SUPPLEMENT ARTICLE

The Role of Conserved N-Linked Glycans on Ebola Virus Glycoprotein 2

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Background. N-linked glycosylation is a common posttranslational modification found on viral glycoproteins (GPs) and involved in promoting expression, cellular attachment, protection from proteases, and antibody evasion. The GP subunit GP2 of filoviruses contains 2 completely conserved N-linked glycosylation sites (NGSs) at N563 and N618, suggesting that they have been maintained through selective pressures.

Methods. We assessed mutants lacking these glycans for expression and function to understand the role of these sites during Ebola virus entry.

Results. Elimination of either GP2 glycan individually had a modest effect on GP expression and no impact on antibody neutralization of vesicular stomatitis virus pseudotyped with Ebola virus GP. However, loss of the N563 glycan enhanced entry by 2-fold and eliminated GP detection by a well-characterized monoclonal antibody KZ52. Loss of both sites dramatically decreased GP expression and abolished entry. Surprisingly, a GP that retained a single NGS at N563, eliminating the remaining 16 NGSs from GP1 and GP2, had detectable expression, a modest increase in entry, and pronounced sensitivity to antibody neutralization.

Conclusions. Our findings support the importance of the GP2 glycans in GP expression/structure, transduction efficiency, and antibody neutralization, particularly when N-linked glycans are also removed from GP1.

Keywords. filovirus; Ebola virus; glycoprotein; antibody neutralization; virus entry; mutagenesis; N-linked glycosylation; glycan.

Ebola virus and Marburg virus are members of the family Filoviridae that cause highly lethal, sporadic outbreaks of viral hemorrhagic fever throughout central Africa and, most recently, in western Africa. There are 5 *Ebolavirus* species with the type viruses named after geographic regions where they were first discovered: Ebola virus (EBOV), Sudan virus, Taï Forest virus, Bundibugyo virus, and Reston virus [1]. Currently, there are no vaccines or antivirals available for prevention or treatment of filoviral hemorrhagic fevers.

Filovirus entry requires the viral glycoprotein (GP), composed of a trimer of cysteine cross-linked GP1/GP2 heterodimers. The GP1 subunit contains a core region, composed of a receptor-binding domain and glycan cap, and a mucin-like domain. The transmembrane GP2 subunit mediates fusion through the formation of a 6-helix bundle [2]. EBOV GP is extensively glycosylated, with more than half of the molecular weight attributed to carbohydrates [3]. The EBOV GP1 subunit contains 15 N-linked glycosylation sites (NGSs; N-X-S/T) with the mucin-like domain containing another 80 potential O-linked glycosylation sites [4]. Lennemann et al [5] have shown elsewhere that mutagenesis of all NGSs in the GP1 subunit had a minimal impact on expression and increased GP-mediated entry into Vero cells and primary murine macrophages, which correlated with an increase in protease sensitivity. Furthermore, removal of N-linked glycans surrounding the highly conserved receptor-binding domain resulted in enhanced antibody-mediated neutralization of GP-mediated entry.

The GP2 subunit of all filoviruses contains 2 completely conserved NGSs at residues N563 and N618 which are located in the heptad repeat (HR) 1 and HR2 regions of GP2, respectively (Figure 1). Previous work has shown that both of these sites are occupied by glycan modifications [6]. The conservation of these sites

