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Deglycosylation of the NS1 protein of dengue 2 virus, strain 16681: Construction and characterization of mutant viruses

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Summary. The dengue 2 virus (DENV-2) NS1 glycoprotein contains two potential sites for N-linked glycosylation at Asn-130 and Asn-207. NS1 produced in infected cells is glycosylated at both of these sites. We used site-directed mutagenesis of a DENV-2, strain 16681, full length infectious clone to create mutant viruses lacking the Asn-130, Asn-207 or both of these NS1 glycosylation sites in order to investigate the effects of deglycosylation. Ablation of both NS1 glycosylation sites resulted in unstable viruses that acquired numerous additional mutations; these viruses were not further characterized. Viruses altered at the Asn-130 site exhibited growth characteristics similar to the wild-type (WT) 16681 virus in LLC-MK_2 cells and reduced growth in C6/36 cells. Viruses mutated at the Asn-207 site achieved similar titers in LLC-MK_2 cells compared to WT, however, the appearance of cytopathic effect was delayed and growth of these viruses in C6/36 cells was also reduced compared to WT virus. The plaque size of mutant viruses altered at the Asn-130 site did not differ from that of the WT virus, while mutants altered at the Asn-207 site exhibited a reduced and mixed plaque size. Temperature sensitivity studies comparing the growth of the viruses at 37 °C and 39 °C showed no significant differences compared to the WT virus. Immunofluorescent antibody staining of infected cells showed that for WT 16681 virus or the Asn-130 site mutant viruses NS1 was located throughout the cytoplasm, however, Asn-207 site mutant virus NS1 protein appeared to be localized to the perinuclear region. Viruses deglycosylated at either site exhibited a significant reduction in mouse neurovirulence compared to the WT virus. The results of our studies indicate that glycosylation of the DENV-2 virus NS1 protein may influence NS1 protein processing/transport as well as the pathogenicity of the virus.

Introduction

Dengue 2 virus (DENV-2) is a mosquito-borne virus in the genus *Flavivirus*, family *Flaviviridae*. The four serotypes of *Dengue virus* (DENV) are responsible for significant human disease in tropical and sub-tropical regions of the world. The disease consists of either a self-limiting febrile illness, known as dengue fever, or the more severe dengue hemorrhagic fever-dengue shock syndrome. DENV-2 is an enveloped virus containing a positive-sense RNA genome that codes for three structural proteins (capsid, membrane and envelope (E) and seven nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5).

The flavivirus NS1 protein is an approximately 46 kDa glycoprotein that contains 12 highly conserved cysteine residues, two or three potential sites for N-linked glycosylation, and regions of high sequence homology [2]. The flavivirus NS1 protein was first described as a soluble complement-fixing antigen found in infected cell cultures [1]. In mammalian cells, NS1 exists as dimers and multimers in intracellular, cell-surface and secreted extracellular forms, although it is mostly retained in the cell and has been localized to sites of RNA replication [8, 18, 32, 33]. Extracellular NS1 can elicit a strong humoral immune response, and immunization with NS1, passive immunization with anti-NS1 antibody or immunization with DNA encoding NS1 can be protective [6, 30, 35]. High levels of NS1 have been found in the sera of patients with confirmed secondary DENV infections suggesting that this protein may contribute to the formation of immune complexes that are postulated to be involved in the pathogenesis of severe dengue disease [37]. The specific function of the NS1 protein has not yet been fully elucidated. However, studies have demonstrated a link between NS1 and viral RNA accumulation and replication. *Trans*-complementation studies of yellow fever virus (YFV) NS proteins have revealed a requirement for NS1 in early RNA replication and a genetic interaction between NS1 and NS4A, a putative replicase component, that affects RNA replication [16, 17, 22, 23]. Additionally, Jacobs et al. have demonstrated expression of a glycosyl-phosphatidylinositol-anchored form of DENV-2 NS1 that is capable of antibody-induced signal transduction, suggesting a role for NS1 in cell activation [12].

Processing of the flavivirus NS1 protein begins with translocation of the premembrane-envelope-NS1 portion of the viral polyprotein into the lumen of the endoplasmic reticulum (ER) where a signal peptidase cleaves the E/NS1 junction. The NS1/NS2A precursor is N-glycosylated and NS2A is then released from the carboxy terminus of NS1 by a membrane-bound, ER-resident host protease [2, 7]. Monomeric NS1 is hydrophilic; however, upon dimerization NS1 becomes more hydrophobic and membrane-associated [34]. Dimerization is required for NS1 transport through the Golgi system, where the glycans attached to the Asn-130 sites of the dimer are modified to complex sugars; this is followed by transport to the plasma membrane, where a portion of NS1 is released from the cell. Modification of the Asn-130 carbohydrate to a complex sugar is required for efficient release of NS1 from infected cells; in infected insect cells, which are incapable of producing complex oligosaccharides, NS1 is not secreted [3, 8, 11, 14, 34].

