 20, 0.1% NaAzide) for a minimum of 1 h. Biotto was removed, and WNV hyperimmune ascites fluid (1:500 diluted in Biotto) was added and allowed to bind for 1 h at 37°C or overnight at 4°C. Unbound antibody was removed by three washes with PBS and sheep anti-mouse IgG HRP added (1:10,000 diluted in Biotto without NaAzide). Plates were incubated at 37°C with the secondary antibody for 45 min, washed three times, and developed by adding TMB peroxidase substrate (KPL, Inc., Gaithersburg, MD). Plates were read in a MRX Revelation plate reader at a wavelength of 450 nm.

Glycosylation assays. HEK-293T cells were plated in a six-well plate at 1 × 10^6 cells and transfected the following day with WNV glycoprotein expression plasmids. Forty-eight hours posttransfection, both supernatants and cells were collected. SVPs were harvested from the supernatant by pelleting through a 20% sucrose cushion (HEPES buffered, pH 8.0) and cells solubilized in lysis buffer (50 mM Tris–150 mM NaCl–2 mM EDTA–1% Triton X-100 [pH 7.5] with protease

Downloaded from http://jvi.asm.org/ by guest on September 8, 2017.
inhibitors [Roche Molecular Biochemicals, Indianapolis, IN]. Lysates were cen-
trifuged at 10,000 × g for 10 min to clear cellular debris. Virus and virally infec-
ted cells were treated similarly with the exception that frozen viral stocks were
thawed and used directly. Both SVPs/virus and lysates were denatured in sample
preparation buffer (2% SDS-50 mM Tris [pH 6.8]–10% glycerol) at 55°C for 20 min.
Aliquots from each sample were then treated with 50 U of peptide N-glycosidase F (PNGase F) or endoglycosidase F (Endo H) according to the
manufacturer’s instructions (New England Biolabs, Inc., Beverly, MA) or with
PNGase F buffer without enzyme as a control. Digestions were carried out for 3 h
at 37°C. Samples were then analyzed under denaturing conditions by SDS-PAGE
(7.5% or 10 to 20% gradient) and Western blotting using E MAb S1H10 (1,500,
BioReliance Corp., Rockville, MD) or WNV hypermuscle ascites fluid (1,500)
and an appropriate HRP-conjugated murine secondary antibody. Blots were
imaged using SuperSignal West Femto maximum sensitivity substrate (Pierce
Biotechnology, Inc., Rockford, IL) on a FujiFilm LAS-1000 camera.

Antigen capture ELISA. Flavivirus E-specific antibody 4G2 (15) was bound to
Immunol 2HB ELISA plates at a concentration of 100 ng/well diluted in binding
buffer at 4°C overnight. Plates were washed with washing buffer (PBS, 0.05%
TWEEN 20) three times and then incubated in blocking buffer (1% PBS, 1%
bovine serum albumin, 0.05% TWEEN 20) for 1 h with shaking at room temper-
ature. Buffer was removed, and WNV particles in blocking buffer containing 1%
Triton X-100 were diluted 1:2 across the plate in dilution buffer (blocking buffer
with 0.01% Triton X-100). A truncated, soluble form of WNV E protein was
produced from a recombinant vaccinia virus (53), purified, bicinechonic acid
quantified, and used as a standard in this assay. Twenty-five ng of soluble E was
added to one well on the plate in duplicate and then diluted 1:2 as specified
above to generate a standard curve. Plates and standards were allowed to bind
for 60 min at 37°C. Plates were washed five times with PBS at room temperature.
WNV E protein was detected using a 1:2,500 dilution of streptavidin conjugated to HRP (Sigma) was added to each well and incubated on the shaker
at room temperature for 1 h. Plates were washed as described before, and 100 µl of a
1:2,500 dilution of streptavidin conjugated to HRP (Sigma) was added to each well and
incubated on the shaker at room temperature for 1 h. Plates were then washed six times and visualized using a peroxidase substrate (KPL, Inc., Gaithersburg, MD). Plates were read in an
Infinite M2000 Pro reader at a wavelength of 450 nm.