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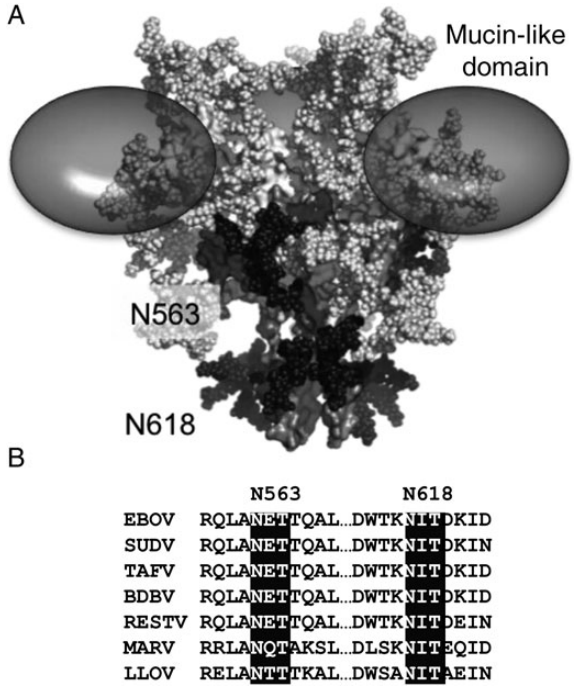

Figure 1. Model of N-glycans at conserved sites in Ebola virus (EBOV) glycoprotein (GP) 2. *A*, Structural model of N-linked glycans on EBOV GP, shown from the side. The heptad repeat 2 of EBOV GP2 (PDB identifier, 2EBO) was modeled at the base of the EBOV $GP_{1,2}$Δmuc structure (PDB identifier, 3CSY). The mucin-like domain for each monomer is shown as a gray sphere as predicted by others [2]. GP1/GP2 subunits are shown in gray, GP1 N-linked glycans in light gray and GP2 N-linked glycans in black. The numbering shown represents the GP2 asparagine residue position to which N-linked glycans are attached. *B*, Alignment of the conserved N-linked glycosylation sites (N-X-S/T, where *X* represents any amino acid except proline), highlighted in black, within the GP2 subunit of representative viruses in the filovirus family. Abbreviations: BDBV, Bundibugyo virus; LLOV, Lloviu virus; MARV, Marburg virus; PDB, Protein Data Bank; RESTV, Reston virus; SUDV, Sudan virus; TAFV, Taï Forest virus.

within the family suggests functional significance, but the importance of these sites for GP expression and function has yet to be investigated.

MATERIALS AND METHODS

Cell Lines and Plasmids

Vero cells and HEK293T cells were maintained in Dulbecco's modified Eagle medium (Gibco) plus 10% fetal bovine serum and 1% penicillin/streptomycin. The pcDNA3.1 expression plasmids for wild-type (WT) EBOV GP (accession No. NP_066246), the N-linked glycan-deficient GP1 mutant (7Gm8G), and pCAGGS Marburg virus Musoke isolate GP (accession No. YP_001531156) have been described elsewhere [5].

Modeling of GP N-Linked Glycans

The prefusion EBOV $GP_{1,2}$ lacking the transmembrane domain and cytoplasmic tail (EBOV $GP_{1,2}$ ΔTM structure (Protein Data Bank [PDB] identifier, 3CSY) is missing the C-terminus (amino acids 600–632), including the C-terminal HR (HR2). Therefore, the HR2 from the postfusion EBOV GP2 structure (PDB identifier, 2EBO) was placed at the base of the ectodomain, using PyMol software version 1.7.2, to serve as a predictive model of the stalk region. In addition, 4 NGSs in GP1 were lacking within the model owing to disordered structures (N204 and N296) or were mutated to promote crystallization (N40 and N228) [7]. To introduce predicted NGSs into our model, the EBOV GP sequence was submitted to the PHYRE2 protein fold recognition server [8], resulting in a structure that contained NGSs at N40 and N228. This structure was then submitted for in silico glycosylation using the GlyProt server, which produced a model containing complex N-linked glycans at all NGSs, except N204 and N296, which are part of disordered regions [2]. Complex glycans at these sites were modeled onto the glycosylated structure in a predictive fashion with PyMol software.

Site-Directed Mutagenesis and Transfections

Primers were designed to mutate the asparagine residues of GP2 NGSs in EBOV GP expression vectors using the QuickChange Site-Direct Mutagenesis Kit (Stratagene), according to the manufacturer's protocol. All mutations were confirmed by sequencing the full length of the EBOV GP open reading frame. All transfections were performed in HEK293T cells seeded in a 6-well plate by polyethylenimine method, as described elsewhere [5, 9].

