INTRODUCTION

West Nile virus (WNV) belongs to the genus Flavivirus within the family Flaviviridae and has caused increasing global concerns in recent years. Infection can result in fatal encephalitis associated with damage to the central nervous system in humans and animals. Like all other flaviviruses, the RNA genome of WNV is approximately 11 kb in length and encodes a single large polyprotein. The order of these proteins on the polyprotein is NH₂-C-prM-E-NS1-length and encodes a single large polyprotein. The order of system in humans and animals. Like all other flaviviruses, global concerns in recent years. Infection can result in fatal

The flavivirus envelope (E) protein is proposed to be involved in a number of biological activities including virus assembly, receptor binding and membrane fusion. It contains potential N-linked glycosylation sites (Heinz, 1986; Chambers et al., 1990). Lad et al. (2000) showed that the glycosylation site of the E protein is important for the native conformation of the epitopes and its proper expression. This glycosylation site is also linked to the neuroinvasiveness (Halevy et al., 1994; Chambers et al., 1998; Beasley et al., 2002; Shirato et al., 2004) and enhanced replication efficiency (Scherret et al., 2001b; Shirato et al., 2004) of several strains of WNV. The rationale behind the impaired replication efficiency of the non-glycosylated variants remains to be elucidated. This study shows the importance of the glycosylation site of the WNV E protein in replication and maturation processes of the virus. The multiple roles played by the glycosylation site of the E protein of the WNV are also discussed.

METHODS

Cells and viruses. Vero cells were grown in medium 199 (M199) containing 10% fetal calf serum. Baby hamster kidney (BHK)-21 and C6/36 cells were grown in RPMI 1640 and L15 media, respectively. WNV, a kind gift from E. G. Westaway (Sir Albert Sakzewski Virus Research Centre, Royal Children’s Hospital, Brisbane, Queensland 4029, Australia), was used as the parental virus.

Sequence analysis. The RNA extracted from WNV was used to generate PCR products for DNA sequencing. The 5'- and 3'-ends of the genome were determined by 5'- and 3'-RACE (GeneRacer; Invitrogen). ScanProsit was used to identify the N-linked glycosylation motif (NYST) in the E protein. Alignment of the nucleotide sequence of WNV with other WNV strains was performed with CLUSTALX 1.8 alignment software. The phylogenetic analysis was performed on the aligned nucleotide sequences with a neighbour-joining

The complete genome of West Nile (Sarafend) virus [WNV(S)V] was sequenced. Phylogenetic trees utilizing the complete genomic sequence, capsid gene, envelope gene and NS5 gene/3' untranslated region of WNV(S)V classified WNV(S)V as a lineage II virus. A full-length infectious clone of WNV(S)V with a point mutation in the glycosylation site of the envelope protein (pWNS-S154A) was constructed. Both growth kinetics and the mode of maturation were affected by this mutation. The titre of the pWNS-S154A virus was lower than the wild-type virus. This defect was corrected by the expression of wild-type envelope protein in trans. The pWNS-S154A virus matured intracellularly instead of at the plasma membrane as shown for the parental WNV(S)V.
Cloning. The construction of full-length cDNA clone (pWNS) is shown in Fig. 1. Modified pBR322 vector was generated as described earlier (Li et al., 2005a). Individual fragments corresponding to the four regions of the full-length genome were separately cloned into the modified vector and served as intermediate clones before sequential ligation into the vector to form pWNS. During the cloning, one silent mutation was engineered to knock out the Xbal site in the parental genome (Xbal*, nt 9474, T to A) and served as a recombination marker. The QuikChange Site-Directed Mutagenesis kit (Stratagene) was used to introduce the substitution at Ser154 of the E protein in pWNS to generate pWNS-S154A so that the expressed E protein would not be recognized by glucosyl transferase. The fragment corresponding to the nt 1–3520 was cloned into pGEM-T-Easy vector (Promega) to generate pGEM-C-prM-E for the complementation study.