Previous research involving deglycosylation of the flavivirus NS1 protein resulted in alteration of virus virulence. Deglycosylation of the first NS1 glycosylation site in a chimeric virus containing the M and E genes of tick-borne encephalitis virus (TBEV) and remaining genes from dengue 4 virus (DENV-4) resulted in reduced growth in cell culture and reduced mouse neurovirulence [26]. Ablation of the second glycosylation site in the chimeric virus increased mouse neurovirulence. Deglycosylation of the first NS1 glycosylation site of the YFV NS1 protein resulted in decreased virulence in cell culture and decreased mouse neurovirulence. Reduced growth in cell culture and decreased mouse neurovirulence have also been observed in DENV-2, New Guinea C strain, derived from an infectious clone that had been mutated to ablate the second NS1 glycosylation site [22, 28]. In the work presented here, we investigated the effects of deglycosylation of DENV-2 NS1 by using site-directed mutagenesis of a DENV-2 16681 infectious clone.

Materials and methods

Viruses and cells

Wild-type DENV-2 16681 (WT) virus was obtained from the virus collection at the Centers for Disease Control and Prevention, Fort Collins, CO, and has been previously described [9, 36]. Viruses were grown in LLC-MK_2 (Rhesus monkey kidney), Vero (African green monkey kidney), BHK-21 (baby hamster kidney), and C6/36 (*Aedes albopictus* mosquito) cells in Dulbecco's modified minimal essential medium (DMEM) containing gentamycin and 5% fetal bovine serum (FBS). Plaque titration assays were performed in 6-well plates of Vero cell monolayers as previously described [21]. Plaques were counted at 8–11 days after infection.

Construction of glycosylation mutant viruses

The mutations listed in Table 1 were introduced into the intermediate plasmid F2 at either or both of the DENV-2 NS1-130 and NS1-207 glycosylation sites by using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The derivation of the DENV-2 16681 F2 plasmid and the full-length DENV-2 infectious clone have been previously described [13]. Mutated F2 plasmids were grown in *Escherichia coli* XL-2 Blue cells (Stratagene) and were screened by sequencing to select those containing the desired mutation. The *SphI-KpnI* fragment from each mutated F2 plasmid was then subcloned into the full-length DENV-2 16681 infectious clone to construct the mutated full length clones. Viral RNA was transcribed and LLC-MK_2 cells were transfected by electroporation as previously described [13, 15]. Production of viral proteins in transfected cells was monitored by using an indirect immunofluorescence assay (IFA) with monoclonal antibodies 3H5 (DENV-2 type-specific), 4G2 (flavivirus group-specific), and 1H10 (DENV-4 type-specific, negative control) as the primary antibodies and fluorescein isothyocyanate-conjugated goat anti-mouse IgG as the secondary antibody [10]. The complete genomes of all mutated viruses were sequenced to confirm the presence of introduced mutations and the absence of additional spurious mutations.

Characterization of replication phenotypes

Mutated viruses were compared to WT virus with regard to plaque size in Vero cells, replication in LLC-MK_2 and C6/36 cells and temperature sensitivity in LLC-MK_2 cells. Additionally, replication of viruses altered at the Asn-207 NS1 glycosylation site was assessed in Vero and

Table 1. NS1 glycosylation site mutants of DEN-2 16681 virus

Virus	Amino acid changed[a]	1st NSI glycosylation site[b]	2nd NS1 glycosylation site[b]
DENV-2 16681		N Q T AAC CAG ACC	N D T AAT GAC ACA
N1	130	***Q*** Q T ***C***A***G*** CAG ACC	
N1C	132	N Q ***N*** AAC CAG A***A***C	
N2[c]	207		***Q*** D T ***C***A***G*** GAC ACA
N2C	209		N D ***N*** AAT GAC A***AC***
N12	130	***Q*** Q T ***C***A***G*** CAG ACC	
	207		***Q*** D T ***C***A***G*** GAC ACA
N12C	132	N Q ***N*** AAC CAG A***A***C	
	209		N D ***N*** AAT GAC A***AC***

[a]NS1 protein amino acid position

[b]Top = amino acid sequence; bottom = nucleotide sequence; nucleotide and amino acid changes in bold italics

[c]and N2p3

BHK-21 cells. Growth curves were performed by infecting cell monolayers at a multiplicity of infection (m.o.i.) of 0.1 plaque-forming unit (PFU) per cell. Samples were removed for titration by plaque assay every other day for 10 (BHK-21 cells) or 12 (all other cell types) days. The temperature sensitivity of the viruses was assayed by infecting LLC-MK_2 cells at an m.o.i. of 0.1 and incubating the cells at either 37 °C or 39 °C. Virus was harvested and titrated by plaque assay 8 days after infection.