Production of Replication-Incompetent Vesicular Stomatitis Virus–Green Fluorescence Protein Pseudovirions

Pseudovirions were produced in HEK293T cells, as described elsewhere [5]. Briefly, HEK293T cells were transfected with the various EBOV GP constructs and at 24 hours transduced by a replication-incompetent vesicular stomatitis virus (VSVΔG–green fluorescence protein [GFP]) pseudotyped with Lassa virus (LASV) GPC. In the genome of VSVΔG-GFP, the GP G gene is replaced with the GFP gene. After 24 hours, cell supernatants were collected and filtered through 0.45-μm syringe-filters, followed by storage at −80°C. When indicated, pseudovirions were purified through a 20% sucrose cushion for 2 hours at 83 000 × *g* and resuspended in phosphate-buffered saline.

Pseudovirion EBOV GP and VSV-Matrix Quantification

Assessment of EBOV GP to VSV-matrix (M) ratios of pseudovirion preparations was performed as described elsewhere [5]. Briefly, pseudovirion stocks were passed through a dot blot apparatus onto nitrocellulose. EBOV GP was detected with anti–EBOV GP human monoclonal antibody (mAb) KZ52 [10], mouse anti–EBOV GP1 mAb 5E6 [11], or rabbit polyclonal antiserum (IBT 0301-015). The dot blot was assessed for VSV-M in parallel using mouse anti–VSV-M mAb 23H12 [12]. Signals were quantified using the Odyssey Imaging Station and Image Studio software version 4.0 (LI-COR) [13].

To determine the amount of soluble EBOV GP present in stocks of our WT and mutant viruses, we pelleted virions

through a 20% sucrose cushion at 26 000 rpm for 2–4 hours in a SW32Ti rotor. The virion pellet was resuspended in phosphate-buffered saline. The spun supernatant after centrifugation was collected and concentrated approximately 10–15-fold in a centrifugal concentrator (molecular weight cutoff, 30 000 daltons). Two-fold serial dilutions of the pellet (starting with 2 μL) and supernatant (starting with 35 μL) were passed through a dot blot apparatus onto nitrocellulose. EBOV GP was detected with the rabbit polyclonal antisera, and VSV matrix with mouse VSV-M mAb 23H12 [12]. Signals were quantified using Odyssey Imaging Station and Image Studio software (LI-COR) [13].

Transduction Assays

Vero cells were seeded in 48-well plates 24 hours before transduction. Pseudovirions were normalized to matrix levels found in the WT EBOV GP/VSVΔG-GFP pseudovirion stocks before addition to Vero cell monolayers (WT multiplicity of infection, approximately 0.1). Transduction was determined by quantification of GFP expressing cells by flow cytometry 16–20 hours after addition of pseudovirions.

Neutralization Assays

Anti-EBOV immunoglobulin (Ig) G was purified from pooled antiserum from EBOV-challenged, convalescent cynomolgus macaques vaccinated with a Venezuelan equine encephalitis virus replicon expressing EBOV GP (a kind gift from John M. Dye, US Army Medical Research Institute of Infectious Diseases). Serial dilutions of IgG were incubated with WT or glycosylation mutant EBOV GP VSVΔG-GFP pseudovirions (WT multiplicity of infection, approximately 0.1), normalized to matrix expression, for 30 minutes at 37°C. Reactions were then diluted 5-fold in media and used to transduce confluent monolayers of Vero cells. Inhibition curves and half-maximal inhibitory concentrations were determined using GraphPad Prism 5 software.