In vitro transcription and transfection. In vitro transcription was performed as described by Li et al. (2005a) to generate RNAs from pWNS and pWNS-S154A clones. Twenty micrograms of the purified RNA was transfected into BHK-21 cells by electroporation as described by Shi et al. (2002). RNA quantification was performed using a Nanodrop ND-1000 Spectrophotometer (Peqlab). The regions spanning the S154A mutation and recombination marker were sequenced to confirm the presence of the S154A mutation and the recombination marker in the recovered viruses.

Western blot and complementation analyses. Western blot analysis was performed as described by Bhuvanakantham & Ng (2005). Briefly, Vero cells were infected with parental, pWNS and pWNS-S154A viruses for 24 h. The cell lysates were then subjected to SDS-PAGE analysis followed by Western blot analysis with an anti-E monoclonal antibody (Microbix). Complementation analysis was done by transfecting the RNA synthesized from the plasmid pGEM-C-prM-E into Vero cells by electroporation as described by Brandt et al. (2001). Five hours post-transfection, the cells were infected with the pWNS-S154A virus at an m.o.i. of 5–6. The expression of the glycosylated and non-glycosylated E proteins in the cell lysates was determined by Western blot analysis.

Growth kinetics of the pWNS-S154A virus. Vero, BHK-21 and C6/36 cells were inoculated with either parental WN(S)V, pWNS or pWNS-S154A virus at an m.o.i. of 5–6. Virus was allowed to be absorbed for 1 h and productive virus in the supernatant was sampled at 6, 12, 18 and 24 h post-infection (p.i.) for plaque assay. Complementation analysis was performed as described above.

Determination of growth kinetics using transcribed RNAs. Twenty micrograms of RNAs transcribed from the pWNS and pWNS-S154A clones was used to transfect Vero cells. After transfection, the cells were seeded into 24-well plates and the culture supernatant was sampled at 8, 16, 24, 32, 40, 48 and 56 h for plaque assay and real-time RT-PCR. Viral RNAs extracted from the culture supernatant were then reverse transcribed with SuperScript II (Invitrogen) using a primer corresponding to nt 11000–11020. The reverse transcripts were applied to a real-time PCR assay using iTaq Supermix (Bio-Rad) with forward (5'-GGTTCGCCACATCAGTACTTAGAG-3') and reverse (5'-GATCAATGCGCGATACAAGCTG-3') primers corresponding to nt 9716–9849 of the WNV genome. The kinetics of cDNA amplification were monitored with an ABI Prism 7000 sequence detection system (Applied Biosystems) using a dual labelled probe (5'-TCGAACGCTGCGACAAAAATGGTTGTA-3') conjugated with 6-carboxyfluorescein at the 5'-end and 6-carboxy-tetramethylrhodamine at the 3'-end. An in vitro transcribed RNA from pWNS clone was used as a standard for quantification.

Transmission electron microscopy (TEM). Vero cells infected with parental WN(S)V, pWNS or pWNS-S154A virus at an m.o.i. of 5–6 were processed at 14, 18 and 24 h.p.i as described by Li et al. (2005a) and viewed under a Philips electron microscope (CM 120, BioTwin). Vero cells transfected with wild-type E protein and infected with pWNS-S154A virus (complementation) were also directly processed for TEM analysis.

Fig. 1. Schematic representation of the construction of the full-length cDNA clone of WN(S)V. The genome organization and position of the four synthesized cDNA fragments, by RT-PCR, covering the full-length genome for sequential cloning are indicated. One silent mutation was engineered to knock out Xbal (Xbal*, nt 9474, T to A) in the wild-type virus to form a recombination marker. Two restrictive sites were created at both ends (BsiWI at the 5'-end and Xbal at the 3'-end) of the cDNA sequence for cloning. The full-length cDNA is positioned at the 3'-end of a T7 promoter sequence for in vitro transcription. Plasmid pBR322 was modified by replacing the AaflI–SalI fragment in the tetracycline resistance gene (Tc') with a linker sequence 5'-TGAACGCTGCAACCATCCGATCATAAGTG-GAGTCGACAACTGGTGCCGAGTACTGTCGACAGCTGCTGCTCCGGATCCCGGACGCTGCTGCTCTAGATACGT-3' to form the plasmid pBR322-linkerS containing BsiWI, AaflI, SalI, SnaI and Xbal (underlined) for restrictive digestions. The resulting pBR322-linkerS is used for individual cDNA fragment cloning and sequencing before sequentially assembled into the full-length cDNA clone, pWNS.

