Comprehensive Functional Analysis of N-Linked Glycans on Ebola Virus GP1

Nicholas J. Lennemann, Bethany A. Rhein, Esther Ndungo, Kartik Chandran, Xiangguo Qiu, Wendy Maury

Department of Microbiology, University of Iowa, Iowa City, Iowa, USA; Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, New York, USA; Special Pathogens Program, National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, Manitoba, Canada

ABSTRACT Ebola virus (EBOV) entry requires the virion surface-associated glycoprotein (GP) that is composed of a trimer of heterodimers (GP1/GP2). The GP1 subunit contains two heavily glycosylated domains, the glycan cap and the mucin-like domain (MLD). The glycan cap contains only N-linked glycans, whereas the MLD contains both N- and O-linked glycans. Site-directed mutagenesis was performed on EBOV GP1 to systematically disrupt N-linked glycan sites to gain an understanding of their role in GP structure and function. All 15 N-glycosylation sites of EBOV GP1 could be removed without compromising the expression of GP. The loss of these 15 glycosylation sites significantly enhanced pseudovirion transduction in Vero cells, which correlated with an increase in protease sensitivity. Interestingly, exposing the receptor-binding domain (RBD) by removing the glycan shield did not allow interaction with the endosomal receptor, NPC1, indicating that the glycan cap/MLD domains mask RBD residues required for binding. The effects of the loss of GP1 N-linked glycans on Ca\(^{2+}\)-dependent (C-type) lectin (CLEC)-dependent transduction were complex, and the effect was unique for each of the CLECs tested. Surprisingly, EBOV entry into murine peritoneal macrophages was independent of GP1 N-glycans, suggesting that CLEC-GP1 N-glycan interactions are not required for entry into this important primary cell. Finally, the removal of all GP1 N-glycans outside the MLD enhanced antiserum and antibody sensitivity. In total, our results provide evidence that the conserved N-linked glycans on the EBOV GP1 core protect GP from antibody neutralization despite the negative impact the glycans have on viral entry efficiency.

IMPORTANCE Filoviruses cause outbreaks of severe hemorrhagic fever, with associated fatality rates as high as 90% (1). There are five antigenically distinct species of Ebola viruses (2, 3): Zaire ebolavirus (EBOV), Sudan ebolavirus (SUDV), Bundibugyo ebolavirus (BDBV), Tai Forest ebolavirus (TAFV), and Reston ebolavirus (RESTV) (4). All of these viruses cause disease in humans except RESTV, which is pathogenic in nonhuman primates (1). Currently there are no licensed antiviral therapies or vaccines, and a better understanding of the structure and function of the viral glycoprotein may lead to novel approaches for the development of antivirals.

Filloviruses express a single viral envelope glycoprotein (GP), which is highly glycosylated and forms a trimer of GP1/GP2 heterodimers on the surface of virions (Fig. 1A). The GP1 subunit is required for receptor interactions, and the transmembrane-associated GP2 subunit is required for membrane fusion. GP1 contains four different domains: base, receptor-binding domain (RBD), glycan cap, and mucin-like domain (MLD) (Fig. 1B). The first three domains compose the core of GP1 and are required for the expression and function of the prefusion glycoprotein. EBOV GP with the MLD deleted (GP1Δmuc) has increased protein expression and provides higher viral titers when pseudotyped onto retroviral (5) or vesicular stomatitis virus (VSV) particles (Nicholas J. Lennemann, personal observations). Although Ebola viruses are antigenically distinct, the RBD is highly conserved, with an average of 87% amino acid identity. This conservation suggests an absence of selective pressures driv-
ing diversification of this region of the protein. In contrast, the glycans attached to viral glycoproteins. Glycans can serve as ligands for Ca$^{2+}$-dependent (C-type) lectins (CLECs), facilitating viral attachment and internalization in a variety of cell types (10). Additionally, glycans promote protein folding/stability and virion incorporation of GP, as demonstrated in studies with Newcastle disease virus and Lassa virus (11, 12). In the case of Nipah virus G/F proteins, not only does glycosylation help protein expression, it also decreases membrane fusion efficiency, thereby controlling premature fusion events (13, 14). Furthermore, the glycans on human immunodeficiency virus (HIV) gp120/gp41, Nipah virus G/F, hepatitis C virus E1/E2, and influenza A virus hemagglutinin (HA) protect virions from antibody-mediated neutralization (15). Despite the high degree of glycosylation found on filovirus GPs, the importance of the N-linked glycans on EBOV GP1 to the structure and function of the protein has not been well studied. Here, we assess the structural and functional importance of N-linked glycans on EBOV GP1.

