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Both E Protein Glycans Adversely Affect Dengue Virus Infectivity but Are Beneficial for Virion Release▽

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The E protein of most flaviviruses is modified by Asn-linked glycosylation at residue 153/154 and in the case of the four dengue virus (DENV) serotypes by a second glycan at residue 67. However, the absence of E protein glycosylation among numerous natural isolates of different flaviviruses suggests that the glycan, *per se*, is not critically important in the virus life cycle. Consistent with this notion, we show that ablation of both glycans from the DENV-2 E protein reduces but does not prevent growth of the variant in mammalian and mosquito cells. We found a pronounced and opposing effect of glycan ablation on two stages of the virus growth cycle: infectivity and release. Loss of either of the two DENV E protein glycans markedly enhanced infectivity of variants for mosquito cells at the expense of efficient virion release. The variants also displayed reduced release in mammalian cells, which was more prominent for viruses lacking the Asn 67-linked glycan than for those lacking the Asn 153-linked glycan, without a marked change in infectivity. Mutations, which compensated for the defect in virus morphogenesis associated with ablation of the Asn 67-linked glycan in mammalian cells but interestingly not in mosquito cells, were identified at the glycosylation acceptor motif and a second site in E protein domain II. The dueling influences of infectivity and release on virus growth affected by the glycans may explain the plasticity in E protein glycosylation among the flaviviruses.

The mature flavivirus particle is composed of an ~11-kb plus-strand RNA genome packaged by the viral capsid (C) protein into a host-derived lipid bilayer and surrounded by 180 copies of two integral membrane proteins, membrane (M) (8 kDa) and envelope (E) (54 kDa). The E protein covers most of the virion surface and contains receptor-binding sites, the fusion peptide, and the majority of epitopes recognized by antibodies against the virus particle. Cryo-electron microscopy (Cryo-EM) of flavivirus particles shows an icosahedral organization consisting of 30 rafts, each composed of 3 E protein dimers with an antiparallel orientation and lying flat on the virus surface (18). The E protein has a 3-domain structure: a central domain (DI) flanked on one side by an elongated dimerization domain (DII) and on the other by an immunoglobulin-like domain (DIII), which makes the highest protrusion from the otherwise smooth particle surface (33, 38). The E protein of most flaviviruses is N-link glycosylated at residue 153/154, although the absence of the carbohydrate in some flaviviruses or natural flavivirus isolates (1, 2, 4, 32, 37, 44) shows that optimal stability and function can be achieved without glycan attachment. Furthermore, loss of the Asn 153-linked glycan observed during mosquito cell adaptation of dengue virus (DENV) suggests that its absence is associated with a selective growth advantage in some cell types, which may be related to an altered fusion phenotype (15, 23). The glycan at Asn 153 on DI extends laterally across the interface of the metastable E protein dimer and covers the fusion peptide at the distal end of DII on the dimer partner (33, 34, 38). This stabilization of the dimer by the oligosaccharide is consistent with the property of loss-of-glycan mutants, which fuse with target cell membranes at higher pH and display lower stability at mildly acidic pH (3, 11, 23). Several reports on West Nile virus (WNV) suggest that the Asn 154-linked glycan is a virulence determinant (3, 26, 40, 42). This contrasts with an increase in neurovirulence of DENV-4 when the Asn 153-linked glycan is ablated (16).

DENV differs from all other flaviviruses in that its E protein contains two N-linked glycans, one at Asn 153 and a second at Asn 67 in E protein DII, which is uniquely present in all strains of the 4 DENV serotypes. One of the physiological functions of the Asn 67-linked glycan may be its involvement in virus attachment to the C-type lectin DC-SIGN, expressed on dendritic cells and a subset of macrophages (34, 35), thus conferring virus tropism to monocyte/macrophage-derived cells thought to play a role in DENV pathogenesis (45). Cryo-EM of DENV binding to DC-SIGN shows that attachment is solely through the interaction of the Asn 67-linked glycan with the carbohydrate recognition domain of DC-SIGN, and it is proposed to exclude involvement of the second E protein glycan (36). Further on the role of the Asn 67-linked glycan as a potential DENV tropism determinant, two recent reports describe an essential function of the carbohydrate in virus growth in mammalian cells (5, 35). They show that ablation of the glycan abolishes growth of DENV-2 in mammalian cells and reduces growth in mosquito cell culture. While infection and viral macromolecular synthesis occurred in mammalian cells, virus release was severely defective in the absence of E protein glycosylation at Asn 67 (35). This loss-of-glycan-mediated defect in virus release was only marginally apparent

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following ablation of the Asn 153-linked glycan and was species or cell type dependent, since in mosquito cells secretion of the DENV-2 E protein lacking either the Asn 67- or Asn 153-linked glycan was comparable to that of wild-type (wt) E protein, albeit recombinant expression vectors rather than viral infections were employed (35). The importance of the carbohydrate on the E protein and/or its dimerization partner, prM, in efficient morphogenesis/release of virus or subviral particles has also been described for other flaviviruses (8, 12, 17) and is consistent with the proposition that glycan interaction with endoplasmic reticulum (ER) chaperones facilitates these stages of the virus life cycle.

The studies on flavivirus E protein glycan ablation did not critically address whether removal of the carbohydrate, *per se*, or associated E protein structural changes accounted for the observed growth phenotypes. The latter scenario is supported by the existence of natural flavivirus isolates devoid of E protein glycosylation. Therefore, we hypothesized that the reported deleterious effects on growth of DENV associated with glycan ablation can be complemented by compensatory mutations, rendering glycan addition redundant for growth. We describe here the production of DENV-2 variants lacking either one or both E protein glycans that are viable and stable in mosquito vector and mammalian host-derived cell lines and report on the opposing influences of the E protein glycans on virus infectivity and release.

MATERIALS AND METHODS

Cells. African green monkey kidney (Vero) and baby hamster kidney (BHK-21) cells were maintained at 37°C in medium M199 (Gibco) plus 5% fetal calf serum (FCS) and Glasgow's minimum essential medium (Gibco) supplemented with tryptose phosphate broth (Sigma-Aldrich) and 5% fetal calf serum, respectively. *Aedes albopictus* (C6/36) mosquito cells were grown at 28°C in minimum essential medium supplemented with nonessential amino acids (Gibco) and 10% fetal calf serum.

Plasmids. Construction of a chimeric plasmid comprising the prM-E coding regions of DENV-2 strain PUO-218 in DENV-2 NGC full-length infectious cDNA (pDVWS601) (10) has been described (24). A chimeric plasmid encoding the prM-E coding regions of DENV-2 strain 16681 (GenBank accession number u87411) in the NGC full-length cDNA clone was constructed using a 2.4-kb synthetic cDNA fragment (GenScript) flanked by the restrictions sites Bsp1407 (nucleotide [nt] 171) and NheI (nt 2544). To introduce amino acid substitutions into the E protein, a fusion PCR approach was employed (20). Plasmid pACprME5′ (24), which encodes prM and the NH_2-terminal quarter of E, was used for introduction of changes at the Asn 67-linked glycosylation acceptor site, whereas plasmid pGEME3′, encoding the remaining COOH-terminal E protein sequence (24), was used for introducing changes at the Asn 153-linked glycosylation acceptor site. Full-length DENV-2 plasmids were generated by ligating three DNA fragments from a pACprME5′ plasmid derivative, a pGEME3′ plasmid derivative, and the pDVWS601 plasmid, respectively, as described previously (24). To produce loss-of-glycan variant E proteins of strain 16681, the cloned synthetic cDNA fragment was used as template DNA for mutagenesis. All full-length plasmid constructs were verified by sequencing the entire prM and E protein genes (24).

