

# Impact of Ebola Mucin-Like Domain on Antiglycoprotein Antibody Responses Induced by Ebola Virus-Like Particles

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**Ebola virus (EBOV) glycoprotein (GP), responsible for mediating host-cell attachment and membrane fusion, contains a heavily glycosylated mucin-like domain hypothesized to shield GP from neutralizing antibodies. To test whether the mucin-like domain inhibits the production and function of anti-GP antibodies, we vaccinated mice with Ebola virus-like particles (VLPs) that express vesicular stomatitis virus G, wild-type EBOV GP (EBGP), EBOV GP without its mucin-like domain ( $\Delta$ MucGP), or EBOV GP with a Crimean–Congo hemorrhagic fever virus mucin-like domain substituted for the EBOV mucin-like domain (CMsubGP). EBGP-VLP immunized mice elicited significantly higher serum antibody titers toward EBGP or its mutants, as detected by western blot analysis, than did VLP- $\Delta$ MucGP. However, EBGP-,  $\Delta$ MucGP- and CMsubGP-VLP immunized mouse sera contained antibodies that bound to cell surface-expressed GP at similar levels. Furthermore, low but similar neutralizing antibody titers, measured against a vesicular stomatitis virus (VSV) expressing EBGP or  $\Delta$ MucGP, were present in EBGP,  $\Delta$ MucGP, and CMsubGP sera, although a slightly higher neutralizing titer (2- to 2.5-fold) was detected in  $\Delta$ MucGP sera. We conclude that the EBOV GP mucin-like domain can increase relative anti-GP titers, however these titers appear to be directed, at least partly, to denatured GP. Furthermore, removing the mucin-like domain from immunizing VLPs has modest impact on neutralizing antibody titers in serum.**

Ebola viruses (EBOVs) are enveloped, single-stranded, negative-sense RNA viruses belonging to the family *Filoviridae* that cause a hemorrhagic fever in humans, with a mortality rate of up to 90%. EBOV attachment and entry into host cells is mediated by the viral envelope glycoprotein (GP) [1–3]. The membrane-anchored GP is a trimer. GP is first produced as a 676-residue-long precursor, GP0, which is cleaved by furin into disulfide-bonded GP1 and GP2. These GP1/GP2 heterodimers assemble into a chalice-shaped trimer that is

expressed on the virion surface [4]. GP1 is distal to the membrane surface and contains the receptor-binding domain (N-terminal residues 54–201) and a heavily glycosylated mucin-like domain. GP2 possesses a trans-membrane domain, the fusion peptide, and heptad repeats required for virus–cell membrane fusion [5, 6].

When expressed from 293T cells, the EBOV matrix protein, virion protein 40 (VP40), induces the production of virus-like particles (VLPs) that are biochemically and morphologically similar to EBOV. If coexpressed with VP40, GP becomes incorporated into the VLPs and can mediate entry into target cells [7, 8]. GP-mediated entry likely occurs via macropinocytosis [9, 10], although other endocytic pathways have also been implicated in entry [9–14]. For productive entry, GP is cleaved by cellular cathepsins B and L in acid endosomes such that a substantial portion of the protein is removed and the remaining approximately 19 kDa cleavage product is sufficient to mediate membrane fusion reactions [15–19].

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Among the regions of GP removed by cathepsin cleavage is the highly glycosylated mucin-like domain. The mucin-like domain is not required for viral entry, because mucin-domain-deleted GP is able to mediate viral attachment and entry in pseudotyped virus systems. EBOV GP interacts with lectin-binding receptors present on some cell types, including antigen-presenting cells, thereby promoting virus attachment and entry [20–24]. The mucin-like domain has specifically been shown to be important for interaction of GP with the human macrophage galactose-specific and N-acetylgalactosamine-specific C-type lectin (hMGL), promoting EBOV infection [25]. In addition, it may serve immune-modulating functions. For example, its presence is correlated with an ability to alter cellular signaling, including mitogen-activated protein kinase signaling [26, 27] and has been shown to enhance Ebola VLP-mediated cytokine secretion from stimulated dendritic cells [27]. Furthermore, VLPs with wild-type but not mucin domain-deleted GP can activate toll-like receptor 4-dependent responses [28].

The GP mucin-like domain obstructs access to GP epitopes and epitopes expressed on other surface proteins [29]. For example, expression of high levels of GP [30] blocks access to surface major histocompatibility complex class 1 molecules (MHC1) and  $\beta$ -integrins [29], resulting in loss of specific anti-MHC1 or  $\beta$ -integrin antibody binding, decreased CD8 T cell access to MHC1 [31] and induction of cell rounding as a consequence of anchorage loss [32]. Furthermore, removal of the mucin-like domain with cathepsin L uncovers epitopes for neutralizing antibodies [16, 33]. However, a mouse model of EBOV infection has shown that immunization of mice with a GP-expressing vaccine elicited mucin domain-specific monoclonal antibodies, some of which were protective [34].