Production of reporter virus particles. WNV reporter virus particles (RVPs)
were produced by complementation of a DNA-launched WNV replication with
structural gene products provided in trans (48a). Briefly, HEK-293T cells were
plated at 2 × 10⁷ cells per 10-cm² dish. The following day, cells were transfected
with a total of 20 µg of three different plasmids (33 µg replication plasmid
[pWIIrep-GFP], 10 µg WNI capsid [pWcap(s)], and 6.7 µg polyprotein plasmid
encoding envelope proteins) using a calcium phosphate transfection kit (Invitro-
gen, Carlsbad, CA). Medium was replaced on the following day with a low
plaque-forming medium by using the QIAmp viral RNA kit (QIAGEN, Valencia,
CA). Medium was replaced on the following day with a low
volume for 1 h at 37°C. Virus inoculum was then placed in 24-well plates in a volume of 2 ml per well and allowed to adhere overnight. Cells were harvested,
fixed in 2% paraformaldehyde in PBS, and analyzed by flow cytometry 40 h
postinfection for BHK-21 and QT6 cells and 72 h postinfection for C6/36 cells.

Metabolic labeling. HEK-293T cells were transfected with a plasmid encoding
WNV envelope polyprotein for SVPs or with replication, capsid, and envelope
polyprotein for RVPs, as described above. Medium was replaced from cells 12 h
posttransfection as described above and analyzed under denaturing conditions
by SDS-PAGE and the WNV E, prM, or M protein band for each sample quantified
with a Storm-860 phosphorimaging system (Molecular Dynamics, Sunnyvale,
CA).

Quantitative PCR. Viral RNA was isolated from 140 µl clarified RVP or virus
containing medium by using the QiAmp viral RNA kit (QIAGEN, Valencia, CA)
with the addition of two DNase treatments. Prior to the addition of lysin
buffer, particle containing medium was incubated for 30 min with 100 U RNAse-
free DNase I (Roche Molecular Biochemicals, Indianapolis, IN) at room temper-
ature. In addition, prior to elution, an on-column digestion of DNA was
performed for 30 min at room temperature using 100 U RNAse-free DNase using
a modification of the manufacturer’s protocol (QIAGEN, Valencia, CA). RNA
was eluted in 50 µl of elution buffer and stored at −20°C until used. A primer was
designed to bind to the 3’ untranslated region (UTR) of lineage I (5’TCTTACA
GCTTACGAACTTTA-3’) for use in cDNA synthesis. Reverse transcrip-
tase-PCR was performed using the Superscript III First-Strand synthesis system
according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA). The WNV
TaqMan primers and probe were designed using a modification of the approach
described by Lanciotti et al. (34) (forward, 5’-CAGTGTGCAAGCACCACACTTTA
ATGT-3; reverse, 5’-CATAGCCAGGTTATGGGGCCTGGTTG-3; and probe,
5’-FAM-TCTTACGAACTTTA-3’-TAMRA). A primer was

RESULTS

Intracellular localization of WNV lineage I and II envelope proteins. Most studies on flavivirus assembly indicate that
these viruses bud into the lumen of the ER (40, 41, 60). How-
ever, the Sarafend strain of WNV, a lineage II strain, has been
reported to bud at the plasma membrane (47). We examined the intracellular distribution of prM and E in HeLa cells infec-
ted with a lineage I (NY99-6480) and a lineage II (956 D117
3B) strain of WNV and cells transfected with plasmids encoding
lineage I and II envelope polyproteins. Detection by indi-

Downloaded from http://jvi.asm.org/ by guest on September 8, 2017

J. VIROL. 13264 HANNA ET AL.
rect immunofluorescence showed that both prM and E colocalized with calnexin, a molecular chaperone that resides within the lumen of the ER. Consistent with our light microscopic results, and as noted below, intracellular glycosylated forms of WNV prM and E remained Endo H sensitive, supporting localization in the ER. Finally, transmission electron microscopy of Vero cells infected with lineage II strain 956 D117 3B of WNV clearly showed virus particles within the rough ER and contained in membrane vesicles within cells (Fig. S1, http://www.med.upenn.edu/micro/domslab/hannajv.png). Thus, consistent with the results of others (40, 41, 60), we conclude that in HeLa and Vero cells, the WNV strains used in this study assemble by budding into the ER and not at the plasma membrane.

