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Mutagenesis of the N-Linked Glycosylation Sites of the Yellow Fever Virus NS1 Protein:

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The flavivirus nonstructural glycoprotein NS1 is highly conserved and contains two N-linked glycosylation sites which are both utilized for addition of oligosaccharides during replication in cell culture. NS1 has been shown to contain epitopes for protective antibodies; however, its roles in virus replication and pathogenesis remain unknown. To study the function of NS1 during yellow fever virus replication, six mutant viruses which lack either one or both glycosylation sites and another one containing silent mutations at both sites were generated by site-directed mutagenesis. Mutants lacking the second glycosylation site and those bearing silent mutations were similar to the parental virus in their cell culture properties. Ablation of the first or both glycosylation sites generated mutants exhibiting small plaque phenotypes, decreased virus yields, reduced cytopathic effects, impaired NS1 secretion, and depressed RNA accumulation. In addition, mutants lacking the first or both glycosylation sites exhibited significant reduction in mouse neurovirulence after intracerebral inoculation. These defects appear to result from the lack of N-linked glycans rather than the introduction of deleterious amino acid substitutions or disruption of *cis*-acting RNA elements important for RNA replication. These results suggest an important role for NS1 in flavivirus RNA replication and pathogenesis.

INTRODUCTION

The family *Flaviviridae* includes three genera of enveloped RNA viruses, the flaviviruses, the pestiviruses, and the hepatitis C viruses (for review, ref. Rice, 1996). Many of these viruses are pathogenic for man and other vertebrates (Monath, 1986). Yellow fever virus (YF) is the prototype member of the *Flavivirus* genus and its genome consists of a single, positive-stranded RNA molecule of 10,862 bases, with a 5′ CAP structure and a nonpolyadenylated 3′ terminus (Rice *et al.,* 1986b). The viral RNA encodes a polyprotein of over 350 kDa which is processed co- and posttranslationally by host and viral proteases to produce the mature structural and nonstructural (NS) proteins (Chambers *et al.,* 1990a; Rice and Strauss, 1990). The gene order is 5′-C-prM(M)-E-NS1-NS2A-NS2B-NS3-NS4A-2K-NS4B-NS5-3′, where C (capsid), prM(M) (membrane), and E (envelope) represent the structural proteins or their precursors and NS1 through NS5 are NS proteins (Chambers *et al.,* 1989, 1990b; Lin *et al.,* 1993a; Rice *et al.,* 1986a).

In contrast to nonstructural proteins encoded by other RNA viruses, the flavivirus NS1 protein possesses several interesting and unusual properties (for review, see Chambers *et al.,* 1990a). NS1 is glycosylated (Smith and Wright, 1985), exists as a homodimer, and is present in cell-associated, cell-surface, and secreted forms (Mason, 1989; Winkler *et al.,* 1989, 1988). During flavivirus infections, NS1-specific antibodies with complement-fixing activity are produced and the secreted form of NS1 occurs as a soluble complement-fixing antigen (Russell *et al.,* 1980; Schlesinger and Brandriss, 1983; Smith and Wright, 1985). NS1 contains protective epitopes and both active immunization with NS1 and passive immunization with NS1-specific monoclonal antibodies can protect animals against lethal challenge with the homologous flavivirus (Gould *et al.,* 1986; Jacobs *et al.,* 1992, 1994; Schlesinger *et al.,* 1986, 1985, 1993). NS1 is highly conserved with 12 invariant cysteine residues and two potential N-linked glycosylation sites whose positions are conserved for all mosquito-borne flaviviruses. These properties have led to the suggestion that NS1 might play a role in virus assembly and/or maturation (Mason, 1989; Rice *et al.,* 1986b).

Nascent NS1 is inserted into the endoplasmic reticulum (ER) via a hydrophobic internal signal sequence located near the C terminus of the E protein, followed by signalase cleavage at the E/NS1 junction and core glycosylation (Falgout *et al.,* 1989). Studies on the cleavage of NS1-2A, using recombinant vaccinia and baculovirus expression systems, indicate that the C-terminal 8 residues of NS1 (Chambers *et al.,* 1990b; Falgout *et al.,* 1989; Hori and Lai, 1990) and as few as 26 amino acids

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from the N-terminus of NS2A (Leblois and Young, 1995) are sufficient for cleavage by an unidentified proteolytic activity in the ER lumen (Falgout and Markoff, 1995). Following dimerization (Winkler *et al.,* 1989), NS1 transits from the ER to the Golgi where at least one of the glycans is modified to a complex form (Després *et al.,* 1991; Flamand *et al.,* 1992; Jacobs *et al.,* 1992; Mason, 1989; Post *et al.,* 1991; Pryor and Wright, 1994), and the secreted form of NS1 is released from the cell. Given the absence of a C-terminal hydrophobic anchor, the mechanism by which NS1 associates with the cellular membranes is unknown.

Several functions for N-linked glycans have been suggested, including protein folding and stability, protection against denaturation and proteolysis, modulation of biological activities, and transport through the secretory pathway (Fiedler and Simons, 1995). Although both N-linked glycosylation sites of NS1 appear to be utilized *in vivo* (Chambers *et al.,* 1990b; Smith and Wright, 1985), the functional role of these sugars has not been determined. Tunicamycin treatment of flavivirus-infected cells (Chambers *et al.,* 1990b; Després *et al.,* 1991; Mason, 1989; Winkler *et al.,* 1989) or transiently expressed NS1 (Jacobs *et al.,* 1992) indicates that lack of N-glycans impairs NS1 processing and secretion. These results must be interpreted cautiously, however, since tunicamycin might also inhibit glycosylation of cellular proteins which may be involved in NS1 transport. Recent studies using site-directed mutagenesis have provided evidence that glycosylation may be required for proper function of the NS1 protein. For instance, ablation of the first glycosylation site of NS1 in a chimeric virus, containing the prM and E proteins of tick-borne encephalitis virus (TBE) and the remaining proteins and RNA elements of dengue 4 virus (DEN4), resulted in reduced growth in vertebrate and mosquito cells and a significant reduction in mouse neurovirulence. In contrast, ablation of the second site of NS1 increased neurovirulence (Pletnev *et al.,* 1993). Elimination of one or both N-linked glycosylation sites in transiently expressed DEN2 NS1 did not abolish dimerization or secretion but lack of the second site reduced dimer stability as well as secretion (Pryor and Wright, 1994). Although these data provide some information on the involvement of the NS1 glycans in maturation of the protein and in flavivirus pathogenesis, the precise role of this protein in replication remains poorly characterized.

