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Regulation of Ebolavirus Surface Glycoprotein Expression and its Role in Infection and Host Immune Evasion

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Abstract

The Ebola virus (EBOV) is the etiologic agent of Ebola Hemorrhagic Fever (EHF), a highly lethal disease with up to 90% mortality. There are currently no approved vaccines or antivirals for treatment of EBOV infection. While great strides have been made over the last two decades, our ability to treat and prevent EBOV hinges on expanding our understanding of EBOV biology and the mechanisms by which the virus induces such severe disease while evading host immunity. The EBOV surface glycoprotein ($GP_{1,2}$), which mediates host cell attachment and fusion and is also the primary target of host antibodies, is a central player in the pathogenesis of EHF. The work presented in this dissertation examines regulation of glycoprotein expression by EBOV as it pertains to host immune evasion and virus infectivity – two primary determinants of viral fitness.

In Chapter 2, we examine the role of the EBOV secreted glycoprotein (sGP) in altering the host antibody response. EBOV uses an RNA editing mechanism to generate two primary isoforms of glycoprotein (GP). Only 20% of GP transcripts encode the surface glycoprotein $GP_{1,2}$, while 80% encode a truncated glycoprotein named sGP, because it is secreted in large quantities by EBOV-infected cells. Many pathogens generate secreted antigens as a mechanism of host immune evasion, since these antigens can absorb antibodies that would otherwise target the pathogen itself. However, such activity has never been conclusively determined for EBOV sGP. Here, we demonstrate that sGP can efficiently compete for anti-$GP_{1,2}$ antibodies, but only from hosts that have been previously exposed to sGP. We term this phenomenon "antigenic subversion", and propose a model whereby sGP diverts the host antibody response to focus on epitopes which it shares with membrane-bound $GP_{1,2}$, thereby allowing it to absorb anti-$GP_{1,2}$ antibodies. Unexpectedly, we found that sGP can also subvert a previously immunized host's anti-$GP_{1,2}$ response resulting in strong cross-reactivity with sGP. This finding is particularly relevant to EBOV vaccinology since it underscores the importance of eliciting robust immunity that is sufficient to rapidly clear an infection before antigenic subversion can occur. Antigenic subversion represents a novel virus escape strategy that likely helps EBOV evade host immunity, and may represent an important obstacle to EBOV vaccine design.

In Chapter 3, we examine the effect of $GP_{1,2}$ expression levels on production and infectivity of virus. We demonstrate that high levels of $GP_{1,2}$ expression impair the production and release of EBOV virus-like particles, as well as the infectivity of $GP_{1,2}$-pseudotyped viruses. Importantly, we show that this pattern holds true for $GP_{1,2}$ from different filoviruses but not for the HIV Env. We further found that these effects are mediated primarily by two mechanisms. First, high levels of $GP_{1,2}$ expression impair synthesis of other virus proteins. Second, viruses that contain high levels of $GP_{1,2}$ are intrinsically less infectious. Interestingly, proteolytic treatment with thermolysin rescues the infectivity of high-$GP_{1,2}$ viruses. We propose a model in which high $GP_{1,2}$ content impairs proper host receptor binding, as well as endosomal processing of $GP_{1,2}$ required for the final steps of infection. We hypothesize that this effect is due to steric shielding, resulting from dense packing of $GP_{1,2}$ trimers at the virion surface. We further propose that proteolysis relieves this shielding, thus promoting host receptor engagement while circumventing the impairment of endosomal processing. Taken together, our findings indicate that $GP_{1,2}$ expression levels have a profound effect on virus fitness, and that RNA editing may be an important mechanism employed by EBOV to regulate $GP_{1,2}$ expression in order to both optimize virus production and infectivity, while evading the host immune response.

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Abbreviations

AA	Amino Acid
ADCC	Antibody-Dependent Cell-Mediated Cytotoxicity
ADCVI	Antibody-Dependent Cell-Mediated Viral Inhibition
ASLV	Avian Sarcoma and Leukosis Virus
BEBOV	Ebola Virus (Bundibugyo Strain)
BSA	Bovine Serum Albumin
BST2	Bone Marrow Stromal Antigen 2
CD	Cluster of Differentiation
CR	Complement Receptor
CTL	Cytotoxic T-Lymphocyte
DC-SIGN	Dendritic Cell-Specific Intercellular Adhesion Molecule-3-Grabbing Non-integrin
DDRP	DNA-Dependent RNA Polymerase
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EBOV	Ebola Virus
EDTA	Ethylenediaminetetraacetic Acid
EHF	Ebola Hemorrhagic Fever
ELISA	Enzyme-Linked Immunosorbent Assay
Env	HIV Envelope Glycoprotein
ER	Endoplasmic Reticulum
ERK2	Extracellular Signal-Regulated Kinase 2 (Also known as MAPK1)
FBS	Fetal Bovine Serum
FcγRIIIb	(CD16B) Human Immunoglobulin Gamma Fc Receptor IIIb
GFP	Green Fluorescent Protein
GP	Glycoprotein
$GP_{1,2}$	Filovirus Surface Glycoprotein
$GP_{1,2}\Delta TM$	EBOV $GP_{1,2}$ Lacking Transmembrane Domain
GPI	Glycosylphosphatidylinositol
GPΔMuc	EBOV $GP_{1,2}$ Lacking Mucin Domain
GTP	Guanosine Triphosphate
HA	Influenza Hemagglutinin
HIV	Human Immunodeficiency Virus
hMGL	Human Macrophage Galactose-Specific C-Type Lectin
HRP	Horseradish Peroxidase
IACUC	Institutional Animal Care and Use Committee
IL	Interleukin
ISD	Immunosuppressive Domain
K_d	Dissociation Constant
kD	Kilodalton
KSHV	Kaposi's Sarcoma-Associated Herpesvirus
L	Viral RNA-dependent RNA polymerase gene
LLOV	Cuevavirus (Lloviu Strain)
L-SIGN	Liver/Lymph Node-Specific Intercellular Adhesion Molecule-3-Grabbing Non-integrin
MAPK	Mitogen-Activated Protein Kinase
MARV	Marburg Virus (Marburg Strain)
MFI	Mean Fluorescence Intensity
MHCI	Major Histocompatibility Factor I
NHP	Nonhuman Primate

NP	Nucleoprotein
NPC1	Niemann-Pick C1 Cholesterol Transporter
OAS	Original Antigenic Sin
p24	HIV p24 capsid protein
PBS	Phosphate-Buffered Saline
PBST	PBS + 0.05% Tween 20
PE	Phycoerythrin
PFU	Plaque-Forming Unit
RBD	Receptor-Binding Domain
RNA	Ribonucleic Acid
SDS-PAGE	Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis
SEBOV	Ebola Virus (Sudan Strain)
SeV	Sendai Virus
sGP	Filovirus Secreted Glycoprotein
ssGP	Small Secreted GP
TACE	TNFα Converting Enzyme
TNFα	Tumor Necrosis Factor Alpha
VEE	Venezuela Equine Encephalitis
VLP	Virus-Like Particle
VP24	Filovirus minor matrix protein
VP30	Filovirus minor nucleoprotein
VP35	Filovirus phosphoprotein
VP40	Filovirus major matric protein
VSV	Vesicular Stomatitis Virus
ZEBOV	Ebola Virus (Zaire Strain)

Chapter 1: Introduction

The Ebola virus (EBOV) is a single-stranded negative-sense RNA virus of the order *Mononegavirales*, which along with the Marburg virus (MARV) and newly-discovered Cueva virus (CUEV), forms the Filovirus family. Ebola virus (EBOV) is the etiologic agent of Ebola Hemorrhagic Fever (EHF), a highly lethal disease with up to 90% mortality[1]. Current treatment for EHF is purely supportive, and the dearth of effective interventions underscores our current lack of understanding of EBOV biology and the mechanisms by which the virus induces such severe disease while avoiding clearance. Specifically, the EBOV surface glycoprotein ($GP_{1,2}$), which mediates host cell attachment and fusion and is also the primary target of host antibodies, plays an important role in the pathogenesis of EHF. The work presented in this dissertation seeks to expand our understanding of how EBOV as well as other filoviruses regulate expression of $GP_{1,2}$ in order to optimize viral fitness and evade host immunity. The answers to these questions are of primary importance to ongoing efforts to develop effective antivirals, as well as a broadly-protective and long-lasting filovirus vaccine.

Epidemiology, transmission, and threat of pandemic

Since its discovery in 1976 in Zaire (now the Democratic Republic of Congo), five distinct strains of EBOV have been recognized (Zaire, Sudan, Ivory Coast, Reston, and Bundibugyo), with all but one strain (Reston) causing disease in humans[2]. Over the last 35 years, EBOV has cause sporadic outbreaks throughout Sub-Saharan Africa, often resulting in death tolls in the hundreds. To date, there have been no natural (non-laboratory) infections in humans outside of Africa. The virus's natural host and route of transmission are both widely

debated, although mounting evidence points to fruit bats as the primary reservoir in the wild[3-6]. The virus is known to be highly pathogenic in nonhuman primates, which may contract the disease from sharing food with bats. It is hypothesized that humans become infected from direct contact with infected primates or bats, possibly through hunting and bushmeat collection. The primary route of human-to-human transmission is unclear, but probably requires direct contact with bodily fluids, though there is some evidence the virus can spread by aerosol[7-9].

The severity and rapidness of disease onset combined with the remoteness of most outbreak foci may be factors in limiting the spread of recent outbreaks. Nevertheless, with expansion of global trade, international travel, and the looming specter of bioterrorism, the threat of a large-scale EBOV epidemic is ever more tangible. In addition to the threat to humans, recent data has demonstrated the potentially catastrophic threat filoviruses present to African great ape populations[10,11]. Huge kill-offs of chimpanzee and gorilla colonies have been documented in Democratic Republic of Congo, Gabon, and Cameroon. In addition to improved public health infrastructure and enhanced surveillance, our ability to contain future EBOV outbreaks in both humans and other primates hinges on the development of effective treatments and efficacious vaccines.

Structure, Biology, and the Surface Glycoprotein

Filoviruses get their name from their filamentous morphology. Virions are approximately 80nm in diameter, but can be over 1000 nm in length. The negative-sense RNA genome consists of seven genes each of which encodes a structural protein. As with other *Mononegavirales*, EBOV employs a very simple regulatory mechanism of gene expression whereby the order of genes (on the negative strand) from 3' to 5' dictates the extent to which

genes are expressed [12,13]. At the core of the virion is the nucleocapsid, composed of the genomic negative-sense RNA complexed with nucleoprotein (NP), phosphoprotein (VP35), a minor nucleoprotein (VP30), and the RNA-dependent-RNA-polymerase (L). Virion assembly, envelope formation, and budding is coordinated by the primary matrix protein (VP40), while a secondary matrix protein (VP24) may facilitate interaction between the nucleocapsid with the envelope[14]. The surface glycoprotein ($GP_{1,2}$), mediates attachment to and fusion with host cells, and is the only viral component that is expressed on the virus surface.

Ebola $GP_{1,2}$ is a type-1 transmembrane protein that forms homotrimeric spikes on the virion envelope in a similar manner to Influenza HA and HIV Env. Also like HA and Env, $GP_{1,2}$ is first synthesized as a pro-protein which is then cleaved in the Golgi into two functional subunits by furin protease. GP_1 contains the putative receptor-binding domain, while GP_2 contains the fusion apparatus and transmembrane domain [15]. Each $GP_{1,2}$ heterodimer has a mass of approximately 170kD, about half of which is comprised of glycans. Uniquely among negative-sense RNA viruses, the Ebola and Cueva viruses use an RNA-editing mechanism to generate two primary isoforms of GP. Membrane-bound $GP_{1,2}$ represents only ~20% of the total GP transcripts, while ~80% of transcripts encode a 50kD truncated secreted GP (sGP) that corresponds to the N-terminal half of $GP_{1,2}$.

Ebola $GP_{1,2}$ first mediates attachment to target cells via binding to host lectins such as DC-SIGN. This initial attachment event is followed by internalization via macropinocyotosis and trafficking to the endo-lysosomal pathway[16]. In the acidified endosome, cysteine proteases cathepsin B and cathepsin L cleave away large portions of GP_1 including the bulky and highly glycosylated mucin domain, revealing the buried receptor binding pocket [17-19]. Low pH combined with binding to a host receptor triggers fusion, whereby GP_2 ejects a fusion loop in

the host membrane and a rapid conformational change brings the virus and host membranes in close apposition induce fusion [20]. The identity of the fusion-inducing receptor remains to be determined, though it has recently been found that the Nieman-Pick Cholesterol Transporter (NPC1) is absolutely required for EBOV egress from the acidified endosome into the host cell cytoplasm[21,22].

Because it is the only structural component exposed on the virion surface, $GP_{1,2}$ is highly visible to the host immune system, and is the primary target for antibodies. Indeed, most EBOV vaccine efforts focus on eliciting a robust and broadly-reactive antibody response to $GP_{1,2}$. Not surprisingly, EBOV has evolved several mechanisms that are believed to suppress, divert, or otherwise evade the host anti-GP response. Importantly, EBOV must regulate GP expression to optimize infectivity while simultaneously evading host immunity. In the following sections, these mechanisms by which EBOV achieves this balance will be discussed, along with their relevance to EHF pathogenesis and the development of an effective EBOV vaccine.

RNA Editing and Secreted Glycoproteins

In the EBOV genome, $GP_{1,2}$ is encoded in two disjointed reading frames. The germline GP gene has a premature stop codon, resulting in production of a truncated secreted glycoprotein, sGP. On the other hand, EBOV uses an RNA editing mechanism to generate the full-length membrane-bound glycoprotein, $GP_{1,2}$. Through slippage of the viral polymerase at a tract of 7-A's (the editing site), an 8^{th} A is inserted, bringing the two $GP_{1,2}$ reading frames into register and allowing read-through translation of $GP_{1,2}$. However, this only happens ~20% of the time, while the remaining transcripts are unedited, directing synthesis of sGP[23,24].

The function of sGP has been widely debated though sparsely studied. GP RNA editing and the generation of sGP are conserved in all EBOV strains, as well as in the Cuevavirus, but is notably absent among Marburgviruses. Moreover, several features of sGP are present among all EBOV strains, strongly suggesting some important conserved function. All sGP's are ~320AA, and correspond linearly with the N-terminal receptor binding region of GP_1, while terminating before the highly O-glycosylated mucin domain[25]. It has been previously demonstrated that ZEBOV sGP forms dimers between Cysteines 53 and 306[26,27]. These cysteines and their flanking regions are almost perfectly conserved in all other EBOV strains (and well conserved in Cuevavirus as well), suggesting that sGP also forms dimers in these viruses. Additionally, the ZEBOV sGP preprotein has a polybasic furin cleavage sequence at residue 324, resulting in cleavage in the Golgi into sGP and the 39AA Δ-peptide, both of which are secreted by EBOV-infected cells. The generation of Δ-peptide is also predicted for all other EBOV strains, and for Cuevavirus, though surprisingly there is a high degree of divergence in Δ-peptide sequence. Most work regarding the function of sGP has focused on its role in modulating host immunity and inflammation. More recently, the finding that EBOV mutates to a primarily non-sGP-producing phenotype in cell culture, while reverting to a primarily sGP-producing phenotype *in vivo* lends weight to the idea that that sGP plays an important role in virus fitness within its host[28].

sGP as a decoy

The use of secreted antigens as decoys for host antibodies has been studied in several viruses. Both vesicular stomatitis virus (VSV) and respiratory syncytial virus (RSV) generate soluble forms of their surface glycoprotein (G) which have been demonstrated to absorb anti-G

antibodies and interfere with antibody-mediated mechanisms of viral clearance [29-31]. Accordingly, it has long been suggested that sGP similarly serves as a decoy to absorb host antibodies that would otherwise bind to $GP_{1,2}$ on virions. Efforts to elucidate such activity have yielded mixed results, with several studies finding that anti-GP antibodies often do not to cross-react between different glycoprotein isoforms [32-36]. Furthermore, it appears that immunization against $GP_{1,2}$ primarily generates antibodies that recognize epitopes not shared with sGP [37-40]. Nevertheless there have been several antibodies identified that bind to both sGP and $GP_{1,2}$, and for which exogenous sGP can interfere with antibody-mediated neutralization[33,34]. These findings demonstrate that sGP can functionally compete for anti-$GP_{1,2}$ antibodies in an *in vitro* assay.

It is worthwhile to note that in all of the aforementioned reports, the authors studied monoclonal antibodies, or antibodies from animals immunized primarily against $GP_{1,2}$. Neither of these experimental approaches truly mimics natural infection, in which hosts are exposed to primarily sGP, and generate a polyclonal response against multiple viral epitopes. Therefore, it is possible that patterns of antibody reactivity that are normally induced in natural infection were missed with these experimental systems. Indeed, in one study, mice were immunized with a Venezuelan Equine Encephalitis (VEE) replicon expressing both $GP_{1,2}$ and sGP, and most resulting monoclonal antibodies were found to cross-react between both GP isoforms[41]. Furthermore, antibodies generated during natural infection appear to preferentially react with sGP[32]. Thus, it is possible that sGP plays a more active role in altering the host antibody response, a finding that would explain the discrepancies with previous studies in which animals were immunized primarily against $GP_{1,2}$.