RESULTS
Loss of the N-linked glycan shield does not affect EBOV GP expression but does enhance virion transduction. To determine the role of GP1 N-glycans in EBOV GP-dependent entry, a library of over 40 individual and combinatorial mutations were made to disrupt the N-linked glycan sites (NGS; N-X-S/T sequons) within the GP1 subunit. We generated NGS mutations in GP1 of both GP and the MLD-deleted GP (GP1Δmuc) (for details of all mutations, see Table S1 in the supplemental material). NGS mutants were expressed in HEK293T cells and pseudotyped onto VSVΔG-eGFP (expressing enhanced green fluorescent protein). The relative expression levels of wild-type (WT) or NGS mutant GP were determined by assessing the GP-to-VSV matrix ratios present in cell supernatants by dot blot analysis. In addition, the transduction efficiencies of the pseudovirions were assessed in Vero cells.

Initial studies were performed to disrupt the seven NGS within the GP1 core of GP1Δmuc and GP. Reduced levels of glycosylation on mutant GPs that were pseudotyped onto VSV were apparent in immunoblots, evidenced by faster migration of the mutant GP with immunoblot analysis confirmed that all N-linked glycans were disrupted at the N-linked glycan sites (NGS; N-X-S/T sequons) within the GP1 subunit. (A) A molecular model of EBOV GP1/2 shown in a face-on view. Complex N-glycans are shown in orange, GP is shown in light gray, RBD is shown in red, and MLD structure that has not been solved is represented as a gray sphere. PBD ID 3CSY. (B) Linear model of EBOV GP. The disulfide bond between GP1 and GP2 is indicated, as well as the locations of N-linked glycans (marked with “Ys”) in the GP1 and -2 domains, and the known protease cleavage sites are noted. SP, signal peptide; RBD, receptor-binding domain; MLD, mucin-like domain; IFL, internal fusion loop; HR1 and -2, heptad repeats 1 and 2; TM, transmembrane domain. (C) Alignment of predicted N-linked glycan sites within the GP1 core of the five Ebola virus species. N-X-S/T sequons are highlighted with a black background.
virion transduction was further enhanced by the removal of all NGS within the GP1 core (7G) or the core and MLD (7Gm8G). Consistent with our findings with 7Gm8G, the elimination of N-glycans solely within the MLD (GPM8G) also led to a modest but consistent increase in transduction, suggesting that NGS in both the core and MLD decreased transduction. In total, these results indicated that N-linked glycans are not required for entry into Vero cells but, rather, decrease the efficiency of entry.

**GP1 N-glycans do not affect virion attachment to Vero cells.** As extensive N-linked deglycosylation of GP led to greater transduction, we sought to identify which step(s) in EBOV entry were affected by the loss of N-linked sugars. Removing the GP1 N-glycans within the GP1 core (7G) or throughout GP1 (7Gm8G) did not affect the binding of pseudovirions to the cell surface (see Fig. S3, top, in the supplemental material) and the binding of all virions tested was decreased to similar levels (~50% reduction) in the presence of a calcium chelator, EGTA (Fig. S3, middle). This finding was not unexpected, since EBOV entry into Vero cells has been shown to be mediated by the phosphatidylinositol 4-kinase, TIM-1, which binds to virion-associated phosphatidylinositol in a calcium-dependent manner (16).

**Removal of GP1 N-glycans imparts CatB independence and increases protease sensitivity.** Endosomal processing of EBOV GP by cathepsin B (CatB) is an important step in EBOV entry that results in the removal of both the MLD and glycan cap, exposing the RBD (17–20). Additionally, it has been shown that disruption of the N40 NGS (T42A mutation) leads to CatB independence. Pseudovirus entry mediated by GP1Δmuc, GP, the 6G mutant, containing a fully deglycosylated glycan cap, and the GPM8G mutant containing a MLD that was fully deglycosylated for N-linked glycans were abrogated by treating Vero cells with the CatB inhibitor CA-074, which profoundly blocked CatB activity (Fig. S4A). These data indicated that these GPs were dependent upon CatB processing for subsequent transduction steps (Fig. 3A). VSV pseudotyped with the native glycoprotein (VSV-G) served as a control in these studies and was completely resistant to the drug. As anticipated, the mutant with a mutation of T42 in the absence of the MLD was significantly less sensitive to CA-074.