The contribution of antibody responses to EBOV infection and its role in protective immunization remain unclear [35]. Studies have demonstrated the presence of EBOV-neutralizing antibodies in the sera of human survivors of infection [33, 36]. However, passive transfer of neutralizing anti-EBOV antibodies has shown mixed efficacy, failing to be protective in the more relevant nonhuman primate models of EBOV infection but exhibiting some efficacy in rodent models. Furthermore, there is also evidence of enhanced EBOV binding to target cells in the presence of anti-EBOV antibodies [37–39].

EBOV VLPs are immunogenic and have been used to vaccinate and protect mice and nonhuman primates in experimental animal models of EBOV infection [40, 41]. This immunogenicity is due, at least in part, to the mucin domain of the GP, yet studies have shown that the mucin-like domain itself may block access to the GP and adjacent immune molecules potentially inhibiting immune responses, including neutralizing antibody, raising the question of whether vaccines would be more efficacious with or without the GP mucin-like domain. Therefore, to test the hypothesis that the mucin-like domain modulates antibody responses to GP, including neutralization, mice were

immunized with VLPs expressing EBOV wild-type GP, GP lacking the mucin-like domain, and a GP expressing a heterologous mucin-like domain; the ensuing antibody response was assessed.

## METHODS

### Cell Culture and VLP Plasmids

CHO-K1 (generously provided by Dr. Matthew Evans (Mount Sinai School of Medicine) and HEK 293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and 1% L-Glutamine. Plasmids used in transfection were pCAGGS containing a Zaire EBOV VP40 gene. GP expression plasmids were in pcDNA with inserts expressing 1 of the following: wild-type Zaire EBOV strain Mayinga GP (EBGP), EBOV GP with a deletion of the mucin-like domain ( $\Delta$ 308–477,  $\Delta$ MucGP), vesicular stomatitis virus (VSV) GP (VSV-G), or EBOV GP with a Crimean-Congo hemorrhagic fever virus mucin domain substituted for the EBOV mucin-like domain (CMSubGP).

### VLP Production and Purification

To produce VLPs, 293T cells were cotransfected with 2 plasmids: pCAGGS EBOV VP40, and a pcDNA plasmid expressing EBGP,  $\Delta$ MucGP, VSV-G, or CMSubGP, using Lipofectamine 2000. VLP were harvested 3 days posttransfection; the supernatant was layered over 10 mL of 20% sucrose in NTE buffer (100 mM NaCl, 20 mM Tris-hydrochloride, and 1 mM ethylenediaminetetraacetic acid [EDTA]). VLP were pelleted at 25,000 rpm ( $\sim$ 80,000  $\times$  g) for 2 hours at 4°C, then washed with 25 mL NTE and centrifuged again under the same conditions. VLP were resuspended in a total of 150  $\mu$ L NTE and stored on ice at 4°C.

### VLP Entry Assays

VLP entry assays were performed as outlined in a previous study [8].  $\beta$ -lactamase equivalents of different beta-lactamase-VP40 containing purified VLPs were used in each entry assay. VLPs were "spinoculated" onto human dendritic cells at 2000 rpm for 45 minutes at 4°C before incubation at 37°C in 1% Roswell Park Memorial Institute medium (RPMI) for 3.5 hours. CCF2-AM substrate was then loaded onto cells for 1 hour at room temperature. A violet laser from an LSR II (BD Bioscience) was used to excite the CCF2-AM substrate and events (cells) were assayed for green and blue fluorescence. Those cells fluorescing blue as compared with control (for example, no VLP control, not shown) were infected with VLPs.

### VLP Protein Assays

VLP protein content was quantified using the DC protein assay (Bio-Rad). Equivalent amounts of purified VLPs were boiled in 1X sample buffer and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using PAGER

Gold precast 4%–20% glycine gradient gels (Lonza). These gels were then either stained with GelCode Blue Staining Reagent (Thermo) or transferred to polyvinylidene fluoride (PVDF) membranes (Roche) and subjected to western blot analysis. All membranes were blocked with 2% skim milk in phosphate-buffered saline (PBS), all antibodies were diluted in 1% skim milk in PBS, and all blots were washed with 0.05% Tween 20 in PBS. Specificity was assessed using 9c11 [27], a mouse monoclonal  $\alpha$ -EBOV mucin-like domain antibody, a rabbit polyclonal  $\alpha$ EBGP targeted to the N-terminus of EBOV GP [8], or a mouse monoclonal  $\alpha$ VSVG antibody (AbCam). Antibody binding was detected using Pierce enhanced chemiluminescence (ECL) western blotting substrate (Thermo). Relative band density was determined using ImageJ image processing software (National Institutes of Health).