Analysis of NS1 proteins

NS1 protein in virus-infected LLC-MK_2 cell lysates and the protein secreted into infected cell culture medium were analyzed by SDS-PAGE. For preparation of cell-associated NS1, cells were infected at an m.o.i. of 1.0 PFU per cell, maintained in DMEM with 2% FBS,

and harvested when there was evidence of cytopathic effect (CPE) in the monolayer. Cells were washed twice with phosphate buffered saline (PBS), solubilized in 1X NuPage LDS sample buffer (Invitrogen/Life Technologies, Baltimore, MD), passed 4–7 times through a 30 gauge needle to shear the DNA and stored at −20 °C. Glycosylation status of cell-associated proteins was analyzed by peptide:N-glycosidase F (PNGaseF) deglycosylation of proteins prior to electrophoresis using the GlycoShift kit (Oxford Glycosystems, Rosedale, NY) per manufacturer's instructions. For preparation of secreted NS1 protein, cells were infected as described above and cell culture medium was removed at 24 h and replaced with DMEM without FBS. Culture medium was harvested four days after infection, clarified by centrifugation at 8,000 rpm for 10 min at 4 °C, and concentrated approximately 10-fold using a Centricon-30 concentrator (Millipore Corp., Bedford, MA). One part 4X LDS sample buffer was added to 3 parts concentrated protein and samples were stored at −20 °C. Prior to electrophoresis, samples were denatured by adding 0.1 volume of NuPage reducing agent (containing 0.5 M dithiothreitol (DTT), Invitrogen/Life Technologies) followed by incubation at 70 °C for 10 min. Denatured samples were electrophoresed on 10% NuPage Bis/Tris gels with MOPS buffer using the XCell SureLock electrophoresis apparatus according to manufacturers instructions (Invitrogen/Life Technologies). A protein standard marker, MultiMark or Magic Mark (Invitrogen/Life Technologies) was included on each gel to aid in molecular weight estimation. Following electrophoresis, proteins were blotted onto nitrocellulose using the XCell II Blot Module (Invitrogen/Life Technologies). Immobilized NS1 protein was detected using the Western blot technique with the Western Breeze kit (Invitrogen/Life Technologies) and an anti-NS1 monoclonal antibody, 3D1.4 (generously provided by Dr. A. Falconar, Department of Infectious Disease Epidemiology, Imperial College of Medicine, University of London) diluted 1:1200 as the primary antibody [5, 31]. Blots were analyzed to estimate molecular weights (mw) by using a GS710 Calibrated Imaging Densitometer and Quantity One quantitation software (Bio Rad Laboratories, Hercules, CA).

PAGE analysis was also used to compare the stability of NS1 dimers from the WT and mutant viruses. Cell-associated viral protein was subjected to treatments of 22 °C for 2 h, 37 °C for 1 h, 55 °C for 30 min, or 70 °C for 10 min in the presence of 0.8% DTT, followed by electrophoresis, blotting and detection as described above. Relative quantities of NS1 monomer and dimer were determined for each virus using the Imaging Densitometer and quantitation software described above (Bio Rad Laboratories).

Immunofluorescence studies

Distribution of NS1 protein in infected LLC-MK_2 cells was visualized by using the immunofluorescent antibody technique (IFA). LLC-MK_2 cells grown on 8-well Lab-Tek Chamber Slides (Nalge Nunc International, Rochester, NY) were infected with WT or mutant viruses at an m.o.i. of 1.0. Five days after infection, slides were rinsed with ice-cold PBS and wells and gaskets were removed. Slides were then rinsed twice more in cold PBS, fixed for 10 min in ice-cold acetone and air dried. NS1 protein was detected by using the 3D1.4 anti-NS1 monoclonal antibody diluted 1:800 as the primary antibody followed by fluorescein isothyocyanate-conjugated goat anti-mouse IgG (Gibco/BRL Life Technologies, Baltimore, MD) as the secondary antibody.

Mouse neurovirulence studies

Newborn outbred white ICR mice were inoculated intracranially with 10,000 PFU of WT or mutant virus in 20 µl of Minimal Essential Medium (MEM) containing 15% FBS. Control mice were inoculated with 20 µl of diluent only. Mice were observed for signs of encephalitis and mortality for 28 days.

Results

Production of mutant viruses

Two sets of mutants were constructed by altering either the 1st or 3rd amino acid (aa) in each glycosylation motif (Table 1). Mutants designated N1, N2 or N12 were altered at the 1st aa of the motif of the Asn-130 glycosylation site, the Asn-207 glycosylation site or both sites, respectively. Mutants designated N1C, N2C or N12C were confirmation mutants that were altered at the 3rd aa of the Asn-130 glycosylation motif, the Asn-207 glycosylation motif or both motifs, respectively. Confirmation mutants were constructed to verify that phenotypic changes observed for the original mutant viruses were due to the deglycosylation of the protein and not due to the specific aa change introduced. Virus was produced by transfection of LLC-MK_2 cells with RNA transcribed from each of the mutant full-length clones. Virus infection was demonstrated by IFA detection of viral antigen in transfected cells and by plaque titration assay.

Characterization of replication phenotypes

Growth of the N1 and N1C mutant viruses, altered at the Asn-130 NS1 glycosylation site, was similar to the WT virus in LLC-MK_2 cells, however, reduced titers were observed in C6/36 cells (Table 2, Fig. 1A, B). Additionally, a significant difference in maximum C6/36 cell titer was observed between the N1 and N1C mutants (6.0 and 7.4 $\log_{10}$ PFU/ml, respectively; $p = 0.015$, Welch's Modified t-test) (Table 2). Both of these mutant viruses caused CPE similar to that caused by