**FIG 3** Effects of N-glycan removal on entry processes. (A) CatB inhibition assay. Vero cells were transduced with pseudovirions bearing the indicated GPs in the presence of 80 μM CA-074. Data are presented as percentage of cells transduced compared to results for DMSO control. Shown are averages ± SEM of three independent experiments. (B) Thermolysin sensitivity assays. Pseudovirions, normalized for GP expression, were incubated with 1.25 μg/ml of THL and used to transduce Vero cells. Transduction is presented as percentage of transduction observed at 200 μg/ml THL. Data represent the averages ± SEM of three independent experiments, performed with three independent stocks of pseudovirus. (A and B) Significance was determined by Student’s t-test, comparing each mutant to its respective WT control (either GP or GPΔmuc). *, P < 0.01; **, P < 0.001; ***, P < 0.0001. (C) NPC1 C loop binding assay. VSV pseudovirions bearing the indicated GP were bound to an enzyme-linked immunosorbent assay (ELISA) plate and incubated with soluble NPC1 C loop. The amount of bound protein was quantified by ELISA. As a positive control, VSV pseudotyped with GP was treated with THL (GPThr) (200 μg/ml) for 1 h at 37°C prior to incubation with C loop. Graph is representative of two independent experiments.
Removing glycans increased the protease sensitivity of GP. Previous work has shown that treatment with 200 μg/ml thermolysin (THL) results in an ~20-fold increase in GP-mediated transduction associated with complete removal of the glycan cap and MLD (20, 21). To initially assess THL sensitivity, we evaluated the transduction efficiency of GP and 7Gm8G following treatment with increasing concentrations of THL up to a high concentration (200 μg/ml). 7Gm8G was dramatically more sensitive to low concentrations of THL than GP, providing evidence that the deglycosylated GP was more readily proteolytically processed (Fig. 4B). Given these results, a panel of NGS mutants was evaluated for the relationship between entry and sensitivity of deglycosylated GP to low concentrations (1.25 μg/ml) of protease (Fig. 3B). A correlation between the number of mutations and the THL sensitivity was observed. The results from 7G and GPm8G indicated that the removal of glycans from the core has a greater effect on protease sensitivity, possibly due to the large number of O-glycans still present in the MLD. However, 7Gm8G showed the greatest protease sensitivity, suggesting an additive effect when the mutations of 7G and GPm8G were combined. These findings indicated that N-glycans in the glycan cap and, to a lesser extent, in the MLD control the efficiency of proteolytic processing, thereby regulating subsequent steps in entry, such as binding to the endosomal receptor, NPC1.

Removal of glycans shielding the RBD does not allow for NPC1 binding. Enzymatic removal of the glycan cap and MLD allows interaction of GP with the endosomal receptor NPC1 (23, 24). Consequently, we postulated that exposing the RBD by disrupting NGS within the GP1 core and MLD might enhance the binding of GP to NPC1. In one NPC1 binding assay, a soluble form of the second luminal domain (C loop) of NPC1, which directly interacts with residues in the RBD of EBOV GP (23), was used to bind VSV pseudovirions. Treatment of GP with THL, which is commonly used to mimic GP cleavage by CatB/L in removing the MLD and glycan cap (20, 21), resulted in binding of C loop over a wide range of concentrations (Fig. 3C). However, exposure of the RBD through deglycosylation (7Gm8G) did not lead to binding of the NPC1 C loop above the background level, indicating that loss of the N-linked glycans on GP1 does not abrogate the need for endosomal proteolysis that is required for receptor interactions. This result suggests that, while N-glycans on GP may interfere with the ability of the RBD to interact with this endosomal receptor, glycinerference is not the sole explanation for the inability of GP to bind to NPC1.

Domain-specific N-linked deglycosylation alters C-type lectin utilization. CLECs, which mediate GP-dependent entry, are expressed on a wide variety of EBOV target cell types, including macrophages, dendritic cells, and hepatic cells, and each binds to specific glycan moietyes (25–29). To better understand the role of EBOV GP N-linked glycans in CLEC-dependent tropism, we determined the receptor utilization of mutants that lack N-linked glycans on the GP1 core (7G), the MLD (GPm8G), or both (7Gm8G). Constructs expressing Myc-tagged CLECs were transfected into poorly permissive HEK293T cells. To enhance expression, the ectodomains of CLECs DC-SIGN and human MGL (hMGL) were fused to the transmembrane and cytoplasmic domain of L-SIGN (resulting in constructs 1DC-SIGN and hMGL, respectively). All constructs expressed well in transfected cells (Fig. S5).

In these experiments, transfection of a TIM-1-expressing plasmid served as a transduction control, since TIM-1-dependent entry into endosomes is GP independent and should be independent of the GP glycosylation status (16, 30). Consistent with this, the entry of all pseudovirions was similarly enhanced by transfection of TIM-1 (Fig. 4A).