A full-length Murray Valley encephalitis virus (MVEV) cDNA clone (21) was used for the introduction of a T155A change to abolish N-linked glycosylation in the E protein. The full-length West Nile virus (WNV) (strain Kunjin, MRM61C) cDNA clone, FLSDXpro HDVr (29) contains no N-linked glycosylation site in the E protein. A laboratory-derived glycosylated variant of Kunjin virus, strain MRM 61C, was used as a source of cDNA for introduction of an F156S change (CTT to CTC) into the WNV (KUN) full-length cDNA clone by substitution of an ~1.2-kb cDNA fragment flanked by MfeI (nt 269) and SacII (nt 1478) restriction endonuclease sites.

RNA transcription and electroporation of BHK-21 cells. Plasmids containing the full-length DENV-2 cDNAs were digested with XbaI and transcribed using T7 RNA polymerase, and full-length RNA transcripts were electroporated into BHK-21 cells as described previously (21), with the following modifications. For determination of electroporation efficiency, 200 μl, 20 μl, or 2 μl of electroporated cells (25 ml at 4×10^5 cells/ml) were plated on Vero cell monolayers in 6-well trays (3.5×10^5 cells/well; Nunc) and incubated for 3 h, and agar overlay medium comprising M199 medium, 1% agar (BD Biosciences), 0.02% DEAE-Dextran (Sigma), and 2% FCS was added. Neutral red (0.02% in Hanks' balanced salts solution; 1 ml) was added at 5 to 7 days postinfection (p.i.), and plaques were counted after overnight incubation. Plaques were picked after 7 days and amplified on C6/36 cell monolayers (2×10^5 cells in 35-mm tissue culture dishes; Nunc) for generation of working stocks. For determination of intracellular DENV-2 RNA levels, BHK-21 cells (1×10^7) were washed twice after electroporation by centrifugation ($800 \times g$ for 5 min), removal of supernatant, and resuspension in growth medium (10 ml). Live cells were counted and seeded in 35-mm tissue culture dishes (1×10^6 cells/dish); total cellular RNA and supernatants were collected at indicated times postelectroporation for analysis of DENV-2 RNA content by quantitative reverse transcription-PCR (qRT-PCR) (see below).

Infectivity determinations by flow cytometry. Infectivity of virus stocks was measured using a fluorescence-activated cell sorter (FACS)-based assay, as described previously (22). Briefly, Vero cells were fixed in ice-cold 75% ethanol for 0.5 h and stained with NS1 protein-specific monoclonal antibody (MAb), 4G4 (6), followed with fluorescein isothiocyanate (FITC)-conjugated anti-mouse Ig; C6/36 cells were fixed in 2% paraformaldehyde-phosphate-buffered saline (PBS) for 0.5 h on ice, permeabilized in 0.5% saponin-PBS for 10 min at room temperature, and stained as described above. Dose response in the FACS assay was examined using 3-fold serially diluted virus samples for infection: linearity was observed between 3% and 40% infection rates, while at higher virus doses the infection rates achieved underestimated virus infectivity.

Specific infectivity determinations. Virus released from infected C6/36 cells (multiplicity of infection [MOI] of ~0.1) was collected between 70 and 72 h p.i. in fresh growth medium, which was added to the infected monolayers following two washes with medium, and cellular debris was removed by centrifugation ($8{,}000 \times g$, 5 min). Infectivity for Vero cells was determined by plaque assay; 50% tissue culture infective dose ($TCID_{50}$) assays using C6/36 cells were performed by infecting monolayers in 96-well trays (1×10^4 cells/well) using serially diluted virus (8 wells per dilution). Cells were incubated for 5 days and fixed in acetone-methanol (1:1 [vol/vol]), and enzyme-linked immunosorbent assay (ELISAs) were performed using an NS1 protein-specific MAb, 4G4 (6), for detection of virus-infected cells, as described previously (27). Virion RNA content in 2-h virus release stocks was determined by real-time RT-PCR following treatment of virus stocks with RNase (see below).

Real-time RT/PCR. Infected culture supernatants (100 μl) were treated with RNase (20 μg/ml, 30 min at 37°C), and virion RNA was extracted using Trizol (Invitrogen) for quantitation of viral genome copy numbers by qRT-PCR (20, 24). A plus-strand DENV-2 RNA standard was generated from XbaI-linearized pDVWS601 infectious clone DNA, using T7 RNA polymerase and a trace amount of [^{3}H]UTP for determination of the yield of RNA transcript (39). The RNA standard was treated with DNase I (Roche), purified, and resuspended in water as described previously (20). Serial dilutions of the *in vitro*-transcribed DENV-2 RNA were performed to yield standards of 10^6, 10^5, 10^4, and 10^3 RNA copies/μl. Reverse transcription was performed at 43°C for 90 min in a 10-μl mixture containing 2 μl sample RNA, Expand reverse transcriptase (Roche), RNase inhibitor (Invitrogen), 10 mM deoxynucleoside triphosphate (dNTP), 10 pmol downstream primer (5′-AGC GTC TCT CCT ATG TTG CCA GTT −3′), 10 mM dithiothreitol and the manufacturer's recommended buffer conditions. Real-time PCR was performed using IQSybr qPCR mixtures (Bio-Rad) and 0.2 nM downstream and upstream primers (5′-GGG AAG GAA ATC CAG GGA GGT T −3′) under cycling conditions of 95°C for 3 min for 1 cycle and 95°C for 30 s, 63°C for 30 s, and 72° for 60 s using 40 cycles. Each RNA sample was tested in duplicate, and virion RNA content was determined by extrapolation from the standard curve generated within each experiment.

Quantitation of DENV minus-strand RNA. For quantitation of minus-strand RNA, mixtures (5 μl) comprising RNA sample (2 μl; extracted as described above) and the upstream tagged DENV-2 primer (10 pmol; 5′-CTC ATG GTG GCG AAT CAG TTC TAA GGA AAT CCA GGG AGG TT-3′) were denatured at 80°C for 5 min and immediately placed on ice for 10 min. Enzyme mix (5 μl) comprising Expand reverse transcriptase (Roche), 10 mM dNTP, and RNase inhibitor (Invitrogen) was added to each tube and incubated at 50°C for 1 h. The samples were diluted by the addition of 30 μl PCR-grade water (Sigma-Aldrich) and then inactivated at 90°C for 10 min prior to qRT-PCR using the DENV-2 downstream primer (0.2 nM) and the tag primer (0.2 nM; 5′-CTC ATG GTG GCG AAT CAG TTC T-3′) and conditions as described above. The minus-strand DENV-2 RNA standard was generated from a plasmid comprising pGEM-5Zf+ and a 3.9-kb fragment flanked by NsiI and AatII sites from the

TABLE 1. Biological properties of recombinant DENV-2 with PUO-218 or NGC strain structural proteins lacking the Asn 67-linked, Asn 153-linked, or both E protein glycans

