Virology 387 (2009) 222–228

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

West Nile virus envelope protein glycosylation is required for efficient viral transmission by *Culex* vectors

Robin M. Moudy [a,*], Bo Zhang [b], Pei-Yong Shi [b,c], Laura D. Kramer [a,c]

[a] *Arbovirus Laboratories, Wadsworth Center, New York State Department of Health, 5668 State Farm Road, Slingerlands, NY 12159, USA*
[b] *Laboratory of Viral Disease, Wadsworth Center, New York State Department of Health, Albany, NY 12208, USA*
[c] *Department of Biomedical Sciences, School of Public Health, State University of New York at Albany, Albany, NY 12202, USA*

ARTICLE INFO

Article history:
Received 5 January 2009
Returned to author for revision
20 January 2009
Accepted 28 January 2009
Available online 27 February 2009

Keywords:
West Nile virus
Glycosylation
Virus–vector interactions

ABSTRACT

Many, but not all, strains of *West Nile virus* (WNV) contain a single N-linked glycosylation site on their envelope (E) proteins. Previous studies have shown that E-glycosylated strains are more neuroinvasive in mice than non-glycosylated strains. E protein glycosylation also appears to play a role in attachment and entry of WNV into host cells *in vitro*; however, studies examining how E protein glycosylation affects the interactions of WNV with its mosquito vectors *in vivo* have not yet been performed. We mutated the E protein glycosylation site from NYS to IYS in a previously described full-length clone of the NY99 genotype of WNV (WT), resulting in a virus that lacked the glycan at aa154. WNV-N154I replicated less efficiently than WNV-WT in *Culex* mosquito tissues, although the extent of the decrease was greater in *Cx. pipiens* than in *Cx. tarsalis*. Following peroral infection, mosquitoes infected with WNV-N154I were less likely to transmit virus than those infected with WNV-WT. Interestingly, all but one of the mosquitoes infected with WNV-N154I transmitted a revertant virus, suggesting that there is strong selective pressure toward E protein glycosylation. Together these data suggest that loss of the glycan at aa154 on the WNV E protein can severely restrict viral spread in the mosquito vector.

Introduction

West Nile virus (WNV) is a member of the Japanese encephalitis virus (JEV) serocomplex of the *Flavivirus* genus (family *Flaviviridae*) (Gubler et al., 2007; Lindenbach et al., 2007). Other members of this genus include the mosquito-borne viruses *St. Louis encephalitis virus* (SLEV), *Dengue virus* (DENV), and *Yellow fever virus* (YFV), as well as tick-borne viruses such as *Tick-borne encephalitis virus* (TBEV) and other viruses that do not appear to be transmitted by an insect vector. WNV is maintained in nature via an enzootic transmission cycle between *Culex* mosquito vectors and avian reservoir hosts. Although periodic outbreaks occur in horses and humans, infection of mammals does not appear to contribute substantially to the maintenance of WNV in nature (Weaver and Barrett, 2004). Of the two main lineages of WNV, lineage 1 has the larger geographic range and includes the viruses currently circulating in the Americas (Gubler et al., 2007). Viruses in this lineage are generally more pathogenic and can lead to severe central nervous system infection and death. Lineage 2 viruses are found only in sub-Saharan Africa and Madagascar; infection with WNV belonging to this lineage rarely progresses to severe disease in humans.

WNV contains a conserved N-linked glycosylation site in the prM envelope protein. This glycosylation site is located within the ectodomain of prM that is cleaved by cellular enzymes during viral particle maturation. The prM proteins of flaviviruses form heterodimers with the viral envelope (E) protein (Zhang et al., 2003) and appear to act as chaperones for correct and efficient folding of E protein (Konishi and Mason, 1993; Lorenz et al., 2002; Ocazionez Jimenez and Lopes da Fonseca, 2000). Studies with WNV and TBEV have found that deletion of the N-linked glycosylation site in prM led to a decrease in virus release (Goto et al., 2005; Hanna et al., 2005). These results suggest that prM glycosylation may be required for efficient particle assembly, possibly due to a requirement for glycosylated prM as a chaperone for E protein folding. However, the precise role of prM glycosylation in particle assembly has not yet been elucidated.

In contrast to the conserved glycosylation of prM protein, WNV E protein is not universally glycosylated. Many strains possess an N-linked glycosylation site at aa position 154, while others contain mutations at aa154 or aa156, or a four aa deletion, that ablate this site (Adams et al., 1995; Berthet et al., 1997; Davis et al., 2005; Ebel et al., 2004; Lanciotti et al., 2002). Many other flaviviruses also contain a glycosylation site in this region of the E protein (Ryman et al., 1997). Interestingly, it was noted that many of the major outbreaks of human disease were caused by strains containing this glycosylation site (Hanna et al., 2005). Studies in mice have found that although the E-

* Corresponding author. Fax: +1 518 869 4530.
E-mail address: rmoudy@wadsworth.org (R.M. Moudy).

0042-6822/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2009.01.038

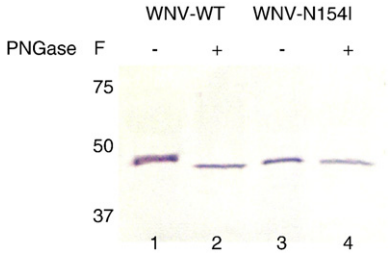

Fig. 1. Effect of N154I mutation on WNV E protein glycosylation. WNV-WT and WNV-N154I viruses were incubated with PNGase F (+) or buffer only (−). Viral E proteins were visualized by Western blot using an anti-E MAb.

glycosylated and non-glycosylated strains of WNV are equally neurovirulent, viruses containing the E protein glycosylation site are more neuroinvasive (Beasley et al., 2005; Shirato et al., 2004). Deletion of the glycosylation site in the TBEV or WNV E proteins resulted in substantially decreased viral particle release from mammalian cells (Goto et al., 2005; Hanna et al., 2005). However, particle infectivity was increased threefold for mammalian and avian cells and 30-fold for mosquito cells when WNV lacked the glycosylation site (Hanna et al., 2005). It is interesting to speculate that differentially glycosylated WNV may not be equally infectious for or transmitted equally by their mosquito vectors or avian reservoir hosts. In the current study, we examined the effect of E protein glycosylation on the interaction of WNV with *Culex* mosquito vectors. We first examined whether glycosylated and non-glycosylated viruses exhibited replicative differences *in vitro* and *in vivo*. We then examined whether WNV infectivity and spread *in vivo* was affected by the presence or absence of a glycan on the E protein. These are the first studies to date examining how E protein glycosylation affects WNV–vector relationships.