**Secretion of subviral particles by expression of envelope proteins.** Expression of the prM and E glycoproteins of several flaviviruses in the absence of other viral proteins or the viral genome results in the production and secretion of recombinant SVPs (2, 28, 30, 31). SVPs are also a natural by-product of flavivirus infection (52) and, for those studied thus far, are structurally (albeit smaller) and antigenically similar to infectious virus (11, 23, 55). Since WNV is classified as a BSL-3 agent, WNV SVPs represent a useful tool to study envelope protein structure and function. It has already been shown that expression of WNV prM and E results in the release of viral antigen into the medium (13). To further characterize this soluble antigen, we transfected human HEK-293T cells with plasmids expressing lineage I prM and E singly, in combination, or as part of the polyprotein to determine if viral envelope release into the medium is dependent on the expression of both envelope proteins and whether they must be expressed in cis. The presence of prM and E in both cell lysates and pelleted media collected from transfected cells was determined by Western blot analysis. Lineage I WNV E, prM, and the mature M protein were recovered only from pelleted cell media when both proteins were expressed (Fig. 1A and 1B), independent of whether they were expressed from one plasmid or in trans. The same experiments were carried out with lineage II envelope proteins with similar results (data not shown). When analyzed by equilibrium density gradient ultracentrifugation, the SVPs sedimented as a discrete population with an apparent density of 1.16 g/cm³ (Fig. 1C), which corresponds to the relative density for tick-borne encephalitis virus SVPs (55).

**Glycosylation profile of envelope proteins within cells and incorporation into particles.** N-linked glycosylation plays an important role in both the assembly and the infectivity of many viruses (12, 17, 37, 49, 57, 58, 64, 66, 67). All strains of WNV examined thus far encode a potential N-linked glycosylation site in the ectodomain of the prM protein at amino acid 15. In contrast, the presence of a potential N-linked glycosylation site in E is variable in both WNV lineages, although only lineage II isolates contain a 4-amino-acid deletion at this position that ablates the N-linked glycosylation site. To determine if these glycosylation sites are utilized, HEK-293T cells were transfected with a plasmid expressing a lineage I prM/E polyprotein (NY99-6480) containing potential glycosylation sites in both prM and E. Cell lysates were prepared and analyzed by Western blotting, with aliquots digested with either PNGase F (removes all N-linked carbohydrate structures) or Endo H (removes only immature carbohydrate structures). Two different types of SDS-PAGE gels were used to resolve the E and prM proteins obtained from HEK-293T cells (Fig. 2A). In cell lysates, both prM and E migrated more quickly following PNGase F digestion, indicating that the single N-linked sites in prM and E were utilized. Similar results were obtained with Endo H, indicating that in cells, WNV glycoproteins were predominantly in high mannose form, consistent with their localization to the ER (Fig. 2A). Identical results were obtained with lysates from either BHK-21 or Vero cells infected with lineage II virus (Fig. 2B).

In contrast to their cell-associated forms, prM and E released from cells either as SVPs or as viruses were largely or completely Endo H resistant (Fig. 2C), consistent with modification of the carbohydrate side chains as the envelope proteins were transported through the Golgi apparatus. These data show that when present, the glycosylation sites on prM and E are utilized and suggest that the rate-limiting step of particle assembly is folding and association in the ER (39), while transport and processing through the Golgi network and secretory pathway occurs quickly, resulting in the majority of Golgi-processed particles being released into the supernatant.