Virus	Electroporation efficiency (PFU/μg RNA)	Plaque size (mm)	Specific infectivity	
			Vero cells (genomes/PFU)	C6/36 cells (genomes/$TCID_{50}$)
Expt 1				
rPUO (parent)	1.5×10^5	3	885 ± 215	4,900 ± 1,500
rPUO.N67D	1.1×10^5	2	1,250 ± 250	615 ± 295
rPUO.N67Q	1.5×10^5	2.5	1,600 ± 500	490 ± 230
rPUO.T69A	1.1×10^5	1.5	1,800 ± 1200	440 ± 160
rPUO.T69L	1.5×10^5	3	2,150 ± 450	615 ± 235
rPUO.T69V	2.5×10^5	3	NT[a]	NT
rPUO.T69V/N124S/T155A	1.3×10^5	0.5	1,850 ± 150	190 ± 60
Expt 2				
rNGC (parent)	5.6×10^4	2	NT	NT
rNGC.T69A	2.0×10^2	1–3	NT	NT
rNGC.T155A	3.6×10^5	1	NT	NT
rPUO (parent)	9.5×10^4	4	1,500 ± 230	4,900 ± 140
rPUO.T155A	3.3×10^5	1.5	3,200 ± 950	48 ± 4.9

[a] NT, not tested.

DENV-2 NGC strain (nucleotides 4696 to 8571), using T7 RNA polymerase and [^{3}H]UTP as described above, and serially diluted to 10^6, 10^5, 10^4, and 10^3 RNA copies/μl to set up a standard curve in each experiment. Negative controls included a "no RNA" control, "no cDNA" control, and a corresponding plus-strand RNA (10^7 copies/μl); this last control was always below the detection limit (10^3 minus-strand RNA copies) for the assay.

Metabolic labeling, immunoprecipitation, and SDS-PAGE. Metabolic labeling of proteins was performed by starving BHK-21 or Vero cells (infected and uninfected control) for 0.5 h by replacement of the growth medium with 1.0 ml of methionine- and cysteine-free minimal essential medium (MEM) (Gibco). After removal of medium, 0.5 ml of methionine- and cysteine-free MEM containing 100 μCi of Tran^{35}S-label (ICN)/ml was added to the cell monolayers, followed by incubation for 2 h. For chases, the monolayers were washed with PBS, and MEM containing 5% FCS was added for 1 h. Lysis, immunoprecipitation using monoclonal antibody (MAb) 4G2 (kindly provided by J. Aaskov, Queensland University of Technology, Australia), SDS-polyacrylamide gel electrophoresis (PAGE), and fluorography were as described previously (43), with the modification in some experiments of addition of a mammalian protease inhibitor cocktail (Biosciences) to the lysis buffer to prevent partial E protein degradation, reflected in the appearance of a dimer band in SDS-PAGE profiles. For endoglycosidase digestion, the immunoprecipitated samples were incubated in 17 μl of a buffer containing PNGaseF (1 U; Roche) in 50 mM sodium phosphate (pH 7.0), 0.5% NP-40 (Sigma-Aldrich), and 10 mM EDTA for 3 h at 37°C prior to gel loading.

RESULTS

Recovery from mammalian cells of DENV-2 variants lacking the E protein Asn 67-linked glycan. Glycosylation at Asn 67 in the E protein of the four serotypes of DENV is unique among the flaviviruses. Two recent studies using the DENV-2 16681 strain showed that ablation of Asn 67-linked glycosylation prevented virus growth in mammalian cells due to a defect in virus morphogenesis/release (5, 35). However, these investigations did not establish whether loss of glycosylation *per se* or a structural alteration to the E protein specific to the introduced amino acid substitution (N67Q) accounted for the growth-defective phenotype. We addressed this issue by introducing different residues varying in size and chemical properties at position 67 (N67D; N67Q) or 69 (T69A, T69L, and T69V) in the E protein of DENV-2 strain PUO-218 in order to prevent glycan addition. To generate these variants, the chimeric NGC/PUO-218 prM-E derivative (24) of the full-length infectious cDNA clone of the DENV-2 NGC strain (10) was used. Introduction of all infectious clone-derived wt and mutant RNAs into BHK cells by electroporation yielded viable virus. The loss-of-Asn 67-linked glycan variants did not markedly differ from the results for the parent NGC/PUO-218 chimera (rPUO) in electroporation efficiency measured by infectious center assays of transfected BHK cells on Vero cell monolayers (Table 1). However, plaque size differences were noted, with rPUO.T69V and rPUO.T69L producing wt-size plaques (~3 mm), while the rPUO.N67Q, rPUO.N67D, and rPUO.N69A variants gave rise to smaller plaques (2.5, 2.0, and 1.5 mm, respectively) (Table 1).

To confirm loss of the Asn 67-linked glycan from the E protein of the variants, electroporated BHK-21 or infected Vero cells were metabolically labeled, and the E proteins were immunoprecipitated from cell lysates and analyzed by SDS-PAGE with or without prior treatment with the glycanase PNGase F. Consistent with ablation of Asn 67-linked glycosylation, the variant E proteins showed faster mobility during electrophoresis than the fully glycosylated rPUO control. Moreover, PNGase F treatment resulted in identical electrophoretic mobility between rPUO and variant E proteins (Fig. 1 and data not shown).

Sequence analysis was performed on plaque-purified virus stocks of rPUO.N67D, rPUO.N67Q, rPUO.T69A, rPUO.T69L, and rPUO.T69V. The genotypes of each of the variants, except that of rPUO.T69A, were stable and showed the introduced mutations in the absence of additional changes in the prM-E protein genes. Only propagation of rPUO.T69A on Vero cell

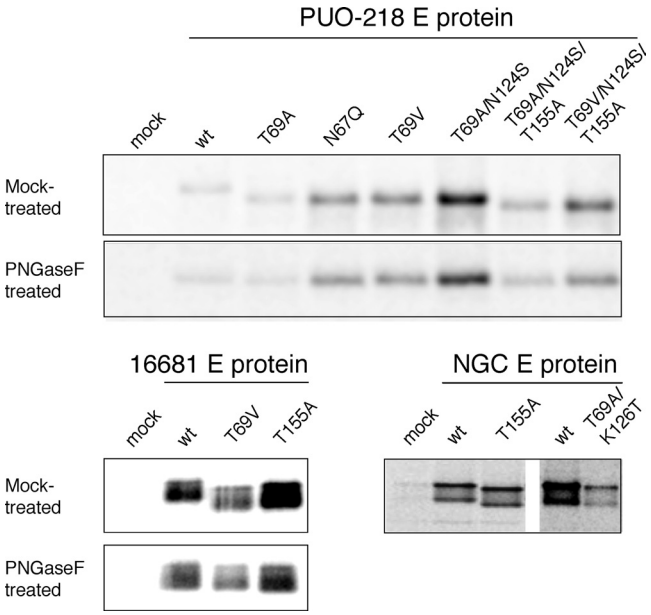

FIG. 1. E protein glycosylation status for DENV-2 wt and loss-of-glycan variants in infected cell lysates. Infected Vero cells and an uninfected control were metabolically labeled from 21 to 24 h p.i., and the E protein was immunoprecipitated from cell lysates using MAb 4G2, followed by treatment with or without PNGaseF and electrophoresis on a 15% SDS-polyacrylamide gel. The DENV-2 E protein in cell lysates is sensitive to partial degradation by proteases, which can give rise to an E protein dimer band in SDS-PAGE profiles.