Transmission electron microscopy (TEM). Vero cells infected with parental WN(S)V, pWNS or pWNS-S154A virus at an m.o.i. of 5–6 were processed at 14, 18 and 24 h.p.i as described by Li et al. (2005a) and viewed under a Philips electron microscope (CM 120, BioTwin). Vero cells transfected with wild-type E protein and infected with pWNS-S154A virus (complementation) were also directly processed for TEM analysis.

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RESULTS

Sequence analysis

The complete genomic sequencing of WN(S)V showed that the genome was 11057 bp in length, and its open reading frame begins at nt 97 and encodes a 3434 aa polypeptide. The putative anchored capsid (C) protein is located between nt 97 and 465. The prM protein is encoded from nt 466 to 966, with the M protein encoded between nt 742 and 966. The E protein is encoded between nt 967 and 2469, followed by the non-structural proteins: NS1 (nt 2470–3525), NS2a (nt 3526–4218), NS2b (nt 4219–4611), NS3 (nt 4612–6468), NS4a (nt 6469–6846), putative 2K protein (nt 6847–6915), NS4b (nt 6916–7683) and NS5 (nt 7684–10398), respectively.

As an initial step to examine the genetic relationship of WN(S)V to other WNV isolates, multiple nucleotide sequence alignments were made utilizing the corresponding sequences of WNV lineages. The HNY1999 strain was chosen as a representative of lineage Ia viruses and the MRM61C strain represented lineage Ib viruses. The WNFCG strain represented lineage II viruses. The Rabensburg strain was included since it was considered as a new lineage of WNV (Bakonyi et al., 2005). When the complete nucleotide sequences of these virus were aligned, WN(S)V exhibited 79 % nucleotide identity to the HNY1999 and MRM61C strains, and 78 % identity to Rabensburg strain and 93 % identity to WNFCG strain. The alignment of C, E and NS5/3′ untranslated region (UTR) nucleotide sequences showed 93–97 % identity with WNFCG, 78–85 % with HNY1999, 78–84 % with MRM61C and 78–79 % with Rabensburg. Taken together, these results demonstrate that WN(S)V exhibited the greatest percentage identity with the WNFCG strain.

Phylogenetic trees were constructed from the aligned nucleic acid data by utilizing the entire genome or the selected genes (C, partial E, NS5/3′-UTR) of 27 WNV strains and one Japanese encephalitis virus strain (JEV – used as an outgroup), and the analysis was performed in 1000 bootstrap replicates (Fig. 2). Since the complete genomic sequence for most of the lineage II viruses is not available for comparison, the analysis was limited to nucleic acid data by utilizing the entire genome or the other additional mutations except the intentionally introduced mutations.

Generation of recombinant viruses

To analyse the effect of the glycosylation site of the E protein on the virus phenotype, full-length cDNA clones of pWNS and pWNS-S154A were constructed. After transfection of pWNS and pWNS-S154A RNAs, the progeny viruses were collected from the supernatant. The titre of amplified pWNS virus in Vero cells at 24 h p.i. was 2–6 × 10^9 p.f.u. ml^-1, which was similar to that of the parental virus. The titre of amplified pWNS-S154A virus was lower but still reached 2–5 × 10^9 p.f.u. ml^-1 at 48 h p.i. The plaques formed by parental WN(S) and pWNS viruses ranged from 2.1 to 2.6 mm on day 3, while pWNS-S154A virus displayed smaller size plaques (1.5 to 1.7 mm). However, the plaque size of 1.9 to 2.3 mm could be achieved by day 5 for pWNS-S154A virus. The genetic stability of pWNS and pWNS-S154A viruses was monitored by direct sequencing of the full-length genome by RT-PCR. Sequencing of the virus stocks from passages 1 and 3 confirmed that there are no other additional mutations except the intentionally introduced mutations.