### Mouse Immunization and Serum Harvesting

Mice ( $n = 5$ ) were immunized with 10  $\mu$ g of each VLP (EBGP,  $\Delta$ MucGP, VSV-G, and CmsubGP) in 100  $\mu$ L of PBS, or 100  $\mu$ L of PBS only as a control. Blood was collected from the tail vein once prior to immunization, 3 weeks after the first immunization, and then 2 weeks after each subsequent injection (see Figure 2). Sera were isolated by allowing blood to coagulate at room temperature for 3 hours, then subjecting it to centrifugation at  $500 \times g$  for 10 min. Individual sera were stored at  $-20^{\circ}\text{C}$  and pooled prior to use. The extended time gap between the 4th and 5th bleeds reflected a need to restock serum that was depleted during preliminary experiments. All animal procedures were performed in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines and have been approved by the IACUC of Mount Sinai School of Medicine.

### Titration of Immunized Mouse Sera for Western Blot

Each of the 4 VLP preparations were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a PVDF membrane, and blocked using the same protocol as described under VLP Protein Assays section. Varying concentrations of sera were diluted in 1% skim milk in PBS, and binding detected with HRPO-conjugated goat  $\alpha$ Mouse immunoglobulin G (IgG) and Pierce enhanced chemiluminescence (ECL) Western blotting substrate.

### Surface-GP Flow Cytometric Assays

CHO-K1 cells were transfected with pcDNA plasmids expressing EBGP,  $\Delta$ MucGP, VSV-G, or CmsubGP using FuGene HD Transfection reagent (Roche) and pCAGGS containing green fluorescent protein (GFP). Transfection was performed using 8  $\mu$ L of Fugene HD to 1.5  $\mu$ g of GP plasmid and 0.5  $\mu$ g pCAGGS-GFP (2  $\mu$ g total plasmid). Cells were harvested 72 hours post-transfection and aliquoted into 96-well round-bottom well plates at 200,000 cells per well, pelleted and washed. Immunized mouse sera from the final bleed were heat-inactivated at  $50^{\circ}\text{C}$  for 15 minutes and added to each well at a dilution of 1:120 in 50

$\mu$ L PBS with 2% heat-inactivated fetal calf serum (PBS-FCS) and incubated on ice for 2 hours. Cells were washed twice with phosphate-buffered saline–fetal calf serum (PBS-FCS) and stained with PE-conjugated  $\alpha$ -mouse immunoglobulin G (IgG) for 1 hour on ice. After an additional wash with PBS-FCS, cells were assayed by flow cytometry for PE fluorescence gated on cells that were positive for GFP fluorescence.

### Neutralization Assays

Recombinant VSVs (rVSVs) encoding GFP and either an EBOV GP (rVSV-EBGP) or an EBOV GP with its mucin-like domain deleted (rVSV-EBOV  $\Delta$ MucGP) were used for all neutralization experiments [42]. EBGP,  $\Delta$ MucGP, CmsubGP and PBS sera were serially diluted using Hank's balanced salt solution beginning at 1:5 or 1:10 and diluted from 2- to 2.5-fold up to approximately 1:400. Sera were incubated at room temperature for 1 hour with either rVSV-EBGP or rVSV-EBOV  $\Delta$ MucGP before adding to Vero cells in replicate wells of a 96-well culture plate. Vero cells were infected with 60  $\mu$ L of the sera and virus mixture for 45 minutes, washed, and replenished with 5% DMEM. At 48–72 hours postinfection, Vero cells were examined for cytopathic effect (CPE) and GFP fluorescence. GFP fluorescence was assayed using a fluorescence plate reader. The average GFP fluorescence of triplicate wells were compared with PBS-serum virus mixture. Those averaged wells with decreased GFP fluorescence and CPE, compared with PBS sera-virus mixture, were deemed to contain neutralizing activity. Endpoints of serum-neutralizing activity are shown in Table 1. The neutralization assay shown in Table 1 was performed 3 times with similar results.

## RESULTS

VLPs were generated by cotransfecting 293T cells with expression plasmids for VP40 and 1 of 4 glycoproteins; VSV-G, EBGP, Zaire EBOV with a deleted mucin-like domain ( $\Delta$ MucGP), or Zaire EBOV in which the mucin-like domain is substituted with a Crimean-Congo hemorrhagic fever virus mucin-like domain