TABLE 2. Pseudoreversions and suppressor mutations selected in the E protein of 2 strains of DENV-2 lacking the Asn 67-linked glycan

Variant (bold) or mutant derived therefrom	Growth in mammalian cells	Nucleotide change[a]	Amino acid change[b]
rPUO.T69A	Yes	$A_{1141} \rightarrow G$	$Thr_{69} \rightarrow Ala$
rPUO.T69A/N124S	Yes	$A_{1141} \rightarrow G$	$Thr_{69} \rightarrow Ala$
		$A_{1307} \rightarrow G$	$Asn_{124} \rightarrow Ser$
rNGC.T69A	No	$A_{1141} \rightarrow G$	$Thr_{69} \rightarrow Ala$
rNGC.T69V	Yes	$A_{1141} \rightarrow G$	$Thr_{69} \rightarrow Val$
		$C_{1142} \rightarrow T$	silent
rNGC.T69A/K126T	Yes	$A_{1141} \rightarrow G$	$Thr_{69} \rightarrow Ala$
		$A_{1313} \rightarrow C$	$Lys_{126} \rightarrow Thr$

[a] Numbering from the 5′-terminal nucleotide in the DENV-2 NGC genome (9).
[b] Numbering from the N-terminal residue in the E protein.

monolayers gave rise to plaque size heterogeneity. Sequencing of a large plaque confirmed the presence of the T69A mutation originally introduced but uncovered the additional N124S mutation in DII of the E protein (Table 2). The smaller plaques obtained for rPUO.T69A showed the expected sequence without additional changes in the prM and E protein genes.

Growth and replication in BHK cells of DENV-2 variants lacking the Asn 67-linked glycan. The growth kinetics in BHK cells of the loss-of-Asn 67-linked glycan variants was determined by quantitation of viral genome copies released into the extracellular fluid. To guard against potential differences in specific infectivity between the wt and variants, infection of BHK cells was by *in vitro*-synthesized genomic RNA transfection. This resulted in infection of a similar number of cells (9 to 15%) for each of the viruses, demonstrated by NS1 protein-specific staining and flow-cytometric analysis at 20 h after electroporation (Fig. 2A). Growth of the variants rPUO.T69L and rPUO.T69V was comparable to that of the wt in terms of plateau virus titers reached at 3 and 4 days after transfection, although the growth kinetics was marginally slower, with ~5-fold-lower virus titers on days 1 and 2 p.i. (Fig. 2B). In contrast, the growth of 2 variants, rPUO.T67D and rPUO.T69A, was significantly poorer and virus titers produced were 50- and 100-fold reduced, respectively, relative to the parent. The variant rPUO.N67Q demonstrated an intermediate growth phenotype, which was reduced by ~5-fold relative to that of the wt virus (Fig. 2B).

The second site mutation, N124S, was identified in the E

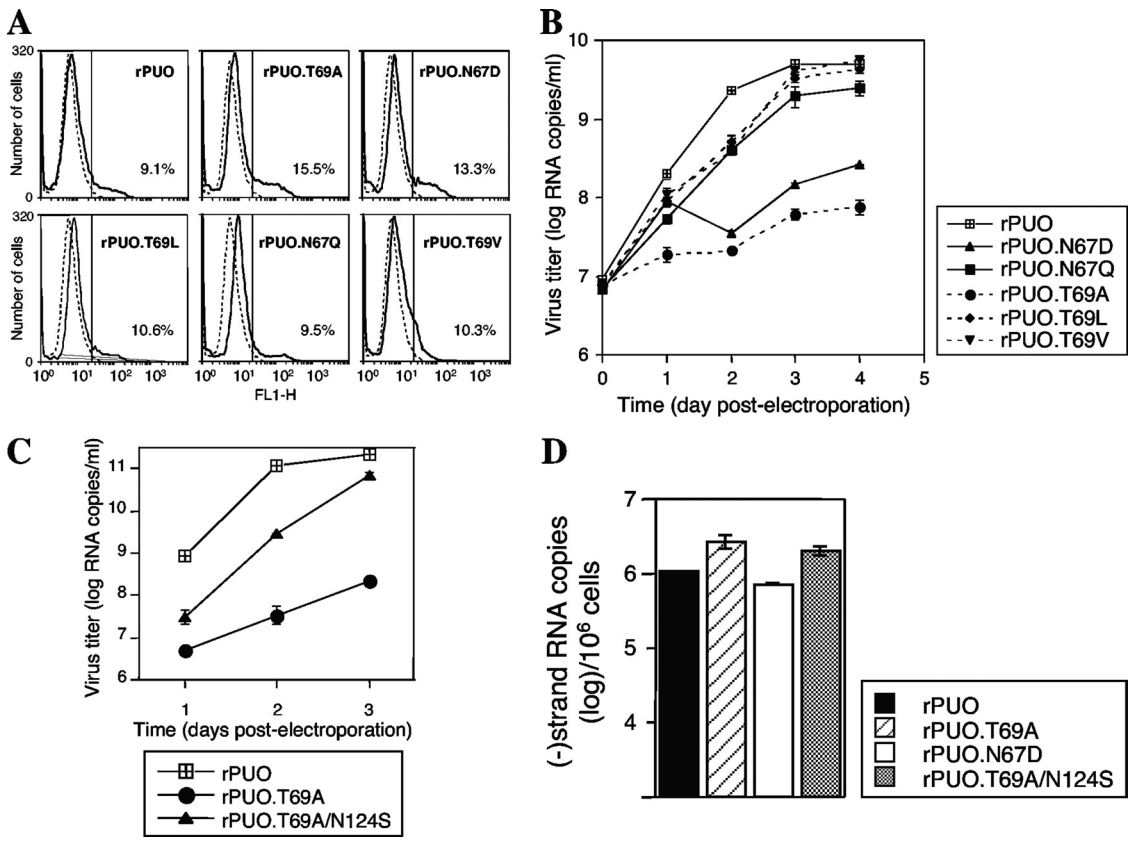

FIG. 2. Growth properties of loss-of-glycan variants relative to rPUO parent virus in mammalian cells. (A) FACS histograms showing BHK-21 cells electroporated with 1 μg wt or variant RNA transcripts (solid lines) or left untreated (dotted line) and stained with anti-NS1 MAb 4G4 at 20 h after electroporation, as described previously (22). The percentage of NS1-expressing cells is shown in the panels. (B and C) Virion-associated RNA release from BHK cells electroporated with PUO parent or loss-of-glycan variant RNA transcripts is shown. Supernatant samples were collected at the indicated time points, and virion-associated DENV-2 RNA was measured by quantitative real-time RT-PCR. (D) DENV-2-specific minus-strand RNA in BHK-21 cells electroporated with rPUO or representative loss-of-glycan variant RNA transcripts is shown. Total RNA was extracted at 18 h postelectroporation, and minus-strand DENV-2 RNA was estimated by strand-specific real-time RT-PCR. Error bars indicate the standard error of the mean (SEM) for two real-time PCR determinations for each sample shown. Representative data from 2 experiments are shown.

protein of a large-plaque variant derived from rPUO.T69A (Table 2). To confirm that this additional change compensates for the deleterious impact of the T69A mutation on growth in mammalian cells, an infectious cDNA clone for the double mutant virus, rPUO.T69A/N124S, was produced, and virus growth was measured by quantitation of virion RNA released from BHK cells electroporated with genomic RNA, as described above. The presence of the N124S change in rPUO.T69A/N124S improved growth by ~100-fold relative to that of the variant rPUO.T69A, although it remained less efficient than that of wt virus (Fig. 2C). The N124S mutation did not introduce a second glycosylation motif at a novel site in the E protein and did not result in addition of a second E protein glycan, as expected (Fig. 1).