Western blot analysis

To examine the potential molecular size change caused by the silencing of E-NYST, the cell lysates from the Vero cells
infected with WN(S)V, pWNS or pWNS-S154A viruses were subjected to Western blot analysis. As shown in Fig. 3(a), differences in electrophoretic mobility of the E proteins were observed, which indicated the presence (lanes 2 and 3) and absence (lane 4) of glycosylation of the E protein of the above-mentioned viruses. These results are consistent with previous reports showing the differences in the molecular mass of glycosylated and non-glycosylated forms of the E protein (Johnson et al., 1994; Chambers et al., 1998; Lorenz et al., 2003; Shirato et al., 2004). Complementation analysis was
performed by transfecting Vero cells with the RNA from pGEM-C-prM-E followed by infection with the pWNS-S154A virus. The expression of both glycosylated and non-glycosylated forms of the E proteins (lane 5) was observed. The non-glycosylated E protein, shown in lower intensity (lane 5), migrated faster than the glycosylated form.

Fig. 3. (a) Western blot analysis showing the glycosylation status of the WNV E proteins. Cell culture supernatants harvested at 24 h p.i. from mock-infected Vero cells (lane 1) and from Vero cells infected with parental WN(S)V (lane 2), pWNS (lane 3) or pWNS-S154A (lane 4) virus, were analysed by SDS-PAGE followed by Western blot analysis. The blot was probed with WNV-specific anti-E monoclonal antibody. The non-glycosylated E protein (lane 4) migrated faster than the glycosylated forms (lanes 2 and 3). Complementation analysis performed by transfecting the Vero cells with the RNA from pGEM-C-prM-E, 5 h prior to infection with the pWNS-S154A virus, showed both the glycosylated and non-glycosylated forms of the E proteins (lane 5). (b) Comparison of the growth kinetics of parental (●), pWNS (■), pWNS-S154A (▲) and pWNS-S154A plus complementation (■) viruses at an m.o.i. of 5-0 in Vero cells determined at 6, 12, 18 and 24 h after the inoculation. The mean values ± SD are representative of three independent experiments. (c and d) Comparison of the growth kinetics for pWNS (■) and pWNS-S154A (▲) infectious clones derived viruses by transfecting Vero cells with transcribed RNAs. The culture supernatant was sampled at selected time points (8, 16, 24, 32, 40, 48 and 56 h) and were used for titration of infectious virus by plaque assay (c) and for detection of viral RNA copies by real-time RT-PCR (d). The mean values ± SD are representative of two independent experiments. The detection limit for real-time assay was > 10^2 copies. (e and f) Comparison of the growth kinetics of parental (●), pWNS (■) and pWNS-S154A (▲) viruses at an m.o.i. of 5-0 in BHK-21 and C6/36 cell lines, respectively, determined at 6, 12, 18 and 24 h after the inoculation. The mean values ± SD are representative of three independent experiments.
Growth kinetics of pWNS-S154A virus

In order to examine whether the efficiency of the virus replication was affected by the S154A mutation, the virus growth kinetics were determined. Fig. 3(b) shows the comparison of growth curves between parental WN(S), pWNS and pWNS-S154A viruses. The growth kinetics in Vero cells showed no significant difference between the parental WN(S) and pWNS viruses. However, the titre of the pWNS-S154A virus was approximately 100-fold lower than that of pWNS virus. In addition, the pWNS-S154A replicated relatively slower during the early stages after infection. The impaired replication was reversed by wild-type E protein complementation as shown in Fig. 3(b), which indicated that the expression of the wild-type E protein is able to compensate for the propagation of the pWNS-S154A virus. These results proved that the glycosylation site of the E protein is important for efficient virus replication in mammalian cells.