While all five CLECs enhanced the transduction of WT GP between 6- and 13-fold, the impact of the loss of GP1 N-glycans on transduction varied depending on the CLEC examined (Fig. 4B to F). Entry mediated by L-SIGN, 1DC-SIGN, and LSECtin was highly dependent on N-glycans present throughout GP1; however, LSECtin-dependent entry was less dependent on N-glycans present in the GP1 core (Fig. 4B to D). Asialoglycoprotein receptor 1 (ASGPR1)-mediated entry was completely independent of N-glycans in the GP1 core. While the loss of NGS in the MLD resulted in a trend of decreased transduction, this did not achieve statistical significance (Fig. 4E). Furthermore, this CLEC was able...
transduction, with the reduction being more modest for transduction studies to be performed (Fig. S6). All five CLECs enhanced ~3-fold over entry mediated by the WT, which is more consistent with our Vero cell data than with our CLEC utilization studies to be performed (Fig. S6). All five CLECs enhanced entry by 4.5- to 22-fold (Fig. 4B to F). In all cases, the loss of five of the N-linked glycans (5G\textsubscript{muc}) on the glycan cap resulted in a decrease in transduction, with the reduction being more modest for transduction mediated by 1\textsubscript{DC-SIGN}, ASGPR\textsubscript{I}, and 4\textsubscript{hMGL}. As the better transduction of 5G\textsubscript{muc} into cells expressing 1\textsubscript{DC-SIGN}, ASGPR\textsubscript{I}, and 4\textsubscript{hMGL} cannot result from interactions with MLD glycans, this enhancement must be due to either the two remaining intact N-glycans on the GP core at N40 and N204 or the two N-glycans on GP2. Additionally, the differences in 5G\textsubscript{muc}-mediated transduction observed between L-SIGN and 1\textsubscript{DC-SIGN} indicate that these CLECs have different ligand specificities, despite both binding to high-mannose oligosaccharides.

N-linked glycans on GP1 are not required for entry into macrophages. Macrophages are major, early targets during filovirus infections, and others have suggested that CLECs may be important for entry into these cells (31–33). Previous work has shown that murine MGL and SIGNR1, the murine homolog of DC-SIGN, are expressed on peritoneal macrophages (34, 35). We used our most extensively deglycosylated mutants to determine the role of N-linked and O-linked glycans in entry into these cells. Since VSV is highly sensitive to the type I interferon response (36), resident peritoneal cells were isolated from IFNAR\textsuperscript{−/−} mice for these studies. In addition, these mice lacked TIM-1, which eliminated the possibility for entry in epithelial cells that may have contaminated the preparations. Cells were treated for 3 days with murine macrophage colony-stimulating factor (M-CSF) and their phenotypes were determined prior to use. Eighty-five percent of adherent cells from the peritoneal cavity were CD11b\textsuperscript{+/+}/F480\textsuperscript{−/−}, indicative of matured macrophages (see Fig. S7 in the supplemental material).

These cell types were highly permissive for VSV pseudotyped with GP (Fig. 5). Entry mediated by 7Gm8G was enhanced ~3-fold over entry mediated by the WT, which is more consistent with our Vero cell data than with our CLEC utilization data. Furthermore, macrophages supported entry mediated by the 5G\textsubscript{muc} mutation, although this was ~15-fold decreased compared to the results for the WT. These findings demonstrated that neither O- or N-linked glycans on GP1 are absolutely required for entry into peritoneal macrophages.

Removal of N-linked glycans enhances antiserum sensitivity. Our studies indicated that the most deglycosylated EBOV GP1 mutants were expressed at WT levels and provided the highest levels of transduction into Vero cells and peritoneal macrophages. Nonetheless, the conservation of the glycan sites, particularly in the GP1 core sequences, across the species suggested that positive selection for these sites was occurring. Therefore, our deglycosylated EBOV GPs were evaluated for their sensitivity to antiserum neutralization, since deglycosylation may increase the exposure of potential EBOV GP epitopes. Purified anti-EBOV IgG from convalescent cynomolgus macaques that were vaccinated prior to challenge with Ebola virus (a gift from John M. Dye, USAMRIID) was incubated with VSV pseudotyped with the indicated GP. TCID\textsubscript{50} values were calculated from two independent experiments performed at least in quadruplicate and are presented as the averages ± SD.