Immunomodulatory effect of sGP

In addition to a possible role as an antibody decoy, sGP has demonstrated several biological properties that likely help EBOV modulate host inflammation and immunity. sGP can rescue endothelial barrier function following TNFα- treatment, and may play an important role in moderating vascular permeability during infection, since loss of endothelial barrier function is one of the primary mechanisms of EHF pathogenesis[27,42]. Interestingly, proper tertiary and quaternary structure are vital for this function, as dimer-impaired sGP, as well as ssGP, are severely diminished in their ability to rescue barrier function[43]. sGP has also been demonstrated to bind to neutrophils via the FcγRIIIb receptor (CD16b), altering binding of CD16b to αMβ2 integrin (CR3) [44,45]. These receptors are important in mediating early inflammatory signals induced by immune complexes and complement-opsonized targets. Additionally, sGP prevents downregulation of CD62L (L-selectin) in activated neutrophils. Taken together, these findings indicate that sGP plays an important role in dampening early inflammation that might alert the host immune system to the virus's presence as well as prevent efficient dissemination of the virus within the host.

Modulation of $GP_{1,2}$ expression

While much work has focused on the role or RNA editing and sGP production in host immune evasion, it is also possible that RNA editing serves to modulate expression of $GP_{1,2}$. High levels of $GP_{1,2}$ expression are toxic to host cells, and induce cell rounding, detachment from surrounding tissues and monolayers, and loss of detection of cell surface markers [46-48]. These cytotoxic activities of $GP_{1,2}$ are thought to contribute to endothelial dysfunction characteristic of EHF[49]. Given that RNA editing results in only 20% of transcripts encoding

full-length $GP_{1,2}$, it is possible that RNA editing has evolved to attenuate $GP_{1,2}$ expression and $GP_{1,2}$-mediated cytotoxicity. Indeed, a recombinant EBOV in which the editing site was mutated to direct production of solely $GP_{1,2}$, displayed considerably higher cytopathicity than wild-type EBOV, and grew to much lower infectious titers in cell culture because it killed host cells so rapidly[50].

Other secreted glycoprotein products

In addition to sGP, EBOV generates several other secreted glycoprotein products. GP_1 can be released into the extracellular space via detachment of the disulfide bond with GP_2[51]. Additionally, cell surface proteases such as TNF-α converting enzyme (TACE) cleave $GP_{1,2}$ just N-terminal of the transmembrane domain, resulting in large quantities of $GP_{1,2}\Delta TM$ shed from EBOV infected cells[52]. These secreted antigens can compete for anti-$GP_{1,2}$ antibodies and interfere with antibody-mediated neutralization in a manner similar to that proposed for sGP. In addition to sGP, RNA editing also produces small amounts of a third GP product, short sGP (ssGP), which is generated by insertion of two extra adenosines at the editing site [43]. The function of ssGP is unknown, and because it represents only a tiny fraction of the total GP gene product, it may simply be an "accidental" byproduct of RNA editing. Finally, all Ebolaviruses produce Δ-peptide, a 35-40AA polypeptide cleaved from pre-sGP and secreted into the extracellular space[53]. While the sequence of Δ–peptide is highly divergent among EBOV strains, all Δ–peptides are predicted to be O-glycosylated (sGP has little to no O-glycosylation), and share other common features despite a lack of sequence identity[54]. No role has been determined for Δ-peptide in host immune evasion, though Δ–peptides tagged with human F_c's

were recently found to specifically inhibit filovirus infection, suggesting that they may play a role in regulating viral spread.

The Mucin Domain and Glycan Shielding

GP_1 accounts for almost 90% of the mass of the $GP_{1,2}$ heterodimer. The N-terminal half of GP_1 contains the putative receptor-binding domain (RBD), while the C-terminal half consists of a highly O-glycosylated mucin-like domain that wraps around and covers the RBD. The mucin domain plays no role in the machinery of $GP_{1,2}$ fusion, as it is cleaved and degraded in the acidified endosome. In fact, the mucin domain is completely dispensible for infection, and pseudoviruses generated with mucin-deleted $GP_{1,2}$ ($GP_{1,2}\Delta$Muc) can still attach to and infect cells, and surprisingly, with several-fold higher efficiency than pseudoviruses generated with wild-type $GP_{1,2}$. Furthermore, it has been demonstrated that digestion of the 150kD $GP_{1,2}$ with cathepsins or other proteases generates a 19kD core protein that is still stable and capable of mediating infection.

Immunodominance of the mucin domain

The dispensability of the mucin domain for virus infection indicates that it plays some other important role in virus survival. Considerable research has focused on the role of the mucin domain in host immune evasion, and it has been suggested that an important function of the mucin domain is to shield critical regions of $GP_{1,2}$ from host immune surveillance[55]. Sequence alignment of $GP_{1,2}$ from all known filovirus strains reveals that the mucin domain is the most divergent region of $GP_{1,2}$, while GP_2 and the N-terminal half of GP_1 are highly conserved among filoviruses. Furthermore, the mucin domain has been demonstrated to exhibit

intrinsic adjuvant activity[56], and a large proportion of antibodies induced by $GP_{1,2}$ are directed against the mucin domain[57,58]. The hypothesis that the mucin domain functions to shield conserved epitopes from immune surveillance follows the logic of HIV Env's "glycan shield"[59]. The virus "lures" the host response towards domains that are not critical for GP function, sparing more conserved functionally critical regions from attack. If this mechanism is at play in Ebola, it is likely not relevant to human infections, where hosts rarely have an opportunity to mount an adaptive immune response in the first place and most outbreaks are evolutionary dead ends for the virus. Instead, it is more likely that the mucin domain helps the virus survive in its chronically infected natural reservoir, and that divergence in this region emerged through chronic immune pressure. Nevertheless, shielding by the mucin domain has important implications for filovirus vaccine design, since broadly protective vaccines will likely have to focus the host immune response on regions that are both conserved among filovirus strains, yet are still accessible to antibody binding.

Interference with cell surface proteins

Another mechanism by which the $GP_{1,2}$ mucin domain may help EBOV evade the host immune response is by interfering with communication between virus-infected cells and the host immune system. It has been demonstrated that expression of $GP_{1,2}$ in both EBOV infected cells, and transiently transfected cells, results in global loss of detection of surface proteins including MHC1 and integrins, leading to rounding and detachment of cells [47,48]. Furthermore, this effect is dependent on the mucin domain, as cells transfected with $GP_{1,2}\Delta Muc$ do not exhibit rounding or disappearance of surface proteins. In fact, one study demonstrated that substitution of the EBOV $GP_{1,2}$ mucin domain onto membrane-bound surface glycoprotein of the unrelated

Avian Sarcoma and Leukosis Virus (ASLV) was able to recapitulate the same loss of detection of β1-integrin and MHC1 seen with wild-type EBOV $GP_{1,2}$[46].

Mechanism of surface protein loss: active downregulation vs. steric shielding

The mechanism underlying $GP_{1,2}$ mediated loss of surface protein expression has been somewhat controversial. Some studies have indicated that EBOV $GP_{1,2}$ induces active downregulation of adhesion and immunostimulatory molecules by infected cells. One in particular observed that GP-mediated loss of αVβ3 integrin and MHC1 occurred via a pathway regulated by dynamin, a GTPase involved in endocytosis and cycling of cell surface proteins [60]. The authors found that $GP_{1,2}$ can physically interact with these host surface proteins, altering their trafficking and inducing their active internalization, thus allowing virus-infected cells to hide from the host immune system. Separate studies found that $GP_{1,2}$ also reduces activation of ERK2, a member of the MAP kinase family that regulates a variety of cell processes including adhesion, cytoskeletal remodeling, and endosome cycling[61,62]. Lack of ERK2 phosphorylation was correlated with cell rounding and downregulation of integrins, and suppression of ERK2 activity with a dominant-negative ERK2 mutant or via siRNA potentiated $GP_{1,2}$-induced downregulation of integrins. On the other hand, complementation with constitutively active ERK2 significantly attenuated $GP_{1,2}$-induced loss of cell surface proteins. Both of these studies support a model in which $GP_{1,2}$ induces host cells to actively downregulate expression of specific surface factors, and both studies also found that the respective $GP_{1,2}$-mediated effects were completely dependent on the mucin domain.

More recently, another study has suggested that the loss of detection of integrins and MHC1 in $GP_{1,2}$-expressing cells is primarily due to masking of these proteins via steric

hindrance[63]. The authors demonstrated that the $GP_{1,2}$ mucin domain and its associated glycans form a dense canopy that renders buried host cell surface proteins undetectable to antibody-based assays such as flow cytometry. In fact, the authors were able to demonstrate that the glycan shield is so effective that it can shield buried epitopes on EBOV $GP_{1,2}$ itself. Digestion of $GP_{1,2}$ glycans, as well as removal of GP_1 via DTT treatment, both resulted in significant increases in detection of surface β1 integrin and MHC1 expression. Most importantly, the authors demonstrated that expression of $GP_{1,2}$ by antigen-presenting target cells prevented them from activating antigen-specific $CD8^+$ T-cells. The debate between the steric shielding and active internalization models will be clarified by further studies, but neither mechanism necessarily precludes the other. It is reasonable to hypothesize that both mechanisms may be at play, and that EBOV has evolved multiple parallel strategies to ensure that virus-infected cells remain minimally visible to the host immune system.

Immunosuppressive Domain

All filoviruses have a highly conserved sequence in the GP_2 subunit that bears a high degree of sequence homology to the immunosuppressive domain of several retroviruses, including HIV[64]. This sequence of approximately 15-20 amino acids was first identified over 30 years ago, and has been demonstrated in retroviruses to inhibit or otherwise interfere with proper activation of macrophages and lymphocytes[65-67]. Peptides corresponding to retrovirus immunosuppressive domains inhibit lymphocyte activation *in vitro,* and induce lymphocytes to elaborate TH_2-cytokines, thereby polarizing the host immune system away from an optimal antiviral response [68,69]. The corresponding peptides from both EBOV and MARV were demonstrated *in vitro* to induce downregulation of markers of activation in both CD4 and CD8

T-cells, as well as inhibit elaboration of inflammatory cytokines (while inducing IL-10 expression), and induce lymphocyte apoptosis[70]. Most interestingly, human lymphocytes were unresponsive to the immunosuppressive effects of the Reston EBOV (REBOV) ISD peptide, while lymphocytes from rhesus macaques displayed the same markers of suppression seen with other EBOV peptides. This finding is consistent with the observation that REBOV causes rapidly fatal disease in macaques, while causing no disease in humans, and strongly suggests that the immunosuppressive domain of EBOV $GP_{1,2}$ plays a central role in the pathogenesis of EHF. Future studies to examine the effect of the ISD on EBOV pathogenesis *in vivo*, as well as the effect of the ISD on generation of anti-$GP_{1,2}$ antibodies, will yield crucial information for EBOV vaccine design.

Tetherin Antagonism

Tetherin, also known as CD317, or Bone Marrow Stromal Antigen 2 (BST2), is a Type-II transmembrane protein that inhibits egress of budding virions. The protein structure consists of an N-terminal transmembrane α-helix, a coiled-coil ectodomain, and a C-terminal GPI anchor, resulting in the protein being attached to the cell membrane at both ends[71]. When enveloped viruses bud from the cell membrane, one end of tetherin is embedded in the nascent virion membrane, while the other end is still attached to the host cell, thus "tethering" the virus to the cell. Tetherin is expressed constitutively in many immune cells including lymphocytes and monocytes, and expression is induced in other cell types by type I interferons. Tetherin orthologs have been identified in several placental mammals, and its activity against a range of enveloped viruses indicates that this innate immune mechanism has existed for quite some time. Given this ancient relationship, several viruses have evolved their own mechanisms of

antagonizing tetherin[72,73]. HIV Vpu and KSHV K5 both target tetherin for ubiquitination and degradation[74,75]. More recently, it was demonstrated *in vitro* that tetherin inhibits budding of EBOV VP40-based virus-like particles (VLP's), and that EBOV $GP_{1,2}$ antagonizes tetherin and rescues VLP budding [76]. The mechanism by which $GP_{1,2}$ mediates this effect is unclear, though it has been demonstrated that EBOV GP_2 physically interacts with tetherin. However, unlike HIV Vpu and KSHV K5, EBOV $GP_{1,2}$ does not result in loss of surface expression of tetherin, suggesting that $GP_{1,2}$ tetherin antagonism is not dependent on ubiquitination and degradation[77].

Interestingly, it has been demonstrated that levels of tetherin expression, as well as the ability of tetherin to mediate its antiviral effects, varies between cell types [78]. Thus, it is tempting to hypothesize how EBOV regulation of GP expression may be related to tetherin antagonism. It has been demonstrated that serial passage of EBOV in cell culture leads to rapid emergence of a predominantly $GP_{1,2}$-producing phenotype, while passaging of mutant virus in naïve guinea pigs results in reversion to a predominantly sGP-producing phenotype within 6 days[28]. This short period suggests that reversion is not the result of adaptive immune pressure. Instead, the tendency to adopt a predominantly sGP-forming versus $GP_{1,2}$-forming phenotype may have more to do with innate immunity, and local environmental factors such as tetherin expression levels. Future studies comparing the emergence of editing site mutants in different cell lines that vary in endogenous tetherin expression levels will further elucidate the connection between $GP_{1,2}$ expression levels, tetherin antagonism, and viral fitness.

Implications for Vaccine Design

Because $GP_{1,2}$ is the most visible filovirus structural component, filovirus vaccinology has centered around inducing a potent anti-$GP_{1,2}$ response in order to neutralize or otherwise clear incoming virus. While great strides have been made in developing an EBOV vaccine, strategies that are highly effective in rodent models of EHF have generally yielded less promising results in nonhuman primate studies. One of the reasons for this is likely that rodent models incompletely recapitulate EHF seen in primates[79]. For example, mouse-adapted EBOV must be delivered intraperitoneally in order to induce disease, while mice inoculated subcutaneously with high doses of virus are able to clear the infection without prior immunization or supportive treatment[80,81]. On the other hand, low-dose mucosal inoculation of EBOV in NHP's induces rapidly fatal fulminant EHF. Furthermore, mice do not appear to exhibit the same degree of global immunosuppression, coagulopathy, and inflammatory dysregulation as observed in primates. Thus, it is likely that the mechanisms EBOV uses to evade and suppress host immunity are more effective in primates than in rodents, and that therefore the "threshold" for immunity in rodents is both lower and less stringent than that required for protection in primates.

All of these factors point to the importance of better understanding the mechanisms by which EBOV avoids immune clearance in order to better tailor vaccines to counteract or circumvent those mechanisms. What is the role of sGP in immune evasion? Are anti-$GP_{1,2}$ antibodies that cross-react with sGP more likely to be absorbed and therefore less likely to protect? On the other hand, sGP corresponds to a well-conserved region of $GP_{1,2}$, which means that sGP-reactive antibodies may also react well with other strains of EBOV $GP_{1,2}$. Also, how protective are antibodies directed against the mucin domain? It has previously been

demonstrated that the mucin domain contains some neutralizing epitopes, and mucin-reactive antibodies have been shown to protect mice from lethal EBOV challenge [57,82,83]. However, because of the high degree of sequence variation in this region among EBOV strains, mucin-reactive antibodies are unlikely to be broadly protective. Thus, the desire for a broadly-reactive EBOV vaccine may be at odds with experimental data that suggests mucin-reactive antibodies are the most easily-induced protective antibodies. Finally, the high degree of conservation of GP_2 among EBOV strains, combined with its functional importance, suggests that this region is a prime target for broadly-neutralizing antibodies. However, the location of GP_2 proximal to the cell membrane where it is shielded by GP_1, means that it may not be easily accessible to antibodies. Furthermore, the immunosuppressive domain of GP_2 may inhibit the generation of anti-GP_2 antibodies in the first place. The series of conundrums presented here illustrates exactly how much work remains to fully elucidate the pathogenesis of and the useful correlates of protection against Ebola hemorrhagic fever. As these problems are gradually unraveled, EBOV vaccinologists will be able to pursue more rationally designed immunization strategies.

The next chapters seek to answer some of these important questions, with the unifying theme of better understanding how EBOV regulates GP expression to optimize virus survival within the host. The data we present here sheds light on the importance of GP RNA editing in host immune evasion and virus infectivity, and we further discuss the implications of these findings to EHF pathogenesis and Ebola vaccinology. Specifically, we examine the role of sGP in altering the host anti-$GP_{1,2}$ immune response, and how sGP may work with the GP_1 mucin domain to ensure that the majority of host antibodies either do not bind $GP_{1,2}$, bind $GP_{1,2}$ but can be absorbed by sGP, or bind to functionally dispensible regions of $GP_{1,2}$. We then examine the importance of $GP_{1,2}$ expression levels in optimizing virus infectivity, and how RNA editing may

also represent a mechanism for optimizing $GP_{1,2}$ expression in order to both maximize infectivity and antagonize host defenses. We believe that these findings illuminate important virus survival strategies that have previously been sparsely studied. Furthermore, the mechanisms discussed here have important implications for our understanding of Ebola hemorrhagic fever pathogenesis, and it is our hope that these novel findings will help to inform future strategies to develop a potent and broadly-protective EBOV vaccine.