Results

Confirmation of E protein glycan removal from WNV-N154I

To begin our studies of the effects of WNV E protein glycosylation, we made a recombinant virus in which the asparagine at aa154 was changed to an isoleucine (WNV-N154I), using a double-

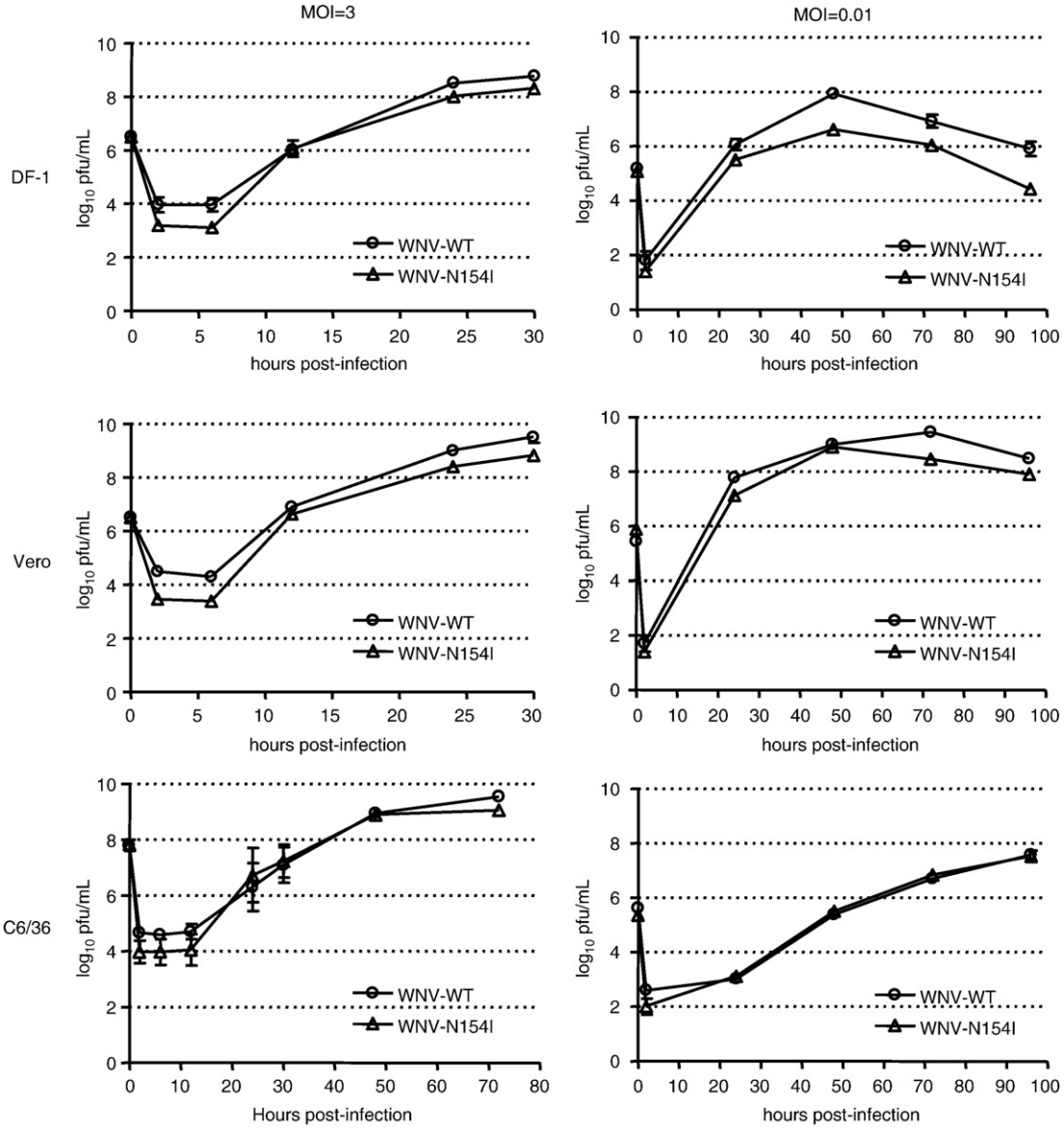

Fig. 2. Replication efficiencies of WNV-WT and WNV-N154I *in vitro*. Confluent cells were infected with WNV-WT or WNV-N154I at an MOI of 3 or 0.01, and samples of supernatant were taken at the indicated times for virus titration in Vero cells. Viral titers at each time point are shown as $\log_{10}$ PFU/mL.

nucleotide change in the codon (AUA to AAC) to reduce the likelihood of reversion. We then confirmed that mutation of the E-N154 residue resulted in a WNV with a non-glycosylated E protein. Following peptide *N*-glycosidase F (PNGase F) digestion of the WNV-WT E protein, a mobility shift was observed, consistent with removal of the single N-linked glycan from the protein (Fig. 1, lanes 1 and 2). However, both the mock- and PNGase F-digested WNV-N154I E proteins (lanes 3 and 4) migrated at the same position as the PNGase F-digested WNV-WT E protein (lane 2), indicating that the E-N154I substitution resulted in a non-glycosylated E protein.

Replicative ability of WNV-WT and WNV-N154I in cell culture

We first examined whether the WNV-WT and WNV-N154I viruses differed in their replication efficiencies and/or kinetics *in vitro*. At an MOI of 3, both viruses replicated with similar kinetics and to similar peak titers in mosquito cells (Fig. 2). However, in vertebrate cells, WNV-WT replicated to titers up to1 log higher than those of WNV-N154I. Similar results were obtained at an MOI of 0.01. There were no detectable differences between the plaque phenotypes of WNV-WT and WNV-N154I (data not shown).

Previous studies have suggested that WNV entry and release may be affected by a lack of E protein glycosylation. We therefore compared the ratios of genome copies per PFU for the glycosylated and non-glycosylated viruses. WNV-N154I had more genome copies per PFU (135) than WNV-WT (70, $p<0.0001$), suggesting that a significantly larger proportion of the viral particles produced by WNV-N154I are non-infectious.