**DISCUSSION**

EBOV GP1 is highly glycosylated, yet limited studies have investigated the role of these sugar chains on GP function to date. In this report, we determined the effects of removing the N-linked glycans present on the EBOV GP1 subunit by examining the impact on protein expression/stability, viral entry, and antiserum/antibody sensitivity. Surprisingly, the elimination of all N-linked glycans on GP1 had no effect on the expression levels of GP, suggesting that, in the presence of the MLD, N-linked glycans attached to GP1 are not critical for GP folding. However, as our
studies were performed with pseudovirions in tissue culture, it remains possible that these N-linked glycans are critical for GP stability in the context of infectious EBOV virions and/or in vivo. Future studies to test our deglycosylated GPs in the context of recombinant filoviruses to verify our findings are warranted. The loss of GP1 NGS significantly decreased the utilization of four of the five C-type lectins known to be used by EBOV GP for entry, while it enhanced EBOV transduction into Vero cells and peritoneal macrophages. The enhanced levels of transduction were associated with enhanced sensitivity to thermolysin cleavage, but deglycosylation did not alleviate the need for proteolysis prior to NPC1 binding. Finally, the loss of highly conserved glycans on the core of GP1 increased virus sensitivity to antibody neutralization. Therefore, we propose that the strong conservation of individual glycosylation sites in the GP1 core across the Ebola virus genus results, at least in part, from selective pressures to protect the virus against immune responses, despite the fact that these sites collectively can have a negative impact on GP-dependent entry.

Modeling N-linked glycans onto the GP1 muc structure suggests that the highly conserved RBD of Ebola viruses is effectively masked and protected by glycan cap glycans attached to well-conserved NGS, likely protecting the RBD against selective immune pressures, as previously proposed (6, 8, 9, 15, 18, 37). However, we provide the first experimental evidence to support this hypothesis. Our results suggest that there are neutralizing epitopes in the GP1 core that are normally masked by N-glycans. Surprisingly, the removal of N-glycans from the MLD, which is highly targeted by neutralizing antibodies (7–9), did not affect antigenic sensitivity. Neutralizing epitopes within the MLD that do not appear to be affected by the loss of N-linked glycans may be obstructed by the large number of O-glycans attached to the MLD. Alternatively, neutralizing epitopes within the MLD may target O-linked glycan sequons. Since filovirus hemorrhagic fevers are acute and often lethal infections in primates, it is likely that antibody-driven positive selection for NGS surrounding conserved regions occurs in nonprimate reservoirs, such as bats. Thus, it is important to understand filovirus infection and persistence in bat populations.

A role for CLECs in EBOV GP-dependent entry has been firmly established by others (32, 33, 38, 39). We have shown for the first time that the removal of N-glycans from either the glycan cap (7G) or MLD (GPm8G) dramatically decreases the utilization of DC-SIGN, L-SIGN, and LSECtin. Surprisingly, these same CLECs enhanced the transduction mediated by GP1 muc, despite this form of the protein sharing exactly the same pattern of NGS as GPm8G. Another surprising finding was that hMGL utilization was largely unaffected by the loss of GP1 N-glycans. This provided indirect evidence that hMGL-dependent EBOV transduction is strongly dependent on O-linked glycans in GP. However, despite the apparent utilization of O-linked glycans by hMGL, the transduction mediated by GP1 muc was greatly enhanced by the expression of this CLEC (32). In combination, these two sets of results suggest that deletion of the MLD alters the species of glycans present on GP1. Such a conclusion is consistent with previous work showing that glycosylation differences exist between GP and the small, soluble form of GP (sGP), which shares the same NGS with GP1 muc (40). More glycan processing was found on sGP, indicated by the higher percentage of galactose residues and lower percentage of high-mannose glycans. Since the types of glycans present on proteins are largely dependent on the environment in which the protein is produced (41), it is possible that the cytotoxicity associated with overexpression of GP (42, 43) or the large number of O-glycans present changes the glycosylation machinery in the ER/Golgi.