RESULTS

Altered Monoclonal Antibody Detection of EBOV GP2 Containing N-Linked Glycosylation Site Mutations

To investigate the role of GP2 N-linked glycans during entry, the asparagine residues to which the glycan is added were mutated to aspartic acid residues, as has previously been performed at GP1 NGSs [5, 7], to disrupt NGSs within GP2 individually and in combination. Plasmids expressing WT GP or GP mutants were transfected into HEK293T cells and pseudotyped onto VSVΔG-GFP. To evaluate GP expression, pseudovirion containing supernatants were spotted on to nitrocellulose and the ratio of VSV matrix to GP was determined within a single well using a mouse anti–VSV matrix mAb (23H12) and a conformation-specific human anti–EBOV GP mAb (KZ52). Detection of mAb binding was assessed using 2 infrared dyes conjugated to species-specific secondary antibodies [5].

The N563D mutation abrogated KZ52 antibody binding (Figure 2*A*), whereas disruption of the N618 site modestly decreased binding of this mAb. Not surprisingly given the absence of KZ52 binding to the N563D mutant, binding of the mAb to the GP containing both mutations was indistinguishable from virions produced in the absence of GP. Because part of the KZ52 epitope lies within GP2, loss of the N-linked glycan at 563 may affect the ability of this antibody to bind [2]. Therefore, we also used a GP1-specific mAb that targets the GP1 mucin-like domain, 5E6 [11], to detect GP levels.

In contrast to KZ52, 5E6 readily detected the N563D GP in virus stocks, albeit at approximately 60% of WT levels, whereas N618D binding of 5E6 was indistinguishable from WT pseudovirion binding (Figure 2*B*). Moreover, the N563D/N618D double mutant could also be detected with 5E6 but was significantly decreased compared with WT or either mutant individually. Similar findings to those obtained with 5E6 were observed with rabbit polyclonal antiserum against EBOV virus like particles (VLPs; data not shown). Overall, because KZ52 is known to recognize the prefusion GP conformation [2], these results raise the possibility that both GP2 NGSs that significantly affect KZ52 binding are important for stabilizing the prefusion conformation of GP, with the 563 position more critical for this than the 618 residue. Alternatively, because N563 has a greater impact on KZ52 binding, and is located near the KZ52/GP interface and because the 563 glycan extends from GP in parallel with the body of KZ52 [2], this glycan structure may support the interaction of these 2 molecules.

Effect of Removing GP2 N-Linked Glycans on GP-Mediated Transduction

Next, we assessed the ability of these GPs to mediate VSV pseudovirion transduction of Vero cells. Transduction mediated by the N563D mutant pseudotyped onto VSVΔG-GFP was enhanced 2-fold (Figure 2*C*), despite the decrease in expression and the loss of KZ52 binding. The enhanced entry by N563D mutant indicates that GP resides on virions in a prefusion conformation and suggests that this N-linked glycan impedes at least one entry step. Transduction mediated by VSVΔG-GFP pseudotyped with the N618D mutant was decreased almost 2-fold; however, this was somewhat variable in our studies and not significantly different from WT levels of transduction. Despite low levels of detection of the double mutant, we were unable to detect transduction mediated by the N563D/N618D mutant above background, suggesting that mutant GP present on virions was not in a fusion-competent conformation.

Functional Competence of EBOV GP Containing a Single N-Linked Glycan

Lennemann et al [5] have shown elsewhere that a GP with all 15 N-linked glycans from the GP1 subunit removed (7Gm8G) has