Table 2. Peak titers and cytopathic effects observed during 12-day growth curves

Virus	Cell type							
	LLC-MK_2		BHK-21		Vero		C6/36	
	Titer[a]	CPE[b]	Titer[c]	CPE	Titer[a]	CPE	Titer[a,d]	CPE
DENV-2 16681	7.8	+	5.5	+	7.2	+	8.0	−
N1	7.8	+	nd[e]	nd	nd	nd	6.0	−
N1C	7.5	+	nd	nd	nd	nd	7.4	−
N2p3	7.6	+[f]	5.0	+	6.6	+	5.9	−
N2C	7.2	+[f]	5.5	+	6.3	+	7.0	−

[a]Peak titers, $\log_{10}$ PFU/ml, achieved at 6–8 days after infection at an m.o.i. of 0.1

[b]Presence or absence of gross cytopathic effect

[c]Peak titers, $\log_{10}$ PFU/ml, achieved at 4 days after infection at an m.o.i. of 0.1

[d]Each C6/36 cell mutant virus titer significantly different from WT (ANOVA, $p < 0.001$, Dunnett's method, $\alpha = 0.05$)

[e]Not done

[f]Delayed, slightly decreased level of CPE relative to WT 16681, N1 and N1C viruses

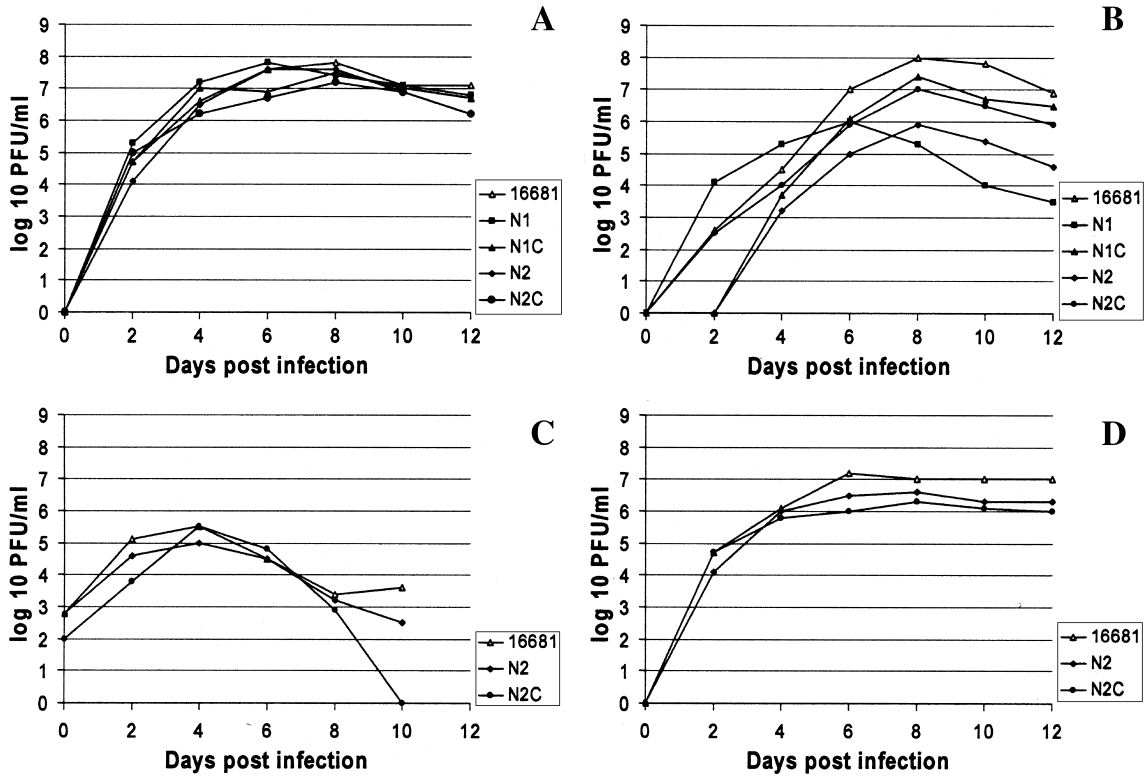

Fig. 1. Twelve-day growth curves. **A**, LLC-MK2 cells; **B**, C6/36 cells; **C**, BHK-21 cells; **D**, Vero cells

WT virus in LLC-MK_2 cells and, like WT virus, they did not cause CPE in C6/36 cells. The morphology of plaques produced by the WT virus and the N1 and N1C mutant viruses was also similar (1.5–5.0 mm, 1.0–4.5 mm, and 1.0–4.5 mm plaque size range, respectively). Temperature sensitivities of the N1 and N1C mutants were not significantly different from that of the WT virus (data not shown).