To study the functional importance of N-linked glycosylation of NS1 in flavivirus replication, we used site-directed mutagenesis to engineer mutants lacking either one or both YF NS1 glycosylation sites and an additional mutant containing multiple silent mutations at both sites. In this report, we have examined the effects of these mutations on virus growth, RNA and protein synthesis, and mouse neurovirulence.

TABLE 1

NS1 Mutations[a]

Mutant designation	Amino acid changes	Nucleotide changes
SB	—	$T_{2842}C$, $A_{2845}C$, $A_{2846}T$, $G_{2847}C$, $C_{2848}A$, $T_{3076}C$, $G_{3079}T$, $A_{3082}C$
G1a	$S_{132}A$	$A_{2846}G$, $G_{2847}C$, $C_{2848}T$
G1b	$N_{130}A$	$A_{2840}G$, $A_{2841}C$
G2a	$T_{210}A$	$A_{3080}G$, $A_{3082}C$
G2b	$N_{208}A$	$A_{3074}G$, $A_{3075}C$
G1aG2a	$S_{132}A$, $T_{210}A$	$A_{2846}G$, $G_{2847}C$, $C_{2848}T$, $A_{3080}G$, $A_{3082}C$
G1bG2b	$N_{130}A$, $N_{208}A$	$A_{2840}G$, $A_{2841}C$, $A_{3074}G$, $A_{3075}C$

[a] The nomenclature of the NS1 mutants is based on the loss of the first (G1) and/or the second (G2) glycosylation site and on the residue which is substituted in the consensus sequence for N-linked glycosylation, N-X-S/T (a and b for S/T and N, respectively). SB indicates the mutant bearing eight silent mutations, five at the first site and three at the second site.

MATERIALS AND METHODS

Cell culture and YF plaque assays

BHK-21 and human SW-13 cells were propagated in α-minimal essential medium (αMEM) supplemented with 10% fetal calf serum (FCS). Titers of infectious YF virus or RNA transcripts were determined by plaque assay on SW-13 monolayers. Infected or transfected monolayers were overlaid with αMEM containing 2% FCS and 0.9% agarose (GIBCO BRL). Plaques were visualized after 5 days by staining with 0.02% neutral red in phosphate-buffered saline (PBS) and, after fixation of the cells with 7% formaldehyde, by removal of the agarose overlay and staining with 1.3% crystal violet in 20% ethanol.

Plasmid constructions and site-directed mutagenesis

Plasmids were constructed using standard methods (Ausubel *et al.,* 1993), and mutations at both conserved N-linked glycosylation sites were created by oligonucleotide-directed mutagenesis of uridylated phagemid DNA (Kunkel, 1985; Lin *et al.,* 1993b). The phagemid pH2J1-NS1/2A was constructed by subcloning the *Xba*I fragment of pBLSCIISK-NS1/2A(+) (C.M.R., A. Grakoui, and R.G., unpublished) containing the NS1/2A region into pH2J1 (Hahn *et al.,* 1992). The strand rescued with helper phage R408 corresponded to the YF plus strand. Engineered mutations were verified by sequence analysis and are listed in Table 1. Full-length YF 17D cDNA templates were constructed by *in vitro* ligation of restriction fragments from pYF5′3′IV and pYFM5.2 or its mutant derivatives (Rice *et al.,* 1989). pYFM5.2 derivatives with mutations at either N-linked glycosylation site were constructed by replacing the corresponding *Sst*I–*Mlu*I (G1 site; YF nts 2486–2947) and *Mlu*I–*Kpn*I (G2 site; nts 2947–3262) fragments from the clones produced by site-

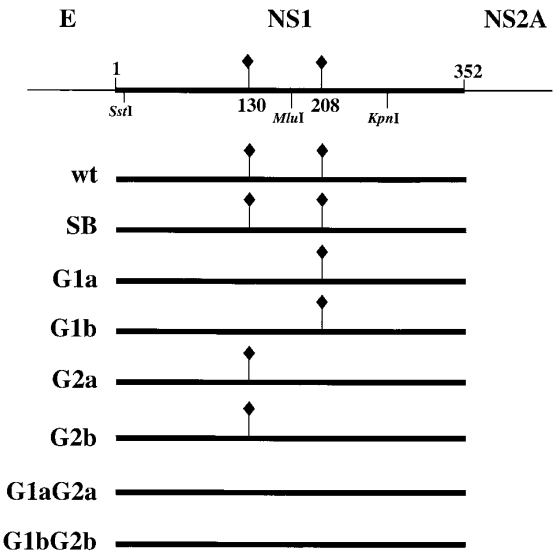

FIG. 1. NS1 glycosylation mutants. The diagram shows the 352-residue YF NS1 glycoprotein, portions of the adjacent E and NS2A proteins, and the positions of the Asn residues (Asn-130 and Asn-208) in the N-linked glycosylation sites (♦) mutagenized in this study (numbered relative to the NS1 N terminus). Mutant NS1 constructs, as described in Table 1 and under Materials and Methods, are diagrammed below.

directed mutagenesis (Fig. 1). To generate pYFM5.2 derivatives with mutations at both sites, the plasmids containing the individual G1 and G2 mutations were digested with *Apa*I (which cuts in the E coding region; nt 1599) and *Mlu*I (which cuts between the G1 and G2 sites; nt 2947) and the appropriate fragments ligated. For all constructs, the sequence of the entire subcloned fragment was verified.