To test if differences in RNA replication efficiency contributed to the different growth phenotypes in BHK cells between the variant and parent viruses, minus-strand RNA synthesis was quantitated by strand-specific qRT-PCR. This analysis showed comparable levels of virus-specific minus-stand RNA between the parent and 3 glycosylation variants at 18 h post-electroporation (Fig. 2D).

Specific infectivity of loss-of-Asn 67-linked glycan variants for mammalian and mosquito cells. To assess the impact of ablation of Asn 67-linked glycosylation on the specific infectivity of the DENV-2 variants for mammalian and invertebrate cells, particle-to-infectivity ratios were determined using 2-h virus release stocks collected from infected C6/36 mosquito cells between 70 and 72 h p.i. RNA genome copy numbers in virus stocks were determined by qRT-PCR, while $TCID_{50}$ and plaque assays were performed on the virus stocks to assess infectivity for C6/36 and Vero cells, respectively. We note that direct comparison of specific infectivity values in the mammalian and mosquito cell lines may not be possible, since different assays were used to measure infectivity, although infectivity determinations by plaque assay and $TCID_{50}$ for parent DENV-2 strains in Vero cells differ by <2-fold (data not shown). A particle-to-Vero-cell PFU ratio of 885 was determined for the parent virus, rPUO, which was only slightly lower than the ratios calculated for the loss-of-Asn 67-linked glycan variants (1,250 to 2,150) (Table 1). Accordingly, the ablation of E protein glycosylation at Asn 67 reduced infectivity of Vero cells by at most 3-fold. In contrast, the particle-to-C6/36-cell $TCID_{50}$ ratio found for rPUO (4,900) was ~10-fold higher than those for the variants (440 to 615), suggesting that DENV-2 particles lacking the Asn 67-linked glycan display markedly greater infectivity for mosquito cells than wt virus.

Effect of ablation of the Asn 67-linked E protein glycan on virus growth in mosquito cells. Ablation of the Asn 67-linked E protein glycan significantly enhanced the specific infectivity of the DENV-2 variants for mosquito cells compared to that of the wt virus. This property of the loss-of-glycan mutants was not reflected in enhanced growth in C6/36 cells: on the contrary, 3 variants tested (rPUO.T69A, rPUO.T69V, and rPUO.T69A/N124S) showed an ~10-fold-poorer particle release in comparison to the parent, rPUO (Fig. 3), despite the ~10-fold-higher specific infectivity for this cell line (Table 1). Figure 3 also shows comparable growth phenotypes for rPUO.T69A and rPUO.T69V in the mosquito cell line, which contrast with an ~100-fold difference in particle release from infected mammalian cells between the 2 variants (Fig. 2B).

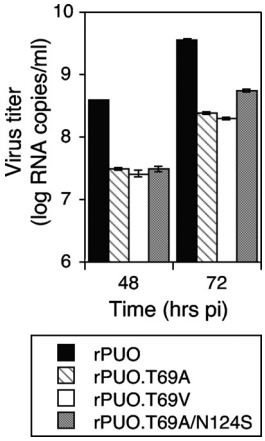

FIG. 3. Growth of rPUO parent and loss-of-glycan variants in C6/36 mosquito cells. Extracellular virus titers in culture supernatants from infected cells (MOI of ~0.2) were measured by quantitation of virion-associated RNA by qRT-PCR at 48 and 72 h p.i. Error bars indicate the SEM for two real-time PCR determinations for each sample shown. Representative data from 2 experiments are shown.

Tolerance to ablation of E protein glycosylation at Asn 67 is DENV-2 strain dependent. The replication competence in BHK and Vero cells of loss-of-Asn 67-linked glycan variants of DENV-2 encoding the PUO-218 strain prM and E proteins contrasts with the reported replication-defective phenotype of DENV-2 strain 16681, lacking Asn 67-linked glycosylation due to an E protein N67Q mutation (5, 35). The latter mutation did not abolish growth in mammalian cells when introduced into a NGC/PUO-218 prM-E protein chimeric virus (Fig. 2). To further investigate the influence of strain origin of the E protein on tolerance to loss-of-Asn 67-linked glycosylation, strain 16681 and NGC variants lacking the Asn 67-linked glycan were produced. The growth kinetics in BHK cells of the chimeric parent and two loss-of-Asn 67-linked glycan variants encoding the DENV-2 strain 16681 prM and E proteins in the NGC backbone were compared, following electroporation of cells with similar amounts of *in vitro*-synthesized genomic RNA (Fig. 4). Relative to that of the parent, r16681, growth of the variants r16681.T69V and r16681.T69A was reduced by 16- and >1,000-fold, respectively, highlighting the potent influence on growth of the type of amino acid introduced at residue 69 to abolish glycosylation, as was found for PUO-218 E protein variants (Fig. 2). Growth of r16681.T69V in BHK cells was similar to that of a mutant lacking the Asn 153-linked glycan (r16681.T155A) (Fig. 4); consistent with this result, ablation of the latter E protein glycan in DENV-2 16681 was shown by others to reduce virus growth by ~10-fold in mammalian cells relative to that of the parent (5, 35). Ablation of an E protein glycan moiety in variants r16681.T69V and r16681.T155A was confirmed by the slightly increased electrophoretic mobility of the variant relative to the r16681 wt E protein (Fig. 1).

The detrimental effect of the T69A substitution on virus growth in mammalian cells was more pronounced in the NGC E protein background than in that of the PUO-218 strain. Electroporation efficiency for rNGC.T69A was reduced by

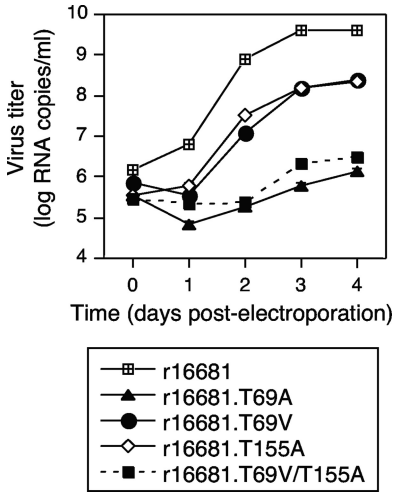

FIG. 4. Growth of 16681 parent and variants lacking one or both E protein glycans in mammalian cells. Virion-associated RNA release from BHK cells electroporated with 16681 parent or loss-of-glycan variant RNA transcripts (1 μg genomic RNA) is shown. Supernatant samples were collected at the indicated time points, and virion-associated DENV-2 RNA was measured by quantitative real-time RT-PCR. Error bars indicate the SEM for two real-time PCR determinations for each sample shown. Representative data from 2 experiments are shown.