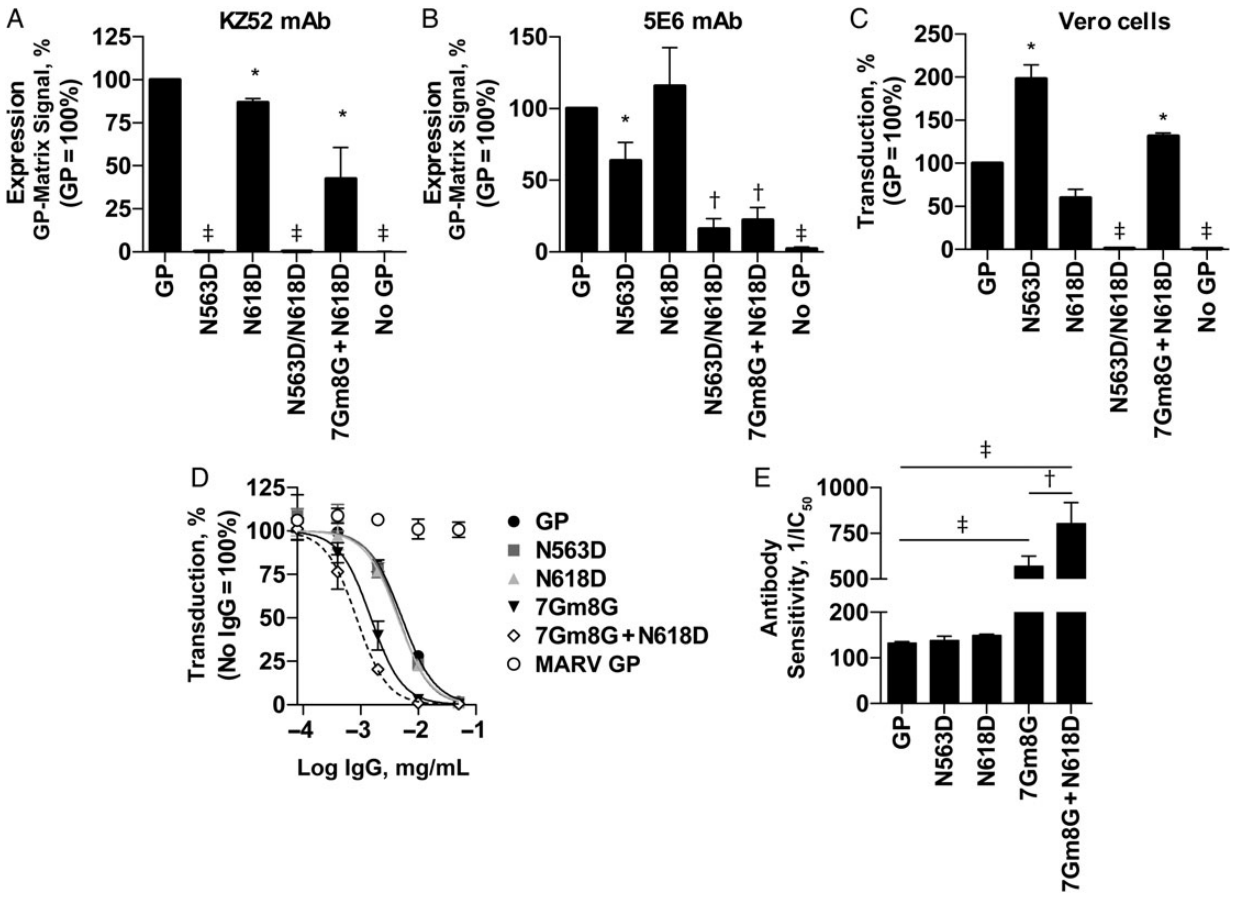

Figure 2. Expression, entry, and antibody-mediated neutralization of Ebola virus (EBOV) glycoprotein (GP) 2 N-linked glycosylation site mutants. *A, B,* EBOV GP to vesicular stomatitis virus (VSV) matrix ratios of the indicated pseudovirion preparations, determined by dot blotting with anti–VSV matrix monoclonal antibody (mAb), 23H12, and either conformation-specific anti–EBOV GP mAb, KZ52 (*A*) or anti–EBOV GP1 mAb 5E6 (*B*). Data represent mean GP/M ratio (with standard deviations [SDs]) for 3 independent stocks of virus, presented as percentage of wild type (WT) EBOV GP pseudovirions (GP). *C*, The indicated pseudovirions, normalized for VSV matrix expression, were used to transduce Vero cells. Green fluorescence protein–positive cells were quantified the next day with flow cytometry. Data represent the mean transduction (with standard error of the mean) observed with 3 independent stocks of virus and tests performed in triplicate, presented as a percentage of WT GP (GP). "No GP" represents pseudovirions produced in the absence of a viral GP. Significance was determined with a 1-sample *t* test. *D, E*, Virions pseudotyped with the indicated GP were normalized to VSV-M and incubated with serial dilutions of purified anti-EBOV immunoglobulin (Ig) G, pooled from convalescent cynomolgus macaques, for 30 minutes at 37°C. *D*, Neutralization curves. *E*, Antibody sensitivity of different mutants tested. Data are presented as the reciprocal of the half-maximal inhibitory concentration (IC_{50}) calculated from neutralization curves in *D*; data in *D* represent means and SDs from 3 independent experiments. Significance was determined with 1-way analysis of variance and a Bonferroni posttest. $^{*}P < .05$; $^{\dagger}P < .005$; $^{\ddagger}P < .001$. Abbreviation: MARV, Marburg virus.