In vitro transcription and RNA transfection

Five-prime-capped transcripts were synthesized *in vitro* from full-length cDNA templates using SP6 RNA polymerase (Rice *et al.,* 1989). Trace quantities of [α-^{32}P]CTP were included in the transcription reactions to permit quantitation of RNA yield and analysis of transcript integrity. Incorporation was measured by adsorption to DE81 (Whatman) filter paper (Sambrook *et al.,* 1989). Subconfluent BHK monolayers (35-mm dishes) were used for RNA transfection with lipofectin (GIBCO BRL; Rice *et al.,* 1989). Transfection mixtures were made by mixing 100 ng of RNA with 200 μl of PBS containing 8 μg of lipofectin followed by incubation on ice for 10 min. During this time, monolayers were washed twice with PBS. The mixtures were added to the cells and incubated at room temperature for 10 min. Following transfection, monolayers were washed once with αMEM and either incubated in αMEM containing 2% FCS at 37° to generate virus stocks or assayed directly for plaque formation. For virus stocks, cell culture supernatants were harvested at 3–4 days posttransfection and clarified at 3000 *g* for 10 min, and aliquots stored at −80°.

Virus stocks and polyethylene glycol (PEG) concentration

To prepare concentrated virus stocks, subconfluent monolayers of SW-13 cells (in 150-mm dishes) were infected at a multiplicity of infection (m.o.i.) of 0.04 PFU (plaque-forming units)/ml with the wild-type parent or its mutagenized derivatives in αMEM and 2% FCS. After 3–4 days at 37°, culture supernatants were harvested, clarified as above, and precipitated by adjusting the solution to 8% PEG 6000 (w/v), 120 m*M* NaCl, 12 m*M* Tris–Cl, pH 8.0, 1 m*M* EDTA, followed by incubation on ice for 1 hr. Virus was recovered by centrifugation at 9000 *g* for 30 min, resuspended in αMEM and 2% FCS, and stored in aliquots at −80°.

Growth curves

Subconfluent monolayers of SW-13 cells (35-mm dishes) were infected at a m.o.i. of 5 PFU/cell with PEG-concentrated virus in αMEM and 2% FCS. After 1 hr at 37°, the inoculum was removed and the monolayers were washed with prewarmed αMEM and incubated with 2 ml αMEM containing 2% FCS. Differential growth curves were performed by harvesting and replacing the culture supernatants at 12, 24, 48, or 72 h postinfection (p.i.). Aliquots were stored at −80° and titers were determined by plaque assay on SW-13 monolayers.

Preparation of labeled proteins

SW-13 monolayers (35-mm dishes) were infected at a m.o.i. of 5 PFU/cell with PEG-concentrated virus stocks in αMEM and 2% FCS. At 24 hr p.i., cells were incubated for 6 hr at 37° in methionine-free αMEM containing 2% FCS and 40 μCi/ml of Tran-^{35}S label (ICN). For pulse–chase experiments, cells were labeled at 23 hr p.i. for 1 hr with methionine-free αMEM containing 2% FCS and 100 μCi/ml Tran-^{35}S label and either harvested immediately or incubated for an additional 6 hr in αMEM containing 2% FCS and 10 times the normal amount of unlabeled methionine. At the desired intervals, supernatants were collected and clarified at 3000 *g* for 2 min and cells were lysed in either SDS- or Triton X-100 (Triton)-containing lysis solution, as described previously (Chambers *et al.,* 1989).

Immunoprecipitation

SDS-treated viral proteins were immunoprecipitated using NS1-specific polyclonal (Chambers *et al.,* 1990b) or monoclonal antibodies 8G4 and 1A5 (Schlesinger and Brandriss, 1983). Triton-solubilized proteins were immunoprecipitated by a YF-specific mouse hyperimmune ascitic fluid (HIAF; kindly provided by Joel Dalrymple) or monoclonal antibodies 5H3 (anti-E; Schlesinger *et al.,* 1983) or 8G4, as previously described (Chambers *et al.,* 1990b). All immunoprecipitates were collected using

Staphylococcus aureus Cowan strain I (Calbiochem) and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Chambers *et al.,* 1990b; Laemmli, 1970). Gels were treated with 1 *M* sodium salicylate, pH 7.0, for fluorographic detection of radiolabeled proteins at −80° (Chamberlain, 1979).

Endoglycosidase digestion

S. aureus pellets containing immunoprecipitated YF NS1 proteins were solubilized and digested with either endoglycosidase H (Endo H) or glycopeptidase F (PNGase F) (Boehringer Mannheim) or were incubated without added endoglycosidase using conditions described by Mason (1989). After incubation, samples were analyzed by SDS–PAGE as described above.