Chapter 2: Antigenic Subversion: A Novel Mechanism of Host Immune Evasion by the Ebola Virus

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All data reported here were generated by the author of this document unless otherwise noted in the figure legends

Abstract

In addition to its surface glycoprotein ($GP_{1,2}$), Ebola virus (EBOV) directs the production of large quantities of a truncated glycoprotein isoform (sGP) that is secreted into the extracellular space. The generation of secreted antigens has been studied in several viruses and suggested as a mechanism of host immune evasion through absorption of antibodies and interference with antibody-mediated clearance. However such a role has not been conclusively determined for the Ebola virus sGP. In this study, we immunized mice with DNA constructs expressing $GP_{1,2}$ and/or sGP, and demonstrate that sGP can efficiently compete for anti-$GP_{1,2}$ antibodies, but only from mice that have been immunized by sGP. We term this phenomenon "antigenic subversion", and propose a model whereby sGP redirects the host antibody response to focus on epitopes which it shares with membrane-bound $GP_{1,2}$, thereby allowing it to absorb anti-$GP_{1,2}$ antibodies. Unexpectedly, we found that sGP can also subvert a previously immunized host's anti-$GP_{1,2}$ response resulting in strong cross-reactivity with sGP. This finding is particularly relevant to EBOV vaccinology since it underscores the importance of eliciting robust immunity that is sufficient to rapidly clear an infection before antigenic subversion can occur. Antigenic subversion represents a novel virus escape strategy that likely helps EBOV evade host immunity, and may represent an important obstacle to EBOV vaccine design.

Author Summary

The function of the Ebola virus (EBOV) secreted glycoprotein (sGP) has been long debated, and the fact that sGP production is conserved among all known EBOV species strongly indicates an important role in the viral life cycle. Furthermore, the recent finding that EBOV mutates to a predominantly non-sGP-forming phenotype in cell culture, while the mutant virus reverts to an sGP-forming phenotype *in vivo,* suggests that sGP is critical for EBOV to survive in its infected host. Here we demonstrate that sGP can function to absorb anti-GP antibodies. More importantly, instead of simply passively absorbing host antibodies, sGP actively subverts the host immune response to induce cross-reactivity with epitopes it shares with membrane-bound $GP_{1,2}$. Immune subversion by sGP represents a distinct mechanism from the use of secreted antigens as antibody decoys, an immune evasion tactic previously proposed for other viruses, and should be an important consideration for future EBOV vaccine design efforts since vaccines may need to be specifically tailored to avoid subversion.

Introduction

Ebola virus (EBOV) is an enveloped single-stranded negative-sense RNA virus in the order *Mononegavirales*, which along with the Marburg virus (MARV) forms the Filovirus family. EBOV is the etiologic agent of Ebola Hemorrhagic Fever (EHF), a highly lethal hemorrhagic fever with up to 90% mortality [84]. Since its discovery in 1976, EBOV has caused sporadic outbreaks in Sub-Saharan Africa with death tolls in the hundreds. Interestingly, while filoviruses have been only recently discovered, they are one of the few non-retrovirus RNA paleoviruses identified in mammalian genomes, suggesting an ancient relationship with mammals [85,86]. Growing evidence suggests that bats are the natural reservoir of EBOV in the wild today [3,4,6].

Current treatment for Ebola hemorrhagic fever is purely supportive, and the lack of effective interventions underscores the importance of developing a broadly-protective vaccine that confers long-lasting immunity. The ability to develop such a vaccine is critically dependent on our understanding of the mechanisms by which EBOV suppresses, distracts, or otherwise evades the host immune response [87]. One widely hypothesized immune evasion mechanism employed by Ebola virus is secretion of a truncated viral glycoprotein by EBOV infected cells. The EBOV surface glycoprotein ($GP_{1,2}$) mediates host cell attachment and fusion, and is the primary structural component exposed on the virus surface. For this reason, $GP_{1,2}$ is the focus of most EBOV vaccine research, and it is generally accepted that a robust anti-$GP_{1,2}$ antibody response is crucial for protection against lethal EBOV challenge [88]. EBOV $GP_{1,2}$ forms trimeric spikes on virion surfaces similarly to influenza HA and HIV Env [15]. Also like HA and Env, GP is first synthesized as an uncleaved precursor (GP_0) which is then cleaved in the Golgi complex by the protease furin [89] into two functional subunits: The N-terminal GP_1

subunit contains the putative receptor-binding domain (RBD), and the C-terminal GP_2 subunit contains the fusion apparatus and transmembrane domain. $GP_{1,2}$ is encoded in two disjointed reading frames in the virus genome. The two reading frames are joined together by slippage of the viral polymerase at an editing site (a tract of 7-A's) to insert an 8^{th} A, generating an mRNA transcript that allows read-through translation of $GP_{1,2}$ [23,24]. However, only about 20% of transcripts are edited, while the remaining 80% of unedited transcripts have a premature stop codon, resulting in synthesis of a truncated glycoprotein product (sGP) which is secreted in large quantities into the extracellular space.

Though its production is conserved in all EBOV species, there has been considerable debate regarding the function of sGP. Unlike $GP_{1,2}$, sGP forms homodimers and appears to have some intrinsic anti-inflammatory activity [26,27,42,90,91]. The recent finding that EBOV quickly mutates to synthesize primarily $GP_{1,2}$ in cell culture, while this mutant virus reverts to a primarily sGP-producing phenotype *in vivo,* suggests an important role for sGP in virus survival within the host [28]. Because sGP shares over 90% of its sequence with the N-terminal region of $GP_{1,2}$, it was initially hypothesized that sGP functions as a decoy for anti-$GP_{1,2}$ antibodies. Early efforts to identify such activity yielded mixed results, and the observation that antibodies often do not cross-react between sGP and $GP_{1,2}$ had cast doubt on this hypothesis [32-36]. Furthermore, recent studies demonstrated that immunization against $GP_{1,2}$ elicits antibodies largely against epitopes not shared with sGP [37-40]. However, most of these studies investigated monoclonal antibodies from animals immunized with vaccines containing or expressing primarily $GP_{1,2}$, which does not represent the state of natural infection. Of note, one early study examined monoclonal antibodies from mice immunized with a Venezuelan equine encephalitis replicon that produces both $GP_{1,2}$ and sGP, and found that many of these antibodies

cross-reacted between $GP_{1,2}$ and sGP [41]. Further, monoclonal antibodies isolated from human EHF survivors have been shown to preferentially react with sGP [32]. These studies suggest that sGP may play an important role in altering the host antibody response.

In this study, we demonstrate that sGP induces a host antibody response that focuses on epitopes it shares with $GP_{1,2}$, thereby allowing it to bind and compete for anti-$GP_{1,2}$ antibodies. We describe a mechanism that we term "antigenic subversion", which is distinct from previously proposed "decoy" mechanisms in which secreted glycoprotein simply passively absorbs anti-glycoprotein antibodies. Importantly, we demonstrate that sGP can also subvert an existing anti-$GP_{1,2}$ immune response that was only weakly cross-reactive with sGP. Antigenic subversion represents a novel host immune evasion mechanism that has important implications for EBOV vaccine design, and may shed light on how the virus survives in its natural reservoir.

Materials and Methods

Ethics statement - Animal ethics approval for the immunization studies in mice was obtained from the *Institutional Animal Care and Use Committee* (IACUC) at Emory University. All animal studies were performed under approval from the *Institutional Animal Care and Use Committee* (IACUC) at Emory University. Female BALB/c mice (8-week old) were purchased from the Jackson Laboratory and housed in the animal facility at the Emory University.

Cell Lines and Plasmids – 293T cells and HeLa cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Mediatech) supplemented with 10% fetal bovine serum (Hyclone, ThermoFisher) and penicillin/streptomycin. All Ebola glycoprotein constructs were based on the Ebola Zaire strain (ZEBOV), Mayinga Subtype (GenBank accession# U23187.1). Editing site mutants were generated in pBlueScript II K/S+ vector through site-directed mutagenesis using the QuickChange XL kit (Stratagene). Constructs were then subcloned pCAGGS mammalian expression vector. Protein expression was carried out by transfecting 90% confluent cells in 6-well plates with 5 μg DNA + 12 μL Fugene HD (Roche) per well, as per manufacturer instructions, and detected at 48 h post transfection by Western blot using rabbit anti-ZEBOV polyclonal antibodies.

Vaccine Preparation and Immunization – Mutant ZEBOV GP plasmids for DNA immunization experiments were prepared using the EndoFree Plasmid Mega Kit (Qiagen) as per manufacturer instructions and redissolved in pure endotoxin-free water at a concentration of 4-6 μg/μL, and purity was verified by restriction analysis and spectrophotometry. For immunization, DNA was diluted in sterile PBS to 0.5 μg/μL and filter sterilized. Female BALB/C mice (Charles River Laboratory) at six mice per group received 50 μg of DNA intramuscularly

(25 μg/leg) per immunization. Anesthetized mice were bled retro-orbitally two weeks after each immunization and serum samples were stored at -80^{o}C until use.

Recombinant Protein Production and ELISA – Production of purified histidine-tagged HA has been described previously [92]. Soluble histidine-tagged $GP_{1,2}$ and sGP were generated by C-terminal addition of a single 6x histidine tag. Soluble $GP_{1,2}$ was generated by truncation of the transmembrane domain and cytoplasmic tail. Recombinant vaccinia viruses (rVV) were generated as described elsewhere to synthesize soluble His-tagged $GP_{1,2}$ (His- $GP_{1,2}$) and sGP (His-sGP), as well as membrane-bound $GP_{1,2}$ [93]. For production and purification of His-$GP_{1,2}$ and His-sGP, rVV-infected cell supernatant was clarified and purified using a PrepEase His Purification Kit (Affymetrix) and purity of recombinant protein was verified by SDS-PAGE followed by Western blot or coomassie stain. Further, purified His-$GP_{1,2}$ and His-sGP were tested for reactivity to pre-immune sera or sera from unvaccinated mice by ELISA and Western blot, and they were found to be unreactive. For ELISA, flat-bottom Immulon 4-HBX 96-well plates (Thermo) were coated overnight with 0.1 μg/well of His- $GP_{1,2}$ or His-sGP. A standard curve was generated by coating control wells with known concentrations of mouse IgG. Plates were washed 5x in PBS+Tween (PBST), blocked in PBST+2%BSA, and then incubated in duplicate for two hours with antisera diluted in PBST+2%BSA. Plates were washed again, and incubated with 1:1000 (pooled anti- IgG subtype) HRP-conjugated goat anti-mouse secondary antibody. After final wash, plates were developed with 3,3',5,5'-Tetramethylbenzidine (TMB, Thermo) and stopped at 5 minutes with 0.2M HCl. Plates were read and antibody concentration was calculated using the standard curve.

Competition ELISA – Competition ELISA was performed by modifying the above protocol. Plates were coated with His- $GP_{1,2}$. Pooled antisera were diluted in PBST+2%BSA to a

concentration corresponding to an OD of 1.0 by anti- $GP_{1,2}$ ELISA. Diluted antisera were then mixed with decreasing concentrations of purified His-sGP or His- $GP_{1,2}$ and immediately added to His- $GP_{1,2}$-coated wells. The ELISA was then developed as described above and competition was calculated as percent of signal compared to no competing antigen.

Competition Immunoprecipitation – Competition immunoprecipitation was performed by incubating pooled antisera (normalized for anti-$GP_{1,2}$ titer as determined by ELISA) with 200ng of purified His- $GP_{1,2}$ and increasing amounts purified His-sGP at molar ratios of 0.25:1, 1:1, and 1:4 sGP:$GP_{1,2}$. Antisera incubated with His-sGP alone, His-$GP_{1,2}$ alone, or with no GP was used as controls. Samples were incubated on ice for 20 minutes, followed by addition of protein-G coupled agarose beads (Thermo Scientific) to further incubate at 4^{o}C for an additional two hr with agitation. Samples were then centrifuged and washed three times with with lysis buffer, and then mixed with 6x Laemmli SDS sample buffer with 12% b-mercaptoethanol. The samples were heated at 95^{o}C for 5 minutes and then used for SDS-PAGE followed by Western blot analysis using antibodies gainst both sGP and $GP_{1,2}$.

Affinity of Polyclonal Antisera – Apparent affinity of polyclonal antisera was determined by quantitative ELISA using purified IgG from immunized animals. IgG was purified using Melon Gel (Thermo) as per manufacturer instructions and purity of IgG was verified by ELISA and coomassie gel staining. Since quantitative affinity ELISA requires that coating antigen be incubated with increasing dilutions of antibodies until coating antigen becomes saturated, we found that high antibody concentrations can result in signals that exceed the plate reader's range of detection. Thus, we titrated the amount of coating antigen down to 0.05 μg/well to avoid signal saturation. Wells were coated overnight with 0.05 μg of purified His-$GP_{1,2}$ or His-sGP and after washing and blocking were incubated with purified IgG diluted in PBST + 2%BSA, at

dilutions ranging from 1:10 to 1:1280 (based on original serum volume). ELISAs were developed as described above and the signal converted to nM concentration of IgG by comparison to a standard curve. Apparent K_d's of polyclonal sera were calculated by nonlinear regression analysis using GraphPad Prism. These results were verified manually by analysis of linearized binding curves as detailed elsewhere [94].

Pseudovirus Generation and Neutralizing Assay – EBOV-GP pseudotyped HIV was generated as described elsewhere[95]. Briefly, 293T-cells were cotransfected with Env-defective HIV backbone and ZEBOV GP in pCAGGS vector using Fugene HD (Roche). Supernatants were harvested 48 h post-transfection, clarified, and filtered using a 0.45 micron filter. Pseudoviruses were titered by infecting JC53 cells [96], which express b-galactosidase and luciferase under a *tat*-activated promoter, causing infected cells turning blue with X-Gal staining. Neutralization assays were performed as described elsewhere [95] with minor modifications. Briefly, pseudoviruses were pre-incubated with dilutions of heat-inactivated antisera, and supplemented with heat-inactivated naïve mouse sera (Innovative Research) so that 5% of the total volume was mouse serum. Pseudovirus-antiserum mixtures were then added to 30% confluent JC53 cells and incubated for 48 h. Virus infection and neutralization was measured by luciferase reporter assay, and neutralization was measured by decrease in luciferase expression compared to virus-only controls [96].

We performed a competition neutralization assay by selecting the antiserum concentration that corresponded to 80% neutralization for each antiserum sample. Fixed antiserum concentrations were incubated with dilutions of purified His-sGP or with soluble influenza PR8 hemagluttinin (HA) as a control (GenBank Accession# JF690260). Antiserum mixtures were then mixed with pseudovirus and the neutralization assay was developed as

described above. Interference with neutralization was measured by percent drop in neutralization compared to antiserum-only controls.

Results

Immunogenicity of EBOV GP Editing Site Mutant DNA Vaccines

We first generated EBOV GP constructs to individually express $GP_{1,2}$ and sGP. In natural infection, EBOV directs the synthesis of sGP and $GP_{1,2}$ through differentially edited mRNA transcripts (Fig 1A). However, it has been observed that DNA-dependent RNA polymerases (DDRP) do not edit with the same efficiency as the EBOV RNA polymerase [24]. Furthermore, in addition to polymerase slippage, it is possible that the 7-A editing site can also serve as a premature poly-adenylation signal, as well as a ribosomal slippage signal [97-99]. We thus generated a panel of EBOV GP editing site mutants in order to control the levels of sGP and $GP_{1,2}$ expression (Fig 1B). GP-8A was made by inserting an 8^{th} A into the wild type (GP-7A) editing site, resulting in $GP_{1,2}$ as the dominant gene product. Silent A→G mutations were introduced into the GP-8A editing site to ablate transcriptional slippage, resulting in $GP_{1,2}$Edit, that expresses $GP_{1,2}$ as the sole gene product. The same mutations were also introduced into GP-7A to generate sGPEdit, that expresses sGP as the sole gene product. These constructs were subcloned into a mammalian expression vector (pCAGGS) and protein expression was examined in both HeLa cells (Fig 1C) and 293T cells (data not shown). Cells transfected with GP-8A and $GP_{1,2}$Edit expressed $GP_{1,2}$ intracellularly and on their surfaces, and secreted $GP_{1,2}$ into the supernatant through previously characterized TACE-dependent cleavage [52]. Interestingly, $GP_{1,2}$Edit produced higher amounts of $GP_{1,2}$ than GP-8A. GP-7A and sGPEdit expressed high levels of sGP, which was secreted efficiently into the supernatant. $GP_{1,2}$ expression by GP-7A was undetectable, likely because of minimal DDRP-mediated editing [24]. These expression experiments demonstrate that mutation of the editing site has a significant effect on GP expression.