**Table 1. Pseudotyped Vesicular Stomatitis Virus–Neutralizing Sera Titers**

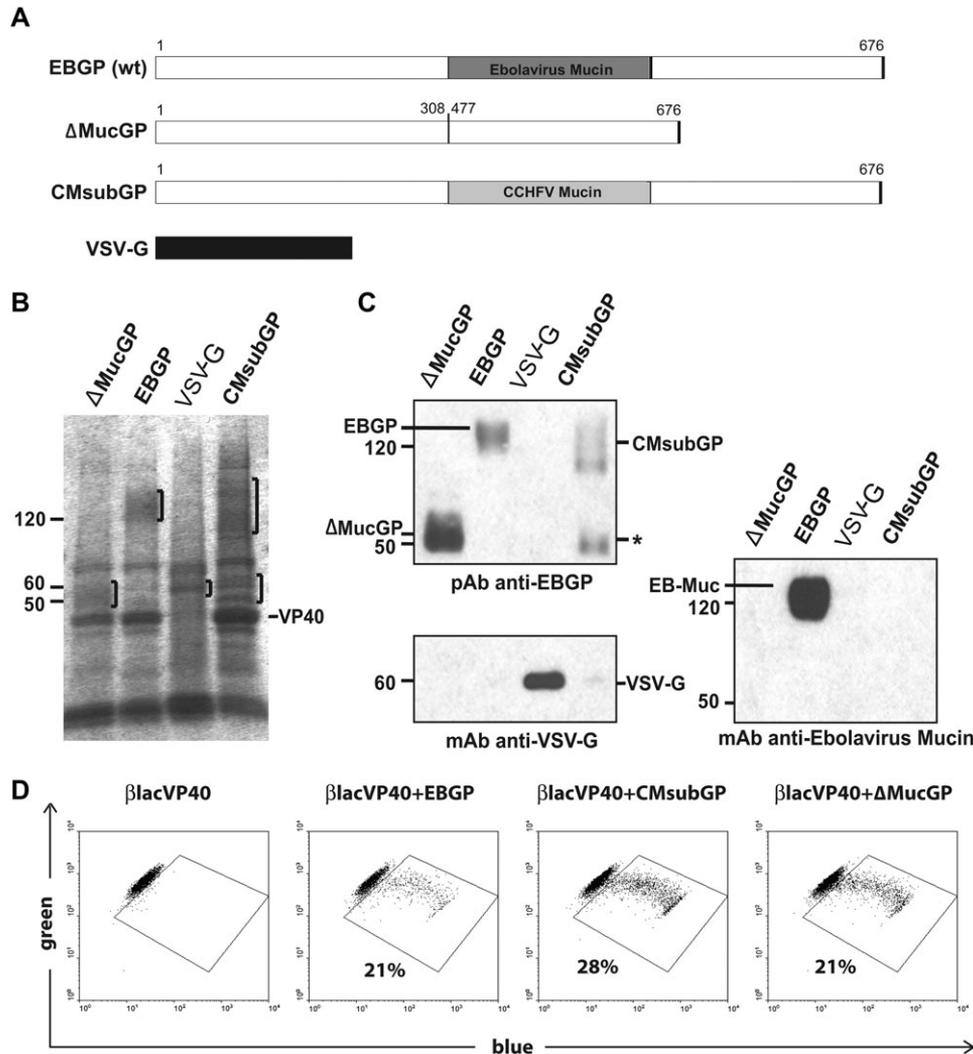
Sera	rVSV- EBGP <sup>a</sup>	rVSV- $\Delta$ MucGP <sup>a</sup>
PBS	...	...
EBGP	10	10
$\Delta$ MucGP	20	25
CmsubGP	10	10

**NOTE.** rVSV, recombinant vesicular stomatitis virus; EBGP, Ebola virus glycoprotein;  $\Delta$ MucGP, Ebola virus glycoprotein without its mucin-like domain; PBS, phosphate-buffered saline; CmsubGP, Ebola virus glycoprotein with a Crimean–Congo hemorrhagic fever virus mucin-like domain substituted for the EBOV mucin-like domain.