The N2 and N2C mutant viruses, altered at the second NS1 glycosylation site, were phenotypically different from WT virus in plaque morphology and growth in cell culture. Both mutant viruses formed smaller plaques overall (0.5–4.0 mm), compared to WT virus, and when the cell culture source of the virus being assayed was infected LLC-MK_2 or C6/36 cells, a mixed plaque phenotype was observed that included pinpoint plaques of less than 0.5 mm size. When the cell culture source of the virus being tested by plaque assay was infected BHK-21 or Vero cells these pinpoint plaques were not observed. Four N2 mutant viruses from plaques of different sizes were isolated, amplified and sequenced. Each of the plaque-picked viruses contained one to five additional nucleotide mutations, most of which were silent (Table 3). There was no correlation between the number or nature of the mutations and the size of the plaque from which they were isolated. One plaque isolate, designated N2p3, that contained only a single additional silent mutation (nucleotide 1677; A to G), was selected for further characterization of

Table 3. Additional mutations accumulated in N2 plaque-picked viral isolates

Plaque isolate designation	Plaque size[a]	Nucleic acid		Amino acid change	Gene
		Site	Mutation		
N2p1	3.5 mm	1881	A to C		E
		4830	A to C		NS3
		7296	A to C		NS4b
		9915	A to C		NS5
N2p2	1.0 mm	3804	A to G		NS2a
		5829	T to G	Asp to Glu	NS3
		6491	G to A	Arg to Lys	NS4a
		8090	A to G	Asn to Ser	NS5
		9363	A to G		NS5
N2p3	0.5 mm	1677	A to G		E
N2p4	0.5 mm	1881	A to C		E
		4830	A to C		NS3
		5023	A to G	Thr to Ala	NS3
		7296	A to C		NS4b

[a]Plaque size range = 0.5–4.0 mm

the N2 mutant virus. This isolate, as well as the N2C mutant virus from the original LLC-MK_2 cell transfection, was shown to be genetically stable based on full-length sequencing of virus passaged three times in LLC-MK_2 cells. The N2p3 and N2C mutant viruses were passaged once in LLC-MK_2, Vero, BHK-21 and C6/36 cells and the resulting virus from each cell type was sequenced. No additional mutations were acquired during these passages. Both mutant viruses continued to exhibit a mixed plaque phenotype different from that of the WT virus.

Although growth rates and maximum virus titers of the Asn-207 site mutant viruses, N2p3 and N2C, in LLC-MK_2 cells were similar to those observed with WT virus (Table 2, Fig. 1A) less CPE was observed and the time required for development of CPE was increased. Whereas WT virus caused gross CPE including 70–80% cell death by 6–8 days after RNA transfection, cells transfected with Asn-207 site mutant virus RNA initially exhibited a moderate increase in the formation of vacuoles and multinucleate cells, followed by cell rounding and death of approximately 50% of cells in the monolayer by day 7–10. However, the mutant virus RNA-transfected cells were 80–95% IFA positive for DENV-2 antigen by 5–6 days after transfection, which was similar to antigen levels detected in WT virus RNA-transfected cells. In contrast to growth in LLC-MK_2 cells, the N2p3 and N2C mutant viruses caused a more cytopathic infection in Vero cells, similar to that caused by the WT virus although peak titers achieved were not significantly different (ANOVA) (Table 2, Fig. 1D). Infection of BHK-21 cells with N2p3 or N2C resulted in massive CPE with peak titers, similar to WT, reached 4 days following infection compared to 6–8 days following infection in the other cell

types (Table 2, Fig. 1C). Day-12 growth curve samples were not obtained from BHK-21 cells due to complete CPE in the infected cell monolayers. Peak titers of the N2p3 and N2C mutant viruses in C6/36 cells were reduced compared to the WT virus (Table 2, Fig. 1B). Similar to the N1 and N1C mutant viruses, there was a significant difference between the N2p3 and N2C mutant viruses in maximum C6/36 cell titers (5.9 and 7.0 $\log_{10}$ PFU/ml, respectively; $p = 0.017$) (Table 2). As seen with the WT virus, neither the N2p3 nor the N2C mutant viruses caused CPE in C6/36 cells (Table 2). No significant difference in temperature sensitivity was observed for the N2p3 or N2C viruses compared to WT.

The double mutant viruses, N12 and N12C, which were altered at the first or third aa of both NS1 glycosylation site motifs, respectively, were genetically unstable. LLC-MK_2 cells were transfected with transcribed N12 RNA in two separate experiments. Each time the resulting virus was of low titer (approximately 3.8 $\log_{10}$ PFU/ml), did not cause CPE and cells did not become IFA positive (1–2%) until 12–14 days after transfection. Upon passage of the virus in LLC-MK_2 cells to amplify the titer, one to five additional mutations were acquired, most of which were non-silent, making the virus too unstable to characterize further. In contrast, the N12C double mutant virus initially appeared to be stable and the virus grew to high titer (6.0 $\log_{10}$ PFU/ml) following transfection. The growth rate and maximum titer achieved in LLC-MK_2 cell growth curve studies were similar to WT virus (max. titer = 7.2 $\log_{10}$ PFU/ml) and, as seen with the single mutant viruses, the maximum N12C virus titer in C6/36 cells was reduced compared to WT virus (max. titer = 5.6 $\log_{10}$ PFU/ml). However, protein characterization and subsequent re-sequencing revealed that the virus had acquired an Asn-to-Ser mutation at aa 209. This mutation reconstituted the 2nd glycosylation site, although it did not revert to the WT aa 209 (Thr). Attempts to reisolate the original mutant virus were unsuccessful and this virus was not characterized further.