We next investigated the immunogenicity of editing site mutant DNA vaccines. Female BALB/c mice were immunized with $GP_{1,2}$ or sGP-producing constructs (Fig. 2A). Mice immunized with sGPEdit, GP-7A, and GP-8A constructs developed similar titers of anti-$GP_{1,2}$ antibodies as measured by ELISA, while mice immunized with $GP_{1,2}$Edit developed four-fold higher titers of anti-$GP_{1,2}$ antibodies (Fig. 2B). Mice immunized with constructs expressing predominantly sGP (GP-7A and sGPEdit) developed much higher titers of anti-sGP antibodies than mice immunized with constructs expressing predominantly $GP_{1,2}$ (GP-8A and $GP_{1,2}$Edit) (Fig 2C). As shown in Fig. 2D, $GP_{1,2}$-immunized mice developed much higher titers of $GP_{1,2}$-binding antibodies than sGP-binding antibodies. On the other hand, sGP-immunized mice developed much higher titers of sGP-binding antibodies than $GP_{1,2}$-binding antibodies, despite the fact that sGP shares roughly 95% of its linear sequence with $GP_{1,2}$. These results suggest that in sGP-immunized animals, either many sGP-binding antibodies are directed against conformational epitopes not shared with $GP_{1,2}$, or they are directed against shared epitopes that are inaccessible in $GP_{1,2}$.

sGP can compete for binding of anti-$GP_{1,2}$ antibodies induced by sGP but not by $GP_{1,2}$

Given that animals immunized by $GP_{1,2}$ or sGP develop antibodies that preferentially bind to different GP isoforms, we performed Western blot analysis to determine if there is a difference in the linear epitopes targeted by antibodies in $GP_{1,2}$ versus sGP-immunized mice. As shown in Fig. 3A, antisera from $GP_{1,2}$-immunized mice reacted strongly with $GP_{1,2}$ but only weakly with sGP. On the other hand, antisera from sGP-immunized mice reacted strongly with sGP, but only weakly with $GP_{1,2}$. This suggests that most linear epitopes targeted by anti-$GP_{1,2}$ antibodies from $GP_{1,2}$-immunized mice are unshared with sGP. To investigate whether the $GP_{1,2}$-binding and sGP-binding antibodies in immunized mice were cross-reactive between the

two GP isoforms or were separate populations of antibodies, we performed a competition ELISA to determine if sGP could compete with $GP_{1,2}$ for $GP_{1,2}$-binding antibodies (Fig 3B). Similar to the Western blot data, sGP was unable to compete for binding of anti-$GP_{1,2}$ antibodies from $GP_{1,2}$ immunized mice (Fig. 3C). On the other hand, sGP was able to efficiently compete for anti-$GP_{1,2}$ antibodies from sGP-immunized mice. As expected, $GP_{1,2}$ was able to compete with itself in all groups (Fig. 3D). Furthermore, we observed an identical reactivity pattern with native membrane-anchored EBOV $GP_{1,2}$ using a cell surface competition ELISA (Supplemental Fig. S1). We further examined the ability of the two GP isoforms to compete with each other for antibodies by performing competition immunoprecipitation. Purified $GP_{1,2}$ in the presence of sGP at varying molar ratios was immunoprecipitated with antiserum from $GP_{1,2}$-immunized or sGP-immunized mice, and analyzed by Western blot using a polyclonal rabbit antibody that reacts with both GP isoforms. As a negative control, recombinant influenza HA was allowed to compete with $GP_{1,2}$ for anti-$GP_{1,2}$ antibodies. Antiserum from $GP_{1,2}$-immunized mice precipitated both $GP_{1,2}$ and sGP, and increasing concentrations of sGP did not attenuate the amount of $GP_{1,2}$ signal (Fig 3E), suggesting the presence of two separate populations of antibodies that do not cross-react between $GP_{1,2}$ and sGP. However, while antiserum from sGP-immunized mice also precipitated both $GP_{1,2}$ and sGP, increasing concentrations of sGP significantly attenuated the amount of $GP_{1,2}$ precipitated (Fig 3F), indicating that $GP_{1,2}$-reactive antibodies in these mice are cross-reactive with sGP. Recombinant HA had no effect on the amount of $GP_{1,2}$ precipitated. Taken together, these data suggest that anti-$GP_{1,2}$ antibodies induced by $GP_{1,2}$ are directed primarily against epitopes not shared between $GP_{1,2}$ and sGP, whereas such antibodies induced by sGP are directed against epitopes shared between $GP_{1,2}$ and sGP.

sGP differentially interferes with antibody-mediated viral neutralization by antisera from sGP and $GP_{1,2}$ immunized mice

We further investigated whether there was a difference in the ability of antisera from the immunization groups to neutralize EBOV $GP_{1,2}$-mediated virus infection, and whether sGP could interfere with antibody-mediated neutralization. Pseudoviruses were generated using an Env-deficient HIV backbone pseudotyped with Zaire EBOV $GP_{1,2}$. In order to achieve consistent neutralization, we pooled sera from the four highest responders among $GP_{1,2}$-immunized animals and among sGP-immunized animals. Antisera from both groups were able to effectively neutralize pseudoviruses as measured by a luciferase reporter assay (Fig. 4A), although antisera from $GP_{1,2}$-immunized mice exhibited more potent neutralizing activity than antisera from sGP-immunized mice, probably due to higher overall anti-$GP_{1,2}$ titer. To determine if sGP interferes with neutralization, we used an antiserum dilution corresponding to 80% neutralizing activity in each group and preincubated antisera with different amounts of sGP. Consistent with the competition ELISA results, sGP was able to completely attenuate neutralizing activity of antisera from sGP-immunized mice, while it had no effect on neutralizing activity of antisera from $GP_{1,2}$-immunized mice (Fig. 4B). Purified influenza HA was used as a control and had no effect on neutralizing activity of either antiserum group. Similar results were observed when we used an antiserum dilution corresponding to 50% neutralizing activity (Supplemental Fig. S2). These data confirm that sGP can compete with $GP_{1,2}$ for anti-$GP_{1,2}$ antibodies and interfere with antibody-mediated neutralization, but can only do so in animals that have been immunized against sGP.

Anti-$GP_{1,2}$ and anti-sGP antibodies induced by different GP isoforms exhibit similar average affinity

The inability of sGP to compete with $GP_{1,2}$ for antibodies from $GP_{1,2}$-immunized mice was intriguing considering that $GP_{1,2}$ shares almost half of its ectodomain sequence with sGP. We reasoned that some of these antibodies may be directed solely against $GP_{1,2}$ epitopes not shared with sGP, while other antibodies may be directed against shared epitopes, but preferentially bind $GP_{1,2}$ because of conformational differences between the two GP isoforms resulting from tertiary and quarternary structure and steric shielding. To address this possibility, we used quantitiative ELISA to determine the relative titers and estimate the average affinity of antibodies from $GP_{1,2}$ and sGP-immunized animals for $GP_{1,2}$ and sGP. We individually examined purified polyclonal IgG from the five highest responders in $GP_{1,2}$-immunized and sGP-immunized groups, and calculated the apparent dissociation constant (K_d) of anti-$GP_{1,2}$ and anti-sGP antibodies. This apparent K_d was calculated by Scatchard analysis as described elsewhere [94,100] and represents an estimate of the average affinity of anti-GP antibodies, with lower apparent K_d correponding to higher average affinity. Consistent with previous ELISA data, mice immunized against $GP_{1,2}$ had higher titers of anti-$GP_{1,2}$ antibodies than anti-sGP antibodies (Fig. 5A). However, there was no measurable difference in the apparent K_d's of $GP_{1,2}$-binding vs. sGP-binding antibodies (Fig 5B), indicating that preferential binding of antibodies from these animals to $GP_{1,2}$ is not due to affinity differences for different GP isoforms. In mice immunized against sGP we again observed very high titers of anti-sGP antibodies, and very low levels of anti-$GP_{1,2}$ antibodies. However, those antibodies that did bind to $GP_{1,2}$ appeared to have modestly lower K_d (higher average affinity) than did sGP-binding antibodies (Fig. 5B). Future studies with monoclonal antibodies directed against shared epitopes between sGP and $GP_{1,2}$ will provide further information on whether specific antibodies bind to the two GP isoforms with different affinities. Nonetheless, the present data provide evidence that differences in affinity are

not responsible for antibodies from $GP_{1,2}$ and sGP-immunized mice reacting preferentially with different GP isoforms.

Expression of $GP_{1,2}$ in the context of sGP allows sGP to compete for anti-$GP_{1,2}$ antibodies

The secretion of surface glycoproteins as a mechanism of absorbing antiviral antibodies has been hypothesized before for several viruses including vesicular stomatitis virus (soluble G) and respiratory syncytial virus (secreted G) [29,30]. It has been demonstrated that RSV secreted G can absorb anti-G antibodies and interfere with both neutralization and antibody-dependent cell-mediated virus clearance. However, we observed that EBOV sGP can only compete for anti-$GP_{1,2}$ antibodies in mice immunized against sGP. This led us to hypothesize that sGP may serve a role in altering the repertoire of epitopes against which the host immune response is directed, in order to divert the host immune response towards epitopes shared between sGP and $GP_{1,2}$. To test this hypothesis, we vaccinated mice with a 3:1 ratio of sGPEdit:$GP_{1,2}$Edit (Fig 6A) to simulate antigen expression during EBOV infection. Control groups were immunized with either sGPEdit or $GP_{1,2}$Edit plus empty pCAGGS vector to keep the total amount of DNA constant. As a proxy for *in vivo* antigen expression, HeLa cells were transfected with corresponding ratios of sGPEdit, $GP_{1,2}$Edit, and pCAGGS. As measured by Western blot analysis, the levels of sGP and $GP_{1,2}$ expression in both lysate and culture supernatant of cells co-transfected with sGPEdit and $GP_{1,2}$Edit were similar to cells transfected with sGPEdit or $GP_{1,2}$Edit alone (Fig S3). All immunization groups generated similar titers of anti-$GP_{1,2}$ antibodies (Fig. 6B). However, when we performed a competition ELISA using antisera from sGPEdit+ $GP_{1,2}$Edit-immunized mice, sGP was able to compete with $GP_{1,2}$ for over 50% of the anti-$GP_{1,2}$ antibodies (Fig. 6C). Mice immunized with $GP_{1,2}$Edit + vector or sGPEdit + vector displayed the same serum reactivity patterns we had observed previously in mice

immunized against only one of the GP isoforms. Further, after boosting mice a second time, almost 70% of $GP_{1,2}$-antibodies in week 12 antisera from sGPEdit+ $GP_{1,2}$Edit-immunized mice were absorbed by sGP. Interestingly, in mice immunized with lower ratios of sGPEdit:$GP_{1,2}$Edit, significant sGP cross-reactivity was also observed, with almost 70% of anti-$GP_{1,2}$ antibodies being susceptible to competition in mice immunized with a 1:1 ratio of sGP:$GP_{1,2}$, and about 25% being susceptible to competition in mice immunized with a 1:3 ratio of sGP:$GP_{1,2}$ (Figure S4). Similar results were also obtained with a competition immunoprecipitation assay. As shown in Fig. 6D, antiserum from sGPEdit+$GP_{1,2}$Edit-immunized mice was able to precipitate both $GP_{1,2}$ and sGP, but increasing concentrations of sGP attenuated the amount of $GP_{1,2}$ precipitated. Furthermore, while sGPEdit+$GP_{1,2}$Edit antiserum was able to effectively neutralize pseudovirus infectivity (Fig. 6E), the addition of exogenous sGP almost completely inhibited pseudovirus neutralization (Fig 6F), indicating that sGP can effectively interfere with antibody mediated neutralization in these mice. Similar observations were also made at an antiserum concentration corresponding to 50% neutralization (Fig S5). Taken together, these data confirm that sGP can direct the host antibody response to focus on shared epitopes between $GP_{1,2}$ and sGP, thereby allowing sGP to compete for antibodies and interfere with antibody-mediated virus neutralization. Furthermore, the observation that sGP can compete for a greater proportion of $GP_{1,2}$ antibodies from week 12 antisera compared to week 6 suggests that iterative exposure to sGP gradually drives the host to a dominantly sGP-reactive response.

sGP can subvert the $GP_{1,2}$-specific antibody response

In order to test the hypothesis that overexpression of sGP can overwhelm the $GP_{1,2}$-specific antibody response, we primed and boosted mice with either sGPEdit or $GP_{1,2}$Edit, and then boosted again at week 10 with the opposite GP isoform (Fig 7A). Control groups were

boosted with the same GP isoform. As shown in Fig. 7B, anti-$GP_{1,2}$ antibodies were induced in all groups at week 12. However, in mice immunized with $GP_{1,2}$Edit and then boosted with sGPEdit, sGP was able to efficiently compete for anti-$GP_{1,2}$ antibodies in competition ELISA (Fig 7C). Furthermore, sGP was also able to efficiently compete for anti-$GP_{1,2}$ antibodies from mice primed against sGPEdit and boosted with $GP_{1,2}$Edit,. . Neutralizing activity of antisera from animals primed and boosted with opposite GP isoforms was determined as previously described (data not shown). Interestingly, sGP was able to interfere with neutralization only from animals primed against sGP and boosted with $GP_{1,2}$ (Fig 7D), while antisera from animals primed against $GP_{1,2}$ and boosted with sGP maintained neutralizing activity in the presence of sGP. Furthermore, comparison of the antisera titers corresponding to 50% neutralizing activity (NT_{50}) in groups before boosting with the opposite GP isoform (week 6) and after boosting with the opposite GP isoform (Week 12) (Fig 7E) reveals that neutralizing activity is not boosted despite a dramatic increase in overall $GP_{1,2}$-binding antibodies. The observation that most anti-$GP_{1,2}$ antibodies in $GP_{1,2}$-primed, sGP-boosted mice can be competed away by sGP in competition ELISA, while sGP does not interfere with neutralizing activity of these antibodies suggests that neutralizing antibodies may only represent a small proportion of anti-$GP_{1,2}$ antibodies.Nevertheless, the ability of sGP to alter the reactivity profile of the anti-$GP_{1,2}$ response has important implications for EBOV vaccinology, since during a subsequent infection, sGP could subvert the immune response of a previously vaccinated individual if the virus is not cleared rapidly.

Discussion

The putative role of sGP in EBOV host immune evasion has not been clearly defined. In this study, we analyzed antibody responses in mice immunized against sGP, $GP_{1,2}$, or both GP isoforms and present evidence that sGP serves to redirect the immune response towards epitopes that are either not present or inaccessible in $GP_{1,2,}$ or epitopes that are shared between the two GP isoforms, thereby allowing sGP to effectively absorb anti-$GP_{1,2}$ antibodies. We term this phenomenon "antigenic subversion", because it is distinct from previously proposed mechanisms in which sGP passively absorbs anti-glycoprotein antibodies. In antigenic subversion, the ability of sGP to absorb anti-$GP_{1,2}$ antibodies is critically dependent on exposure to sGP during induction of the anti-$GP_{1,2}$ immune response. In mice immunized against $GP_{1,2}$ in the presence of sGP, an immunization strategy designed to simulate antigen exposure during natural infection, we observed that most resulting anti-$GP_{1,2}$ antibodies were cross reactive to and thus susceptible to competition by sGP, even though the titers of anti-$GP_{1,2}$ antibodies in these mice were similar to the titers in mice immunized against $GP_{1,2}$ alone. On the other hand, in mice immunized against $GP_{1,2}$ alone, we observed only low cross-reactivity of anti-$GP_{1,2}$ antibodies with sGP, a finding consistent with previous studies, indicating that antibodies in these mice are largely directed against epitopes not shared with sGP [36,37].

The model we propose for the mechanism of antigenic subversion by sGP assumes that before immunization, the host begins with a repertoire of naïve B-cells that recognize epitopes distributed throughout $GP_{1,2}$ and sGP (Fig 8A). However, because sGP is generated in much higher quantities than $GP_{1,2}$, B-cells that recognize sGP epitopes and epitopes shared between sGP and $GP_{1,2}$ are more likely to encounter their cognate antigens as compared with B-cells that recognize $GP_{1,2}$-specific epitopes. Furthermore, as the sGP-reactive B-cell population expands,

it will outcompete other B-cells for antigen and survival signals. Thus, the humoral response is skewed towards sGP, and epitopes of $GP_{1,2}$ that are shared with sGP. Antigenic subversion represents a novel viral escape strategy that has some similarities to original antigenic sin (OAS). In classical OAS, initial exposure to a pathogen results in a population of memory B-cells that recognize antigens specific to that pathogen strain. Upon subsequent exposure to a different strain of the same pathogen, cross-reactive memory B-cells will respond preferentially, producing antibodies with high affinity to the initial pathogen which may not bind to the new strain as effectively [101,102]. Furthermore, these memory B-cells can compete for antigen and survival signals with naïve B-cells that might otherwise produce higher affinity or more protective antibodies to the new strain. Similarly, overexpression by Ebola virus of sGP ensures that sGP-reactive B-cells preferentially expand and outcompete $GP_{1,2}$-specific B-cells for antigen and survival signals, resulting in a suboptimal host response that is directed away from membrane-bound $GP_{1,2}$ on the virion surface. However, unlike classical OAS, this process does not require temporal separation of antigen encounters, but can also occur during simultaneous exposure to two partly identical antigens.

Our model for antigenic subversion can also explain how anti-$GP_{1,2}$ antibodies from animals primed against sGP and then boosted with $GP_{1,2}$ maintain cross-reactivity with sGP. In these animals, priming with sGP elicits antibodies against sGP epitopes, some of which are shared with $GP_{1,2}$ (Fig 8B). When these animals are boosted with $GP_{1,2}$, memory B-cells that recognize shared epitopes vastly outnumber (and express higher affinity receptors than) the naïve B-cells that recognize unshared epitopes. Thus, the anti-sGP memory B-cells will be preferentially activated and expanded, boosting the anti-sGP response. This situation is analogous to one in which previously-infected individuals are vaccinated against $GP_{1,2}$, and

raises the possibility that immunizing such individuals may simply boost an already unprotective antibody response. While filovirus infection is rare, our findings suggest that it may be necessary to devise alternate strategies for immunizing previously-infected individuals in a way that specifically boosts the anti-$GP_{1,2}$ response and avoids subversion.