**Effects of glycosylation on SVP formation.** To assess the effects of N-linked glycosylation on WNV SVP assembly and release, we individually removed or added the glycosylation sites in prM and E of lineage I (NY99-6480) and II (956 D117 3B) envelope polyproteins. The lineage I construct contained glycosylation sites in both prM and E that were individually removed by changing the asparagines in each N-linked site to glutamine. The lineage II polyprotein encoded a glycosylation site in prM but contained a 4-amino-acid deletion in E found only in lineage II strains, resulting in the loss of the E glycosylation site. Therefore, we made one construct removing the glycosylation site in prM and another in which we introduced the N-linked site commonly found in lineage I strains (NYST) into the lineage II E protein. Western blots of cell lysates and media from HEK-293T cells transfected with lineage I constructs showed that the loss of either the prM or E protein glycosylation sites resulted in a marked decrease in the secretion of SVPs (Fig. 3A). Changes in amino acid sequence or polypeptide modifications can adversely affect protein folding; therefore, we repeated the experiment at a lower temperature, 32°C, which can sometimes rescue a misfolding phenotype, but obtained similar results (Fig. 3A). In agreement with results obtained with the envelope proteins from the lineage I strain, when lineage II proteins were expressed, we found that the loss of glycosylation in prM resulted in a decrease in SVP release. However, the introduction of an N-linked glycosylation site in lineage II E protein had no obvious effect on SVP release, as judged by nonquantitative Western blot analysis (Fig. 3B).

To quantify the release of SVPs, we utilized an antigen capture ELISA where pelleted medium from transfected cells was bound to wells coated with a flavivirus E antibody (4G2) and the amount of bound SVPs detected by another noncompeting E monoclonal antibody (MAb) (E1). Quantitation based on ELISA data confirmed a dramatic decrease in SVP release with the removal of either the prM or E glycosylation sites in lineage I (3% and 10% of wild-type levels, respectively) (Fig. 3C). Removal of the N-linked glycosylation site in lineage II prM also resulted in a dramatic decrease in SVP release.
However, SVP release was greatly enhanced with the introduction of an N-linked glycosylation site in the lineage II E protein (approximately 1 log). Since these mutations could potentially alter the reactivity of these proteins with the antibodies used in the Western blot and ELISA, we also analyzed SVP release by metabolically labeling particles with [35S]methionine-cysteine. SVPs were pelleted from the medium of transfected cells by ultracentrifugation, separated by SDS-PAGE, and quantified by phosphorimage analysis. Quantitation of the prM and E band densities confirmed the effects of removing or adding carbohydrate sites on particle release seen using the antigen capture ELISA (Fig. 3D).

**Effects of glycosylation on virus production.** We have developed methods for the production of WNV particles capable of a single round of replication (RVPs) by complementation of a subgenomic replicon with structural genes in trans (24, 25, 48a, 59). RVPs were produced by transfection of HEK-293T cells with plasmids expressing WNV capsid, a DNA-launched replicon encoding GFP, and either wild-type or glycosylation mutant envelopes from lineage I and lineage II strains of WNV (Fig. 4A). Particle-containing medium was collected 48 h post-transfection and particle release quantified by both WNV antigen capture ELISA and quantitative PCR (qPCR) using primers and a probe specific for the 3' UTR of the WNV
Viral RNA genome copies within a sample were calculated using a DNA plasmid as a standard. This sensitive qPCR assay made it possible to measure the relative number of viral genome copies in a sample rather than an absolute number. Thus, we were able to measure the levels of viral antigen as well as the viral genome number (Fig. 4B). Such an analysis is required because mutations in the envelope proteins that impact folding or overall expression levels could alter the ratio of infectious to noninfectious particles by affecting the levels of SVP production and this shift would not be appreciated using only an antigen ELISA (48a). We found that removal of N-linked glycosylation from either prM or E reduced the production of RVPs, as measured by either viral antigen or WNV genome copies, though not always to the same extent (Fig. 4B). Removal of the N-linked site from either lineage I or lineage II prM had a greater impact on the release of viral antigen than on the release of viral genome copies. Altering the glycosylation status of the E protein, however, had little effect on the ratio of genome-carrying virus particles to subviral particles. Thus, N-linked glycosylation had similar effects on SVP and RVP secretion.