RNA analysis

SW-13 monolayers (35-mm dishes) were infected at a m.o.i. of 5 PFU/cell with PEG-concentrated viruses in αMEM and 2% FCS. At 4, 12, 24, and 36 h p.i. cells were washed once with ice-cold PBS and total cellular RNA was isolated by using the RNAzol method (TEL-TEST, Inc.). The RNase protection assay for analysis of positive- and negative-strand RNAs was performed essentially as described by Novak and Kirkegaard (1991). For positive-strand analysis, 5 μg total cytoplasmic RNA was resuspended in 30 μl hybridization buffer containing 1.5 pmol of ^{35}S-labeled (or ^{32}P-labeled) RNA probe (see below) followed by denaturation at 85° for 5 min, hybridization overnight at 55°, and treatment with RNases A and T1. Negative-strand RNAs were analyzed using a two-cycle RNase protection assay (Novak and Kirkegaard, 1991). Five micrograms of total cytoplasmic RNAs were resuspended in 30 μl of hybridization buffer and hybridized overnight at 55° in the presence of 10 ng of RNA transcribed from pTM3/YF/NS3 which had been linearized with *Xho*I (Chambers *et al.,* 1993). After RNase treatment and alcohol precipitation, the samples were resuspended in 30 μl of hybridization buffer containing 1.5 pmol of ^{35}S-labeled RNA probe (see below), denatured at 85° for 5 min, hybridized overnight at 55°, and digested with RNases. Protected fragments were separated by electrophoresis in 5% polyacrylamide–urea sequencing gels which were fixed, dried, and subjected to autoradiography (Sambrook *et al.,* 1989).

^{35}S-labeled (or in some cases ^{32}P-labeled) RNA probes were synthesized by *in vitro* transcription of pProteus31/5.2$_{3839-4089}$ (positive-sense probe) or pProteus31/5.2$_{4089-3839}$ (negative-sense probe) (as described in the Promega *Protocols and Applications Guide*). These plasmids were constructed by subcloning the *Bgl*II (3839)–*Bam*HI (4089) fragment of pYFM 5.2 (Rice *et al.,* 1989) into *Bgl*II-digested pProteus 31 (kindly provided by H. V. Huang). Plasmids were linearized with *Acc*I to allow transcription from the SP6 RNA promoter of positive-sense or negative-sense RNA probes of 284 nucleotides.

Animal experiments

Four-week-old female mice (ICR strain; Harlan Sprague–Dawley) were used for mouse neurovirulence studies. All animal handling was conducted according to approved standards for the care and use of laboratory animals. Mice were anesthetized with methoxyfluorane and injected in the left frontal cortex with approximately 5000 PFU of the parental virus or G1b, G2b, or G1bG2b NS1 mutants in 30 μl of pyrogen-free PBS (GIBCO BRL). This dose was selected because a similar dose of the parent virus (YFiv5.2) (Rice *et al.,* 1989) has been observed to cause nearly uniform mortality in young adult CD-1 mice (Schlesinger *et al.,* 1996). Actual titers of infectious virus in each inoculum were determined by plaque assay on SW-13 cells at the time of inoculation. Mice were observed until found dead or sacrificed when found in an advanced moribund condition. When possible, sera were collected and stored at −80° until use for determination of plaque reduction neutralization titers.

Neutralization titers

Virus neutralization titers were measured by plaque reduction neutralization assays using the parent as a test virus and SW-13 cells to monitor plaque formation. Mouse sera were collected upon sacrifice or between 3 and 6 weeks following infection, for mice which survived. Briefly, 20 μl of sera was heat-inactivated and diluted serially in minimal essential media containing 2% FCS. These dilutions were mixed with 200 PFU virus and incubated for 6 hr at 4° prior to plaque assay. The neutralization titer is given as the highest serum dilution capable of neutralizing 50% of the input virus compared to samples containing no mouse serum.

RESULTS

Oligonucleotide-directed mutagenesis of both conserved N-linked glycosylation sites (Asn-130 and Asn-208) was used to probe the function of NS1 glycosylation in YF replication. Mutations were introduced to abolish glycosylation at the first (G1), the second (G2), or both (G1G2) N-linked glycosylation sites by replacement of the Asn (series b) or Ser/Thr (series a) residues with Ala residues (Table 1 and Fig. 1). To control for the possibility that *cis* RNA elements important for replication might be located in these regions of the YF genome, we created an additional mutant (SB) with eight silent mutations, five at the first glycosylation site and three at the second (Table 1).

Recovery of NS1 mutant viruses after RNA transfection

As shown in Table 2, RNA transcripts derived from the parent and mutant templates had similar specific

TABLE 2

Effects of Mutations on Viral Replication[a]

Virus	RNA-specific infectivity[b] (PFU/20 ng RNA)	Virus yield[c] (PFU/ml)	Plaque size[d]
Wild type	25	1.3×10^7	L
SB	31	1.0×10^7	L
G1a	33	6.5×10^5	S
G1b	30	2.0×10^5	S
G2a	38	9.5×10^6	M
G2b	32	1.0×10^7	M
G1aG2a	35	6.0×10^5	T
G1bG2b	30	1.3×10^5	T

[a] BHK-21 cells were transfected with RNA transcripts as described under Materials and Methods.

[b] Number of plaques obtained at 5 days posttransfection with 20 ng of RNA.

[c] Viral titer in the media harvested from the monolayers at 72 hr post RNA transfection. Titers were determined by plaque assay on SW-13 cells.

[d] Relative plaque size at 5 days posttransfection. L, large; M, medium; S, small; T, tiny.

infectivities and produced viable virus after RNA transfection of SW-13 monolayers. These results show that NS1 glycosylation is not required for the initiation of YF RNA replication. Virus yields and plaque sizes, however, differed considerably for some of the mutants. The SB mutant containing the eight silent nucleotide substitutions was phenotypically identical to the parental virus (Table 2). G2a and G2b produced similar virus yields compared to the parent but exhibited slightly smaller plaques. In contrast, mutants lacking either the first site (G1a and G1b) or both glycosylation sites (G1aG2a and G1bG2b) produced depressed virus yields (20- to 100-fold less) and small plaques compared to the parent.