Perhaps the most striking finding in this study is that boosting $GP_{1,2}$-immunized mice with sGP could effectively subvert the anti-$GP_{1,2}$ response and render it susceptible to competition by sGP. We hypothesize that while the majority of B-cells activated in mice immunized against $GP_{1,2}$ are directed against epitopes not shared with sGP (Fig 8C), there is a small population of activated B-cells that react with sGP. This is supported by our observation that even though sGP cannot measurably compete in ELISA and immunoprecipitation for anti-$GP_{1,2}$ antibodies from $GP_{1,2}$-immunized mice, these mice still develop low titers of sGP-binding antibodies. When $GP_{1,2}$-immunized mice are boosted with sGP, these sGP-reactive B-cells expand while the remaining $GP_{1,2}$-specific B-cells that recognize unshared epitopes do not, shifting the anti-$GP_{1,2}$ antibody response from mostly $GP_{1,2}$-specific to mostly sGP-cross reactive. This situation is analogous to one in which an individual is immunized against $GP_{1,2}$ is subsequently infected with EBOV. If the individual is unable to rapidly clear the virus, the virus may replicate sufficiently to subvert the host immune response. Thus, it will be critical for vaccines to induce high enough titers of anti-$GP_{1,2}$ antibodies to ensure that the virus is cleared before it is able to effect subversion (Fig 8D). It is interesting to note that while anti-$GP_{1,2}$ antibodies in $GP_{1,2}$-primed, sGP-boosted mice were highly susceptible to competition by sGP as measured by ELISA, sGP did not interfere with neutralizing activity of these antisera. Furthermore, neutralizing activity actually decreased after boosting with sGP, despite an increase in overall anti-$GP_{1,2}$ antibodies.

The inability of sGP to compete for anti-$GP_{1,2}$ antibodies from $GP_{1,2}$-immunized mice is consistent with a growing body of evidence pointing to the immunodominance of the $GP_{1,2}$ mucin domain, a highly glycosylated region of GP_1 not shared with sGP[37,38]. This domain is thought to form a sterically bulky "cloak" that shields the putative receptor binding domain from host antibodies, as suggested for the HIV Env "glycan shield" [59]. The role that the mucin domain plays in host-pathogen interaction is complex and previous studies indicate that this region contains both neutralizing and infection-enhancing epitopes, and can mask epitopes on $GP_{1,2}$ itself by steric occlusion [103,104]. Furthermore, the mucin domain is the most divergent region of $GP_{1,2}$ among EBOV strains, and is dispensible for $GP_{1,2}$ mediated virus attachment and membrane fusion [18,105,106], strongly suggesting a role in protecting more functionally conserved regions of $GP_{1,2}$ from immune attack. Because the linear sequence of sGP corresponds to the putative mucin-shielded receptor binding domain (RBD) of GP_1, it is possible that sGP works together with the mucin domain so that host antibodies are directed either to shared epitopes that are sterically shielded in the $GP_{1,2}$ trimer, or to the mucin domain itself, which is removed in the host cell acidified endosome along with any bound antibodies [17,19]. The possibility that $GP_{1,2}$ epitopes shared with sGP may be shielded in the $GP_{1,2}$ trimer is supported by our observation that very few anti-sGP antibodies in sGP-immunized mice cross-react with $GP_{1,2}$ despite the fact that sGP shares over 90% of its linear sequence with $GP_{1,2}$. Furthermore, antigenic subversion allows sGP to efficiently absorb those antibodies that do recognize unshielded and shared epitopes in $GP_{1,2}$.

The importance of sGP-mediated antigenic subversion to EHF pathogenesis remains to be elucidated. Passive immunization studies with polyclonal sera or monoclonal antibodies will reveal whether sGP-crossreactive antibodies are in fact less protective than $GP_{1,2}$-specific

antibodies. This is particularly important given that passive transfer of anti-EBOV monoclonal antibodies has gained traction recently as a post-exposure therapeutic. If sGP cross-reactivity turns out to be correlated with impaired virus clearance, it would underscore the need to elicit and produce $GP_{1,2}$-specific antisera or monoclonal antibodies for achieving more effective treatment of EBOV infection. Moreover, our findings also suggest that EBOV vaccines should be tailored to target regions not shared between sGP and $GP_{1,2}$. This is particularly relevant to recent efforts to develop a broadly-protective vaccine, since these studies have centered around focusing vaccines on conserved epitopes by deleting highly variable regions of $GP_{1,2}$ such as the mucin domain [37,106,107]. Because sGP actually corresponds to the most highly conserved region of GP_1, antibodies elicited by these constructs may be cross-reactive with sGP and therefore susceptible to sGP-mediated subversion. Candidate pan-filovirus vaccines may need to be focused on regions of $GP_{1,2}$ that are both highly conserved and unshared with sGP, such as the membrane-proximal GP_2 subunit.

It will also be of great interest for EBOV vaccinology to determine whether antigenic subversion correlates with successes and failures to protect vaccinated animals against lethal challenges. It may be critical for an EBOV vaccine to elicit a long lasting immune response with high enough antibody titers so the host can clear the virus before it is able to replicate and effect antigenic subversion. This possibility is consistent with nonhuman primate lethal challenge experiments, in which survival was most closely correlated with maintenance of anti-$GP_{1,2}$ antibody titers above a threshold level, while lower antibody titers only delayed the time to death [108]. Further, while much of EBOV vaccinology has focused on eliciting protective antibodies against the membrane-bound glycoprotein, a robust T-cell response may also improve vaccine efficacy. Immunization of nonhuman primates with a low dose of GP and nucleoprotein (NP)-

expressing recombinant adenoviruses was demonstrated to elicit robust antibody and T-cell responses and confer protection against lethal challenge [109]. More importantly, EBOV-specific T-cells were shown to reduce the threshold of anti-$GP_{1,2}$ antibodies needed for protection. Recombinant vectors expressing CTL epitopes have been demonstrated to confer protection to lethal EBOV challenge in mice, and GP-specific as well as nucleoprotein (NP)-specific CD8 T-cells can control infection even when adoptively transferred to otherwise naïve animals [110,111]. These studies suggest that a robust T-cell response may reduce the threshold of antibodies needed for rapid virus clearance.

It is noteworthy that although the expression of sGP is conserved in Ebola viruses, sGP is not produced by Marburg virus (MARV), another member of the filoviridae.. There are other instances where related viruses often diverge in the mechanisms they employ to survive in their respective hosts. For example, Sendai virus (SeV), a paramyxovirus that causes severe respiratory tract infections in rodents, expresses a V protein via RNA editing of the P gene. V is necessary for *in vivo* survival and pathogenesis of SeV, though V-deficient SeV show no defect in replication *in vitro* [112]. However, the closely related human parainfluenza virus type I (HPIV-1) does not express V, even though its P gene displays a high degree of homology to SeV P, and HPIV-1 causes similar disease in humans as SeV causes in rodents [113]. Similarly, while secretion of GP has not been observed in MARV, it has likely evolved alternative strategies to survive within its host.

While the precise relevance of antigenic subversion to Ebola vaccinology remains to be determined, antigenic subversion represents a novel and elegant solution to the challenge that viruses face of balancing the ability to infect host cells efficiently while evading host immune surveillance. The constraints of a very small genome neccessitate packing a great deal of

functionality into a small space, and sGP-mediated subversion represents a mechanism which, along with glycan-dependent steric shielding, and immunodominance of the $GP_{1,2}$ mucin domain, may help EBOV to survive in its host. Improving our understanding of how these mechanisms work together will eventually open the door to a more rationally designed vaccine. A vaccine directed against highly conserved regions of $GP_{1,2}$, such as the GP_2 subunit, could induce broadly reactive antibodies while also avoiding the potential for sGP-mediated immune subversion. Such a vaccine could protect against multiple strains of EBOV, including strains that have not yet been identified.

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Figures

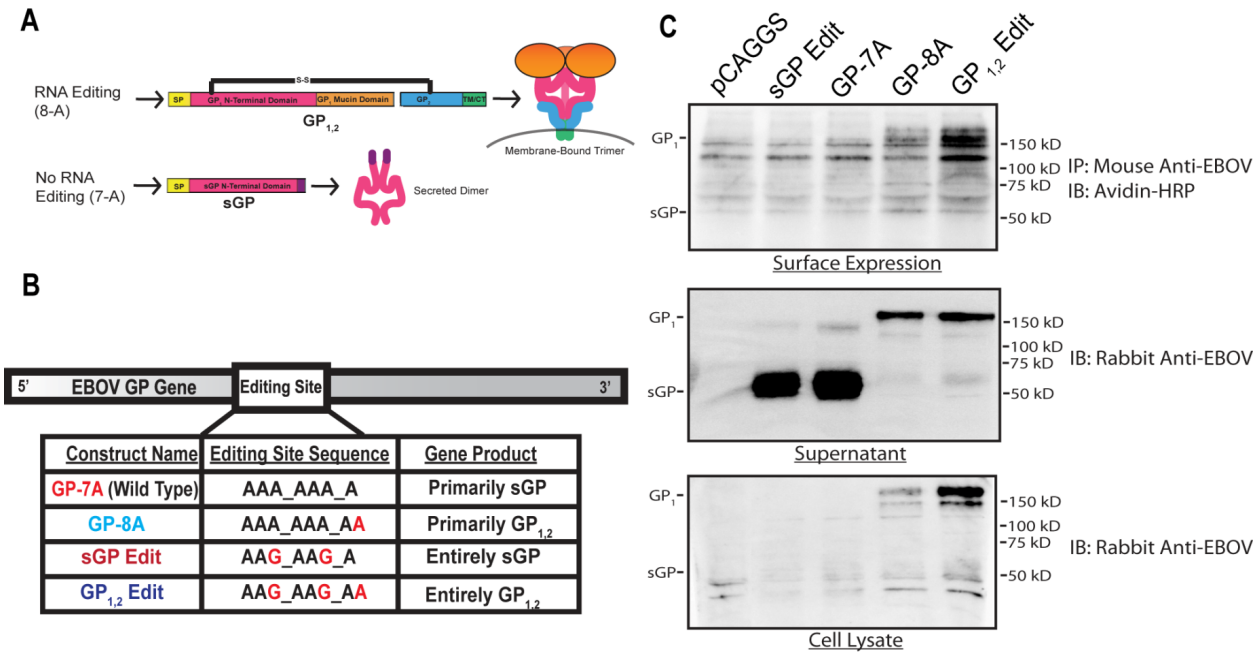


Figure 2-1. Diagram of EBOV RNA editing and construction of EBOV GP mutants.

(A) Schematic diagram of GP1,2 and sGP. Membrane-bound GP1,2 is encoded in the EBOV genome in two disjointed reading frames. The GP editing site is a tract of 7 A's approximately 900 nucleotides downstream of the start codon. Slippage of EBOV RNA-dependent RNA polymerase at the editing site results in insertion of an 8th-A which brings the two GP reading frames in register resulting in read-through translation of full-length membrane-bound trimeric GP1,2. Unedited transcripts contain a premature stop codon and produce truncated dimerized sGP. (B) EBOV GP and editing site mutants. Mutated nucleotides are shown in red and the primary gene products expressed by these constructs are also listed. (C) Expression of EBOV GP by wild type and mutant DNA constructs. HeLa cells were transfected with the wild type GP or editing site mutant constructs and GP expression was assayed by Western blot at 48 h post-

transfection. Surface expression was detected by surface biotinylation followed by immunoprecipitation with anti-EBOV GP mouse polyclonal antibody, SDS-PAGE, and Avidin-HRP blotting. Cell lysate was harvested in cell lysis buffer and cell culture supernatant was collected, spun down to remove cell debris, and concentrated to 10x by a centrifugal concentrator. Cell lysate and concentrated cell culture supernatant were run on SDS-PAGE under denaturing conditions, followed by probing with anti-EBOV GP1,2/sGP rabbit polyclonal antibody.

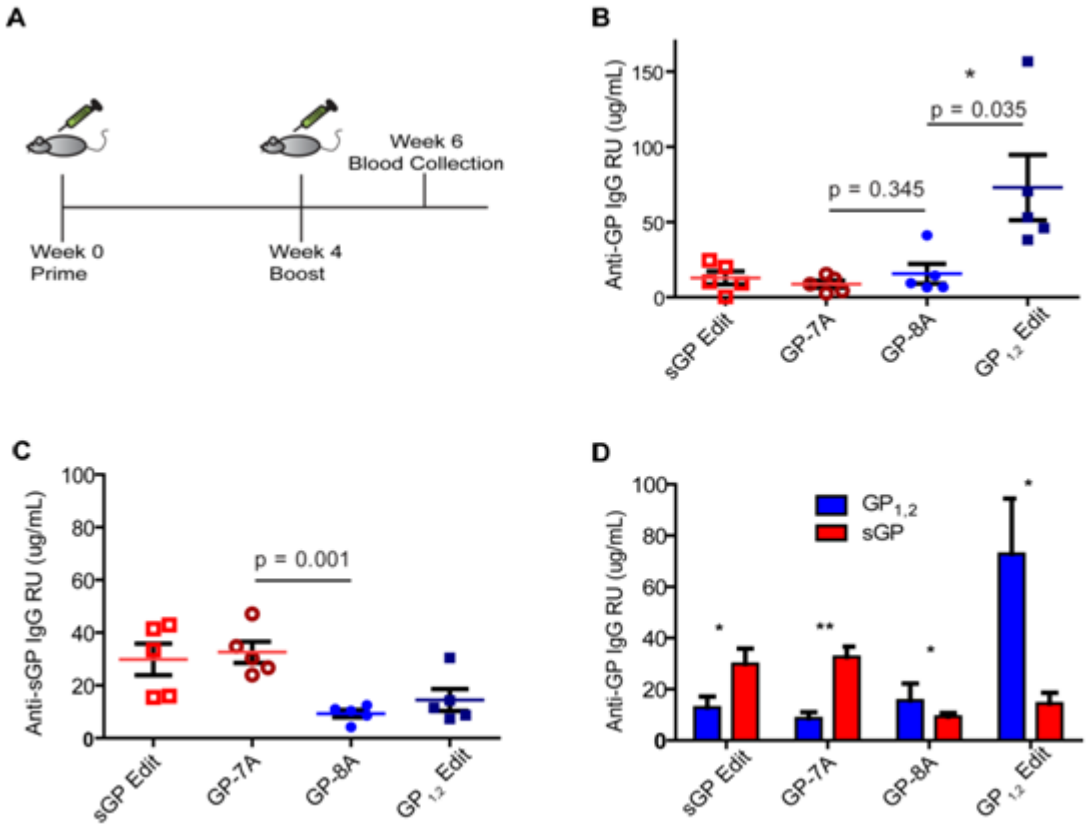


Figure 2-2. Immunogenicity of EBOV GP editing site mutants.

(A) Immunization study design. Female BALB/C mice were immunized with the four editing site mutant constructs in the pCAGGS vector. Mice were vaccinated IM with 50 μg of DNA (25 μg/leg) according to the schedule shown. (B) Antibody response against $GP_{1,2}$. (C) antibody response against sGP. The levels of antibody response induced by EBOV GP DNA constructs in mice were measured by ELISA using His-$GP_{1,2}$ or His-sGP as coating antigen. Antibody concentration was determined from a standard curve and expressed as μg/mL of anti-GP IgG. Asterisks indicate statistically significant difference between groups and P-values are given in red. (D) Comparison of antibody levels against $GP_{1,2}$ and sGP induced by each EBOV GP DNA construct. Average titers of anti-$GP_{1,2}$ (blue) and anti-sGP (red) antibodies within immunization

groups are shown for comparison of the GP isoform reactivity profiles both within and between immunization groups. Asterisks indicate statistically significant differences between anti-$GP_{1,2}$ and anti-sGP titers within groups, as measured by paired, two-tailed Student's t-test (* = $p<0.05$, ** = $p<0.001$)

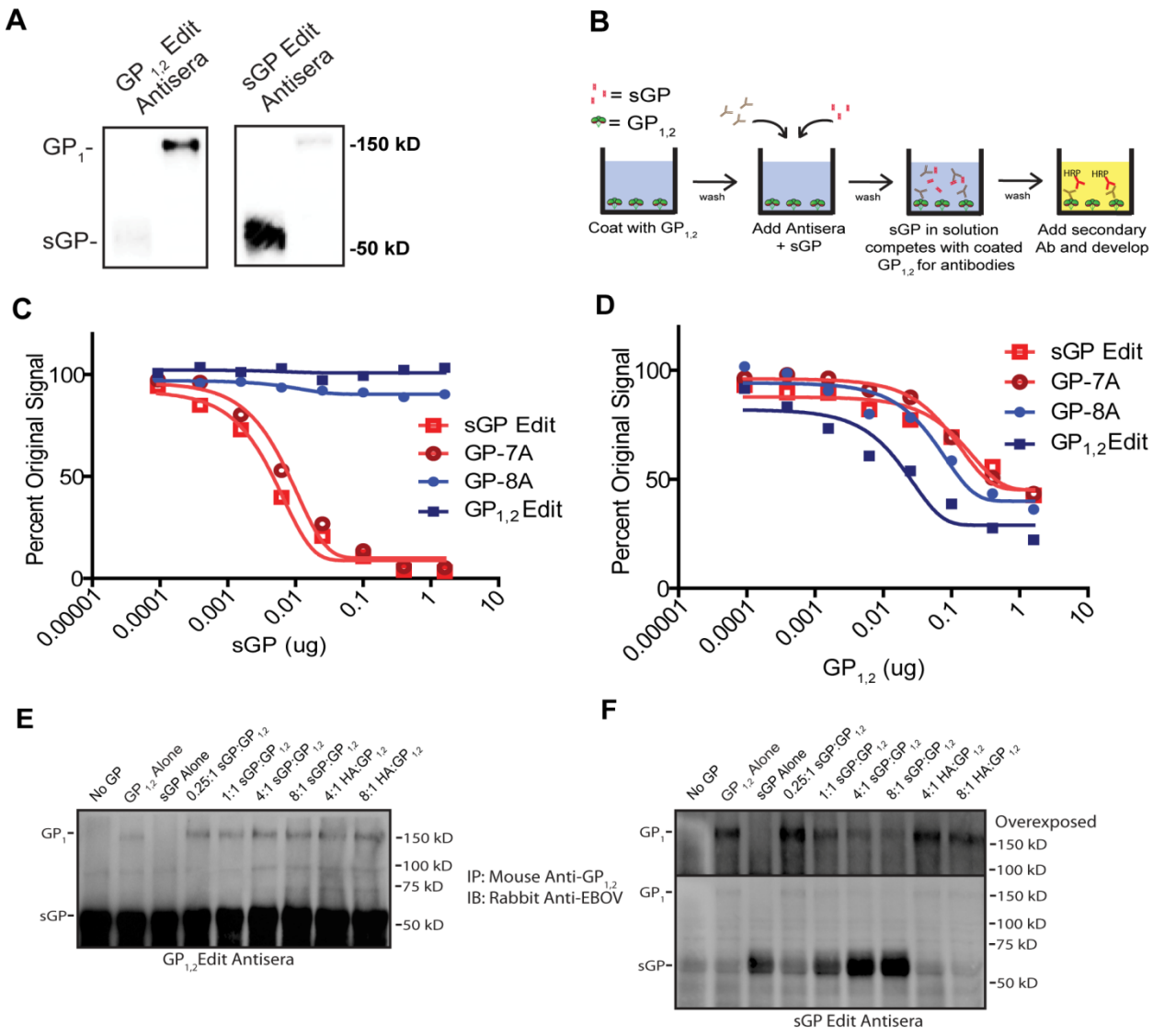


Figure 2-3. Antiserum from mice immunized against $GP_{1,2}$ or sGP display different reactivity patterns.