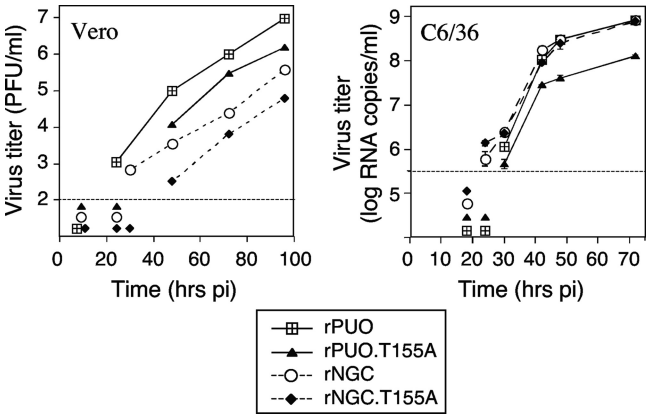

FIG. 5. Growth of parent and loss-of-Asn153-linked glycan variants in mammalian (Vero) and mosquito (C6/36) cells. Extracellular virus titers in culture supernatants from infected Vero (MOI ~0.01) and C6/36 cells (MOI ~0.05) were determined by plaque assay and qRT-PCR, respectively. To ascertain comparable levels of infection for parent and variant viruses, Vero and C6/36 cell monolayers were infected in a parallel experiment with a 5-fold higher and identical virus doses, respectively, harvested at 24 (Vero) and 40 h (C6/36) pi, and the number of infected cells quantitated by flow-cytometry. Vero and C6/36 cell infection for the rNGC pair ranged from 2 to 3% and 3 to 4%, respectively, and for the rPUO pair from 3 to 5% and 4 to 8%, respectively. Dotted lines indicate the detection limits of the assays. Error bars indicate the SEM for two real-time PCR determinations for each sample shown. Representative data from 2 experiments are shown.

250-fold relative to that of the parent, rNGC (Table 1). Furthermore, growth of rNGC.T69A in BHK cells was not detectable by measurement of RNA genome copy numbers in the culture medium over a period of 4 days, while electroporation with a comparable amount of rNGC RNA (0.5 μg) gave rise to a 500-fold increase of virion RNA in the culture supernatant (data not shown).

Despite the much poorer electroporation efficiency of rNGC.T69A than that of the NGC parent, the plaques obtained in infectious center assays were not uniformly smaller than those for rNGC (Table 1). Sequence analysis of the entire prM and E protein genes of plaque isolates failed to identify progeny virus with the expected genotype but revealed a pseudoreversion and a compensatory mutation. Five small-plaque variants that were sequenced each showed a single nucleotide change in the E protein gene, resulting in the T69V pseudoreversion, while a large-plaque variant derived from the rNGC.T69A mutant retained the introduced amino acid change but had acquired a K126T suppressor mutation in the E protein (Table 2). This second-site mutation created a novel glycosylation motif in DENV-2 E protein DII with glycan linkage to Asn 124, based on the electrophoretic mobility of the NGC.T69V/K126T E protein in SDS-PAGE, which corresponded to that of the wt and was marginally slower than that of the variant NGC.T155A, which lacked one E protein glycan (Fig. 1).

Growth and replication of DENV-2 variants lacking the Asn 153-linked glycan in vertebrate and invertebrate cells. E protein glycosylation at Asn153 is not essential for DENV viability. While loss of the Asn 153-linked glycan reduced virus growth in mammalian cells by ~10-fold (5, 35), growth of the variant in mosquitoes was comparable to that of the wt (5); furthermore, serial passage of DENV-3 in mosquito cells selected for variants that lack the glycan (23), suggesting that ablation of Asn 153-linked glycosylation provides a growth advantage in invertebrate cells relative to the parent virus. Passage of DENV-2 strain PUO-218 in C6/36 cells also selected for a loss-of-Asn 153-linked glycan variant (T155A mutation) following 5 passages (data not shown). Here we have introduced the same E protein T155A mutation found in DENV-2 and -3 mosquito cell passage variants into the DENV-2 NGC and chimeric NGC/PUO-218 prM-E cDNA clones to test the influence of loss-of-Asn 153-linked glycosylation on virus growth and explore the possibility of removing both glycans from the E protein. The rPUO.T155A and rNGC.T155A viruses were viable and displayed a BHK cell electroporation efficiency which did not markedly differ from that of the respective parents, although Vero plaque sizes were smaller than those for the parent viruses (Table 1). Ablation of the Asn 153-linked glycan reduced growth of the variants in Vero cells by 5- to 10-fold relative to that of the parent viruses (Fig. 5), while the specific infectivities of the respective pair differed by only ~2-fold (Table 1 and data not shown). The growth of rNGC in Vero cells was markedly poorer than that of rPUO (Fig. 5), which was consistent with the plaque size difference observed (Table 1).

Ablation of E protein glycosylation at Asn 153 resulted in greatly increased specific infectivity for C6/36 mosquito cells: a 100-fold enhancement of infectivity was found for rPUO.T155A relative to the parent (Table 1). This enhanced infectivity as a result of loss of the Asn 153-linked glycan was not reflected in the growth phenotypes in C6/36 cells for the 2 pairs: rNGC and rNGC.T155A displayed comparable growth, while virus yield from rPUO.T155A-infected mosquito cell cultures was reduced by ~10-fold in comparison to that for the parent, where infection

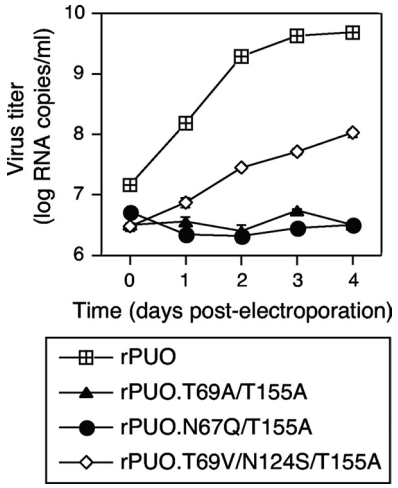

FIG. 6. Growth of rPUO parent and variants lacking both E protein glycans in mammalian cells. Virion-associated RNA release from BHK cells electroporated with PUO parent or loss-of-glycan variant RNA transcripts (1 μg genomic RNA) is shown. Supernatant samples were collected at the indicated time points and virion-associated DENV-2 RNA was measured by quantitative real-time RT-PCR. Error bars indicate the SEM for two real-time PCR determinations for each sample shown. Representative data from 2 experiments are shown.

with rPUO was 2-fold higher than that with rPUO.T155A (Fig. 5). These data resemble those obtained for the loss-of-Asn 67-linked glycan variants in C6/36 cells and suggest that the enhancement of infectivity for the mosquito cells associated with the removal of either of the two E protein glycans is at the expense of markedly reduced virion production and/or release.