Immunofluorescent antibody staining

IFA conducted on LLC-MK_2 cells with anti-NS1 MAb 3D1.4 five days after infection with WT, N1 or N1C virus showed diffuse NS1 antigen staining throughout the cytoplasm (Fig. 2A, B, C). In contrast, IFA staining of N2p3 or N2C-infected LLC-MK_2 cells revealed NS1 antigen localized in the perinuclear region of the cells (Fig. 2D, E). Similar results were also observed at 10 days after infection (data not shown).

Analysis of viral proteins

Polyacrylamide gel electrophoresis followed by Western blot detection of NS1 proteins from WT and mutant viruses confirmed the deglycosylated status of the mutant viruses (Fig. 3A). The mw of cell-associated WT virus NS1 protein was estimated to be 45.0 kDa. Cell-associated NS1 protein from the N1, N1C, N2p3 and N2C mutant viruses migrated faster than WT NS1; mw were estimated to be 43.6 kDa, 43.3 kDa, 42.4 kDa and 42.5 kDa, respectively. These differences in

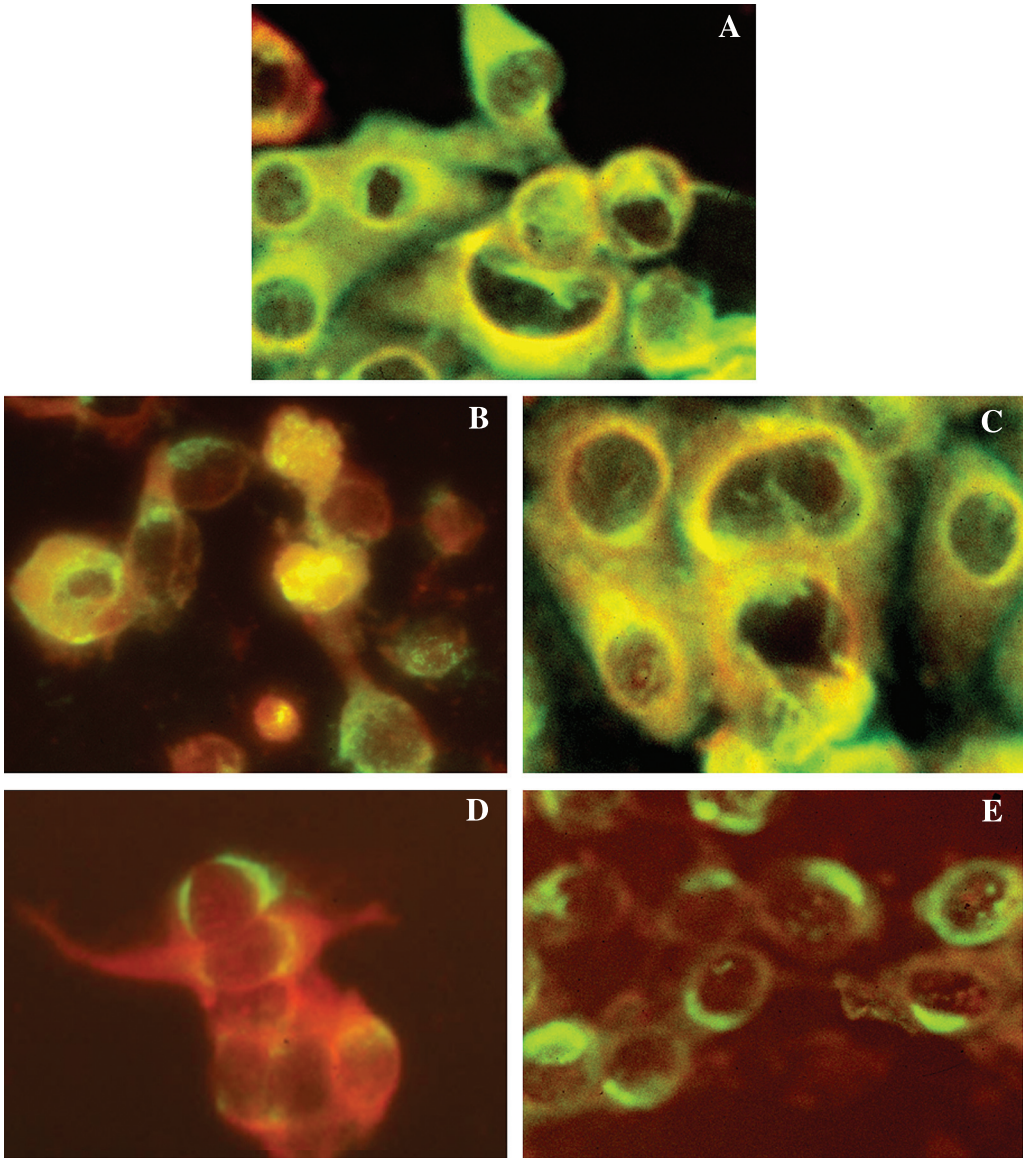

Fig. 2. Immunofluorescent antibody staining of LLC-MK2 cells five days after infection with (**A**) WT DENV-2 16681, (**B**) N1, (**C**) N1C, (**D**) N2p3, (**E**) N2C

mw from WT NS1 of approximately 1.6 kDa for the Asn-130 site mutants and 2.5 kDa for the Asn-207 site mutants suggested the loss of a single carbohydrate.