Growth properties

The NS1 mutants and parental virus were compared for growth rate and yield after high multiplicity infection of SW-13 cells (Fig. 2). Similar to the parental virus, all of the mutants produced the highest yields between 24 and 48 hr p.i. However, the mutants lacking both glycosylation sites produced reduced yields (10- to 20-fold) during the first 24 hr. Cytopathic effects (CPE), which became pronounced between 24 and 48 hr, were similar in infections with the parental virus, SB, G2a, and G2b. Interestingly, in cells infected with mutants lacking the first or both glycans (G1a, G1b, G1aG2a, and G1bG2b), relatively high virus yields were still being produced between 48 and 72 hr p.i. (Fig. 2) and significant CPE was observed only after 72 hr. Similar results were obtained after infection at lower multiplicities (0.5 PFU/cell) except that the yields of the G1 and G1G2 mutants were more dramatically reduced compared to the parent and other mutants (~100-fold), particularly at early time points (data not shown).

Analysis of proteins in infected cells

To characterize the virus-specific proteins produced by these mutants, lysates of metabolically labeled mock- and virus-infected SW-13 cells were immunoprecipitated with a polyclonal YF-specific mouse antiserum (HIAF; Fig. 3). As predicted for mutations ablating one or both glycan acceptor sites, forms of NS1 with faster mobilities were observed for the single and the double mutants (~1 and ~2 kDa increase in mobility, respectively). The latter form of NS1 comigrated with the NS1 produced in YF-infected cells labeled in the presence of tunicamycin. For the G1 mutants, NS1 migrated slightly faster than the form produced by the G2 mutants (see below). As expected, silent mutations in the NS1 coding region of SB did not alter the apparent MW of NS1 by SDS–PAGE. The profiles of the other viral proteins were similar, although a slight decrease in virus-specific protein synthesis was observed for G1a, G1b, G1aG2a, and G1bG2b (Fig. 3).

The nature of the N-linked oligosaccharides at the G1 and G2 acceptor sites was determined by endoglycosidase digestion (Fig. 4). Cell-associated and secreted

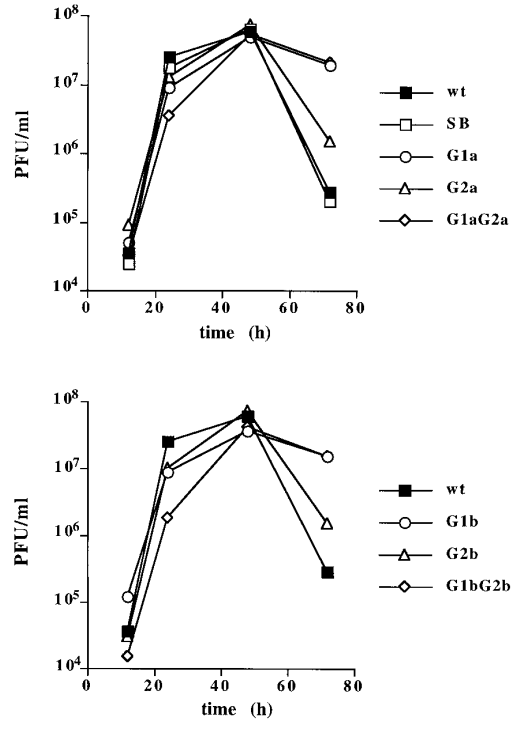

FIG. 2. Single-step growth analyses. Subconfluent monolayers of SW-13 cells were infected at a m.o.i. of 5 PFU/cell with either the wild-type virus (wt) or the indicated NS1 mutant viruses. At 12, 24, 48, and 72 hr p.i. the medium was collected and replaced with fresh medium. Titers of virus recovered from the media over these intervals were determined by plaque assay on SW-13 cells.

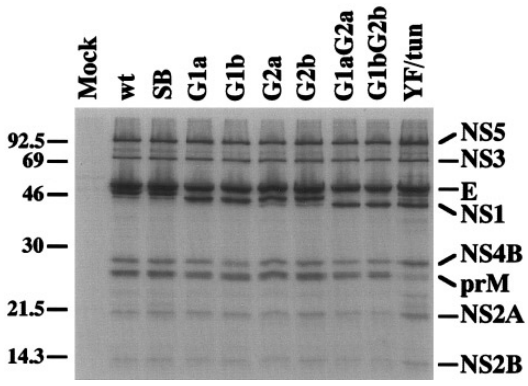

FIG. 3. Immunoprecipitation of metabolically labeled viral proteins. SW-13 cells were mock-infected or infected at a m.o.i. of 5 PFU/cell with either the wild-type virus (wt) or the indicated NS1 mutants. At 24 hr p.i., cells were labeled with [^{35}S]methionine for 6 hr, in the presence or absence of 5 μg/ml of tunicamycin (tun), and then disrupted with lysis buffer. Lysates were immunoprecipitated with YF-specific mouse HIAF and analyzed by SDS–13% PAGE as described under Materials and Methods. The positions of molecular weight markers are indicated on the left (in kDa) and YF-specific proteins on the right.