DENV-2 variants lacking both Asn-linked E protein glycans. Given the viability of DENV-2 recombinants lacking the Asn 67- or Asn 153-linked glycans and our identification of E protein amino acid changes that, at least in part, compensate for the deleterious effect of Asn 67-linked glycan ablation, the following variants lacking both glycans were constructed: rPUO.N67Q/T155A, rPUO.T69A/T155A, and rPUO.T69V/N124S/T155A. Infectious virus was recovered only in the form of small plaques (0.5 mm) on Vero cells for rPUO.T69V/N124S/T155A by infectious center assay after electroporation of BHK cells (Table 1). A growth assay in BHK cells showed that extracellular DENV-2 RNA levels between 1 and 4 days after electroporation did not markedly increase for the two variants, rPUO.T69A/T155A and rPUO.N67Q/T155A but clearly demonstrated growth competence of variant rPUO.T69V/N124S/T155A: extracellular virion-associated genomic RNA levels in the culture fluid for the latter virus rose by ~ 50-fold over a 4-day period but remained significantly below those obtained for the parent, rPUO (Fig. 6). The DENV-2 variants lacking both E protein glycans showed even greater enhancement of infectivity for C6/36 cells than those with single glycan ablation relative to rPUO in the absence of a marked effect on infectivity for mammalian cells (Table 1). Thus, a compounding effect of ablation of both E protein glycans on virion morphogenesis/release in mammalian cells is apparent, given that the variants were not noticeably defective in virus macromolecular synthesis (8 to 14% of BHK cells electroporated with genomic RNA for the different viruses in Fig. 6 expressed similar levels of the nonstructural protein NS1 on the basis of FACS analysis performed at 20 h posttransfection). The absence of Asn-linked glycosylation in the E protein of variant rPUO.T69V/N124S/T155A was confirmed by immunoprecipitation and SDS-PAGE, showing faster E protein mobility than the parent and loss-of-single-glycan mutants (Fig. 1). Sequence analysis of the prM and E protein genes also confirmed the ablation of both Asn-linked glycosylation sites and revealed no additional nucleotide changes. These findings for the DENV-2 strain PUO-218 E protein were extended to that of the 16681 strain using the NGC/16681 prM-E chimeric virus. A variant lacking both E protein glycans, r16681.T69V/T155A, was viable in mammalian cells: (i) the mutant produced plaques on Vero cells, albeit smaller in size than those of r16681 (1 mm and 2 to 3 mm at 7 days p.i., respectively), (ii) produced progeny virus in BHK cells over a 4-day growth period, although the yield was greatly reduced in comparison to that for the parent (Fig. 4), and (iii) and could be passaged (at least twice) in mammalian cells without reverting or acquiring a novel glycosylation motif in the E protein. Taken together, the results show that E protein glycosylation *per se* is not essential for DENV-2 growth in mammalian cells and that the deleterious effect on growth of glycan ablation can be suppressed, at least in part, by amino acid changes in the E protein, which do not generate a novel glycosylation motif.

Dueling effects of E protein glycosylation on infectivity and virion release. Ablation of either of the E protein glycans markedly improved the infectivity of DENV-2 variants for C6/36 cells but failed to enhance virus growth in the insect cells compared to that of wt virus (Table 1 and Fig. 3 and 5). To resolve this discrepancy, we infected mammalian (Vero) and mosquito (C6/36) cell monolayers with the rPUO parent or loss-of-glycan variants, equilibrating virus input based on genome copy numbers, and measured the percentage of infected cells by flow cytometry and virus release into the culture supernatant by qRT-PCR (Table 3). The absence of one of the E protein glycans resulted in an 18- and 24-fold enhancement of infection of C6/36 cells with rPUO.T69V and rPUO.T155A

TABLE 3. Effects of loss of E protein glycosylation in different flaviviruses on infectivity and virus release in mammalian and invertebrate cells

Virus	Strain/variant[a]	% infection ± SEM[b]		Virus release ± SEM[c] (log genome copies/ml)	
		Vero	C6/36	Vero	C6/36
DENV-2	rPUO	21.8 ± 1.2	3.8 ± 0.4	8.2 ± 0.11	8.5 ± 0.08
	rPUO.T69V	30.5 ± 0.4	68.9 ± 0.7	8.0 ± 0.08	8.6 ± 0.07
	rPUO.T155A	14.3 ± 0.6	91.6 ± 2.0	7.8 ± 0.09	9.5 ± 0.11
WNV	rKUN.Ser_{156}	24.0 ± 0.6	3.9 ± 0.5	8.4 ± 0.09	6.1 ± 0.007
	rKUN.Phe_{156}	30.8 ± 2.0	93.5 ± 0.1	8.7 ± 0.06	7.2 ± 0.12
MVEV	rMVEV	27.6 ± 0.5	7.0 ± 0.2	9.1 ± 0.11	7.0 ± 0.05
	rMVEV.S156A	26.8 ± 1.6	73.0 ± 2.6	9.0 ± 0.06	7.6 ± 0.12

[a] Vero and C6/36 (5×10^5) cells in 6-well Nunc plates were infected with DENV-2 (2×10^8 genome copies), WNV (8×10^7 genome copies), or MVEV (6×10^8 genome copies). The Virus rKUN.Ser_{156} or rKUN.Phe_{156}, encodes the glycosylated or unglycosylated E protein, respectively.

[b] Percent infected cells was determined by FACS staining of DENV-2-, WNV-, and MVEV-infected Vero cells collected at 24 h, 22 h, and 22 h p.i., respectively, and infected C6/36 cells collected at 48, 24, and 24 h p.i., respectively.

[c] Virus release was estimated using culture supernatant collected from infected cells at the same time as FACS analysis.

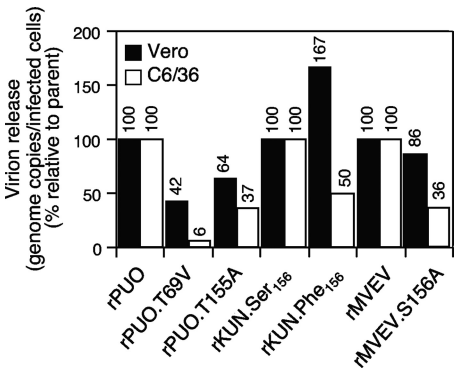

FIG. 7. Impact of flavivirus E protein glycosylation on virion release from Vero and C6/36 cells. The efficiency of virion release was calculated as a ratio of virion-associated genome copies in infected culture supernatants and the percentage of infection using the values shown in Table 3. The results were normalized by setting the values for virion release from cells infected with the parent viruses to 100%.

relative to that of the parent at 48 h p.i. This contrasted with comparable amounts of virus release between the parent and the rPUO.T69V mutant, indicating that Asn 67-linked glycosylation is critically important for virus release from mosquito cells. Deletion of the Asn 153-linked glycan impacted less dramatically on virion release in C6/36 cells: thus, an ~24-fold improvement in infection resulted in release of a 9-fold-greater number of virion-associated genome copies than that for the parent virus. On the other hand, infectivity for Vero cells differed by <2-fold between rPUO and the 2 mutant viruses, and only the ablation of the Asn 67-linked glycan had a noticeably deleterious impact (~2-fold) on virus morphogenesis/release in the mammalian cell line. Virion release of loss-of-glycan variants from infected Vero and C6/36 cells as a percentage relative to parent virus is summarized in Fig. 7.