To determine if changes in glycosylation affected the overall structure of the particles released, we analyzed lineage I wild-type, prM glycosylation-null, and E glycosylation-null RVPs by equilibrium density gradient ultracentrifugation. All RVP preparations sedimented with the same apparent density (1.16 g/cm³), indicating that the bulk of the particles produced with each envelope construct were similar (data not shown). Additionally, we produced [35S]methionine-cysteine-labeled RVPs and assessed the ratio of E to prM/M and M to prM for each of the lineage I and lineage II envelope constructs. We found that changing the glycosylation status of either lineage I or lineage II E protein had no effect on prM processing or on the ratio of E protein to prM. The very low levels of protein recovered from viruses lacking the glycosylation site in prM precluded an accurate assessment of protein content and processing (Fig. S2, http://www.med.upenn.edu/micro/domslab/hannajvi.pdf). Taken together, these data suggest that the glycosylation status of either E or prM does not significantly alter the overall density or organization of the viral particles.

Effect of glycosylation on reporter virus infectivity. To assess the ability of prM and E glycosylation mutant RVPs to infect target cells, we produced RVPs in HEK-293T cells, collected the medium, and quantified the number of viral genome copies by qPCR. Based on these data, RVPs for each construct were normalized to each other based on genome copies and used to infect BHK-21 cells, varying the viral input over a broad range. Infection efficiency was determined by measuring the percentage of GFP-expressing cells using flow cytometry 40 h postinfection (Fig. 5A). All envelopes were capable of mediating the infection of target cells. For lineage I, infectivity increased slightly when the glycosylation site in E was removed. In contrast, introduction of a glycosylation site into the lineage II E protein reduced infectivity. Removal of the prM glycosylation site in either lineage had a negligible effect on viral infectivity.

The natural infection cycle for West Nile virus occurs between mosquitos and birds, with mammals as incidental hosts.
For this reason, we tested RVP infectivity on QT6 (quail) and C6/36 (Aedes albopictus mosquito) cells (Fig. 5B and 5C). Relative infection levels on QT6 cells were consistent with results seen on BHK-21 cells. However, altering glycosylation patterns had a dramatic effect on the infection of C6/36 mosquito cells. For lineage I WNV, the removal of the E protein glycosylation site enhanced RVP infectivity by more than 30-fold. Likewise, introducing a glycosylation site into the lineage II E protein reduced viral infectivity by an equivalent amount (Fig. 5B). Thus, the presence of an N-linked carbohydrate chain at amino acid position 154 on the WNV E protein of both lineages I and II greatly reduced viral infectivity on C6/36 mosquito cells while the presence of a carbohydrate structure on E had only a modest effect on the infection of mammalian or avian cells.

Effect of glycosylation on replication-competent virus infectivity. To examine the effects of prM and E protein glycosylation on WNV infectivity in the context of replication-competent virus, lineage II (956 D117 3B) glycosylation mutant envelopes were cloned into a DNA-launched infectious molecular clone derived from the same viral strain (48). Virus stocks were produced in BHK-21 cells, viral RNA was quantified by qPCR, and an equivalent number of genome copies for each virus was used to infect BHK-21, QT6, and C6/36 cells across a broad range of viral dilutions. To monitor initial virus infec-