WT levels of GP expression, is virion associated, and mediates enhanced entry. Because the N618D mutation had minimal impact on native protein conformation, as determined by the ability of EBOV VLP polyclonal antisera, KZ52, and 5E6 to bind to the mutant, we included this mutation in our GP1 N-linked glycan-deficient construct, 7Gm8G, and assessed expression and entry. Addition of the N618D mutation to 7Gm8G led to a significant decrease in binding of KZ52 or 5E6 mAbs to the mutant GP in virion-containing supernatants (Figure 2*A* and 2*B*). However, virions pseudotyped with this mutant GP exhibited modest but significantly enhanced entry compared with WT (Figure 2*D*). Therefore, 16 of 17 N-linked glycans can be removed from EBOV GP without sacrificing its ability to mediate efficient entry.

In contrast, introduction of the N563D mutation into a GP construct that had 7 NGSs removed from the GP1 core (7G) abolished detectable levels of expression or virion entry (data not shown). Our inability to detect transduction activity of either7G/N563D or N563D/N618D suggested that when N563D is combined with other NGS mutations, those GPs do not support transduction. This precluded further study of NGS combinations containing N563D and emphasized the importance of N563 glycan for GP transduction and, to a large measure, even GP expression.

Absence of Correlation Between GP Glycosylation Status and GP Shedding

GP on the surface of cells can be cleaved by the cellular protease tumor necrosis factor-α – converting enzyme (TACE), leading to increased GP shedding and reduced levels of GP on the surface of producer cells and on virions [6]. Because elimination of glycans on GP might enhance the sensitivity of GP to TACE cleavage, one possible explanation for reduced GP on mutant virions was that GP was being shed. To assess this possibility, we evaluated the ratio of virion-free GP to virion-associated GP in our virus stocks, using VSV pseudovirions bearing WT GP, N563D, N618D, N563D/N618D and 7Gm8G + N618D. We hypothesized that the mutants containing the fewest N-linked glycans would have the greatest shedding of GP into the supernatant. Virions in supernatants from producer cells were pelleted by ultracentrifugation through a 20% sucrose cushion and resuspended. The centrifuged supernatant was collected and concentrated approximately 10–15-fold to allow GP detection. Serial dilutions of virions and centrifuged supernatants were assessed for GP on a dot blot using rabbit polyclonal antisera against EBOV VLPs. The ratios of GP in spun supernatants to virion-associated GP varied between experiments; however, we found that WT GP bearing pseudovirion cultures produced the highest ratios, suggesting that loss of N-linked glycans does not result in greater shedding of GP (data not shown).