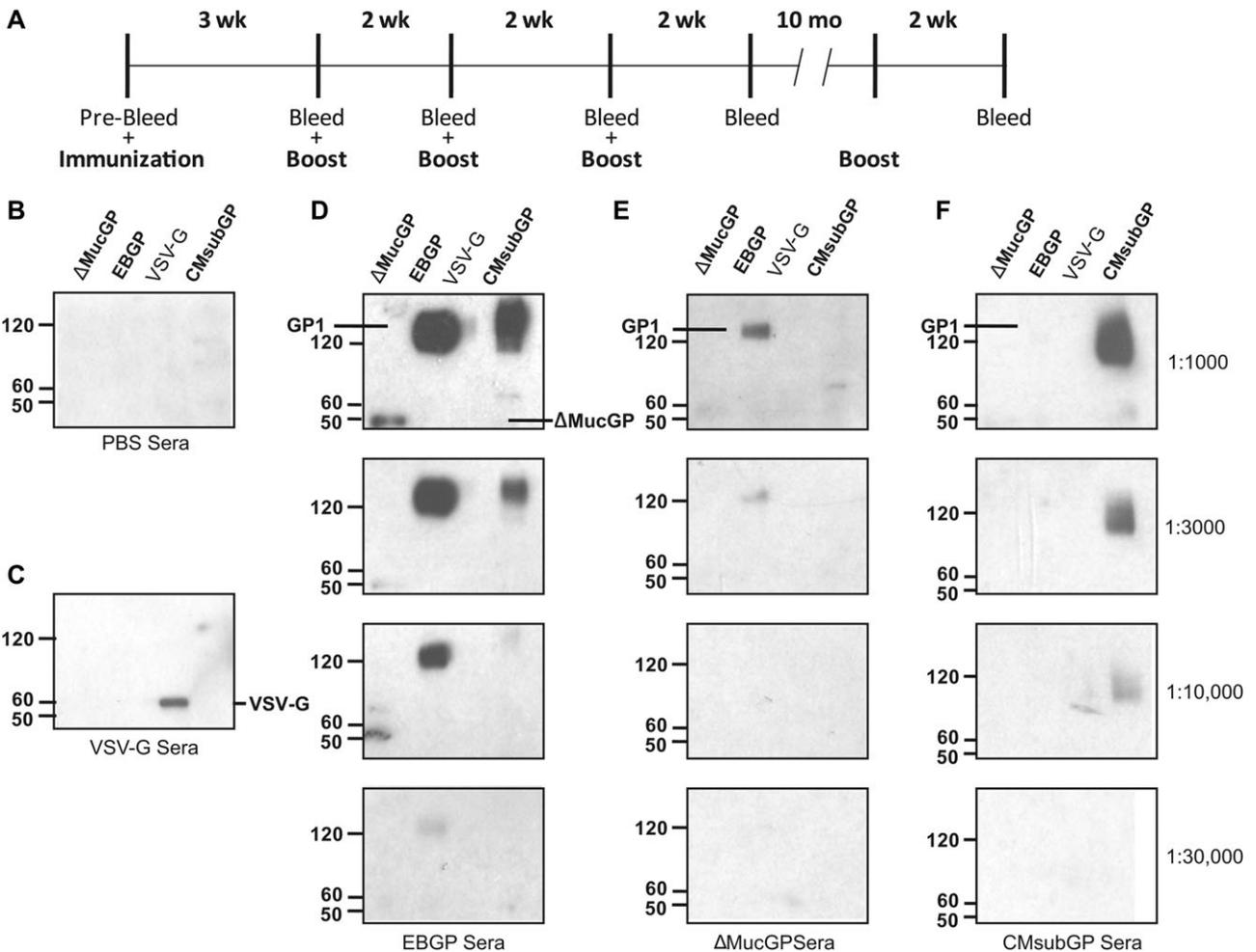
<sup>a</sup> Endpoint neutralizing titer ( $10^{-1}$ ) mL.

(CMSubGP) (Figure 1A). The Crimean-Congo hemorrhagic fever virus (CCHFV), a tick-borne bunyavirus of the *Nairovirus* genus, also expresses a glycoprotein possessing a mucin-like domain [43]. To assess total protein content and GP incorporation, purified VLPs were analyzed by SDS-PAGE and western blot (Figure 1). Total protein staining shows the presence of VP40 at 40 kDa for all VLP preparations except VSV-G. The latter observation likely reflects the ability of VSV-G to bud independently of a viral matrix protein [44]. Total protein staining also shows larger bands corresponding to  $\Delta$ MucGP at

50 kDa, EBGP at  $\sim$ 130 kDa, VSV-G at 60 kDa, and CMSubGP with a large smear that centers at 120 kDa (Figure 1B). VLP preparations were western blotted with polyclonal anti-EBOV GP antibody, monoclonal 9c11 anti-EBOV GP antibody [27], or monoclonal anti-VSV-G antibody to detect EBOV GPs, EBOV mucin-like domain and VSV-G, respectively. Anti-VSV-G antibody yielded a band at  $\sim$ 60 kDa, consistent with the expected molecular weight of VSV-G. Anti-EBOV GP polyclonal antibody bound to  $\Delta$ MucGP at 50 kDa, EBGP at approximately 130 kDa, and CMSubGP at 130 and 40 kDa. The 9c11 monoclonal