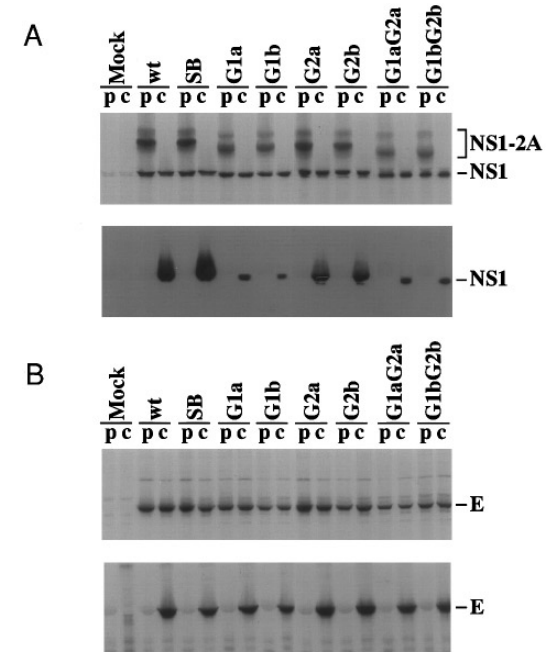

FIG. 5. Pulse–chase analysis of YF glycoprotein processing and secretion. Duplicate SW-13 cell cultures were mock-infected or infected with either the wild type virus (wt) or the indicated NS1 mutants at a m.o.i. of 5 PFU/cell. At 23 hr p.i., cells were pulse-labeled with [^{35}S]-methionine for 1 hr. One set of samples was harvested immediately following the pulse (p). For the remaining cultures, the labeling medium was replaced with αMEM containing 10 times the normal amount of unlabeled methionine and chased for a period of 6 hr (c). (A) NS1 or (B) E were immunoprecipitated from cell extracts (top gel in each part) or cell culture supernatants (bottom gel in each part) and analyzed by SDS–10% PAGE as described under Materials and Methods. A mixture of monoclonal antibodies 8G4 and 1A5 was used to immunoprecipitate NS1; monoclonal 5H3 was used to immunoprecipitate E.

forms of NS1 were immunoprecipitated and treated with Endo H, which removes high-mannose N-linked glycans, or PNGase F, which cleaves both high mannose and complex chains. NS1 from cell lysates was sensitive to digestion with either endoglycosidase (Fig. 4A), demonstrating that cell-associated NS1 possesses predominantly high-mannose oligosaccharide chains, as noted previously (Després *et al.,* 1991; Jacobs *et al.,* 1992; Mason, 1989; Post *et al.,* 1991; Pryor and Wright, 1994; Winkler *et al.,* 1988). NS1 secreted by the G1 mutants was completely Endo H-sensitive, whereas that secreted by the G2 mutants was Endo H-resistant (Fig. 4B). This indicates that glycans linked to Asn-130 (G1) are of the complex type and those linked to Asn-208 (G2) are simple high-mannose chains. These results are consistent with the partial Endo H sensitivity observed for NS1 secreted

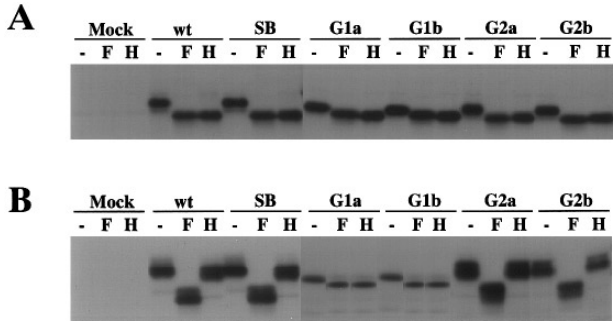

FIG. 4. Endoglycosidase analyses of NS1. SW-13 cells were infected and labeled as described for Fig. 3. Following labeling and treatment with Triton, (A) cell-associated and (B) secreted NS1 were immunoprecipitated using the 8G4 NS1-specific monoclonal antibody. Solubilized immunoprecipitates were treated with Endo H (H) or PNGase F (F) or were incubated without added endoglycosidase (–), and analyzed by SDS–10% PAGE.

by the parental virus and the SB mutant (Fig. 4B), and parallel previous findings for DEN2 NS1 (Pryor and Wright, 1994).

The importance of NS1 glycans for proper processing, stability, and secretion was examined in pulse–chase experiments (Fig. 5A). After a 1-hr pulse, similar levels of mature NS1, as well as higher molecular weight forms, were detected in cells infected with the parent and the mutant viruses (Fig. 5A, top). It has been previously suggested that these high molecular weight proteins represent polyprotein precursors consisting of NS1 and a portion of the NS2A protein (Chambers *et al.,* 1990b). After a 6-hr chase, the NS1-2A polyprotein had disappeared and only mature NS1 was detectable in cell lysates infected with the parent or any of the mutants (Fig. 5A, top). In the cell culture media, similar levels of NS1 had been secreted from cells infected with the parental virus, SB, G2a, and G2b (Fig. 5A, bottom). In contrast, removal of the first (G1a, G1b) or both (G1aG2a, G1bG2b) glycosylation sites dramatically reduced NS1 secretion. No major effect was observed on the appearance of E in the culture media (Fig. 5B), suggesting that impaired NS1