(A) Detection of antibodies against $GP_{1,2}$ and sGP from immunized mice by Western blot. 50 ng of purified His-sGP and His-$GP_{1,2}$ were run by SDS-PAGE under denaturing conditions and probed with 1:1000 pooled $GP_{1,2}$Edit or sGPEdit antisera followed by blotting with HRP-conjugated goat anti-mouse IgG. (B) Schematic of competition ELISA. Wells were coated with $GP_{1,2}$ and incubated with pooled antisera as well as increasing concentrations of competing

antigen (sGP or $GP_{1,2}$) to compete for antibodies. After two hours, plates were washed and then incubated with HRP-conjugated secondary antibody followed by addition of substrate to develop color. (C, D) Competition ELISA. Antisera from mice immunized with sGPEdit, GP-7A, GP-8A, and $GP_{1,2}$Edit were diluted to give similar anti-$GP_{1,2}$ signal. Diluted antiserum was mixed with increasing quantities of purified His-sGP (C) or His-$GP_{1,2}$ (D) and incubated in His-$GP_{1,2}$ coated wells and developed as described above. Experiments were performed in duplicate and repeated at least three times, with representative results shown. (E, F) Competition Immunoprecipitation. Pooled antisera from $GP_{1,2}$Edit-immunized mice (E) or sGP-immunized mice (F) were incubated with no GP, purified sGP or $GP_{1,2}$ alone, or with fixed $GP_{1,2}$ and increasing concentrations of sGP to compete for anti-$GP_{1,2}$ antibodies. $GP_{1,2}$ was incubated with recombinant HA as a negative control. Samples were precipitated, run on SDS-PAGE, and then blotted using a polyclonal rabbit antibody that reacts with both $GP_{1,2}$ and sGP. The upper panel for the sGPEdit antisera shows the $GP_{1,2}$ portion of the blot at a longer exposure time to show the attenuation of signal with increasing sGP concentration. Results are representative of three independent experiments.

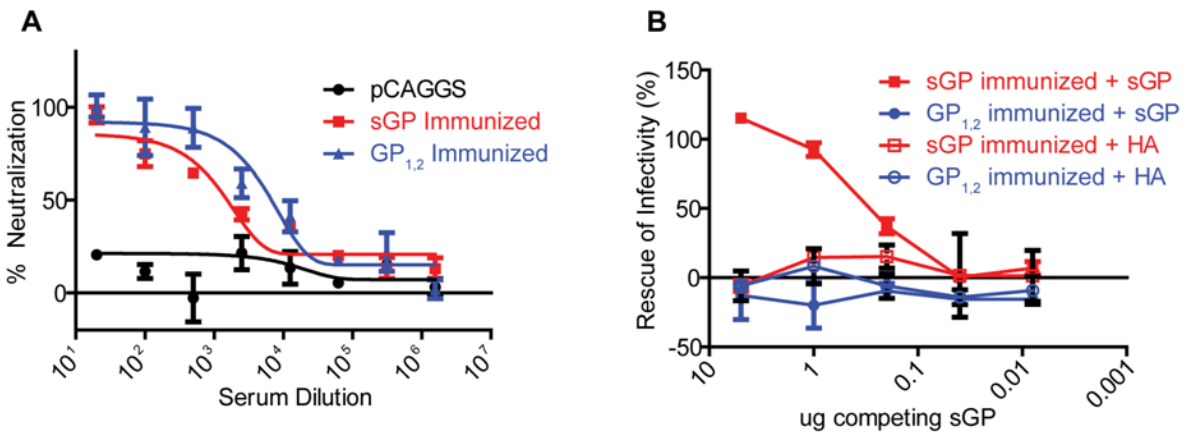


Figure 2-4. Interference with antibody-dependent neutralization by sGP.

(A) Neutralization of EBOV GP pseudovirus. Neutralizing activity of antisera was determined by incubating 500 pfu of $GP_{1,2}$-pseudotyped virus with dilutions of pooled $GP_{1,2}$-immunized (Blue), sGP-immunized (Red), and empty pCAGGS vector-immunized (black) antisera. Neutralization was measured as decrease in luciferase expression compared to virus-only controls after 48 h. (B) Interference of EBOV GP pseudovirus neutralization by sGP. The ability of sGP to interfere with antibody-dependent neutralization was determined by allowing sGP to compete with $GP_{1,2}$ pseudotyped viruses for anti-$GP_{1,2}$ antibodies. Pooled $GP_{1,2}$-immunized (blue) and sGP-immunized (red) antisera were fixed at the dilution corresponding to 80% neutralization. Antisera was co-incubated with increasing dilutions of His-tagged sGP (solid markers) or His-tagged influenza PR8 HA (open markers), and interference with neutralization was measured as a decrease in neutralization compared to antibody-only wells.

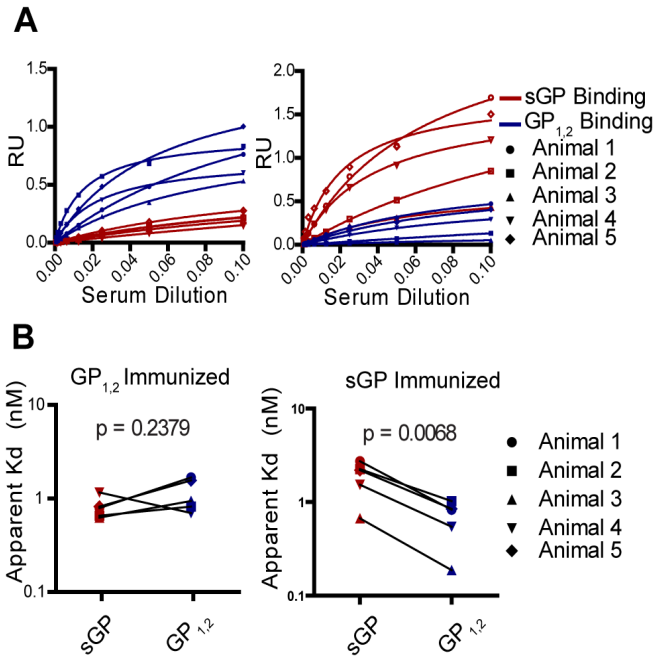


Figure 2-5. Comparison of binding affinity of $GP_{1,2}$-immunized versus sGP-immunized antisera for sGP and $GP_{1,2}$.

(A) Determining apparent K_d value of antibodies from immunized mice for $GP_{1,2}$ and sGP. Antiserum from five mice immunized against $GP_{1,2}$ and five mice immunized against sGP were individually analyzed by quantitative ELISA using $GP_{1,2}$ (blue) or sGP (red) as coating antigen. Scatchard analysis was used to calculate apparent dissociation constants (K_d). (B) Comparison of antibody affinity for $GP_{1,2}$ and sGP. Comparison of apparent K_d's of $GP_{1,2}$-immunized and sGP-immunized polyclonal antisera for sGP (red) and $GP_{1,2}$(blue) was determined by nonlinear regression analysis of Scatchard plots. K_d's for sGP and $GP_{1,2}$ were calculated for five individual mice in each group and values for the same animal are connected by a black line.

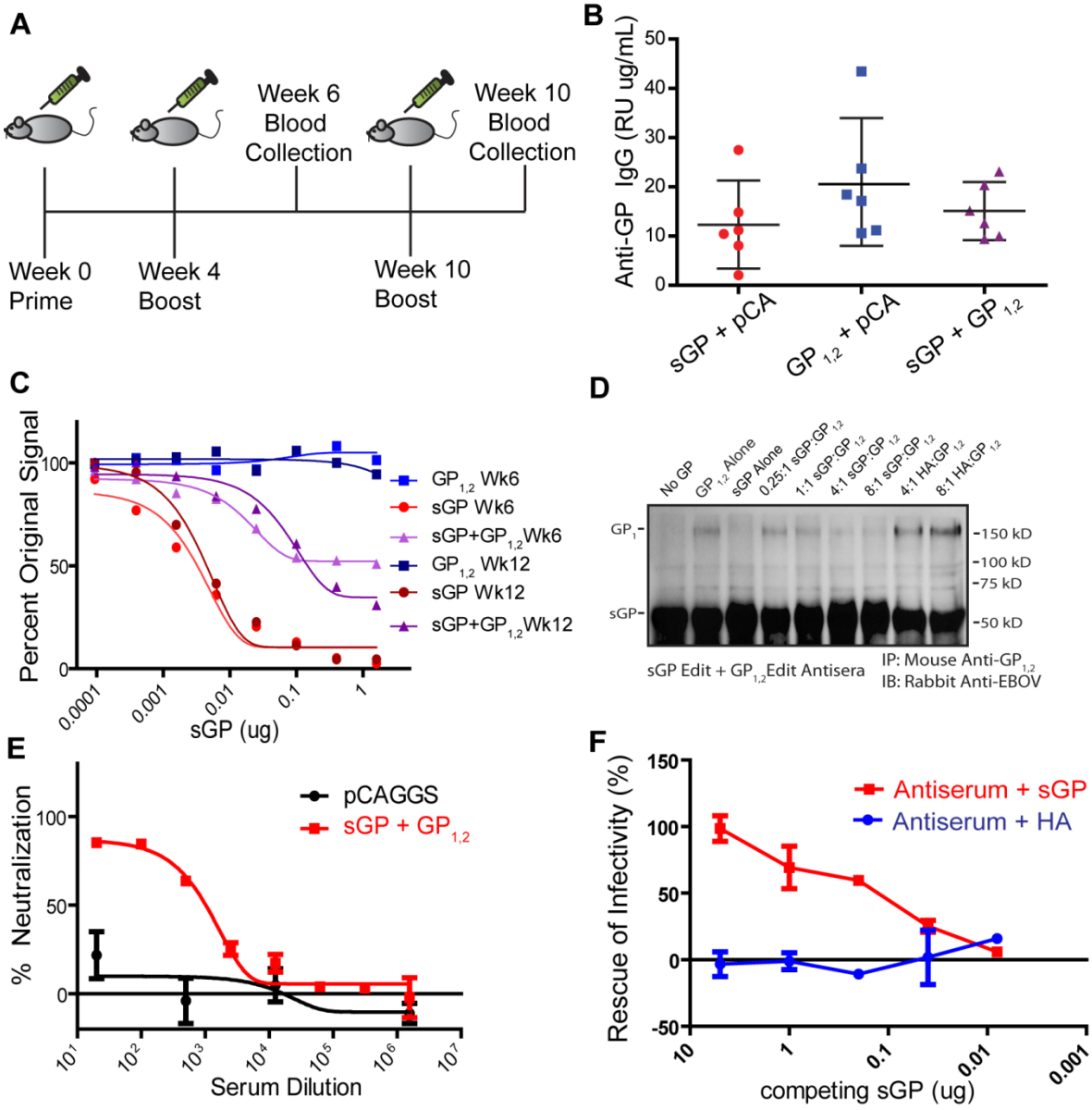


Figure 2-6. The effect of sGP on immune response when antigen exposure mimics natural infection.

(A) Immunization study design. Female BALB/C mice were immunized IM with 50 µg of total DNA per immunization according to the schedule shown. Mice were immunized with a 3:1 ratio of sGP Edit:$GP_{1,2}$ Edit in pCAGGS. Control groups were immunized with sGP Edit or $GP_{1,2}$ Edit alone plus empty pCAGGS vector to keep total amount of immunizing DNA constant. (B)

Comparison of antibody response against $GP_{1,2}$. Mouse sera collected at week 6 were analyzed for anti-$GP_{1,2}$ antibodies by ELISA using $GP_{1,2}$ as coating antigen. (C) sGP competition ELISA. The ability of sGP to compete for anti-GP antibodies was determined by competition ELISA as described in Figure 3B. Pooled antisera were analyzed from mice immunized with a 3:1 ratio of sGP Edit:$GP_{1,2}$Edit (purple), $GP_{1,2}$ Edit (blue), or sGP Edit (red), and were diluted to give roughly equivalent anti-$GP_{1,2}$ signal. Competition ELISA was performed from antisera collected at both week 6 (light color) and week 12 (dark color) according to the immunization schedule. (D) Competition immunoprecipitation. Pooled antisera from sGPEdit+$GP_{1,2}$Edit-immunized mice were incubated with no GP, purified sGP or $GP_{1,2}$ alone, or with fixed $GP_{1,2}$ and increasing concentrations of sGP to compete for anti-$GP_{1,2}$ antibodies. $GP_{1,2}$ was incubated with recombinant HA as a negative control. Samples were precipitated, run on SDS-PAGE, and then blotted using a polyclonal rabbit antibody that reacts with both $GP_{1,2}$ and sGP. (E) Neutralization of EBOV GP pseudovirus. Neutralizing activity of antisera was determined by incubating 500 pfu of $GP_{1,2}$-pseudotyped virus with dilutions of pooled sGP+$GP_{1,2}$-immunized (red), or empty pCAGGS vector-immunized (black) antisera. Neutralization was measured as decrease in luciferase expression compared to virus-only controls. (F) Interference of EBOV GP pseudovirus neutralization by sGP. The ability of sGP to interfere with antibody-dependent neutralization was determined by allowing sGP to compete with $GP_{1,2}$ pseudotyped viruses for anti-$GP_{1,2}$ antibodies. Pooled sGP+$GP_{1,2}$-immunized antisera were fixed at the dilution corresponding to 80% neutralization. Antisera were co-incubated with increasing dilutions of purified sGP (red) or purified influenza PR8 HA (blue), and interference with neutralization was measured as a decrease in neutralization compared to antibody-only wells.

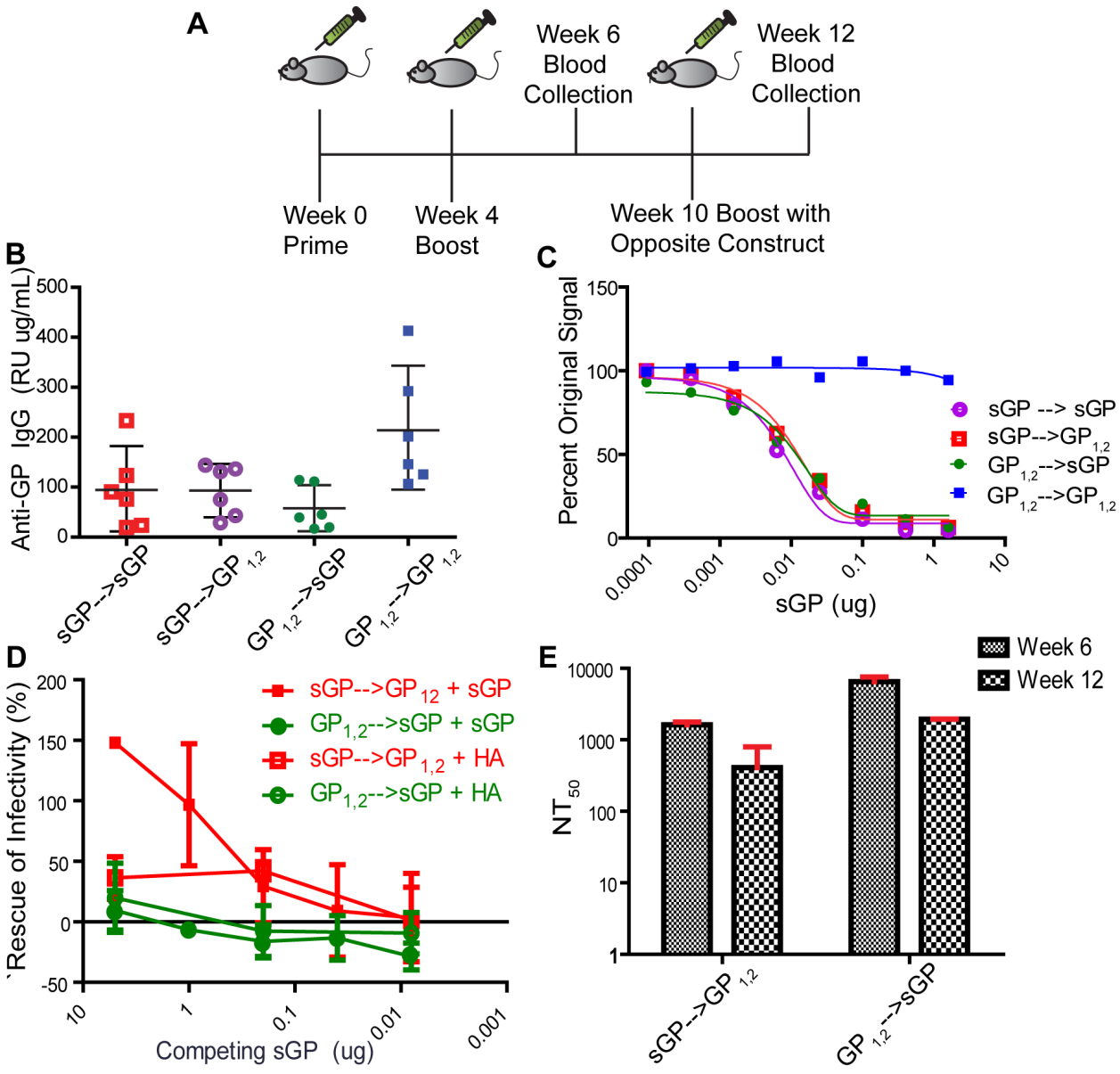


Figure 2-7. Ability of sGP to divert antibody responses against $GP_{1,2}$.