**Figure 1.** Production of virus-like particles. (A) Produced virus-like particles (VLPs) expressed 1 of 4 different glycoproteins (GPs): wild-type Ebola virus GP (EBGP), Ebola virus GP with its mucin-like domain removed ( $\Delta$ MucGP), Ebola virus GP with its mucin-like domain replaced with Crimean-Congo hemorrhagic fever virus mucin-like domain (CMSubGP), and vesicular stomatitis virus GP (VSV-G). Spent supernatant from virion protein 40 (VP40) and EBOV GP plasmid transfected cells were purified, and (B) the GP were identified by size by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and GelCode Blue staining, and (C) antibody specificity with Western blot analysis. VP40 is detectable by total protein stain on  $\Delta$ MucGP, EBGP, and CMSubGP VLP, but not VSV-G VLP. Significant bands present in GelCode Blue stained total protein gels are denoted by brackets, which correspond to the  $\Delta$ mucin GP1 subunit at  $\sim$ 60kDa, wild-type Ebola virus GP1 at  $\sim$ 130 kDa, and CMSubGP smear at  $\sim$ 120 kDa. Polyclonal rabbit  $\alpha$ N-terminus of EBGP antibody was used to stain GP1. VSV-G is present on the stained protein gel at 60 kDa, and is bound by monoclonal  $\alpha$ VSV-G antibody. The 50k Da band detected in the CMSubGP lane (\*) is likely to be a degradation product. Anti-EBOV mucin-like domain antibody (9c11) was used to stain EBOV mucin-like domain. (D) Beta-lactamase equivalents of purified beta-lactamase VP40 (BLAVP40), BLAVP40 + EBGP, BLAVP40 + CMSubGP, and BLAVP40 +  $\Delta$ MucGP VLPs were tested for their ability to infect dendritic cells. The region shows the percentage of cells infected.

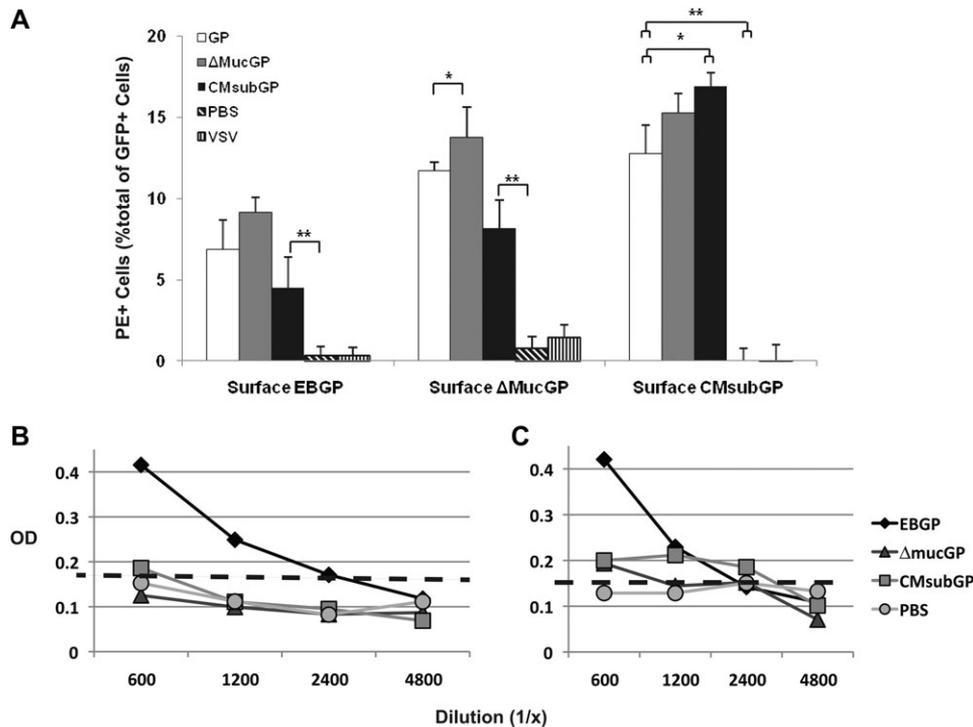


**Figure 2.** Antibody titration of virion protein 40 glycoprotein-immunized mouse sera by Western blot. *A*, Mice were immunized with virus-like particles (VLPs) according to the schedule shown, and sera from the final bleed were used in experiments as shown. *B*, Sera from mice treated with phosphate-buffered saline (PBS sera) and (*C*) sera from mice immunized with vesicular stomatitis virus glycoproteins (VSV-G) VLPs (VSV-G Sera) blots show no binding to any of the 3 Ebola virus glycoprotein (EBGP) constructs at dilutions of 1:250. *D*, EBGP sera, (*E*) Ebola virus glycoprotein without its mucin-like domain ( $\Delta$ MucGP) sera, and (*F*) Ebola virus glycoprotein with a Crimean–Congo hemorrhagic fever virus mucin-like domain substituted for the EBOV mucin-like domain (CMsubGP) sera were diluted 1:1000 to 1:30,000 and used to stain Western blots of VLPs.

anti-EBOV mucin domain antibody bound EBGP GP1, but not  $\Delta$ MucGP or CMsubGP.