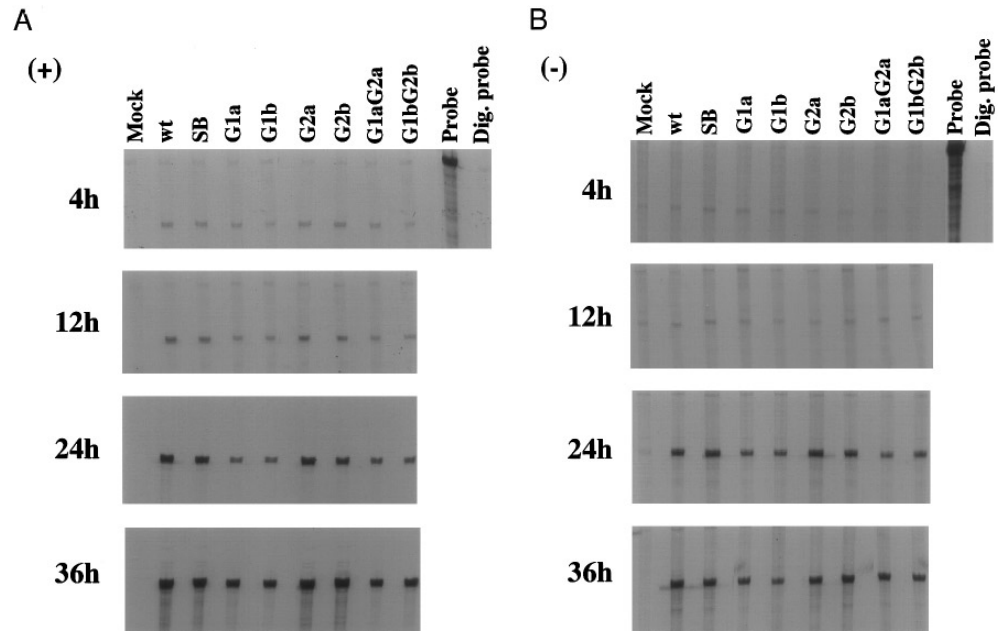

FIG. 6. Accumulation of YF-specific positive- and negative-strand RNAs. SW-13 cells were mock-infected or infected at a m.o.i. of 5 PFU/cell with the wild-type virus (wt) or the NS1 mutants. At 4, 12, 24, and 36 hr, total cytoplasmic RNAs were isolated and the levels of YF-specific RNAs determined using RNase protection as described under Material and Methods. (A and B) Positive- and negative-strand analyses, respectively. The autoradiogram shown in A was exposed for 1 hr, whereas that shown in B was exposed for 48 hr. The sizes of the probe and protected fragments are 284 and 251 nt, respectively. Similar results have been obtained in four independent experiments.

secretion may not influence the production of mature virions or subviral particles (see Discussion).

RNA accumulation in infected cells

The small plaque phenotype and slow growth of the G1 and G1G2 mutants in high m.o.i. infections indicated a possible early defect in replication perhaps at the level of viral RNA synthesis. To investigate this hypothesis, SW-13 cells were mock-infected or infected at a high m.o.i. (5 PFU/cell) with the wild type or the NS1 mutants. At 4, 12, 24, and 36 hr after infection, positive- and negative-strand RNA viral accumulation was analyzed by RNase protection (Fig. 6). Note that a background band present in the mock-infected sample in the negative-strand analysis does not allow a clear assessment of the levels of virus-specific negative-strand RNA accumulated at the early time points (4 and 12 hr). SB, G2a, and G2b exhibited levels of positive- and negative-strand RNA accumulation similar to the wild-type virus (Figs. 6A and 6B). During infections with G1a, G1b, and the G1G2 double mutants, positive- and negative-strand RNA accumulation was diminished relative to the parent up to 24 hr, but approached wild-type levels by 36 hr. These experiments were also performed at low m.o.i. (0.5 PFU/cell) with similar results except that the reduction in the levels of positive- and negative-strand RNA accumulation were more pronounced (approx fivefold lower for the G1 and G1G2 mutants; data not shown). The results of these RNA analyses together with those obtained for the high m.o.i. growth experiments indicate that the lack of a glycan at the first NS1 glycosylation site is associated with a reduced rate of RNA accumulation and a delay in production of infectious virus.

Mouse neurovirulence

A subset of the NS1 mutants was examined in a mouse neurovirulence assay. Given that no differences were observed in the cell culture phenotypes of the a and b series mutants, animal experiments were performed using only G1b, G2b, G1bG2b, and the parent. Table 3 summarizes the mortality endpoints for the parental virus

TABLE 3

Mouse Neurovirulence of NS1 Mutants[a]

Virus	Mouse survival (survived/inoculated)	*P* value[b]
Wild type	3/12	—
G1b	10/12	<0.005
G2b	4/13	NS
G1bG2b	13/13	<0.002

[a] Four-week-old female ICR mice were inoculated intracerebrally with 5000 PFU of wild-type virus or each of the NS1 mutants. Mice were observed daily until found dead or sacrificed when found in advanced moribund condition.

[b] *P* value refers to the significance of the proportion of mice surviving in each group compared with the wild-type group. Statistical significance was evaluated by χ^2 analysis. NS, not significant.

and the three NS1 glycosylation mutants. All mice which died during the experiment exhibited typical signs of encephalitis. Surviving mice were observed for a total of 6 weeks. The parent caused 75% mortality, with the mean time to death of approximately 10.5 days. Among the survivors, two-thirds exhibited signs of illness (ruffling or hindlimb weakness) during the observation period. Mortality in the G2b group (9/13 mice) and mean time to death (11.7 days) were similar to those of the parental virus. All survivors in the G2b group exhibited moderate signs of illness during the observation period. Sixteen percent mortality was observed for the G1b group, with two deaths occurring only after 16 days, and most survivors exhibiting only mild signs of illness. No deaths occurred among the mice infected with the G1bG2b mutant, and minimal signs of disease were observed in this group. Pairwise comparisons of the proportions of mice surviving infection with each mutant relative to the parental virus were made using χ^2 analysis. A statistically significant difference was not observed between the parent and the G2b mutant ($P > 0.1$). Significant differences were observed, however, between the parent and G1b ($P < 0.005$) or G1bG2b ($P < 0.002$).

To verify that infection had indeed occurred, antibody responses were determined where possible for most of the mice in each group (9/12 for the parental virus, 6/13 for the G2b group, 10/12 for G1b, and 12/13 for G1bG2b). Titers among the groups were as follows (reciprocal of the 50% plaque reduction titer): parental virus, range $<$40 to 1280, median of 320; G2b, range 640 to $>$2560, median of 1280; G1b, range $<$40 to $>$1280, median of 640; G1bG2b, range $<$40 to 2560, median of 320 (data not shown).