Results of deglycosylation of the cell-associated WT, N1 and N2p3 viral NS1 proteins with PNGaseF, followed by electrophoresis and Western blot detection, are shown in Fig. 3C. The mw of the WT virus NS1 was reduced from 45.2 kDa to 41.2 kDa, a difference of approximately 4 kDa that correlated with the loss of two carbohydrate units from the protein. N1 mutant virus NS1 was reduced by approximately 2.2 kDa, from 43.3 kDa to 41.1 kDa, and the N2p3 mutant

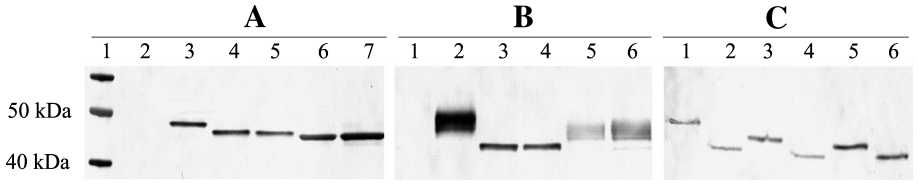

Fig. 3. Analysis of NS1 proteins by SDS-PAGE with Western blot detection using anti-NS1 monoclonal antibody. **A** Cell-associated NS1 protein. *1*, Magic Mark protein standard marker; *2*, mock-infected LLC-MK_2 cells; *3*, WT DENV-2 16681; *4*, N1; *5*, N1C; *6*, N2p3; *7*, N2C. **B** Secreted NS1 protein. *1*, mock-infected LLC-MK_2 cells; *2*, WT DENV-2 16681; *3*, N1; *4*, N1C; *5*, N2p3; *6*, N2C. **C** Untreated (*1*, *3*, *5*) and PNGaseF-treated (*2*, *4*, *6*) cell-associated NS1 protein. *1–2*, WT DENV-2 16681; *3–4*, N1; *5–6*, N2p3

virus NS1 was reduced by approximately 1.4 kDa, from 42.7 kDa to 41.3 kDa, confirming the loss of one carbohydrate unit from each mutant NS1.

Results of PAGE analysis of secreted NS1 are shown in Figure 3B. The secreted WT virus NS1 had a mw higher than the WT cell-associated protein and appeared to consist of three or more closely spaced bands ranging in mw from 46.5 to 49.3 kDa. Similarly, the secreted NS1 from the N2p3 and N2C viruses had a higher mw than either the cell-associated protein from these viruses or the secreted or cell associated NS1 from the N1 or N1C mutant viruses. The N2p3 and N2C secreted NS1 also consisted of multiple bands ranging in mw from 44.7 to 47.5 kDa with an additional faint band of mw 42.8 kDa in the N2C lane equivalent to the mw of the cell-associated NS1 from this virus. Intensity of the N2p3 and N2C secreted NS1 bands appeared reduced. The secreted NS1 protein from cells infected with the N1 and N1C mutant viruses appeared the same in band character and mw (~43 kDa) as that of the cell-associated protein from these viruses although band intensity appeared reduced compared to WT NS1.

Comparison of the stability of NS1 dimers from the WT and mutant viruses was conducted by varying the time and temperature of incubation of cell-associated viral protein in the presence of 0.8% DTT. There was no measurable difference in NS1 dimer stability between the WT and mutant viruses following any of the four treatments tested. For each virus, NS1 dimers remained stable following the 22 °C and 37 °C treatments and were destabilized completely by the 55 °C treatment.

Mouse neurovirulence

Neurovirulence of the mutant viruses in newborn mice was greatly reduced compared to that of WT virus (Table 4). While only 32.1% (18/56) of mice inoculated with WT virus survived to 28 days post inoculation, 92.5% (37/40) and 87.5% (28/32) of mice inoculated with the N1 and N1C mutants survived, respectively. Similarly, 100% (16/16) and 93.8% (15/16) of mice inoculated with the N2p3 and N2C mutant viruses, respectively, survived. Control mice inoculated with media had a 28-day survival rate of 96.9% (31/32). Differences between the WT virus and each mutant virus were significant ($p = <0.001$, Fisher's Exact test); differences

Table 4. Summary of mouse neurovirulence experiments

Virus	n[a]	No. surviving[b]	% Survival	Avg. survival time (days)[c]
DENV-2 16681	56	18	32.1	19.1
N1	40	37	92.5	26.9
N1C	32	28	87.5	26.8
N2p3	16	16	100.0	28.0
N2C	16	15	93.8	27.3
No virus control	32	31	96.9	27.4

[a]Number of mice inoculated
[b]Number of mice surviving 28 days post inoculation
[c]Based on 28-day observation period

among the mutant viruses or between the mutant viruses and the no-virus-control were not significant ($p = 0.54$). Likewise, average survival time was significantly greater (Dunnett's method, $\alpha = 0.05$) for mice infected with the mutant viruses or in the no-virus-control compared to that of mice infected with the WT virus.

Discussion

We successfully constructed viable mutants of the DENV-2 16681 virus that were altered to remove either the Asn-130 or the Asn-207 NS1 protein glycosylation site. Failure to recover stable mutant viruses lacking both NS1 glycosylation sites suggested that glycosylation of at least one of the two NS1 sites was required for replication of the virus although in a previously published study a mutant YFV lacking both sites was viable and exhibited reduction in cell culture virulence and in mouse neurovirulence [22].