We next tested whether each of the mutant EBOV GPs was functional in an entry assay. VLPs were again produced by co-transfecting GP with the EBOV matrix protein, however, the VP40 matrix protein was fused to beta-lactamase. This chimeric beta-lactamase-VP40 protein has been previously used in entry assays where infected cells are identified by the presence of intracellular beta-lactamase enzyme activity [8, 13, 45, 46]. Target cells are initially loaded with a fluorescent substrate that normally fluoresces green, but when cleaved by beta-lactamase then fluoresces blue, which can be assayed with a flow cytometer [8]. As shown in Figure 1*D*, VLPs that do not incorporate a GP (lacVP40), cannot enter target dendritic cells, however wild-type GP and each of its mutants CMsubGP, and  $\Delta$ MucGP mediated significant levels (>20%) of entry into dendritic cells.

Mice were then immunized with 10 $\mu$ g of these VLP preparations in 100  $\mu$ L of sterile PBS a total of 5 times intraperitoneally. 2 weeks after the final immunization, sera harvested from 5 mice from each of the VLP vaccinated groups was pooled and used to stain western blots of VP40-GP VLPs (Figure 2). Sera from mice immunized with PBS (PBS sera) did not exhibit binding to any of the 4 GP variants, whereas sera from mice immunized with VSV-G particles (VSV-G sera), at a dilution of 1:250, detectably reacted with VSV-G, but not other EBOV GP-derived glycoproteins (Figures 2*B* and 2*C*). Titration with EBGP mouse sera showed binding to EBGP,  $\Delta$ MucGP, and CMsubGP GPs at titers of 1:10 000, 1:3000 and 1:3000, respectively (Figure 2*D*).  $\Delta$ MucGP sera only reacted with EBGP and  $\Delta$ MucGP, with detectable binding at the 1:3000 and 1:1000 dilutions, respectively (Figure 2*E*). CMsubGP sera bound only to the CMsubGP up to a dilution of 1:10 000 (Figure 2*F*). In



**Figure 3.** Serum antibody binding against cell surface-expressed glycoproteins. (A) CHO-K1 cells were cotransfected with green fluorescent protein (GFP) and limiting amounts of Ebola virus glycoprotein (EBGP), Ebola virus glycoprotein without its mucin-like domain ( $\Delta$ MucGP), or Ebola virus glycoprotein with a Crimean-Congo hemorrhagic fever virus mucin-like domain substituted for the EBOV mucin-like domain (CMsubGP) expression plasmids and approximately 48 h later were harvested, incubated with mouse sera, and stained with phycoerythrin-conjugated donkey  $\alpha$ Mouse immunoglobulin G. Antibody binding is shown as a percentage of phycoerythrin-positive cells, initially gated on GFP-positive cells by flow cytometry. Binding that is significantly different by 1-tailed *t* test is indicated by brackets labeled with either 2 asterisks (\*\*) for  $P < .01$  or 1 asterisk (\*) for  $P < .05$ . Enzyme-linked immunosorbent assay (ELISA) performed with EBGP,  $\Delta$ MucGP, CMsubGP and phosphate-buffered saline (PBS) sera at indicated dilutions. ELISA plates were coated with either (B) recombinant vesicular stomatitis virus (rVSV)-EBGP or (C) rVSV- $\Delta$ MucGP. The hatched line denotes the average optical density (OD) + 2 standard deviation calculated using the PBS sera binding.

summary, Western blot analysis using VLP-immunized mouse sera revealed that EBGP sera showed robust binding to EBGP, CMsubGP, and  $\Delta$ MucGP, as compared with  $\Delta$ MucGP sera, and the CMsubGP sera showed binding only to CMsubGP.

To determine binding of sera antibody to nondenatured GP, CHO-K1 cells were cotransfected with a GFP expression plasmid and limiting amounts of 1 of the 4 glycoproteins (EBGP,  $\Delta$ MucGP, VSV-G, or CMsubGP). The cells were collected 3 days posttransfection, incubated with a titration of immunized mouse sera (1/120, 1/180, 1/240, 1/300, 1/360) and stained with phycoerythrin (PE)-conjugated anti-mouse IgG. GFP-positive cells were then analyzed by flow cytometry to determine the percentage of GFP-positive cells that also stained positively for PE (indicating binding of IgG). Regardless of sera dilution, EBGP,  $\Delta$ MucGP, and CMsubGP sera exhibited similar levels of binding to surface EBGP,  $\Delta$ MucGP and CMsubGP, binding to ~5%–10% of cells expressing EBGP, ~10%–15% of cells expressing  $\Delta$ MucGP, and ~17%–22% of cells expressing CMsubGP. Surface sera binding at the 1:180 dilution is shown as a representative graph (Figure 3A). VSV and PBS sera exhibited little binding to any of the 3 surface GPs tested.