DISCUSSION

This mutagenesis study has provided new insight into the function of the NS1 glycoprotein in flavivirus replication and pathogenesis. YF 17D mutants lacking the second NS1 glycosylation site or bearing multiple silent nucleotide substitutions were phenotypically similar to the parental virus. In contrast, mutants lacking the first or both N-linked glycans produced small plaques, delayed CPE, depressed virus yields, impaired NS1 secretion, and deficient RNA accumulation. None of the mutants exhibited an obvious defect in either polyprotein processing or E protein secretion. In addition, mutants lacking the first glycosylation site were attenuated in a mouse neurovirulence model. These results suggest that proper glycosylation of NS1 is important for an early function which can influence the efficiency of viral RNA replication which may be related to the pathogenesis of the virus.

The possibility that these defects are mediated directly at the RNA level, via changes in the RNA structure or stability, seems unlikely since (i) the mutant and parental RNA transcripts exhibited similar specific infectivities and (ii) the SB mutant, containing a total of eight silent substitutions at the two N-linked sites, was phenotypically identical to the wild-type parent. Rather, the differences in RNA accumulation and virulence more likely result from altered function of the mutant NS1 proteins. Moreover, mutants in which different amino acid substitutions were used to disrupt the same glycosylation site(s) exhibited similar phenotypes. This suggests that the observed differences in RNA replication and neurovirulence result from the lack of N-linked glycans rather than substitution of alanine for asparagine or serine/threonine in the consensus sequence.

Given that NS1 is glycosylated and secreted, it was previously thought to have a role in virus assembly and/or release. Thus far, no compelling evidence exists to support this notion. The E protein, which is secreted as mature virus or in subviral particles, is readily released from infected mosquito cells which do not secrete NS1 (Mason, 1989). In addition, mutations which impair NS1 secretion do not affect release of E (this study). Rather, our results suggest that NS1 may participate in viral RNA replication. Flavivirus RNA amplification is believed to occur at sites in the perinuclear reticular network (Brinton, 1986; Westaway, 1987) where the putative replicase components (such as the NS3 RNA helicase and NS5 RNA-dependent RNA polymerase) are believed to be membrane-associated (Wengler *et al.,* 1990). Flavivirus infection results in marked hypertrophy of subcellular membrane organelles, producing vesicles which may actually represent sites of RNA replication (Brinton, 1986; Murphy, 1980; Westaway, 1987). How NS1 might participate in RNA replication is unclear. The lumenal rather than cytoplasmic localization of the NS1 glycoprotein makes it unlikely that the mature cleavage product functions as an enzymatic component. We have observed by immunofluorescence that NS1 is localized in the perinuclear region (data not shown), which suggests that it could be involved in the reorganization of cellular membranes to allow formation of active replication complexes. Alternatively, NS1 could function as a replicase component but perhaps as part of an uncleaved polyprotein, or indirectly through continued interaction with NS2A after cleavage at the NS1/2A site. Further studies are needed to understand the mechanism by which NS1 influences the efficiency of RNA replication.

Results of the mouse inoculation experiments indicate that the G1b and G1bG2b mutants are attenuated in neurovirulence when compared with the parental virus or G2b. The observed effects of these mutations on neurovirulence are unlikely to be caused by either differences in challenge inoculum, since equivalent doses of each virus were verified by plaque assay, or failure to initiate infection, since significant levels of neutralizing antibodies were generated in each group of mice. We believe that the differences in mortality associated with the G1b and G1bG2b YF mutants are likely to reflect true quantita-

tive differences in mouse neurovirulence (PFU/LD_{50}); however, this remains to be established. Since the mutations abolishing the first or both glycosylation sites impair virus growth in cell culture and are associated with the most dramatic reductions in mortality, a principal mechanism for the observed attenuation may be reduced replication efficiency, which allows time for development of an effective immune response in the CNS. Another possibility is that the reduced capacity of the G1b and G1bG2b mutants to induce CPE in cell culture may also diminish the capacity of these mutants to cause lethal encephalitis. Our results are similar to those obtained using TBE/DEN4 chimeras, where ablation of the first NS1 glycosylation site significantly attenuated growth in cell culture and mouse neurovirulence, whereas elimination of the second glycosylation site led to a small but measurable increase in virulence (Pletnev *et al.,* 1993).

NS1 has been shown to elicit an immune response during flavivirus infections in experimental animals and antibodies specific for this protein are protective in models of yellow fever encephalitis or hemorrhagic fever (Schlesinger *et al.,* 1986, 1985, 1993). Since immunogenicity depends on structural properties of the NS1 protein, which can oligomerize and exist as cell-associated, cell-surface, or extracellular forms (Mason, 1989; Winkler *et al.,* 1989, 1988), mutations at the conserved glycosylation sites which alter NS1 localization or impair secretion may influence the host immune response to this protein. This could affect phenomena such as antibody-mediated cytotoxicity directed against cell-surface NS1 (Schlesinger *et al.,* 1990, 1993) which might be a factor in determining the extent of cell death within the central nervous system and the progression of encephalitis caused by neurotropic flaviviruses.

The results of this study may be relevant to flavivirus vaccine development. The observation that elimination of the first glycosylation site of NS1 leads to a significant reduction in mouse neurovirulence for both YF 17D (this paper) and the TBE/DEN4 chimera (Pletnev *et al.,* 1993) suggests that targeting mutations to this protein may be an effective approach for attenuating flavivirus virulence. Although the YF 17D human vaccine strain is relatively safe and highly efficacious, postvaccinal cases of encephalitis have occasionally been reported (e.g., Fox *et al.,* 1942) and the use of the vaccine in children of less than 3 years of age is not recommended. Further investigation of the functions of NS1 during viral replication and the effects of engineered mutations on virulence in animal models may lead to improved vaccines against yellow fever and other flaviviruses using cDNA-based technology (Marchevsky *et al.,* 1995; Rice, 1990).

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