FIG. 3. Glycosylation influences SVP production. HEK-293T cells were transfected with polyprotein plasmids encoding prM and E from lineage I or lineage II WNV wild type (wt), glycosylation-mutated prM or E, or an irrelevant protein (GFP) as a control. Mutants used were: prM knockout (k/o), which lacked the glycosylation site in prM; E k/o, which lacked the glycosylation site in the E protein of lineage I WNV; and E k/in, which contained a 4-amino-acid insert (NYST) reestablishing the N-linked glycosylation site in the lineage II E protein. Cell lysates and released SVPs were collected 48 h posttransfection. (A) Western blot of lineage I lysates and SVPs from a standard transfection at 37°C probed with antibodies that recognize the WNV membrane proteins (orPrM/oE). Repeating the experiment at 32°C did not alter the phenotype of either mutant. (B) Western blot of lineage II lysates and SVPs probed with WNV HIAF. (C) SVP quantified using a WNV E antigen capture ELISA. Data represent three separate experiments normalized by setting the sample from each lineage with the highest release of antigen to 100% release. (D) SVP release was also quantified by metabolically labeling cells expressing the indicated constructs with [35S]methionine-cysteine and measuring the density of the resulting WNV E bands using a phosphorimager.
tion, intracellular FACS staining was performed 12 h postinfection for BHK-21 and QT6 cells and 24 h postinfection for C6/36 cells. Consistent with the results seen with RVPs, the absence of glycosylation on prM resulted in a decrease in virus release, as measured by viral antigen levels and genome copies (data not shown), but had only modest effects on initial infectivity (Fig. 6A through C). The presence of the E protein glycosylation site was associated with enhanced virus assembly and release (data not shown) but with a threefold decrease in viral infectivity on BHK-21 and QT6 cells (Fig. 6A and 6B) and with a greater than 30-fold decrease in infectivity on C6/36 cells. Thus, these results using replication-competent virus are consistent with the RVP data.

The dramatic decrease in viral infectivity on C3/36 cells when E protein was glycosylated was somewhat surprising, since this cell line is often used to propagate WNV stocks. We therefore performed viral growth curves on C3/36 cells, BHK-21 and Vero cells, normalized the virus inocula by PFU (multiplicity of infection of 1.0) rather than by genome copy number. Under these conditions, infection with the E-glycosylated mutant produced up to a log more virus, as measured by PFU, than E glycosylation-null wild-type lineage II virus when grown on all cell types tested (Fig. S3, http://www.med.upenn.edu/micro/domslab/hannajvi.pdf). This data is consistent with results previously published (5, 56) indicating that the viruses used in this study exhibit similar growth kinetics when infections are normalized by PFU. The large increase in virus production seen when the E protein is glycosylated (Fig. 3) may partially offset the decrease in viral infectivity (Fig. 6) over the course of a spreading viral infection. Additionally, PFU measures the ability of viruses not only to infect target cells but also to replicate and spread to surrounding cells. Therefore, normalizing virus inocula based on PFU measurement can mask significant differences in virus entry. Not surprisingly, the prM glycosylation-null virus produced viral titers approximately 1 log lower than wild-type virus on BHK-21 and Vero cells and almost 3 logs less when
grown on C6/36 cells, consistent with its association with markedly reduced virus production. Sequencing of viral RNA extracted at 48 h postinfection confirmed that the glycosylation status for all viruses was consistent with the genotype of the virus inoculum.

**DISCUSSION**

Compared to past WNV outbreaks, the current North American WNV epidemic is impressive in both terms of extent and duration (18, 45). While there may be many contributory factors, it is possible that the current WNV strain circulating in North America and those responsible for other significant human outbreaks are more virulent than strains not associated with significant human disease. Thus far, major WNV epidemics have been invariably associated with lineage I strains of WNV while lineage II strains appear to be less virulent. The molecular determinants that are responsible for differential WNV virulence have not been identified, but factors that influence the efficiency of virus production as well as attachment and entry of WNV into cells could play a major role in viral pathogenesis (62).

In comparing E protein sequences between different lineage
I and lineage II WNV strains, perhaps the most significant difference is the presence in some strains of a single N-linked glycosylation site in the E glycoprotein, while in other strains, this site is absent. In lineage II strains, a 4-amino-acid deletion is often responsible for the absence of this glycosylation sequence (6). All North American WNV isolates described to date contain the E protein N-linked glycosylation site, as have some other WNV strains associated with significant human outbreaks (22, 35, 54). In general, carbohydrate structures can have significant effects on both virus production (12, 17, 57, 58, 66, 67) and attachment of virus particles to the cell surface (37, 49, 64). Most flaviviruses have one or two conserved N-linked glycosylation sites in their E glycoproteins and a single N-linked site in prM that is not present in the mature M protein (19). The presence or absence of specific glycosylation sites in flavivirus E glycoproteins has been associated with alterations in virus production (56, 65) and pathogenesis, including neuroinvasiveness (4, 5, 42).