GP2 N-Linked Glycan Protection Against Antibody-Mediated Neutralization

Lennemann et al [5] have shown elsewhere that removal of N-linked glycans surrounding the conserved GP1 receptor-binding domain results in increased sensitivity to antibody-mediated neutralization. To determine whether GP2 N-linked glycans also provide protection against antibody neutralization, we tested whether removal of these modifications resulted increased the sensitivity to purified IgG pooled from EBOV convalescent vaccinated and challenged cynomolgus macaques. Anti-EBOV IgG neutralized Vero cell transduction of VSV pseudotyped with EBOV GP, but not that pseudtyped with Marburg virus GP (Figure 2*E*). Neutralization of pseudovirions bearing GPs with individual glycan site mutations in GP2 was indistinguishable from that in WT (Figure 2*E* and 2*F*). Consistent with previously reported data [5], removal of all N-linked glycans from the GP1 subunit (7Gm8G) significantly increased sensitivity to antibody neutralization, and the addition of N618D to 7Gm8G further enhanced virion sensitivity. These results indicate that removal of individual conserved glycans from GP2 does not significantly increase sensitivity to neutralization by antibodies from convalescent primates, but loss of the HR2 glycan site in combination with all GP1 sites leads to better neutralization.

DISCUSSION

N-linked glycans on viral GPs are known to serve a variety of functions, including C-type lectin binding, antibody evasion, promotion of expression, and protein stability [14]. Lennemann et al [5] have shown elsewhere that relatively conserved N-linked glycans on EBOV GP1 serve as C-type lectin ligands, limit GP proteolysis, and provide virion evasion of antibody neutralization. This study extended the previous work by assessing the 2 NGSs found in GP2 that are conserved across the entire filovirus family. To date, the function of these glycans has not been extensively investigated [3, 6, 15]. We found that the glycan at N563 impedes entry whereas the glycan at N618 may enhance entry. However, the combined removal of the GP2 glycan at N618 along with all GP1 N-linked glycans (7Gm8G + N618D) resulted in similar transduction values to the WT GP. We also showed that removal of either glycan from GP2 had minimal impact on sensitivity to antibody-mediated neutralization, but a EBOV GP containing a combination of N618D and 7Gm8G was approximately 7.5-fold more sensitive to antibody neutralization than WT GP.

N-linked glycans at positions 563 and 618 in GP2 are attached to the HR1 and HR2 regions, respectively. These HR regions are involved in the formation of the 6-helix bundle during fusion, and we postulated that loss of these glycans might affect entry efficiency at this step, because work with paramyxovirus fusion proteins indicated that glycans attached to HR regions limit fusion events [16–18]. Similarly, we found that removal of the glycan at N563 enhanced entry, possibly by partially destabilizing GP, resulting in a more fusion-ready structure. Future studies focusing on the potential roles of these glycans in mediating efficient fusion of virus-host membranes would be informative.

Structural studies of EBOV GP and Sudan virus GP have highlighted a "hot spot" for neutralizing antibody binding located at the base of the GP, where GP1 and GP2 interact [2, 19, 20]. Neutralizing antibodies KZ52 and 16F6 bind to this location in EBOV and Sudan virus, respectively. Interestingly, removal of the glycan at EBOV GP N563 completely abolished binding of KZ52. However, this mutation did not affect neutralization of virions by convalescent serum samples, indicating that a significant proportion of neutralizing antibodies from these primates do not target the KZ52 epitope at the GP1/GP2 interface.

Glycosylation of the stem domain of the Nipah virus fusion protein has been shown to protect from antibody neutralization [17]. Such protection does not seem to occur in the EBOV GP structure, because removal of the individual glycans at N563 or N618 did not increase sensitivity to neutralizing antibodies. However, when the N618 glycan was removed from a GP that also lacked GP1 N-linked glycans, the antibody sensitivity was further increased compared with the GP1 mutant alone. This enhancement cannot be completely explained by decreased protein expression of the 7Gm8G + N618D mutant, because we did not observe enhanced antibody sensitivity with the N563D mutation, which also had decreased expression. It is possible that removal of all N-linked glycans from GP, except for a single

glycan at position 563 in the GP2 subunit, destabilizes the protein and allows better antibody binding, which in turn perturbs the GP structure and renders it nonfunctional. Further work is needed to investigate possible mechanisms of anti-GP antibody–mediated neutralization.

Notes

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