Replicative ability of WNV-WT and WNV-N154I in Culex mosquitoes

Adult female mosquitoes were inoculated with either WNV-WT or WNV-N154I to determine whether E protein glycosylation affected replicative ability *in vivo*. Titers in WNV-WT-inoculated mosquitoes were significantly higher than those inoculated with WNV-N154I beginning at 1 day post-inoculation ($p<0.05$, Fig. 3). WNV-WT reached similar peak titers in both *Cx. pipiens* and *Cx. tarsalis*, while peak titers of WNV-N154I were significantly higher in *Cx. tarsalis* than in *Cx. pipiens* ($p=0.037$).

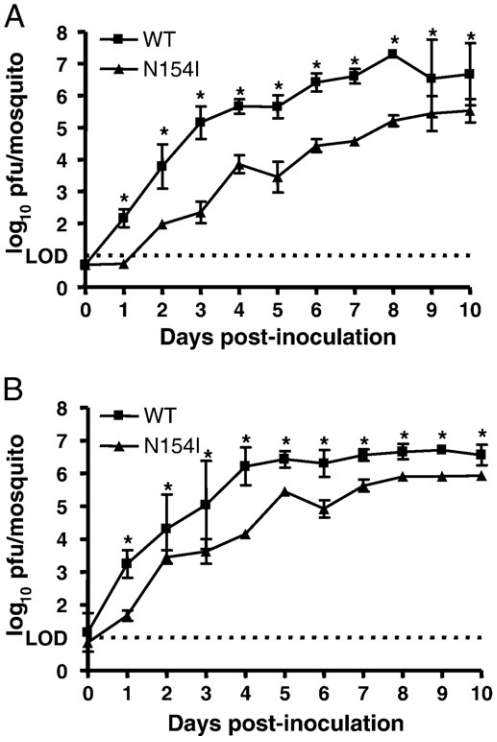

Fig. 3. Viral replication of WNV-WT and WNV-N154I in *Culex* mosquitoes. *Cx. pipiens* (A) and *Cx. tarsalis* (B) were intrathoracically inoculated with 10 PFU of either WNV-WT or WNV-N154I and held for up to 10 days. At daily intervals, 10 mosquitoes per virus were removed and the viral titers present in their bodies determined by plaque titration on Vero cells. Mean viral titers from infected mosquitoes at each time point are shown as $\log_{10}$ PFU/mosquito. LOD: limit of detection. *: $p<0.05$.

We also determined the replication efficiencies of WNV-WT and WNV-N154I in mosquito midguts following peroral infection and salivary glands following inoculation. We did not examine salivary gland replication following feeding due to the potential midgut barrier which would confound our salivary gland results. Viral loads in midguts of *Cx. pipiens* exposed to WNV-WT were significantly higher at all time points than in those exposed to WNV-N154I ($p<0.05$, Fig. 4A). In addition, the peak viral titer in WNV-WT-exposed mosquitoes was significantly greater than that of mosquitoes exposed to WNV-N154I. Although both the viral loads and peak viral titer were greater in midguts of *Cx. tarsalis* exposed to WNV-WT than in those exposed to WNV-N154I, the differences were not statistically significant (Fig. 4B). Viral loads in the salivary glands of both species were significantly higher in mosquitoes infected with WNV-WT ($p<0.05$, Figs. 4C, D).

Vector competence of Culex mosquitoes

To evaluate the impact of E protein glycosylation on the ability of WNV to infect and spread within the mosquito vector following peroral exposure, we examined the vector competence of *Cx. pipiens* and *Cx. tarsalis* for WNV-WT and WNV-N154I. The infection rates and body titers of WNV-WT were higher at 5, 7, and 9 days post-feeding (dpf) than those of WNV-N154I in *Cx. pipiens*, with no significant difference observed at 14 dpf (Table 1 and data not shown). In *Cx. tarsalis*, WNV-N154I was more infectious than WNV-WT at 5 and 14 dpf, while the infection rates were not significantly different at 7 and 9 dpf (Table 2). Interestingly, the body titers of mosquitoes infected with WNV-WT were significantly higher than those infected with WNV-N154 at all dpf (data not shown).

Dissemination rates were significantly reduced in WNV-N154I-infected mosquitoes at 9 and 14 dpf in *Cx. pipiens* (Table 1) and at all dpf in *Cx. tarsalis* (Table 2). Transmission rates of mosquitoes infected with WNV-N154I were reduced in both species, although the difference between the viruses was only significant at 14 dpf in *Cx. tarsalis*. The total transmission rates over the entire experiment were significantly reduced in both species (Tables 1 and 2).

We sequenced a 500 bp region of the E genes of the viruses present in all mosquitoes with disseminated infections that had fed on WNV-N154I ($n=26$). In 20 mosquitoes, the viruses present in the bodies and legs were the virus on which they fed, WNV-N154I (data not shown). Five of these also had infectious virus in their salivary secretions, and in all but one the virus had reverted to contain the glycosylation site in the E protein (Table 3). Six mosquitoes contained revertant viruses in either their legs or both their bodies and legs. One of these mosquitoes also contained revertant virus in its salivary secretions (Table 3).

Discussion

One of the more significant sequence differences among WNV strains is the presence or absence of an N-linked glycosylation site at aa154–156 in the E protein. Previous work examining the potential phenotypic effects of WNV E protein glycosylation has focused on *in vitro* systems and mammalian infection models (Beasley et al., 2005; Davis et al., 2006; Hanna et al., 2005; Li et al., 2006; Scherret et al., 2001; Shirato et al., 2004, 2006). In the current study, we examined whether glycosylation of the E protein affects WNV interactions with mosquito vectors *in vivo*, using two mosquito species important for enzootic viral transmission in the United States.