We found that, when present, the N-linked glycosylation sites in the prM and E glycoproteins of lineage I strain NY99-6480 (14) and lineage II strain 956 D117 3B (68) of WNV were utilized, thus providing a biochemical marker of protein transport. When the glycoproteins were expressed in the context of either subviral or replication-competent virus particles, cell-associated prM and E were sensitive to digestion with Endo H, which is consistent with their localization to the ER. In contrast, the prM and E glycoproteins present on subviral particles as well as WNV released into the medium were resistant to Endo H digestion, consistent with their passage through the Golgi apparatus. Coupled with our electron microscopic observations that revealed virus particles within the rough ER, we conclude that WNV assembles and buds in the ER and quickly transits the Golgi apparatus en route to the cell surface. Our conclusion is consistent with the work of others on both WNV and other flaviviruses (40, 41, 60).

To obtain a clearer understanding of the role that N-linked glycosylation might play on virus production and infectivity, we developed a quantitative E protein ELISA that was used to measure the release of E protein into the medium of transfected or infected cells and coupled this with a quantitative PCR assay to measure viral RNA. Use of both these assays was important since modifications to structural protein processing can affect the relative ratios between SVPs and genome-bearing virus particles (38, 48a), which would not be obvious by ELISA measurements alone. We found that elimination of the highly conserved N-linked glycosylation site in either lineage I or lineage II prM led to a dramatic decrease in the release of SVPs, RVPs, and lineage II replication-competent virus particles. The glycosylation site in the prM protein is in the region of the protein that is removed by proteolysis as WNV transits the Golgi apparatus (19, 63). Therefore, the fact that this glycosylation site is so highly conserved despite its absence in the mature virus particle suggests that its primary role is to assist protein folding in the ER rather than in virus infection. Large, hydrophilic N-linked carbohydrate structures often play important roles in protein folding, both by positioning the polypeptide chain to which they are attached on the outer surface of the folding molecule and by serving as ligands for molecular chaperones such as calnexin and calreticulin (21). In the case of other flaviviruses, prM is known to play a chaperone role by facilitating the correct folding of E and protecting E from premature conformational changes as it traverses the secretory pathway (29, 39). Studies with conformation-dependent antibodies may help reveal whether the folding of E is dependent in part upon proper prM glycosylation. However, such studies will be complicated by the fact that the block to

FIG. 6. Virus infectivity over multiple dilutions of inocula. Lineage II virus infections (strain 956 D117 3B) were normalized based on WNV genome copies per ml in each viral stock and used to infect target cells over multiple relative genome copies ranging from 50,000 to 750 depending on the cell type. Each infection was carried out in duplicate and cells collected for FACS analysis 12 h p.i. for BHK (A) and QT6 (B) and 24 h p.i. for C6/36 cells (C). The x axis shows the relative genome copies. Percent infection is shown on the y axis and represents the number of GFP-expressing cells as measured by flow cytometry analysis. Points outside the linear range for each infection curve were excluded from the presented data.

TABLE 1. Relative genome copy and % infected for each virus infection curve. BHK (A) and QT6 (B) were infected with 956 D117 3B virus at a multiplicity of infection of 10 and harvested at 12 h p.i. and 24 h p.i. respectively. C6/36 (C) was infected at a multiplicity of infection of 5 and harvested at 24 h p.i. The % infected was calculated as the number of GFP-expressing cells divided by the total number of cells and multiplied by 100. The data presented are the average of two independent experiments.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Relative genome copies</th>
<th>% infected BHK (12 h p.i.)</th>
<th>% infected QT6 (24 h p.i.)</th>
<th>% infected C6/36 (24 h p.i.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>956 D117 3B</td>
<td>50,000</td>
<td>20</td>
<td>30</td>
<td>40</td>
</tr>
<tr>
<td>956 D117 3B</td>
<td>10,000</td>
<td>15</td>
<td>25</td>
<td>35</td>
</tr>
<tr>
<td>956 D117 3B</td>
<td>2,000</td>
<td>10</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>956 D117 3B</td>
<td>500</td>
<td>5</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>956 D117 3B</td>
<td>50</td>
<td>0.1</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