The lower replication efficiency of the pWNS-S154A virus might be caused by the presence of a higher proportion of defective viruses. To test this possibility, in vitro transcribed RNAs from pWNS and pWNS-S154A plasmids were used for direct transfection. The supernatant from the transfected cell culture was collected for plaque assay. As shown in Fig. 3(c), the pWNS-S154A virus similarly showed impaired propagations, with the titres being 100-fold lower than those of pWNS virus at 56 h post-transfection. Transfection of the infectious RNA also seemed to increase the latent period compared with infection with intact virus. It showed that the pWNS-S154A RNA is less efficient at replication than the RNA from the pWNS clone.

To investigate the possibility that the production of infectious RNA is less adequate for pWNS-S154A virus, the total number of viral RNA copies (positive-strand) was determined using real-time RT-PCR. As shown in Fig. 3(d), the number of viral RNA copies produced from the pWNS-154A clone was less than that from the pWNS clone at 32 and 40 h post-transfection.

To demonstrate that the defect observed is not limited to the Vero cells, we also examined the growth kinetics of parental WN(S), pWNS and pWNS-S154A viruses in BHK-21 and C6/36 cells, which are shown in Fig. 3(e and f), respectively. These results confirmed the impaired replication of pWNS-S154A virus in both mammalian and mosquito cell lines.

Ultrastructural analysis

To assess the possibility that N-linked glycosylation of the E protein also plays a role in the maturation process of the virus, TEM analysis was carried out. As shown in Fig. 4(a and b), virus particles egressed by budding at the plasma membrane (arrows) of Vero cells infected by parental WN(S) and pWNS viruses, respectively, which is a distinctive feature of this strain (Ng et al., 1994). Fig. 4(c) shows a large proportion of the cell cytoplasm (Cy). There was no accumulation of progeny virus particles intracellularly or at the perinuclear region. Virus particles (arrows) were observed to be budding from the plasma membrane similar to Fig. 4(b).

In the pWNS-S154A virus-infected cells, virus particles were formed within the swollen lumen of the endoplasmic reticulum (Fig. 5a, arrows). This is similar to the trans-

type of maturation seen in many other flaviviruses. The usual smooth membrane vesicles with thread-like structures (Fig. 5a, arrowheads) within swollen endoplasmic reticulum were observed in the infected cells. At higher magnification, mature progeny virus particles (Fig. 5b, arrows) were observed scattered among the smooth membrane vesicles (Fig. 5b, arrowheads). In Fig. 5(a and b), no virus budding was observed from the plasma membrane (PM) after intensive screening of the cells.

When complementation analysis was performed by transfecting the Vero cells with the wild-type E protein followed by infection with pWNS-S154A virus, progeny virus particles were observed budding from the plasma membrane (Fig. 5c, d and inset, arrows) in the majority of the cells, even though the intracellular maturation (Fig. 5c and d, arrowheads) was also observed. This is similar to the parental or the glycosylated recombinant virus [(pWNS) (Fig. 4)]. From the observation in this study, it appears that when the virus matures at the plasma membrane, only a small quantity of paracrystals/convoluted membranes and smooth membrane vesicles are observed, unlike in intracellular maturation. TEM was performed at three different timings (14, 18 and 24 h p.i.) for all the viruses (parental, pWNS, pWNS-S154A viruses and complementation to pWNS-S154A viruses) and similar results were observed irrespective of the timings. These observations suggested that the carbohydrate side chain of the E protein is a determinant of the mode of virus maturation in WN(S)V.