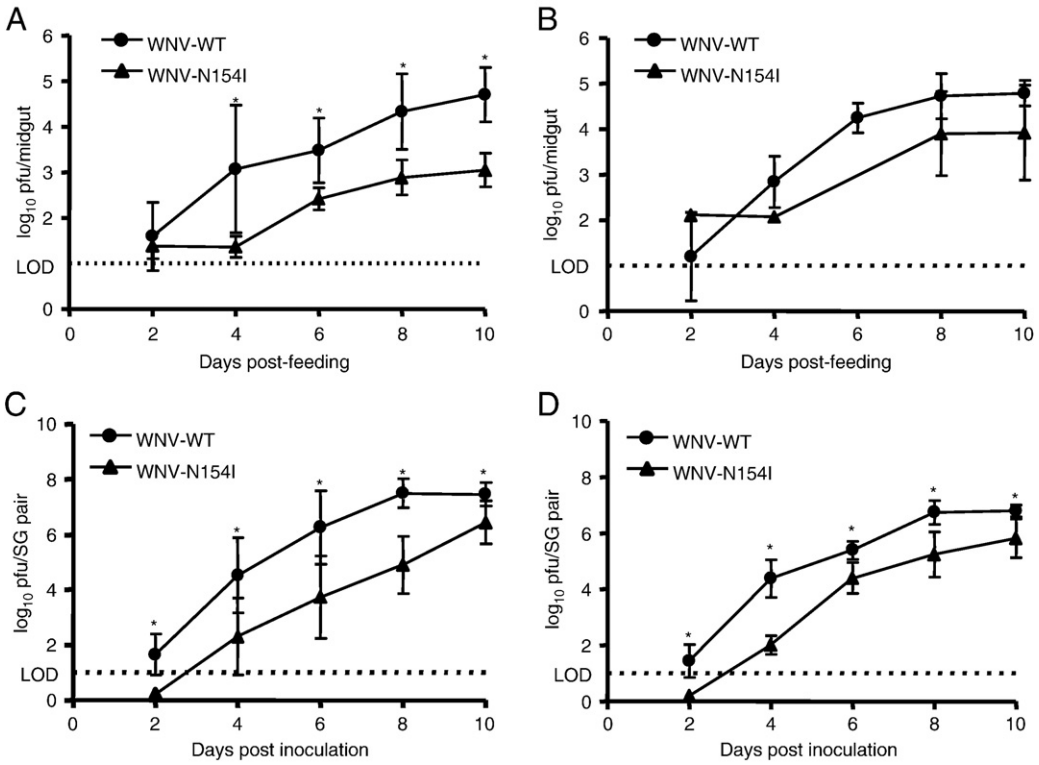

Fig. 4. Replication of WNV-WT and WNV-N154I in mosquito tissues. (A and B) Viral replication in mosquito midguts. *Cx. pipiens* (A) and *Cx. tarsalis* (B) were fed on defibrinated goose blood containing 10^8 PFU/mL of either WNV-WT or WNV-N154I and held for up to 10 days post-feeding. At the indicated time points, midguts were removed from 20 mosquitoes per virus and their viral titers determined by plaque titration on Vero cells. Mean viral titers from infected midguts at each time point are shown as $\log_{10}$ PFU/midgut. LOD: limit of detection. *: $p<0.01$. (C, D) Viral replication in mosquito salivary glands. *Cx. pipiens* (C) and *Cx. tarsalis* (D) were intrathoracically inoculated with 10 PFU of either WNV-WT or WNV-N154I and held for up to 10 days. At the indicated time points, salivary glands were removed from 5–10 mosquitoes per virus and their viral titers determined by plaque assay on Vero cells. Mean viral titers from infected salivary gland pairs at each time point are shown as $\log_{10}$ PFU/SG pair. LOD: limit of detection. *: $p<0.01$.

We first compared the *in vitro* replication efficiencies of two recombinant WNVs, a virus containing the E protein glycosylation site (WNV-WT) and a virus in which the glycosylation site had been mutated (WNV-N154I), and detected no differences in either replication kinetics or peak virus titer in mosquito cells, although minor differences were found in vertebrate cells (Fig. 2). This was unexpected, as work by other groups had found that lack of glycosylation decreased viral peak titer in mosquito cells *in vitro* by at least 10-fold (Hanna et al., 2005; Li et al., 2006; Scherret et al., 2001). However, the previous studies used either lineage 2 viruses or Kunjin virus, which may explain the differences in our results.

As previous work from our laboratory has found that *in vitro* results are not necessarily predictive of *in vivo* differences (Moudy et al., 2007), all further work was done using an *in vivo* system. We first examined whether E protein glycosylation affected viral replication efficiency, either in whole mosquitoes or in individual mosquito tissues. WNV-WT replicated more efficiently and to higher peak titers than WNV-N154I in whole mosquitoes following inoculation (Figs. 3). The differences between the viruses were more pronounced in *Cx. pipiens* (Fig. 3A) than in *Cx. tarsalis* (Fig. 3B), although in both cases the differences in viral titers were significant at all time points post-infection. To examine replication in individual tissues, we used two different methods of infection. Midgut replication was analyzed following peroral infection, as WNV infection of the midgut in these two *Culex* species is unidirectional and does not occur following inoculation (Yongqing Jia, unpublished data). Replication in the salivary glands was examined following intrathoracic inoculation, to ensure that any potential barriers to midgut infection or egress did not confound the salivary gland results. Analysis of replication in both tissues gave similar results, in that WNV-WT replicated more efficiently than WNV-N154I (Fig. 4), although the differences between the viral titers in mosquito midguts were only significant in *Cx. pipiens*. These data indicate that glycosylation of the WNV E protein is important for efficient replication within the mosquito. However, there are apparently species- or colony-specific differences in how glycosylation affects WNV interaction with the mosquito vector. Mosquito colony- and species-specific differences in infectivity, replication efficiency, and vector competence have previously been

Table 1
Vector competence of *Culex pipiens* following peroral infection

Virus	Days post-feeding														
	5			7			9			14			Total		
	I[a]	D	T	I	D	T	I	D	T	I	D	T	I	D	T
WNV-WT	71 ($n=80$)	7 ($n=57$)	0	83 ($n=77$)	3 ($n=64$)	0	79 ($n=77$)	15 ($n=61$)	5	49 ($n=67$)	36 ($n=33$)	12	71 ($n=301$)	12 ($n=215$)	3
WNV-N154I	46 ($n=90$)	5 ($n=41$)	0	58 ($n=90$)	13[b] ($n=48$)	0[b]	68 ($n=60$)	2 ($n=41$)	0	51 ($n=43$)	2 ($n=22$)	0[b]	54 ($n=283$)	7[b] ($n=152$)	0[b]
P value[c]	0.0007	0.735	1.00	0.0004	0.057	1.00	0.147	0.040	0.272	0.845	0.007	0.141	<0.0001	0.105	0.045

[a] I: % infected, D: % disseminated (of infected), T: % transmitting (of infected).
[b] Does not include mosquitoes with revertant virus.
[c] Calculated using χ^2 test.