Previous work has shown that macrophages are important cellular targets during the early days of filovirus infection (31). The effective transduction of murine peritoneal macrophages by N-glycan-deficient GP1-bearing pseudovirions, along with our findings that hMGL-dependent enhancement of transduction was relatively independent of the presence of GP1 N-glycans, supports the idea proposed by others (32) that EBOV entry into macrophages may be mediated by this CLEC. However, additional mechanisms of EBOV entry into macrophages are also likely to be important, particularly in light of our findings that 7Gm8G pseudovirions have ~300% better transduction of macrophages than GP, yet 7Gm8G transduces hMGL-expressing cells at ~60% of the transduction level of the WT. We and others have identified that TIM family members mediate virus uptake into cells by interacting with phosphatidylinerine on the surface of virions (16, 30), and it is possible that this uptake mechanism is important for EBOV entry into macrophages. Future studies need to explore the role for phosphatidylinerine receptors during in vivo filovirus infection.
Our findings that deglycosylation of GP pseudovirions enhances the transduction of Vero cells and peritoneal macrophages provides evidence that the N-glycans on GP1 decrease the efficiency of the entry process. The removal of glycans masking the RBD enhanced proteolytic processing of GP but did not result in the ability of unprocessed EBOV GP to bind the C loop of NPC1, consistent with an earlier study demonstrating that the removal of the MLD did not unmask the RBD for NPC1 interaction (23). This result suggests that there are residues that are critical for NPC1 binding, such as F88 (23), that are masked by the glycan cap/MLD polypeptide rather than being concealed by the heavy glycan shield. Thus, it is likely that the increased sensitivity of our deglycosylated mutants to proteolytic processing results in more efficient transit through the endosomal compartments, leading to greater transduction efficiency.

In these studies, we found that protease sensitivity and CatB dependence are independently controlled within GP1. For instance, GPM8G, which retains the N40 glycosylation site, was completely dependent on CatB but had enhanced thermolysin sensitivity. In contrast, the T42V mutant was found to be partially CatB independent but no more sensitive to thermolysin than WT GP. It was previously hypothesized that mutation of residue T42, which eliminates the N-glycan at N40, allows endosomal proteases other than CatB to process GP within the endosome (21). Extrapolating from these studies, we hypothesized that systematic deglycosylation would yield an EBOV GP that was progressively more CatB independent. However, this was not the case; extensive deglycosylation beyond loss of the N40 glycan sequon did not further alleviate dependence on CatB but did increase thermolysin sensitivity. Additionally, mutants with mutations of N40 and T42 were shown to display different protease sensitivities, suggesting that the glycan is not the only factor in determining CatB dependence (21).

Previous work has shown that cellular receptors influence the intracellular trafficking and endosomal fate of viruses (44). EBOV entry requires a specific intracellular compartment that requires CatB, the HOPS complex, and NPC1 (19, 20, 21, 24, 45). However, similar to our CatB-independent EBOV mutants, not all filoviruses require CatB cleavage for entry (46). Therefore, deglycosylated mutants with distinct protease dependence and sensitivity profiles may be useful in the further characterization of the endosomal compartments that allow for the efficient entry of filoviruses.

**Thermolysin sensitivity assay.** VSV pseudovirions, normalized for GP expression, were incubated with 200 μg/ml, 2-fold serial dilutions starting with 5 μg/ml, or a single concentration of 1.25 μg/ml of thermolysin (TLH) at 37°C for 15 min. The reaction mixtures were immediately placed on ice and diluted 20-fold in growth medium containing 50 μM phosphoramidon (Sigma), a THL inhibitor. THL-treated pseudovirions were evaluated for Vero cell transduction. GFP-positive cells were analyzed by flow cytometry.

**Antibody/antiserum neutralization assay.** Serial dilutions of fractionated IgG pooled from 36 convalescent, vaccinated cynomolgus macaques challenged with EBOV (gift from John M. Dye, USAMRIID) or pooled convalescent sera from mice challenged with mouse-adapted EBOV (gift from Gene Olinger, USAMRIID) were incubated at 37°C for 30 min with pseudotyped VSV bearing the glycoproteins studied and normalized for the amount of matrix protein. The mixtures were diluted 5-fold in growth medium and added to Vero cells plated in a 48-well format. GFP-positive cells were enumerated with flow cytometry. The relative antiserum sensitivities were calculated as the reciprocals of the 50% infective concentration (IC50) values determined with GraphPad Prism 5.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00862-13/-/DCSupplemental.

- Table S1, PDF file, 0.1 MB.
- Figure S1, PDF file, 0.4 MB.
- Figure S2, PDF file, 0.2 MB.
- Figure S3, PDF file, 0.1 MB.
- Figure S4, PDF file, 0.1 MB.
- Figure S5, PDF file, 0.1 MB.
- Figure S6, PDF file, 0.1 MB.
- Figure S7, PDF file, 0.1 MB.
- Figure S8, PDF file, 0.1 MB.
- Text S1, DOC file, 0.1 MB.

**ACKNOWLEDGMENTS**

We thank Richard Roller (University of Iowa) and John M. Dye (USAMRIID) for critical evaluation of the manuscript. We would like to thank Erica Saphire and Dennis Burton (Scripps Institute) for providing the KZ25 MAb, Gene Olinger (USAMRIID) for providing the convalescent mouse sera, and John M. Dye (USAMRIID) for providing the cynomolgus macaque anti-EBOV IgG.