DISCUSSION

Various methods have been employed to address the genomic diversity of different strains of WNV. These mainly include phylogenetic analysis of aligned complete nucleotide sequences and selected gene sequences. Genetic studies have shown that WN viruses can be divided into two lineages. Recently, Rabensburg isolate was suggested to form a new lineage of WNV or a novel member of the JEV group.

Phylogenetic analysis performed previously placed WN(S)V under lineage II (Scherret et al., 2001a). However, this analysis was performed using partial nucleotide sequence of WN(S)V. To extend these findings and to determine more precisely the genetic relationships between the WN(S)V and other WN isolates, the complete genomic sequence of WN(S)V was determined, and its phylogenetic relationship with other WNV strains was analysed utilizing their complete genomes, C, E and NS5/3′-UTR sequences. The outcome of these analyses suggested that WN(S)V was most closely related to WNFCG (Fig. 2). This is the first study showing the phylogenetic trees utilizing the complete
genome, C, E and NS5/3'-UTR nucleotide sequences of WN(S)V for the analyses.

Several groups (Scherret et al., 2001a; Beasley & Barrett, 2002; Li et al., 2005b; Oliphant et al., 2005; Sanchez et al., 2005) have shown that amino acids E306 (Ser), E307 (Lys), E330 (Thr) and E332 (Thr) constituted the epitope on the surface of domain III that can be recognized by lineage-differentiating monoclonal antibodies, and the mutation at residue E332 affected neutralization and membrane receptor preparation-binding characteristics of the virus. Li et al. (2005a or b) demonstrated that T332K and L312A mutations were sufficient to disrupt the epitopes recognized by neutralizing antibodies. In Sarafend, the residues at E312 and E332 are Ala and Lys, respectively. Given the location of these residues in the identified neutralizing epitopes, WN(S)V could probably escape neutralization by the monoclonal antibodies raised against lineage I strain, HNY1999. This awaits further verification.

The mutations identified at residues E126 and E159 were also reported in two other lineage I strains, Italy 1998 and Romania 1996 (Lanciotti et al., 2002) and these mutations have been shown to affect the virus phenotypes in terms of virus neutralization or virulence in other flaviviruses (Lobigs et al., 1987; McMinn et al., 1995; Ryman et al., 1997). Berthet et al. (1997) and Scherret et al. (2001a) defined the signature motifs within the E gene that can differentiate lineage I from lineage II. When we analysed the presence of these signature motifs in WN(S)V, the residues at E128, E129, E131, E172, E199, E205, E208 and E210 concurred with their observations (Fig. 2e). The significance of other mutations identified within the E protein remains to be unveiled.

As demonstrated in this study, the glycosylation site of the WN(S)V E protein plays an important role in regulating the rate of virus replication. Abolishing the glycosylation site of the E protein delayed the replication rate at the early phase (Fig. 3b). Initially, plaque assay was used to determine the infectious virus particles from the supernatant harvested from Vero cells infected with the pWNS and pWNS-S154A viruses. It has been shown that the loss of E protein glycosylation affected the viral stability and infectivity in Dengue virus and St. Louis encephalitis virus (Pletnev et al., 1993; Vorndam et al., 1993; Ishak et al., 2001). Thus, the growth kinetics were also determined using in vitro transcribed RNAs (Fig. 3c). The difference in virus titres between pWNS and pWNS-S154A was observed in both the virus-infected and RNA-transfected samples (Fig. 3b and c). In addition, the total amount of viral RNA measured by real-time RT-PCR was higher for the pWNS virus than the pWNS-S154A virus (Fig. 3d). To gain an insight into how significant the defect (impaired replication) observed in Vero cells was, we extended our studies to BHK-21 and

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C6/36 cell lines using the intact viruses (Fig. 3e and f). Taken together, these results proved that the glycosylation site of the E protein is important for efficient virus replication in both mammalian and mosquito cell lines.