Table 2
Vector competence of *Culex tarsalis* following peroral infection

Virus	Days post-feeding														
	5			7			9			14			Total		
	I[a]	D	T	I	D	T	I	D	T	I	D	T	I	D	T
WNV-WT	25($n=150$)	14($n=37$)	0	31($n=150$)	30($n=47$)	9	22($n=130$)	50($n=28$)	14	21($n=98$)	76($n=21$)	52	25($n=528$)	37($n=133$)	14
WNV-N154I	42($n=130$)	2[b]($n=54$)	0	30($n=130$)	0[b]($n=39$)	0[b]	29($n=130$)	8[b] ($n=38$)	3[b]	34($n=115$)	26($n=39$)	0	34($n=505$)	9[b]($n=172$)	0.06[b]
P value[c]	0.003	0.028	1.00	0.809	0.0002	0.062	0.154	0.0001	0.077	0.044	0.0002	<0.0001	0.003	<0.0001	<0.0001

[a] I: % infected, D: % disseminated (of infected), T: % transmitting (of infected).
[b] Does not include mosquitoes with revertant virus.
[c] Calculated using χ^2 test.

noted for several flaviviruses (Kramer and Ebel, 2003); it is therefore not entirely surprising that we observed them in our studies. Future work examining potential mechanistic differences between glycosylated and non-glycosylated WNV in different mosquito colonies and species should yield additional information regarding the effects of E protein glycosylation on WNV interaction with mosquito vector species.

We next examined how vector competence was affected by E protein glycosylation. These studies examine the ability of the virus to not only infect the mosquito following feeding on an infectious bloodmeal, but also to disseminate from the midgut and eventually be transmitted to a new host. Overall in *Cx. pipiens*, WNV-WT infected a higher proportion of mosquitoes than WNV-N154I, while the reverse was true in *Cx. tarsalis* (Tables 1 and 2), again suggesting species- or colony-specific differences in virus–vector interactions. Our observations in *Cx. tarsalis* were particularly interesting, in that although a larger proportion of mosquitoes were infected following feeding on WNV-N154I, their bodies and midguts contained significantly less virus than mosquitoes that fed on WNV-WT. These results suggest that the presence or absence of the E protein glycan differentially affects multiple steps in the viral replication cycle.

Other members of the *Flaviviridae*, such as TBEV and DENV, also contain at least one glycosylation site in the E protein. DENV normally contains two glycans on its E protein (aa67 and aa153), and removal of the aa67 glycosylation site severely inhibited growth *in vitro*, but not *in vivo* in mosquitoes (Bryant et al., 2007). DENV mutants lacking the glycosylation site at aa153 were found to induce fusion at a higher pH than WT DENV (Guirakhoo et al., 1993; Lee et al., 1997). It has been suggested that carbonic anhydrase (CA) plays a role in maintenance of midgut pH. CA is active to varying extents in the midguts of different species, with the highest activity found in *Ae. aegypti* and the lowest in *Cx. quinquefaciatus* (del Pilar et al., 2005). These studies have shown that mosquito midgut pH varies between species, with the pH of *Cx. tarsalis* midguts ranging from 8.0–9.5 (del Pilar et al., 2005). If the glycan at aa154 has a similar effect on the pH threshold of fusion as has been observed previously for the DENV aa153 glycan, it would be expected that a mosquito with a higher midgut pH could be more easily infected by WNV lacking the aa154 glycan. Unfortunately, the midgut pH range of *Cx. pipiens* has not been determined; however, it is interesting to speculate that the differences we observed in infection and midgut replication between mosquito species are the result of *Cx. tarsalis* having a higher midgut pH than *Cx. pipiens*. As midgut pH would be expected to affect only the initial steps of the viral replication cycle, binding and entry, this also could explain the apparent discrepancy between infection rate and virus titers that we observed in *Cx. tarsalis*.

Table 3
Analysis of WNV reversion in mosquito bodies, legs, and salivary secretions[a]

Mosquito species	Days post-feeding	Input	Body	Legs	Salivary secretions[b]
Cx. pipiens	7	ATA	ATA	ATA	AAC
	7	ATA	ATA	ATA	AAC
	7	ATA	ATA/AAC	AAC	–
	7	ATA	AAC	AAC	–
	14	ATA	ATA	ATA	AAC
Cx. tarsalis	7	ATA	ATA	AAC	–
	7	ATA	ATA	AAC	AAC
	9	ATA	ATA	ATA	ATA
	9	ATA	ATA	ATA	AAC

[a] Only mosquitoes with revertant virus in at least one tissue are shown, out of a total of 9 *Cx pipiens* and 17 *Cx tarsalis* tested from 5–14 dpf.
[b] –: not transmitting.

In both mosquito species, a significantly lower proportion of mosquitoes that fed on WNV-N154I had virus present in their legs and salivary glands. Additionally, in five out of the six mosquitoes that transmitted WNV following feeding on WNV-N154I, the virus present in the salivary secretions contained the glycosylation site, indicating that the virus had reverted to a WT glycosylation phenotype (Table 3). The reversions appear to have occurred at multiple points during WNV spread throughout the mosquito, as we found revertant viruses in the salivary secretions and/or legs without reversion in the mosquito body. As reversion at the glycosylation site had not previously been observed during viral replication in either vertebrate or mosquito cells *in vitro* (data not shown), these data suggest that not only is E protein glycosylation important for efficient WNV spread throughout the mosquito, but that there is strong selective pressure toward glycosylation at aa154 in transmitted viruses. However, E protein glycosylation alone is not sufficient to ensure transmission, as three mosquitoes with revertant viruses in their bodies and/or legs did not have infectious virus present in their salivary secretions. This is not surprising, as there is not a 100% transmission rate for mosquitoes feeding on WNV-WT (Tables 1 and 2).

One caveat to our findings is that we have examined the effect of only a single amino acid change out of several possible mutations that would be predicted to remove the glycosylation site. Other groups examining WNV E protein glycosylation have utilized different mutations to remove the N154 glycosylation site, such as Q154 and S154. In mice, similar phenotypes were obtained regardless of the specific amino acid change (Beasley et al., 2005; Shirato et al., 2004). Therefore, we do not expect that our results are due to the presence of an isoleucine at position 154, rather than the lack of the N154 glycan; however, work is currently underway to examine this possibility.