To further investigate relative EBGP,  $\Delta$ MucGP, and CMsubGP sera binding to EBGP and  $\Delta$ MucGP, an ELISA was performed using plates coated with rVSVs encoding GFP and either an EBOV GP (rVSV-EBGP, Figure 3B) or an EBOV GP with its mucin-like domain deleted (rVSV-EBOV  $\Delta$ MucGP, Figure 3C) [42]. A titration of sera demonstrates that EBGP-sera binds both viruses to a significantly higher level than either  $\Delta$ MucGP or CMsubGP sera (see Figures 3B and 3C).

Neutralization assays were then performed using the same rVSV-EBGP and rVSV-EBOV  $\Delta$ MucGP viruses used for the ELISA. Serial dilutions were performed with EBGP,  $\Delta$ MucGP, CMsubGP and PBS sera and then incubated with either rVSV-EBGP or rVSV-EBOV  $\Delta$ MucGP (moi of 0.01) for 1 hour before infection of Vero cells. At 48–72 hours postinfection Vero cells were examined for cytopathic effect (CPE) and GFP fluorescence. All PBS sera dilutions tested had no effect Vero cell infection. The final dilution at which EBGP,  $\Delta$ MucGP or CMsubGP sera demonstrated a difference as compared with PBS control is shown in Table 1. Modest neutralization activity was seen from EBGP,  $\Delta$ MucGP, CMsubGP sera at a dilution of at least 1:10. There was little difference in the neutralizing activity

of any of the sera, however there was a modest increase in the ability of  $\Delta$ MucGP sera to inhibit rVSV-EBOV  $\Delta$ MucGP and rVSV-EBGP as compared with other sera.

## DISCUSSION

As previously demonstrated wild-type and  $\Delta$ MucGP are readily incorporated into VP40-induced VLPs [8]. Interestingly, we find that we can readily substitute a foreign mucin-like domain from the CCHFV for the EBOV mucin-like domain and still obtain a glycoprotein that is efficiently incorporated into VLPs and can mediate infection into dendritic cells (Figure 1). Furthermore, a strong antibody response was elicited against CSubGP, presumably targeting its own immunogenic mucin-like domain (Figure 2). Therefore, generation of VLPs with the insertion of foreign protein sequences replacing the EBOV mucin-like domain may be a vaccine strategy worth further exploration.

We immunized mice multiple times with each VLP preparation to elicit neutralizing titers. The neutralizing activity induced was modest at best (Table 1). In a previous study, mice immunized three times with 10  $\mu$ g of VLPs induced modestly higher neutralizing antibodies, showing some neutralizing activity in sera diluted at 1:160 and up to 90% at a 1:40 dilution [41]. The reason for the discrepancy is unclear. In our study, C57BL6 mice were immunized, whereas the previous study performed immunizations in BALB/c mice. Furthermore, we used rVSV expressing either  $\Delta$ MucGP or EBGP virus to test neutralization, whereas the previous study made use of EBOV.

The modestly higher anti-rVSV  $\Delta$ MucGP neutralizing titer exhibited by  $\Delta$ MucGP sera compared with EBGP sera (Table 1) was in contrast to the significantly higher anti-GP antibodies titers demonstrated by EBGP, compared with that of  $\Delta$ MucGP sera in western blots (Figure 2) and in an ELISA (Figures 3B and 3C). The mechanism underlying the mucin-like domain induction of higher titers against presumably at least partly denatured GP remains undefined. We have not tested stability of rVSV when plated for ELISA, and it is possible that the plated rVSV may represent a mixture of denatured and nondenatured antigens. Regardless, these higher titers against GP in the context of a western blot or as expressed from rVSV did not result in higher neutralizing activity compared with that of  $\Delta$ MucGP or CSubGP. Given the large difference between Western blot and the neutralizing titers in EBGP sera against EBGP and its mutants, it is possible that the “neutralizing” activity of the sera may just be due to heavy virus coating with antibody or antibody-induced virus aggregation. Taken together, our data demonstrating antibody binding to cell surface-expressed EBGP and its variants (Table 1 and Figure 3A) suggests the presence of antibodies that are not specific to the mucin-like domain. In the context of the western blot data (Figure 2), these antibodies may favor conformational epitopes. The disulfide linkage between GP1 and GP2 may be an important determinant in the conformation or

stability of GP; therefore, future experiments using disulfide bond mutants may be used to further address conformation requirements for the production of efficacious neutralizing antibodies. It will be interesting to determine in future experiments whether these higher titers can impact other non-neutralizing mechanisms of viral clearance or infection.

Further experiments are required to specifically address the efficacy of vaccines that have had the mucin-like domain removed from GP. For example, does removal of the mucin-like domain affect T-cell function and how do vaccinees receiving either the wild-type GP or  $\Delta$ MucGP compare in their ability to neutralize an in vivo EBOV infection?

These data highlight the fact that much of the antibody response to EBOV GP, at least as elicited by VLPs in this system, is nonneutralizing. Removal of the mucin-like domain does not substantially alter the neutralizing antibody response.

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