(A) Immunization study design. Female BALB/C mice were immunized IM with 50 µg of total DNA per immunization according to the schedule. Two groups of mice (n=12) were primed and boosted as in previous experiments with either sGP Edit or $GP_{1,2}$ Edit in pCAGGS vector. Each group was divided in two and subgroups were boosted at week 10 with either the same construct against which they had initially been immunized, or with the opposite editing site mutant

construct. (B) Comparison of antibody response against $GP_{1,2}$. Sera collected at week 12 were analyzed for antibodies against $GP_{1,2}$ by ELISA using $GP_{1,2}$ as coating antigen. (C) sGP competition ELISA. The ability of sGP to compete for anti-$GP_{1,2}$ antibodies was determined by competition ELISA as described in Figure 3B. Pooled antisera were analyzed from mice immunized with sGP Edit and then boosted at week 10 with either $GP_{1,2}$ Edit (red), or sGP Edit (purple), and from mice immunized with $GP_{1,2}$ Edit and then boosted at week 10 with either $GP_{1,2}$Edit (blue) or sGP Edit (green). All ELISA experiments were performed in duplicate at least three times and representative results shown. (D) Interference of EBOV GP pseudovirus neutralization by sGP. The ability of sGP to interfere with antibody-dependent neutralization was determined by allowing sGP to compete with $GP_{1,2}$ pseudotyped viruses for anti-$GP_{1,2}$ antibodies. Pooled sGP-primed, $GP_{1,2}$-boosted (red) and $GP_{1,2}$-primed, sGP-boosted (green) antisera were fixed at the dilution corresponding to 50% neutralization. Antisera was co-incubated with increasing dilutions of His-tagged sGP (solid markers) or His-tagged influenza PR8 HA (open markers), and interference with neutralization was measured as a decrease in neutralization compared to antibody-only wells. (E) Comparison of 50% neutralization titers. Antiserum titers corresponding to 50% pseudovirus neutralization activity (NT_{50}) were calculated for week 6 (fine checkered) and week 12 (coarse checkered) mice.

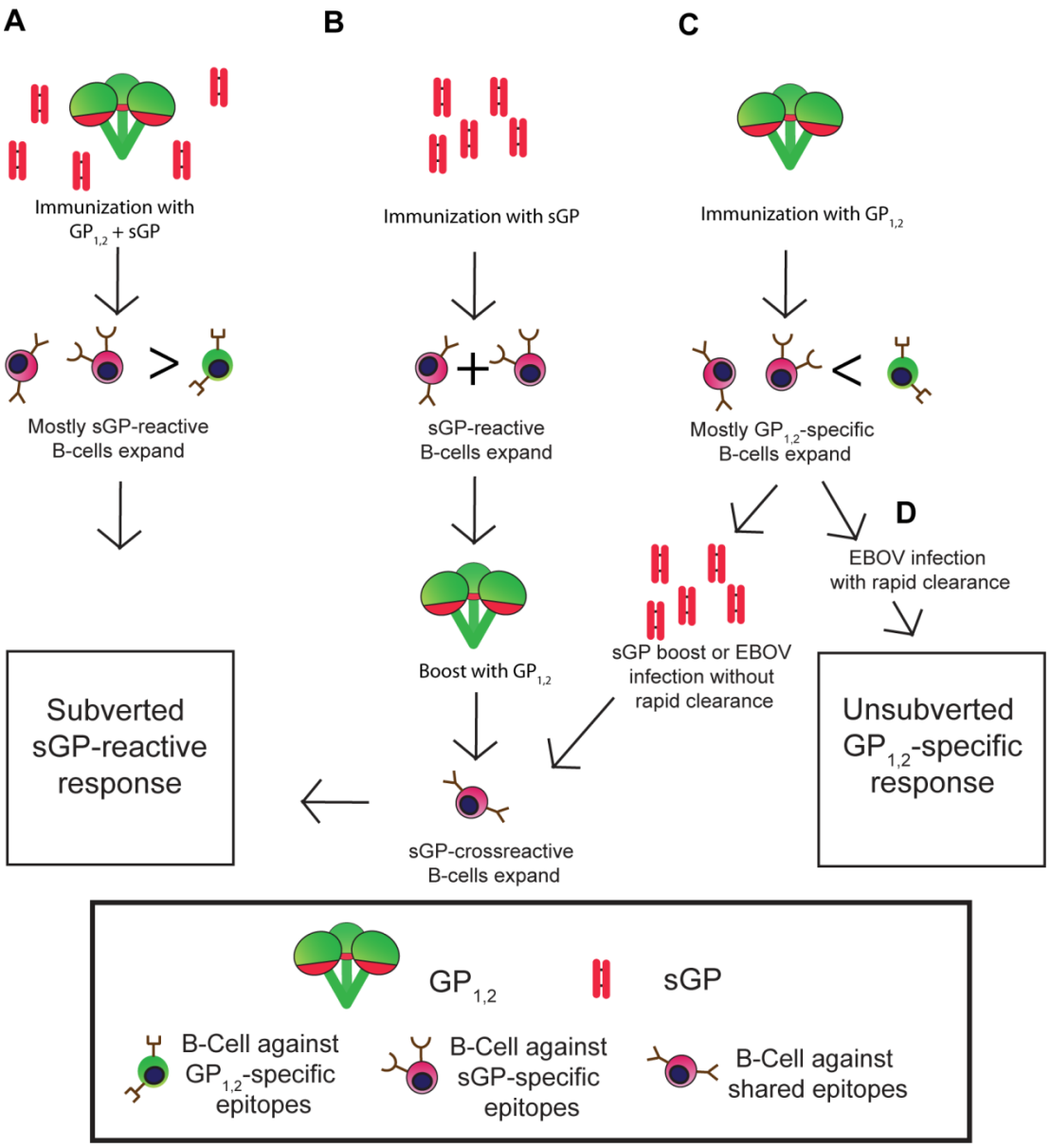
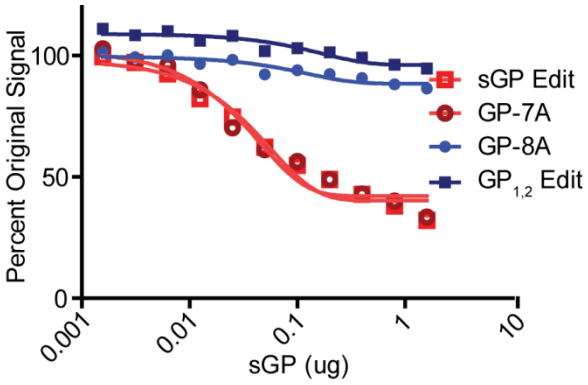


Figure 2-8. Proposed mechanism for antigenic subversion.

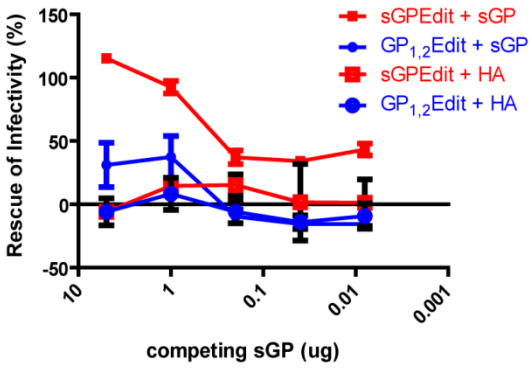
Regions of $GP_{1,2}$ that are shared with sGP are in red, while unshared epitopes are in green. B-cells are colored according to the regions of $GP_{1,2}$ and sGP against which they react. (A) A naïve animal begins with B-cells that can potentially recognize epitopes distributed throughout $GP_{1,2}$ and sGP. When sGP is expressed at much higher levels than $GP_{1,2}$, as occurs during infection, those B-cells that recognize sGP epitopes, many of which are shared with $GP_{1,2}$ (red regions of

sGP and $GP_{1,2}$) are preferentially activated and expanded compared to B-cells that recognize unshared epitopes of $GP_{1,2}$ (green regions of $GP_{1,2}$). Thus, sGP-reactive antibodies dominate the immune response. (B) Prior immunization by sGP. Because sGP shares over 90% of its linear sequence with $GP_{1,2}$, animals primed with sGP generate anti-sGP antibodies, many of which are directed against epitopes shared with $GP_{1,2}$. When these animals (or individuals who have previously been infected and recovered from EBOV infection) are boosted with $GP_{1,2}$, sGP cross-reactive memory cells outnumber and express higher affinity receptors than naïve $GP_{1,2}$ specific B-cells, resulting in preferential expansion of these sGP-cross-reactive B-cells and a predominantly sGP-reactive immune response. (C) Prior immunization by $GP_{1,2}$. Priming naïve animals with $GP_{1,2}$ results in antibodies largely against $GP_{1,2}$ epitopes not shared with sGP, presumably due to the immunodominance and high accessibility of the $GP_{1,2}$ mucin domain and shielding of shared epitopes. When these animals are boosted with sGP, or if they are infected with EBOV and do not have sufficiently high titers of anti-$GP_{1,2}$ antibodies to clear the infection rapidly, memory B-cells that recognize shared epitopes encounter their cognate antigen and expand, while non-cross-reactive $GP_{1,2}$-specific B-cells are not boosted, resulting in subversion of the host immune response towards sGP cross-reactivity. (D) Successful clearance of EBOV infection. In order to avoid sGP-mediated antigenic subversion, high enough titers of non-crossreactive anti-$GP_{1,2}$ antibodies must be maintained to rapidly clear EBOV infection before subversion can occur.



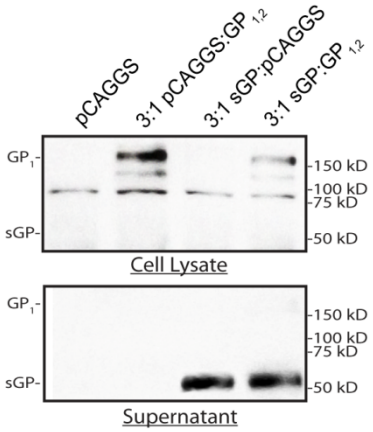
Supplemental Figure 2-S1. Competition cell surface ELISA.

HeLa cells were seeded in a 96-well plate and allowed to grow overnight to 100% confluency. Cells were then infected at an MOI of 5 with a recombinant vaccinia virus that directs infected cells to express membrane-bound EBOV $GP_{1,2}$. At 24 h post-infection, cells were fixed in 2% paraformaldehyde and washed in PBS. Pooled antisera from mice immunized with sGPEdit (light red), GP-7A (dark red), GP-8A (light blue), and $GP_{1,2}$Edit (dark blue) were diluted to give roughly equivalent anti-$GP_{1,2}$ signal. Diluted antiserum was mixed with increasing quantities of purified his-sGP and incubated with fixed $GP_{1,2}$ expressing cells for two hours to allow sGP to compete with $GP_{1,2}$ for antibodies. ELISAs were developed as previously described with the exception that detergent-free PBS was used in washing steps.



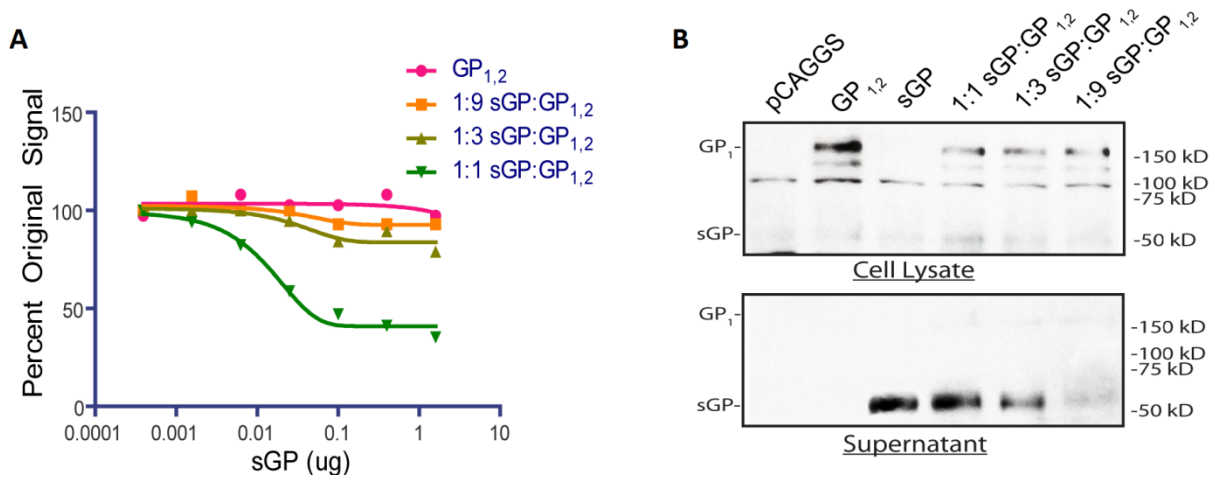
Supplemental Figure 2-S2. Interference with antibody-mediated neutralization by sGP at 50% neutralizing activity.

The ability of sGP to interfere with antibody-dependent neutralization was determined identically to Figure 4B, except that the concentration of antisera was fixed to correspond to 50% neutralization. Pooled $GP_{1,2}$-immunized (blue) and sGP-immunized (red) antisera were co-incubated with increasing dilutions of his-sGP (solid markers) or his-influenza PR8 HA (open markers), and interference with neutralization was measured as a decrease in neutralization compared to antibody-only wells.



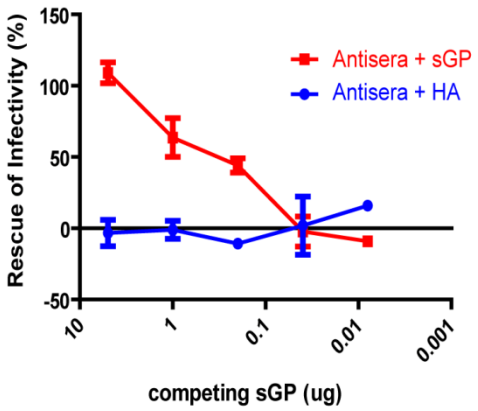
Supplemental Figure 2-S3. Expression of $GP_{1,2}$ and sGP together.

Because antigen expression from DNA vaccines is too low to detect *in vivo*, we measured expression in cell culture as a proxy for *in vivo* expression. HeLa cells in 6-well plates were transfected with $GP_{1,2}$Edit, sGPEdit, and empty pCAGGS vector at the same ratio as used to immunize animals and 5 μg total DNA per well. Expression of sGP and $GP_{1,2}$ was determined 36 h post-transfection in both cell lysate and culture supernatant by Western blot using a polyclonal rabbit antibody that reacts with both GP isoforms. The volume of cell lysate and supernatant analyzed for each sample was proportional to the total amount of lysate and supernatant collected so that the Western blots reflect the relative amounts of total sGP and $GP_{1,2}$ produced.



Supplemental Figure 2-S4. Immunization with lower ratios of sGP:$GP_{1,2}$.

Female BALB/C mice were immunized IM with 50 μg of total DNA per immunization as in previous immunization experiments. The amount of $GP_{1,2}$Edit was fixed at 12.5 μg, and groups were immunized with a 1:1 ratio and 1:9 ratio of sGP Edit:$GP_{1,2}$ Edit, as well as $GP_{1,2}$Edit without sGPEdit. Total immunizing DNA was normalized to 50 μg with empty pCAGGS vector. (Top Panel) sGP competition ELISA. Pooled antisera were analyzed from immunized mice at week 2 post-immunization and the ability of sGP to compete for anti-$GP_{1,2}$ antibodies was determined by competition ELISA as described in Figure 3B. (Bottom Panel) *In Vitro* antigen expression. HeLa cells were transfected with $GP_{1,2}$Edit, sGPEdit, and empty pCAGGS vector at the same ratio as used to immunize animals and 5 μg total DNA per well. Expression of sGP and $GP_{1,2}$ was determined 36 h post-transfection as describe in Figure S3. Both cell lysate and culture supernatant were analyzed by Western blot using a polyclonal rabbit antibody that reacts with both GP isoforms.