Although we did not find a significant difference in the titers of our recombinant virus stocks following recovery in BHK cells, there were nearly twice as many uninfectious particles in the WNV-N154I virus stock as in the WNV-WT virus stock. These data suggest that the N154I mutation inhibits infectious WNV particle production. Hanna et al. (2005) found that non-glycosylated WNV was more infectious for mammalian, avian, and mosquito cells than glycosylated WNV, when the viruses were normalized to genome equivalents. Our data appear to conflict with these findings; however, our study examined the production of infectious particles (PFU), while the previous study defined infectivity as viral entry and initial rounds of replication. When virus production was measured, Hanna et al. observed a significant decrease in viruses lacking the E glycan, similar to our

study. Therefore, the difference in infectivity results is likely due to the difference in experimental design. Work with TBEV also found decreased VLP production when the glycan at E154 was removed (Goto et al., 2005), suggesting that this phenotype is common among the flaviviruses. Future experiments will examine whether the defect in infectivity we observed is due to decreased efficiency of entry into host cells, a defect in prM-E association leading to a decrease in packaging efficiency, or a combination of factors.

Materials and methods

Cells and mosquitoes

Baby hamster kidney (BHK) cells were used for viral growth. *Aedes albopictus* (C6/36), chick embryonic fibroblast (DF-1), and African green monkey kidney (Vero) cells were used for *in vitro* replication analyses. Viruses were titrated on Vero cells. All experiments involving infectious WNV were performed in the Wadsworth Center Arbovirus Laboratories' BSL3 laboratory.

Cx. pipiens and *Cx. tarsalis* mosquitoes were derived from laboratory colonies. The *Cx. pipiens* colony originated in 2002 from egg rafts collected in Pennsylvania, and the *Cx. tarsalis* colony originated in 2003 from egg rafts collected in the Coachella Valley in California. Colonized mosquitoes were maintained on goose blood (for egg laying) and given 10% sucrose *ad libitum*. Larvae were reared and adults maintained under controlled conditions of temperature (27 °C), humidity (70% RH), and light (16:8 L:D diurnal cycle), in 12"×12"×12" cages. All mosquito experiments involving infectious WNV were carried out in the Arbovirus Laboratories' BSL3 insectary.

Construction of mutant WNV cDNA and recovery of infectious viruses

A mutant cDNA plasmid of full-length lineage I WNV, in which the asparagine at amino acid 154 in the E protein was replaced with an isoleucine, was constructed using a modified infectious cDNA clone, pFLWNV (Shi et al., 2002b), and a shuttle vector including the entire E gene sequence. The glycosylation site mutation was first made in the shuttle vector using an overlapping PCR-mediated mutagenesis method. The mutation was confirmed by DNA sequencing prior to transfer of the shuttle vector sequence into pFLWNV. The mutated pFLWNV sequence was also confirmed by DNA sequencing.

To recover infectious viruses with from the WT and mutant pFLWNV plasmids, genome-length RNAs were *in vitro* transcribed and transfected into BHK cells (Shi et al., 2002a). Cell supernatants containing WNV were harvested at 96 h post-transfection and their titers determined by plaque assay on Vero cells. Titers of virus recovered from the WT pFLWNV, henceforth called WNV-WT, were 2–4×10^9 PFU/mL. Titers of virus recovered from the mutant pFLWNV, henceforth called WNV-N154I, were 2×10^9 PFU/mL.

Viral protein analysis

Viral E protein glycosylation states were determined essentially as previously described (Hanna et al., 2005). Briefly, WNV-WT and WNV-N154I were inactivated at 56 °C for 1 h, and aliquots of each virus were digested with 500 U of peptide *N*-glycosidase F (PNGase F, New England Biolabs) for 1 h at 37 °C or mock digested as a negative control. Samples were then analyzed by reducing SDS-PAGE and Western blotting using an anti-E monoclonal Ab (MAb 5H10, BioReliance). E proteins were visualized using WesternBreeze (Invitrogen), according to the manufacturer's instructions.

Viral replication in vitro

Viral replication was analyzed as previously described (Moudy et al., 2007). Briefly, WNV-WT and WNV-N154I were inoculated onto DF-1, C6/36, and Vero cells at an MOI of 0.01 or 3 and virus production examined over a period of up to 120 h post-infection. Viral titers at each time point were determined by plaque titration on Vero cells.

Genome copy number determination

RNA was isolated from 10^8 PFU of WNV-WT and WNV-N154I using the RNeasy mini kit (Qiagen) and viral genome copy number was measured by real-time RT-PCR (7500 RT PCR system, Applied Biosystems) using a primer/probe set targeting the E gene (Shi et al., 2001). Full-length RNA transcribed from an infectious cDNA clone of WNV was used as a reference for the quantitation of the real-time RT-PCR (Shi et al., 2002b). Genome copy number to PFU ratios were determined for six replicates from each virus and compared using a *t*-test.

Viral replication in Culex mosquitoes

WNV-WT and WNV-N154I were diluted in mosquito diluent (MD: 20% heat-inactivated FBS in Dulbecco's phosphate-buffered saline plus 50 μg/mL penicillin/streptomycin, 50 μg/mL gentamicin and 2.5 μg/mL fungizone) to a titer of 10^5 PFU/mL. Mosquitoes (<5 days old) were inoculated intrathoracically with 10 PFU of either WNV-WT or WNV-N154I under CO_2 anesthesia and held at 27 °C, 16:8 light:dark photoperiod for up to 10 days post-inoculation. At daily intervals from 0 to 10 days post-inoculation, 10 mosquitoes inoculated with each virus were removed to individual aliquots of 1 mL MD. All samples were stored at −80 °C. Mosquitoes were homogenized in a mixer mill (Qiagen) and clarified by centrifugation. The viral loads of infected mosquitoes were determined by plaque titration on Vero cells and compared using ANOVA.