Generality of infection enhancement for C6/36 cells associated with loss of E protein glycosylation among mosquito-borne flaviviruses. The E proteins of MVEV (7, 30) and most lineage 1 strains of WNV (19, 41) are glycosylated at Asn 154. Ablation of the glycan, using infectious cDNA clone technology, did not markedly impact on infectivity for mammalian (Vero) cells relative to that of the parent when cells were infected with inocula adjusted for identical numbers of virion-associated genome copies (Table 3). In contrast, the same inocula gave a 10- to 20-fold higher infectivity of mosquito cells for the loss-of-glycan variants than for the parent. Removal of the E protein glycan did not markedly (<2-fold) impact on virus release from Vero cells but reduced release from the insect cell line infected with the MVEV and WNV variants, in comparison to the parent viruses (Table 3 and Fig. 7).

DISCUSSION

The DENV E protein differs from that of all other flaviviruses by the unique presence of a second glycan linked to residue 67. Recently it was suggested that this glycan is critically important for DENV growth in mammalian cells due to facilitating an essential but elusive function required for virus morphogenesis (5, 35). Our present study clearly shows that this is not the case. We find that loss-of-Asn 67-linked glycan variants of DENV-2 can display efficient growth in mammalian cells, depending on the amino acid substitution introduced to abolish glycosylation. Thus, variants with a replacement of Thr 69 (in the Asn_{67}-Thr_{68}-Thr_{69} motif) with a larger and more hydrophobic residue, Val or Leu, retained efficient growth, while the introduction of an Ala residue at the same site reduced virus growth by ~100-fold relative to that of the parent. Similarly, a conservative replacement of Asn 67 with Gln was markedly less detrimental for growth than the nonconservative change to Asp. In addition, a compensatory mutation (N124S) at a nonadjoining region in the E protein partially repaired the growth defect of one of the loss-of-Asn 67-linked variants (T69A) without generating a novel glycosylation acceptor motif. Accordingly, the function of the Asn 67-linked glycan in E protein folding and virus morphogenesis is mostly dispensable for DENV growth in mammalian cell culture in the presence of compensatory changes at the glycosylation site or elsewhere in the E protein. Contrary to the interpretation of Mondotte et al. (35), we did not find that the presence of a glycan at position 67 was obligatory for efficient DENV release from mammalian cells.

In addition to the nature of amino acid substitutions introduced to abolish Asn 67-linked glycosylation, we also found that strain origin of the E protein influenced the impact of glycan ablation on growth. We noted that a T69A mutation produced viable virus in BHK and Vero cells in the context of the PUO-218 and 16681 prM-E protein background, albeit with a poor growth phenotype, but was lethal in the context of the E protein of the NGC strain. Surprisingly, an E protein N67Q mutation, which prevented growth in mammalian cells of the DENV-2 16681 strain (5, 35), was well tolerated in a chimeric virus encoding the PUO-218 prM and E proteins. Furthermore, growth of a T69V loss-of-glycan variant in BHK cells was >10-fold poorer than that of the parent in the context of the DENV-2 strain 16681 E protein but comparable to that of the parent when the mutation was introduced into the PUO-218 E protein. The E proteins of DENV-2 strains NGC and 16681 differ from that of PUO-218 at 6 and 4 residues, respectively (10, 25). A region in DII, between residues 120 and 126, appears to be important in attenuating the deleterious effect on growth associated with ablation of the Asn 67-linked glycan, given that (i) suppressor mutations were identified at residues 124 and 126 and (ii) this region encompasses the nonconservative amino acid differences between PUO-218 and the other two DENV-2 strains at residues 120 (Thr and Arg in the PUO-218 and 16681 strains, respectively) and 126 (Glu and Lys in the PUO-218 and NGC strains, respectively). This DII region not only accommodates amino acid residues that are critical for compensating defects associated with loss of the Asn 67-linked glycan but also impacts on DENV virulence and cell surface receptor binding (10, 24).

The second key finding of this investigation was that of the opposing influences of ablation of either of the two E protein glycans on infectivity and virion release in C6/36 mosquito cell infections. The infectivity of the variants for the invertebrate cell line was increased by up to 100-fold relative to that of wt virus. However, this infectivity enhancement was at the expense of a reduction in virion release, with the consequence that virus growth in C6/36 cells relative to that of wt virus was either comparable to or poorer than that of variants lacking the

Asn 153- or Asn 67-linked glycans, respectively. This finding was extended to two other mosquito-borne flaviviruses, MVEV and WNV, supporting the generality among flaviviruses of improved infectivity for C6/36 cells as a consequence of ablation of E protein glycosylation. The latter conclusion is consistent with the results from an earlier study on WNV (12). Lack of glycosylation at residue 153 in the DENV E protein is associated with an increase in the fusion pH threshold of ~0.5 U (11, 23). Given that this elevated pH threshold would allow fusion to occur at physiological pH, there is the possibility that DENV fusion at the cell surface may give rise to productive infection in mosquito cells. Cell type dependence of such an alternative entry mechanism in establishment of productive virus infection has been reported for another enveloped RNA virus with a mosquito-vertebrate host cycle (31). Thus, the requirement for acid-induced activation of the fusion function of the E protein and entry via the endocytic pathway may be obviated by the ability of variants deficient in E protein glycosylation to mediated fusion at neutral pH. Some EM studies have indeed suggested that flaviviruses can fuse at the plasma membrane (13, 14, 28), although the biological relevance for this entry route remains unclear. It will also be of interest to determine whether ablation of the Asn 67-linked glycan gives rise to an elevated fusion pH threshold, as has been shown for the Asn 153-linked glycan on DENV E protein (11, 23).

Infection enhancement in mosquito C6/36 cells was accompanied by a pronounced reduction of virion release for the loss-of-glycan variants, in particular when the Asn 67-linked glycan was absent. This finding differed from the study by Mondotte et al. (35), which did not show the prominent inhibitory effect of Asn 67-linked glycan ablation on virus production in C6/36 cells, albeit only secretion of recombinantly expressed E protein was measured. In mammalian cells, loss of glycans in the flavivirus E protein can also severely impede virion release (8, 12, 35); however, our investigation clearly shows that this deleterious impact of glycan ablation can be complemented by suppressor substitutions in the glycosylation acceptor site or elsewhere in DII of the E protein. This has allowed us, for the first time, to produce DENV-2 variants lacking both glycans on the E protein which were competent in growth in mammalian cells, although a compounding effect of the absence of glycosylation on virus release was apparent in comparison with variants with single glycan ablation. Growth competence in mosquito cells of a DENV-2 variant lacking both E protein glycans has been reported by others (5, 35). Accordingly, the presence of a glycan, *per se*, on the E protein of DENV and that of other flaviviruses is not critically important for growth in vector and host cells, consistent with the existence of natural isolates from different members of the flaviviruses devoid of glycans on their E proteins (1, 2, 4, 32, 37, 44).

In summary, we show that glycosylation of the E proteins of DENV and that of other flaviviruses strongly influenced two stages in the virus growth cycle: infectivity and virion release. While the enhancement effect of glycan ablation on infectivity was exclusive to a mosquito cell line, the inhibitory effect on release was apparent in both mammalian and insect cells, although suppressor mutations, which complemented glycan ablation in mammalian cells, had no beneficial effect in mosquito cells. This dichotomy in the impact of the E protein glycan(s) at different stages in the flavivirus life cycle may explain, at least partly, why different solutions in terms of modification of the virion surface by glycan addition have evolved among the members of the flavivirus genus.

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