Protein analysis of WT and mutant viruses by PAGE and Western blot, with or without PNGaseF deglycosylation, confirmed that the WT virus was glycosylated at both of the potential NS1 sites and that each of the single-site mutant viruses (N1, N1C, N2p3 and N2C) remained glycosylated at one site (Fig. 3). Molecular weight differences were observed between NS1 proteins deglycosylated at the Asn-130 (N1 and N1C) and the Asn-207 (N2p3 and N2C) sites. Simple, high mannose-type oligosaccharides are attached at both sites of cell-associated NS1. The slightly lower mw of cell-associated NS1 from the N2p3/N2C viruses (deglycosylated at Asn-207) can most likely be attributed to a shorter length mannose chain linked at the Asn-130 site. In contrast, the mw of secreted NS1 from N2p3 and N2C-infected cells was probably higher as a result of modification of the Asn-130-linked glycan to a complex carbohydrate [8, 20, 27]; the secreted WT NS1 protein displayed a similar increase in mw. The presence of multiple secreted NS1 bands from the WT virus and the N2p3 and N2C mutant viruses suggests some variation in the size or type of this complex carbohydrate.

The N1C and N2C mutant viruses were constructed to confirm that any phenotypic changes observed for the N1 and N2p3 mutant viruses were due to deglycosylation of the NS1 protein, and not to the specific amino acid change introduced. With the exception of growth in C6/36 cells, no differences were observed between the original mutant viruses and the confirmation mutant viruses. Maximum titers of all four of the mutant viruses in C6/36 cells were lower than the WT virus. Peak titers of the N1 and N2p3 mutant viruses in C6/36 cells were significantly lower than those of the N1C and N2C viruses (Table 2), suggesting that the specific Asn-to-Gln mutation at the first amino acid position in each glycosylation site motif had an additional effect on replication in these cells.

The effects of flavivirus NS1 deglycosylation reported in previous studies have varied greatly. These dissimilar observations are most likely due to differences in virus or virus strain and/or the specific amino acid changes used to ablate glycosylation in the NS1 protein. Reported results of ablation of the first NS1 glycosylation site have ranged from reduced growth in cell culture and reduced mouse neurovirulence (YFV, TBEV/DENV-4 chimera) to no production of detectable virus (DENV-2, New Guinea C strain) [22, 26, 28]. In this study differences observed between the WT DENV-2 16681 virus and the Asn-130 glycosylation site mutants (N1 and N1C) included reduced NS1 secretion from infected cells, reduced growth in C6/36 cells and attenuated mouse neurovirulence, suggesting that glycosylation at this site can influence NS1 processing/transport as well as virus virulence.

In previous studies removal of the second flavivirus NS1 glycosylation site resulted in an increase in mouse neurovirulence (TBEV/DENV-4 chimera), a decrease in both mouse neurovirulence and replication in C6/36 cells (DENV-2, New Guinea C strain), or no phenotypic difference from the parental virus (YFV) [22, 26, 28]. Our results were similar to those obtained with deglycosylation of the DENV-2, New Guinea C strain NS1. Phenotypic changes associated with deglycosylation of DENV-2 16681 at the Asn-207 glycosylation site (N2p3 and N2C) included reduced CPE in LLC-MK_2 cells, a smaller, mixed plaque phenotype in Vero cells, perinuclear localization of NS1 by IFA, and attenuation of mouse neurovirulence. Additionally, the band intensity of N2p3 and N2C secreted NS1 on Western blots appeared reduced compared to WT suggesting a reduction in NS1 secretion from LLC-MK_2 cells. NS1 has been identified on the plasma membrane and in perinuclear and cytoplasmic regions of infected cells, is associated with intracellular membranes and has been colocalized with viral dsRNA [18, 25, 32]. Our results suggest that ablation of the Asn-207 NS1 glycosylation site may decrease transport of NS1 away from the perinuclear region resulting in reduced secretion from the cell and decreased virulence for mice.

The function of the flavivirus NS1 protein is not known, although it is postulated to play a role in early RNA replication, and the effect(s) of glycosylation on NS1 function have not been fully elucidated [16, 17, 22, 23]. The addition of carbohydrates to viral proteins can influence a variety of characteristics including folding, stability and solubility which in turn may affect expression of immunological epitopes, receptor binding, fusion, intracellular transport, resistance to

proteolytic degradation and heat inactivation, and the host cell range of the virus [4, 19, 24, 29]. We demonstrated that complete ablation of DENV-2 16681 NS1 glycosylation resulted in a genetically unstable virus whereas removal of only one glycosylation site allowed replication of mutated virus with an altered phenotype. Comparison of the WT and mutant viruses demonstrated that of the two NS1 glycosylation sites, deglycosylation at the Asn-207 site had a greater effect on viral phenotype. Our results indicate that deglycosylation of the DENV NS1 protein impairs the processing and transport of NS1 in the infected cell and attenuates the virus' pathogenicity in mice. Further investigation of either of these two attenuating NS1 mutations for potential inclusion in newly designed, live-attenuated dengue vaccine candidates is warranted.

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