Vector competence of Culex mosquitoes

Five to 7 day old adult female mosquitoes were deprived of sucrose for 48 h prior to feeding. WNV-WT and WNV-N154I were added to 5 mL defibrinated goose blood with 2.5% sucrose to a final titer of 10^8 PFU/mL. Mosquitoes were fed for 1 to 2 h using a Hemotek membrane feeding apparatus (Discovery Workshops, Accrington, UK), as directed by the manufacturer. Following feeding, fully engorged mosquitoes were separated under CO_2 anesthesia to 0.5 L cartons, supplied with 10% sucrose *ad lib*, and held at 27 °C, 16:8 L:D photoperiod for up to 14 days post-feeding.

Transmission was evaluated *in vitro*, essentially as previously described (Aitken, 1977). Briefly, mosquitoes were anesthetized with triethylamine (Sigma, St. Louis, MO), and their legs removed into 1 mL MD. Mosquito mouthparts were placed into a capillary tube containing approximately 10 μL of a 1:1 mixture of FBS and 50% sucrose for approximately 30 min, after which time the contents of the capillary tube were expelled into 0.3 mL MD. Mosquito bodies were placed into 1 mL MD, and all samples were stored at −80 °C. Mosquito bodies and legs were homogenized in a mixer mill (Qiagen) and clarified by centrifugation. The proportion of mosquitoes with infected bodies, legs, and salivary secretions was determined by plaque assay on Vero cells. Infection, dissemination, and transmission were defined as the proportion of mosquitoes with infected bodies, legs, and salivary secretions, respectively. Proportions were compared using Chi square analysis.

Viral replication in mosquito midguts

Five to 7 day old adult female mosquitoes were infected perorally with either WNV-WT or WNV-N154I, as described above. Following feeding, fully engorged mosquitoes were held at 27 °C, 16:8 L:D photoperiod for up to 10 days post-feeding. At 2, 4, 6, 8, and 10 days post-feeding, midguts from 20 mosquitoes per virus were removed, washed in MD to remove blood and/or hemolymph, and individually

stored in 0.5 mL MD at −80 °C. Midguts were homogenized using a mixer mill (Qiagen) and clarified by centrifugation. The viral loads of infected midguts were determined by plaque titration on Vero cells and compared using ANOVA.

Viral replication in mosquito salivary glands

Mosquitoes (<5 days old) were intrathoracically inoculated with either WNV-WT or WNV-N154I, as described above, and held for up to 10 days post-inoculation. At 2, 4, 6, 8, and 10 days post-inoculation, salivary glands from 10 mosquitoes per virus were removed, washed in MD to remove hemolymph, and individually stored in 0.5 mL MD at −80 °C. Salivary glands were homogenized using a mixer mill (Qiagen) and clarified by centrifugation. Viral loads of infected salivary glands were determined by plaque titration on Vero cells and compared using ANOVA.

Sequencing of mosquito-derived viruses

Viral genomic RNA was purified from infected bodies, legs, and salivary secretions of mosquitoes that fed on WNV-N154I using RNeasy spin columns (Qiagen) according to the manufacturer's instructions. One-step RT-PCR (Qiagen) was performed using primers designed to amplify the 500 bp region of the envelope gene surrounding the glycosylation site (nt1208–1700). PCR products were purified (Qiagen) and visualized on 1% agarose gels. Automated sequencing of purified PCR products was performed at the Wadsworth Center Applied Genomics Technology Core.

Acknowledgments

The authors would like to acknowledge the excellent technical assistance provided by Mark Meola, Brittany Dodson, and Pamela Chin. We thank Mike Hutchinson for collection of *Cx. pipiens* to begin our colony and William Reisen for providing the *Cx. tarsalis* colony. We also thank the Wadsworth Center Tissue Culture Core facility for providing BHK, Vero, and C6/36 cells and the Wadsworth Center Applied Genomics Technologies Core facility for providing DNA sequencing services. This project has been funded in part with Federal funds from National Institute of Allergy and Infectious Disease contract NO1-AI-25490. Robin Moudy was funded with a Postdoctoral Career Development Award from the Northeast Biodefense Center (AI057518, PI-Lipkin) and a Ruth L. Kirschstein National Research Service Award from the National Institute of Allergy and Infectious Disease, National Institutes of Health (1F32-AI074238-01A2).

References

Adams, S.C., Broom, A.K., Sammels, L.M., Hartnett, A.C., Howard, M.J., Coelen, R.J., MacKenzie, J.S., Hall, R.A., 1995. Glycosylation and antigenic variation among Kunjin virus isolates. Virology 206, 49–56.

Aitken, T.H.G., 1977. An in vitro feeding technique for artificially demonstrating virus transmission by mosquitoes. Mosq. News 37, 130–133.

Beasley, D.W., Whiteman, M.C., Zhang, S., Huang, C.Y., Schneider, B.S., Smith, D.R., Gromowski, G.D., Higgs, S., Kinney, R.M., Barrett, A.D., 2005. Envelope protein glycosylation status influences mouse neuroinvasion phenotype of genetic lineage 1 West Nile virus strains. J. Virol. 79, 8339–8347.

Berthet, F.X., Zeller, H.G., Drouet, M.T., Rauzier, J., Digoutte, J.P., Deubel, V., 1997. Extensive nucleotide changes and deletions within the envelope glycoprotein gene of Euro-African West Nile viruses. J. Gen. Virol. 78 (Pt 9), 2293–2297.

Bryant, J.E., Calvert, A.E., Mesesan, K., Crabtree, M.B., Volpe, K.E., Silengo, S., Kinney, R.M., Huang, C.Y., Miller, B.R., Roehrig, J.T., 2007. Glycosylation of the dengue 2 virus E protein at N67 is critical for virus growth in vitro but not for growth in intrathoracically inoculated *Aedes aegypti* mosquitoes. Virology 366, 415–423.

Davis, C.T., Ebel, G.D., Lanciotti, R.S., Brault, A.C., Guzman, H., Siirin, M., Lambert, A., Parsons, R.E., Beasley, D.W., Novak, R.J., Elizondo-Quiroga, D., Green, E.N., Young, D.S., Stark, L.M., Drebot, M.A., Artsob, H., Tesh, R.B., Kramer, L.D., Barrett, A.D., 2005. Phylogenetic analysis of North American West Nile virus isolates, 2001–2004: evidence for the emergence of a dominant genotype. Virology 342, 252–265.