Downloaded from http://jvi.asm.org on September 8, 2017 by guest
virus assembly is far from complete, with anywhere from about 1% to 10% normal SVP or virus production occurring in the absence of prM glycosylation.

While the loss of glycosylation in prM had a significant effect on virus production, the virus that was produced was nearly as infectious as wild-type WNV. In our hands, cleavage of prM was not complete and we found some prM associated even with replication-competent WNV. Whether residual prM plays any role in virus infectivity is not known. However, the mature M protein lacks the carbohydrate site, so M proteins derived from either wild-type or glycosylation-defective prM may be indistinguishable on the surface of virus particles. The fact that virus produced with glycosylation-defective prM is nearly as infectious as wild-type virus again suggests that the primary function of the prM carbohydrate structure is to assist protein folding and virus assembly. Once the virus is assembled, this domain, which is largely absent in mature virus, plays little role in modulating viral infectivity on the cell types used in this study.

Alterations in E protein glycosylation also affected virus production and infectivity. When the E protein was not glycosylated, as in our mutant lineage I strain or in a naturally glycosylation-deficient lineage II virus, SVP and virus production were reduced approximately 10-fold relative to viruses containing the E protein glycosylation site. This reduction level is similar to that seen in tick-borne encephalitis SVP release upon removal of the E glycosylation site or treatment with tunicamycin, a glycosylation inhibitor (39, 40). Thus, at the level of particle production, absence of E protein glycosylation had effects that were similar to, although less pronounced than, those observed when the prM glycosylation site was removed. Interestingly, when the infectivity of RVPs bearing these envelopes was analyzed, those particles lacking glycosylation on E in both lineages I and II were somewhat more infectious than wild-type RVPs per genome copy on BHK-21 and QT6 cells. When replication-competent lineage II viruses containing the same envelope glycosylation modifications were used, the absence of E protein glycosylation was associated with approximately threefold enhanced infectivity on BHK-21 and QT6 cells.

Since removal of an N-linked glycosylation site appeared to have a greater effect on SVP release than on the release of genome-bearing virus particles, it was possible that the reduced infectivity simply resulted from an altered particle-to-viral genome ratio, with noninfectious particles competing for virus binding sites on the cell surface. However, this trend remained similar over a broad range of viral inocula. Therefore, while the presence of N-linked carbohydrate on E facilitates more robust particle release, perhaps due to more efficient protein folding, this same sugar moiety resulted in a more robust particle release, perhaps due to more efficient protein folding. In addition to virus assembly, E protein glycosylation can influence virus infectivity, sometimes in dramatic ways. The reason for this is not clear, but the deleterious effect of E protein glycosylation on the infection of mosquito C6/36 cells could be related to the presence of different attachment factors or receptors on insect cells relative to those present on the mammalian or avian cell lines studied here. Clearly, further understanding the role of WNV glycosylation in viral tropism and pathogenesis will require the use of multiple cell types, both for the production of virus as well as for virus infection studies.

ACKNOWLEDGMENTS

This research was supported by NIH U54 AI57168 and by a grant provided by the University of Pennsylvania. Sheri L. Hanna received funding support from both Public Health Service Training grants T32-GM-007229 and T32-AI-07324-13. Melissa Sanchez was supported by NIH F31 RR05074.

We thank Michael Diamond for E1 MAb to the WNV E protein, Gwon-Jen J. Chang for the pCBWN plasmid, and Vladimir Yamash-