The exact mechanism behind the impaired virus replication is unknown. Entry of the virus into the cells could possibly be affected by the mutation abolishing the glycosylation site, since the E protein plays a critical role in receptor attachment. The loss of glycosylation could also affect the assembly and secretion of the virus particles (Lorenz et al., 2003). Pulse–chase analysis will be performed in the future to demonstrate the defect in the secretion of the non-glycosylated E protein. Another possible mechanism behind reduced virus replication could be decreased virus–E protein interactions mediated by the glycan residues. Rey et al. (1995) suggested that the glycan residue may interact with domain II of the other E subunit and stabilize the dimeric structure. Lorenz et al. (2003) also linked the function of the carbohydrate moiety to the other glycoprotein, prM, and suggested that this glycan could be necessary in forming the appropriate interface between the E glycoproteins. The glycosylated amino acids were mapped onto the E dimer near domain III (Rey et al., 1995; Modis et al., 2003; Mukhopadhyay et al., 2003; Zhang et al., 2003; Zhang et al., 2004). Moreover, it has been suggested that the carbohydrate moieties on the virus surface might regulate the specificity of virus binding to the cellular receptors (Navarro-Sanchez et al., 2003).

Previously, our study indicated that 5′-UTR and the three structural genes played a role in influencing the mode of maturation for WN(S)V (Li et al., 2005a or b). This concurred with the current observation that the non-glycosylated virus (pWNS-S154A) showed an altered maturation mode. Parental WN(S) and pWNS viruses were observed budding out of the cell plasma membrane (Fig. 4), a characteristic feature of the majority of flaviviruses (Hase, 1993; Serafino et al., 1997; Rahman et al., 1998; Lanciotti et al., 2002). This result indicated that maturation by budding at the plasma membrane was altered in pWNS-S154A recombinant virus.

A more detailed study will be conducted using immunogold labelling to trace the transportation of the synthesized E protein both in the pWNS-S154A-infected cells and in infected cells complemented with wild-type E proteins. These experiments can confirm further whether E proteins synthesized in pWNS-S154A-infected cells are only localized with the virus-induced membranes and not transported to the plasma membrane as in wild-type virus infection.

It was shown previously that the assembly of WN(S)V was affected by brefeldin A (BFA) treatment. Egression at the plasma membrane and filopodia formation, characteristic features of WN(S)V, were not observed in the presence of
BFA (Sreenivasan et al., 1993). BFA is a fungal metabolite known to affect glycoprotein maturation (Whealy et al., 1991). Taken together with the previous reports and the current findings, it is reasonable to conclude that the E protein glycosylation (status) is the major molecular determinant of the budding mode at the plasma membrane for WNV(S)V.

To our knowledge, there are no data to show whether the New York (NY) strain of WNV matures intracellularly or at the plasma membrane. It is therefore tempting to postulate that, if WNV(NY) also buds at the plasma membrane like WNV(S)V, the same glycosylation site might influence the maturation mode. In addition, the replication efficiency of WNV(NY) was also slowed down by the mutation at the glycosylation site of the E protein (Shirato et al., 2004), consistent with our observations in this study.

There have been several studies showing the relationship between N-linked glycosylation in the E protein and neuroinvasiveness of several virus isolates in vivo. The attenuation of neuroinvasiveness after mutation at the glycosylation site of the E protein was proven in NY strains (NY99-6922, BC787 and TM171-03; Beasley et al., 2002; Shirato et al., 2004). However, the loss of glycosylation site did not affect the neurovirulence. Alternatively, Chambers et al. (1998) suggested that glycosylation might not be directly responsible for the attenuation of WNV since neuroinvasiveness was also observed with the non-glycosylated E protein variant of the Israel strain. Wang et al. (2003) showed that WNV(S)V is neuroinvasive and neurovirulent in murine models and that virus replication in peripheral tissues is required for virus invasion of the central nervous system. This observation is consistent with the study on NY strains of West Nile virus: pathogenicity in immunocompetent and SCID mice. The envelope glycoprotein in its low-pH-induced membrane fusion conformation. EMBO J 73, 2293–2297.


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