Davis, C.W., Mattei, L.M., Nguyen, H.Y., nsarah-Sobrinho, C., Doms, R.W., Pierson, T.C., 2006. The location of asparagine-linked glycans on West Nile virions controls their interactions with CD209 (dendritic cell-specific ICAM-3 grabbing nonintegrin). J. Biol. Chem. 281, 37183–37194.

del Pilar, C.M., VanEkeris, L., Salazar, M.I., Bowers, D., Fiedler, M.M., Silverman, D., Tu, C., Linser, P.J., 2005. Carbonic anhydrase in the adult mosquito midgut. J. Exp. Biol. 208, 3263–3273.

Ebel, G.D., Carricaburu, J., Young, D., Bernard, K.A., Kramer, L.D., 2004. Genetic and phenotypic variation of West Nile virus in New York, 2000–2003. Am. J. Trop. Med. Hyg. 71, 493–500.

Goto, A., Yoshii, K., Obara, M., Ueki, T., Mizutani, T., Kariwa, H., Takashima, I., 2005. Role of the N-linked glycans of the prM and E envelope proteins in tick-borne encephalitis virus particle secretion. Vaccine 23, 3043–3052.

Gubler, D., Kuno, G., Markoff, L., 2007. Flaviviruses. In: Knipe, D.M., Howley, P.M. (Eds.), Fields Virology. Lippincott William & Wilkins, Philadelphia, PA, pp. 1153–1252.

Guirakhoo, F., Hunt, A.R., Lewis, J.G., Roehrig, J.T., 1993. Selection and partial characterization of dengue 2 virus mutants that induce fusion at elevated pH. Virology 194, 219–223.

Hanna, S.L., Pierson, T.C., Sanchez, M.D., Ahmed, A.A., Murtadha, M.M., Doms, R.W., 2005. N-linked glycosylation of West Nile virus envelope proteins influences particle assembly and infectivity. J. Virol. 79, 13262–13274.

Konishi, E., Mason, P.W., 1993. Proper maturation of the Japanese encephalitis virus envelope glycoprotein requires cosynthesis with the premembrane protein. J. Virol. 67, 1672–1675.

Kramer, L.D., Ebel, G.D., 2003. Dynamics of flavivirus infection in mosquitoes. Adv. Virus Res. 60, 187–232.

Lanciotti, R.S., Ebel, G.D., Deubel, V., Kerst, A.J., Murri, S., Meyer, R., Bowen, M., McKinney, N., Morrill, W.E., Crabtree, M.B., Kramer, L.D., Roehrig, J.T., 2002. Complete genome sequences and phylogenetic analysis of West Nile virus strains isolated from the United States, Europe, and the Middle East. Virology 298, 96–105.

Lee, E., Weir, R.C., Dalgarno, L., 1997. Changes in the dengue virus major envelope protein on passaging and their localization on the three-dimensional structure of the protein. Virology 232, 281–290.

Li, J., Bhuvanakantham, R., Howe, J., Ng, M.L., 2006. The glycosylation site in the envelope protein of West Nile virus (Sarafend) plays an important role in replication and maturation processes. J. Gen. Virol. 87, 613–622.

Lindenbach, B.D., Thiel, H.J., Rice, C.M., 2007. *Flaviviridae*: the virus and their replication. Fields Virology. Lippincott William & Wilkins, pp. 1101–1152.

Lorenz, I.C., Allison, S.L., Heinz, F.X., Helenius, A., 2002. Folding and dimerization of tick-borne encephalitis virus envelope proteins prM and E in the endoplasmic reticulum. J. Virol. 76, 5480–5491.

Moudy, R.M., Meola, M.A., Morin, L.L., Ebel, G.D., Kramer, L.D., 2007. A newly emergent genotype of West Nile virus is transmitted earlier and more efficiently by Culex mosquitoes. Am. J. Trop. Med. Hyg. 77, 365–370.

Ocazionez Jimenez, R., Lopes da Fonseca, B.A., 2000. Recombinant plasmid expressing a truncated dengue-2 virus E protein without co-expression of prM protein induces partial protection in mice. Vaccine 19, 648–654.

Ryman, K.D., Ledger, T.N., Weir, R.C., Schlesinger, J.J., Barrett, A.D., 1997. Yellow fever virus envelope protein has two discrete type-specific neutralizing epitopes. J. Gen. Virol. 78 (Pt 6), 1353–1356.

Scherret, J.H., MacKenzie, J.S., Khromykh, A.A., Hall, R.A., 2001. Biological significance of glycosylation of the envelope protein of Kunjin virus. Ann. N.Y. Acad. Sci. 951, 361–363.

Shi, P.-Y., Kauffman, E.B., Ren, P., Felton, A., Tai, J.H., Dupuis II, A.P., Jones, S.A., Ngo, K.A., Nicholas, D.C., Maffei, J.G., Ebel, G.D., Bernard, K.A., Kramer, L.D., 2001. High throughput detection of West Nile virus RNA. J. Clin. Microbiol. 39, 1264–1271.

Shi, P.Y., Tilgner, M., Lo, M.K., 2002a. Construction and characterization of subgenomic replicons of New York strain of West Nile virus. Virology 296, 219–233.

Shi, P.Y., Tilgner, M., Lo, M.K., Kent, K.A., Bernard, K.A., 2002b. Infectious cDNA clone of the epidemic West Nile virus from New York City. J. Virol. 76, 5847–5856.

Shirato, K., Miyoshi, H., Goto, A., Ako, Y., Ueki, T., Kariwa, H., Takashima, I., 2004. Viral envelope protein glycosylation is a molecular determinant of the neuroinvasiveness of the New York strain of West Nile virus. J. Gen. Virol. 85, 3637–3645.

Shirato, K., Miyoshi, H., Kariwa, H., Takashima, I., 2006. The kinetics of proinflammatory cytokines in murine peritoneal macrophages infected with envelope protein-glycosylated or non-glycosylated West Nile virus. Virus Res. 121, 11–16.

Weaver, S.C., Barrett, A.D.T., 2004. Transmission cycles, host range, evolution and emergence of arboviral disease. Nat. Rev., Microbiol. 2, 789–801.

Zhang, Y., Corver, J., Chipman, P.R., Zhang, W., Pletnev, S.V., Sedlak, D., Baker, T.S., Strauss, J.H., Kuhn, R.J., Rossmann, M.G., 2003. Structures of immature flavivirus particles. EMBO J. 22, 2604–2613.