The work described in the manuscript was supported by the following NIH/NIAID grants: R01AI077519 (W.M.), T32AI007533 (N.L.), and R01AI101436 (K.C.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**REFERENCES**

1. Feldmann H, Geisbert TW. 2011. Ebola hemorrhagic fever. Lancet 377:849–862. http://dx.doi.org/10.1016/S0140-6736(10)60667-8.
2. Richman DD, Cleveland PH, McCormick JB, Johnson KM. 1983. Antigenic analysis of strains of Ebola virus: identification of two Ebola virus serotypes. J. Infect. Dis. 147:268–271. http://dx.doi.org/10.1093/infdis/147.2.268.
3. Feldmann H, Nichol ST, Klengel HD, Peters CJ, Sanchez A. 1994. Characterization of filoviruses based on differences in structure and antigenicity of the virion glycoprotein. Virology 199:469–473. http://dx.doi.org/10.1006/viro.1994.1147.
4. Kuhn JH, Becker S, Ebihara H, Geisbert TW, Johnson KM, Kawaoka Y, Lipkin WI, Negredo AF, Netesov SV, Nichol ST, Palacios G, Peters CJ, Tenorio A, Volchkov VE, Jahrling PB. 2010. Proposal for a revised naming of the family Filoviridae: classification, names of taxa and viruses, and virus abbreviations. Arch. Virol. 155:2083–2103. http://dx.doi.org/10.1007/s00705-010-0814-x.
5. Jeffers SA, Sanders DA, Sanchez A. 2002. Covalent modifications of the Ebola virus glycoprotein. J. Virol. 76:12463–12472. http://dx.doi.org/10.1128/JVI.76.24.12463-12472.2002.
Wong AC, Sandesara RG, Mulherkar N, Whelan SP, Chandran K.

23. Miller EH, Obernosterer G, Raaben M, Herbert AS, Deffieu MS, Krishnan A, Ndungo E, Sandesara RG, Carette JE, Kuehne AI, Ruthel G, Mbomba Q, Ory D, Chandran K, Cunningham J. 2011. Small molecule inhibitors reveal Niemann-Pick C1 is essential for Ebola virus infection. Nature 477:344–348. http://dx.doi.org/10.1038/nature10380.

24. Croset A, Delafosse L, Gaudry JP, Brechot A, Ory D, Chandran K, Cunningham J. 2011. Small molecule inhibitors reveal Niemann-Pick C1 is essential for Ebola virus infection. Nature 477:344–348. http://dx.doi.org/10.1038/nature10380.

25. Powelson AS, Fisch T, Taylor ME, Smith DF, Tissot B, Dell A, Enborne S, Drickamer K. 2008. A novel mechanism for LSECtin binding to Ebola virus surface glycoprotein through truncated glycans. J. Biol. Chem. 283:593–602.

26. Gao Y, Feinberg H, Conroy E, Mitchell DA, Alvarez R, Blix O, Taylor ME, Weis WI, Drickamer K. 2004. Structural basis for distinct ligand-binding and targeting properties of the receptors DC-SIGN and DC-SIGNR. Nat. Struct. Mol. Biol. 11:591–598. http://dx.doi.org/10.1093/nsr/nwh074.

27. Khoo US, Chan KY, Chan VS, Lin CL. 2008. DC-SIGN and L-SIGN: the SIGNS for infection. J. Mol. Med. 86:861–874. http://dx.doi.org/10.1007/s00109-008-0350-2.

28. Feinberg H, Mitchell DA, Drickamer K, Weis WI. 2001. Structural basis for selective recognition of oligosaccharides by DC-SIGN and DC-SIGNR. Science 294:2163–2166. http://dx.doi.org/10.1126/science.1066371.

29. Meier M, Bider MD, Malashkevich VN, Spiess M, Burkhardt P. 2000. Crystal structure of the carbohydrate recognition domain of the H1 subunit of the asialoglycoprotein receptor. J. Biol. Chem. 300:885–895. http://dx.doi.org/10.1063/1.6100322.

30. Rybchikova EI, Kolesnikova LV, Luchko SV. 1999. An analysis of features of pathogenesis in two animal models of Ebola virus infection. J. Infect. Dis. 179(Suppl 1):S199–S202. http://dx.doi.org/10.1086/514293.

31. Takada A, Fujioka K, Tsuji M, Morikawa H, Higashi N, Ebihara H, Kobasa D, Feldmann H, Irimura T, Kawaoka Y. 2004. Human macrophage C-type lectin specific for galactose and N-acetylgalactosamine promotes filovirus infection. J. Virol. 78:2934–2947. http://dx.doi.org/10.1128/JVI.78.7.2934-2947.2004.

32. Alvarez CP, Lasala F, Carrillo J, Muñiz O, Corbí AL, Delgado R. 2002. C-type lectins DC-SIGN and L-SIGN mediate cellular entry by Ebola virus in cis and in trans. J. Virol. 76:6841–6844. http://dx.doi.org/10.1128/JVI.76.18.6841-6844.2002.

33. Taylor PR, Brown GD, Herre J, Williams DL, Willment JA, Gordon S. 2004. The role of SIGN1R1 and the beta-galactos receptor (dectin-1) in the nonopsonic recognition of yeast by specific macrophages. J. Immunol. 172:1157–1162.

34. Onami TM, Lin MY, Page DM, Reynolds SA, Katayama CD, Marth JD, Ritchie G, Harvey DJ, Stroeher U, Feldmann F, Feldmann H, Wahlberg JH. 2002. DC-SIGN and L-SIGN: the secret life of viral entry glycoproteins: moonlighting in immune evasion. PLoS Pathog. 9e1003258.

35. Müller-Tank S, Kondratowicz AS, Davye RA, Rennert PD, Maury W. 2013. Role of the phosphatidyserine receptor TIM-1 in enveloped virus entry. J. Biol. Chem. 87:8327–8341.

36. Hood CL, Abraham J, Boyington JC, Leung K, Kwong PD, Nabel GJ. 2010. Biochemical and structural characterization of cathepsin L-processed Ebola virus glycoprotein: implications for viral entry and immunogenicity. J. Virol. 84:2972–2982. http://dx.doi.org/10.1128/JVI.01511-09.

37. Dube D, Brecher MB, Delos SE, Rose SC, Park EW, Schornberg KL, Kuhn JH, White JM. 2009. The primed ebolavirus glycoprotein (19-kilodalton GP1,2): sequence and residues critical for host cell binding. J. Virol. 83:2883–2891. http://dx.doi.org/10.1128/JVI.01956-08.

38. Chandran K, Sullivan NJ, Felbor U, Whelan SP, Cunningham JM. 2005. Endosomal proteolysis of the Ebola virus glycoprotein is necessary for infection. Science 308:1643–1645. http://dx.doi.org/10.1126/science.1110656.

39. Schornberg K, Matsuyama S, Kabsch K, Delos S, Bounton A, White J. 2006. Role of endosomal cathepsins in entry mediated by the Ebola virus glycoprotein. J. Virol. 80:4174–4178. http://dx.doi.org/10.1128/JVI.80.11.4174-4178.2006.

40. Wong AC, Sandesara RG, Mulherkar N, Whelan SP, Chandran K. 2010. A forward genetic strategy reveals destabilizing mutations in the Ebola virus glycoprotein that alter its protease dependence during cell entry. J. Virol. 84:163–175. http://dx.doi.org/10.1128/JVI.01832-09.

41. Varki A. 1993. Biological roles of oligosaccharides: all of the theories are correct. Glycobiology 3:297–309. http://dx.doi.org/10.1093/glycob/3.3.297.

42. Miller EH, Obernosterer G, Raaben M, Herbert AS, Defieux MS, Krishnan A, Ndungo E, Sandesara RG, Catterall J, Kuehne AI, Ruthel G, Pfeffer SR, Dye JM, Whelan SP, Brummellkamp TR, Chandran K. 2012. Ebola virus entry requires the host-programmed recognition of an intracellular receptor. EMBO J. 31:1947–1960. http://dx.doi.org/10.1038/embj.2012.53.
45. Krueger RL, Albritton LM. 2013. Characteristics of the cellular receptor influence the intracellular fate and efficiency of virus infection. J. Virol. 87:5916–5925. http://dx.doi.org/10.1128/JVI.00398-13.

46. Misasi J, Chandran K, Yang JY, Considine B, Filone CM, Côté M, Sullivan N, Fabozzi G, Hensley L, Cunningham J. 2012. Filoviruses require endosomal cysteine proteases for entry but exhibit distinct protease preferences. J. Virol. 86:3284–3292. http://dx.doi.org/10.1128/JVI.06346-11.

47. Wang YV, Wade M, Wong E, Li YC, Rodewald LW, Wahl GM. 2007. Quantitative analyses reveal the importance of regulated Hdmx degradation for p53 activation. Proc. Natl. Acad. Sci. U. S. A. 104:12365–12370. http://dx.doi.org/10.1073/pnas.0701497104.