Supplemental Figure 2-S5. Interference with antibody-mediated neutralization by sGP at 50% neutralizing activity from $GP_{1,2}$+sGP antisera.

The ability of sGP to interfere with antibody-dependent neutralization was determined identically to Figure 6F, except the antisera concentration was fixed to correspond to 50% neutralization. Pooled $GP_{1,2}$+sGP-immunized antisera were co-incubated with increasing dilutions of sGP (red) or influenza PR8 HA (blue), and interference with neutralization was measured as a decrease in neutralization compared to antibody-only wells.

Chapter 3: Ebola Surface Glycoprotein Expression Levels Regulate Virus Production and Infectivity

The data presented in this chapter has been submitted for publication as:

Mohan, G.S., Ye, L., Li, W., Monteiro, A.C., Compans, R.W., Yang, C. (2013) "Ebola Surface Glycoprotein Levels Regulate Virus Production and Infectivity"

All data reported here were generated by the author of this document unless otherwise noted in the figure legends

Abstract

The Ebola virus surface glycoprotein ($GP_{1,2}$) mediates host cell attachment and fusion, and is the primary target for host neutralizing antibodies. Expression of $GP_{1,2}$ at high levels disrupts normal cell physiology, and EBOV uses an RNA editing mechanism to regulate expression of the GP gene. In this study, we demonstrate that high levels of $GP_{1,2}$ expression impair production and release of EBOV VLP's and infectivity of $GP_{1,2}$ pseudotyped viruses. We further show that this effect is mediated through two mechanisms. First, high levels of $GP_{1,2}$ expression reduces synthesis of other proteins needed for virus assembly. Second, viruses containing high levels of $GP_{1,2}$ are intrinsically less infectious, possibly due to impaired receptor binding or endosomal processing. Importantly, proteolysis can rescue the infectivity of high-$GP_{1,2}$ containing viruses. Taken together, our findings indicate that $GP_{1,2}$ expression levels have a profound effect on factors that contribute to virus fitness, and that RNA editing may be an important mechanism employed by EBOV to regulate $GP_{1,2}$ expression in order to optimize virus production and infectivity.

Introduction

The Ebola virus (EBOV), a member of the *Mononegavirales* order of enveloped viruses, is the etiologic agent of Ebola hemorrhagic fever (EHF), a highly lethal disease with up to 90% mortality[84]. The EBOV surface glycoprotein ($GP_{1,2}$) is the virion structural component that mediates attachment to and fusion with host cells. EBOV $GP_{1,2}$ is a type-1 transmembrane protein that is presented on the virion envelope as a homotrimeric spike, similar to HIV Env and Influenza HA[114]. Also like Env and HA, EBOV $GP_{1,2}$ is first translated as a pre-protein (GP_0), which is then cleaved into two disulfide-linked subunits ($GP_{1,2}$) in the Golgi complex by the protease furin[89]. The N-terminal GP_1 subunit has a mass of over 150kD and contains the putative receptor binding domain (RBD) as well as a highly glycosylated mucin-like domain, while the C-terminal GP_2 has a mass of roughly 20kD and contains the fusion machinery as well as the transmembrane anchor. It is thought that EBOV $GP_{1,2}$ mediates initial attachment to host cells by binding to lectins such as DC-SIGN, L-SIGN, and hMGL[115-117]. These initial attachment events are followed by internalization of the virus via macropinocytosis and trafficking of virus-containing macropinosomes to the endo-lysosomal pathway[16,118,119]. Within the acidified endosome, GP_1 is digested by the cysteine proteases cathepsins B and L, which cleave off the mucin domain as well as other regions of GP_1, producing a 20kD core and exposing the putative receptor binding domain[19,119,120]. Binding of the RBD to a host receptor then triggers GP_2 to insert its fusion loop into the host cell membrane, at which point a series of conformational changes bring the host and viral membranes together, resulting in fusion[20]. While the definitive host receptor has not yet been determined, recent studies have identified the Niemann-Pick cholesterol transporter NPC1 as indispensable for escape of virus from the acidified endosome following internalization[21,22,121,122].

EBOV $GP_{1,2}$ is also an important virulence factor, and has been implicated in many facets of EHF pathogenesis including cytopathicity, endothelial dysfunction, and immune suppression[49,123]. Previous studies have indicated that EBOV $GP_{1,2}$ is metabolically costly to synthesize and process, and that high levels of $GP_{1,2}$ expression are toxic to host cells[49,50]. While the exact mechanism of toxicity has been debated, it has been observed that over-expression of $GP_{1,2}$ leads to cell rounding and detachment as well as loss of detection of some cell surface markers[46-48]. Importantly, EBOV regulates $GP_{1,2}$ expression via an RNA editing mechanism whereby full-length $GP_{1,2}$ mRNA is produced by slippage of the viral polymerase at an editing site[23,24]. Only around 20% of transcripts are edited, while unedited transcripts contain a premature stop codon and encode a truncated glycoprotein (sGP), which is secreted in large quantities by EBOV infected cells. While the production of sGP has previously been implicated in modulation of host immunity[34,42,45,124], it is possible that RNA editing is also a mechanism by which EBOV could regulate expression of $GP_{1,2}$ in order to moderate $GP_{1,2}$-mediated toxicity. Indeed, recombinant EBOV in which the editing site was mutated to produce only $GP_{1,2}$ exhibited enhanced cytopathicity, and grew to much lower titers in cell culture than wild-type virus[50]. It is therefore of significant importance to understand how regulation of $GP_{1,2}$ expression by EBOV contributes to aspects of viral fitness such as infectivity, virus release, and immune evasion, as this information will help to elucidate how EBOV disseminates within a host and causes disease. Furthermore, a better understanding of how $GP_{1,2}$ expression affects virus production and infectivity may allow us to identify vulnerabilities in the virus life cycle that can be targeted by vaccines and antivirals.

While a great deal of work has examined how modifications to EBOV $GP_{1,2}$ affect its ability to mediate host cell attachment and fusion[117,125,126], there is little information on the

effect that expression levels of $GP_{1,2}$ have on virus production and infectivity. EBOV, like other nonsegmented members of *mononegavirales*, employs a very simple regulatory mechanism for gene expression, in which the order of genes from 3' to 5' on the negative sense genome largely dictates the level of each gene's expression[127]. It has previously been shown that negative strand viruses are extremely sensitive to rearrangement of the gene order, indicating that the stoichiometry of protein expression has a profound effect on viral fitness [128,129]. However, few studies have directly examined the effect of glycoprotein expression viral fitness. One such study used recombinant rabies viruses, in which the surface glycoprotein gene was codon optimized or de-optimized, to demonstrate that lowering glycoprotein expression levels adversely affects the kinetics of virus growth *in vitro* and pathogenicity *in vivo*[130]. Considerable work has also been done regarding the mechanisms by which HIV and other retroviruses regulate Env expression, presumably to balance the generation of infectious virus while minimizing the immune profile of infected cells and progeny virions[131,132]. Two studies in particular demonstrated that very low levels of Env incorporation are sufficient to mediate infectivity, while increasing Env incorporation significantly enhanced infectivity until a plateau was reached[133,134]. These findings are consistent with the idea that because viral glycoproteins are primary targets for host antibodies, viruses must strike a fine balance between optimizing infectivity and evading host immunity. Importantly, EBOV $GP_{1,2}$ has many properties that differentiate it from glycoproteins of other related viruses, including its cytotoxicity, its ability to bind to a nearly-ubiquitously expressed host receptor, and an unique RNA editing mechanism that regulates its expression. Thus, it is of interest to better characterize how expression and incorporation of $GP_{1,2}$ contribute to viral fitness.

In this study we examined the effect of $GP_{1,2}$ expression levels on production of Zaire EBOV virus-like particles as well as the infectivity of $GP_{1,2}$-pseudotyped viruses. We demonstrate that high levels of $GP_{1,2}$ expression can impair both virus production and infectivity, and that expression of sGP may help to optimize virus production and infectivity by attenuating $GP_{1,2}$ expression levels. We further examined how high levels of $GP_{1,2}$ expression affect synthesis of other proteins, virus release, and specific infectivity of pseudoviruses. Additionally, we studied $GP_{1,2}$ from several other filoviruses, as well as mucin-deleted and proteolyzed ZEBOV $GP_{1,2}$, in order to identify the requirements for $GP_{1,2}$-mediated regulation of virus production and infectivity.

Materials and Methods

Cell Lines and Plasmids – 293T cells and JC53 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Mediatech) supplemented with 10% fetal bovine serum (Hyclone, ThermoFisher) and penicillin/streptomycin. The primary Ebola glycoprotein construct used was wild-type Ebola Zaire strain (ZEBOV, Mayinga subtype - GenBank accession# U23187.1). Other filovirus $GP_{1,2}$ constructs used were codon-optimized, and included Sudan Ebolavirus (SEBOV, Gulu subtype - GenBank accession# AY316199.1), Marburg Marburgvirus (MARV, Musoke subtype – GenBank accession# NC_001608.3), and Lloviu Cuevavirus (LLOV - GenBank accession# NC_016144.1). Codon optimization was performed by Biomatik Corporation using proprietary technology. Other plasmids used include Ebola VP40 (ZEBOV, Mayinga subtype – GenBank accession# NC_002549.1), as well as HIV-1 Env (SF162 isolate – GenBank accession# EU123924.1). Additionally, we generated an Ebola VP40-GFP fusion protein by fusing codon-optimized Pontellina Plumata GFP (GenBank accession# AAQ01184) to the C-terminal end of EBOV VP40 as described in[55]. All constructs were subcloned into pCAGGS.MCS mammalian expression vector. Plasmids were grown and purified using the EndoFree Plasmid Mega Kit (Qiagen) as per manufacturer instructions, and redissolved in pure endotoxin-free water at a concentration of 4-6 μg/μL, and purity was verified by restriction analysis and spectrophotometry.

Pseudovirus Generation and Titration – Glycoprotein-pseudotyped HIV was generated as described elsewhere [56]. Briefly, 90% confluent 293T-cells in 6-well plates were cotransfected with 500 ng Env-defective HIV backbone (SG3) DNA, as well as viral glycoprotein DNA in pCAGGS vector (varied between 4 ug and 4 ng). Unless otherwise stated, total DNA was brought to 5 ug per well with empty pCAGGS vector. Fugene HD was used as

the transfection reagent, and transfection complexes were generated in serum-free media with 3 uL Fugene HD per 1 ug DNA. Supernatants were harvested 48 h post-transfection, clarified, and filtered using a 0.45 micron filter. Pseudoviruses were titered by infecting JC53 cells [57], which express β-galactosidase and luciferase under a *tat*-activated promoter. A standard curve was generated by diluting a standard virus and comparing β-galactosidase staining (which requires visual counting of individual blue plaques), with luciferase activity (as measured by a luminometer). Luciferase activity measured from cells infected with unknown virus was then converted to PFU/mL using the standard curve. Importantly, pseudoviruses pelleted through a 20% sucrose cushion demonstrated similar infectivity patterns to those titered directly from producer cell supernatant. Thus, all titration experiments reported were performed with cell supernatant unless otherwise stated.

p24 ELISA – p24 was measured in supernatant and cell lysate of producer cells using a p24 sandwich ELISA kit (ABL Inc). Supernatant was diluted between 1:1000 and 1:10,000 in DMEM + 10% FBS. Cell lysate was collected by lysis with ice-cold RIPA buffer, followed by centrifugation at 20,000xg for 15 minutes at 4^{o}C. Lysate was collected and normalized for β-actin using a β-actin sandwich ELISA kit (Signosis). Lysate was then diluted in DMEM + 10% FBS. Diluted samples were analyzed by ELISA as per manufacturer instructions.

EBOV Virus-Like Particle Generation and Analysis – EBOV VLP's were generated by cotransfection of 293T cells with 100 ng VP40 DNA + varying amounts of EBOV $GP_{1,2}$ DNA. VP40-GFP VLP's were generated with 100 ng of VP40-GFP DNA instead of VP40 DNA. Total DNA transfected per well was normalized to 5 ug with empty pCAGGS plasmid. Supernatants were harvested 48 h post-transfection, clarified, and filtered using a 0.45 micron filter. Cell lysates were collected by lysis with ice-cold RIPA buffer, followed by centrifugation

at 20,000xg for 15 minutes at 4°C. Lysate as normalized for b-actin using a sandwich ELISA kit (Signosis). Cell culture supernatant and cell lysate were run on SDS-PAGE under denaturing conditions, and analyzed by western blotting using a polyclonal rabbit antibody the recognizes EBOV $GP_{1,2}$ and VP40.

Fluorescence Microscopy and Flow Cytometry of VLP and Pseudovirus Producer Cells – For analysis of VP40-GFP VLP producer cells, cells were fixed at 48 hours post-transfection in 0.1% paraformaldehyde and stained for ZEBOV $GP_{1,2}$ using mouse polyclonal anti-$GP_{1,2}$ antisera, followed by incubation with at TRITC-conjugated anti-mouse antibody (Southern Biotech). Confocal fluorescence micrographs were captured using an LSM 510 (Carl Zeiss) laser scanning microscope. LSM software was used for 3D reconstruction of Z-stacked images.

For analysis of EBOV $GP_{1,2}$ pseudovirus producer cells co-transfected with GFP DNA, cells were visualized at 48 hours post-transfection using a Nikon Eclipse TE200 microscope, and photographed. Cells were then treated with 20 mM EDTA, collected, and stained for ZEBOV $GP_{1,2}$ using mouse polyclonal anti-$GP_{1,2}$ antisera, followed by incubation with a PE-conjugated anti-mouse antibody (Sigma). Cells were then analyzed for $GP_{1,2}$ and GFP expression by flow cytometry using an Accuri C6 flow cytometer (BD). Live, single cells were gated by forward and side scatter, and $GP_{1,2}$ as well as GFP expression were recorded as mean fluorescence intensity of gated cells.

Trypsinization of VLP and Pseudovirus Prodcer Cells - 293T cells producing either $GP_{1,2}$-pseudotyped viruses or EBOV VLP's were washed twice in PBS (no Ca^{2+}/Mg^{2+}) and then trypsinized for 10 minutes with 200 uL of 0.25% Trypsin + 0.05% EDTA in PBS (no Ca^{2+}/Mg^{2+}). Trypsinization was stopped by addition of DMEM+10%FBS, at which point

trypsinized supernatant was separated from cells by centrifugation and collected for p24 ELSIA or for SDS-PAGE and western blot analysis.

Thermolysin Treatment of Pseudoviruses – Lyophilized Thermolysin (Sigma) was dissolved to 1 mg/mL in 50 mM Tris + 0.5 mM $CaCl_2$. Thermolysin was then mixed 1:1 with pseudovirus-containing supernatant and incubated at 37^o for 5 minutes. Thermolysin digestion was stopped with 20mM EDTA, at which point viruses were titrated for infectivity on JC53 cells, or denatured and run on SDS-PAGE for western blot analysis.

Results

High levels of $GP_{1,2}$ expression result in lower titers of infectious pseudovirus

One unique feature of Ebola viruses and related filoviruses, is that the expression of EBOV $GP_{1,2}$ is tightly regulated through RNA editing of the GP gene. We have previously shown that RNA editing and sGP production may be important in evading host immunity. In this study, we further investigated whether these mechanisms may also serve to regulate virus production and infectivity. We generated $GP_{1,2}$ pseudotyped viruses using envelope-defective HIV-1 (SG3), by transfection of 293T cells with a fixed amount of SG3 HIV core DNA, plus either 1 ug of $GP_{1,2}$ DNA alone, or 0.25 ug of $GP_{1,2}$ DNA + 0.75 ug of sGP DNA, in order to recapitulate the ratio of GP isoforms produced during natural infection. As a control, we also generated viruses with 0.25 ug of $GP_{1,2}$ + 0.75ug empty pCAGGS plasmid. Pseudovirus-containing supernatants were collected at 48 hours post-transfection and infectivity was measured in JC53 cells by both b-galactosidase staining and luciferase assay. Surprisingly, viruses generated by co-expression of sGP+$GP_{1,2}$ displayed roughly 3-fold higher infectivity than viruses generated with $GP_{1,2}$ only (Fig 1A). Furthermore, sGP+$GP_{1,2}$ viruses demonstrated almost identical infectivity to viruses produced by transfection with $GP_{1,2}$ DNA + empty pCAGGS vector, suggesting that the enhancement of infectivity was due more to the absence of $GP_{1,2}$ than to the presence of sGP.

To further investigate our findings, we determined how $GP_{1,2}$ expression levels affected production of infectious pseudovirus. We generated a panel $GP_{1,2}$-pseudotyped viruses, varying the amount of transfected $GP_{1,2}$ DNA over a 1000-fold range while keeping the amount of the SG3 backbone DNA constant. In parallel, pseudoviruses were also generated with a 1:3 ratio of $GP_{1,2}$:sGP DNA. Unexpectedly, infectious titers of $GP_{1,2}$-pseudotyped viruses were maximum

using 16 ng of transfected $GP_{1,2}$ DNA (Fig 1B). When $GP_{1,2}$ DNA was increased 256-fold to 4ug, titers of the resulting viruses dropped by a factor of over 25. Virus infectivity also dropped when $GP_{1,2}$ transfection was reduced below 16 ng of DNA. Interestingly, when producer cells were matched for transfected $GP_{1,2}$ DNA, viruses generated from sGP+$GP_{1,2}$-expressing cells displayed virtually identical infectivity to those generated with $GP_{1,2}$ DNA alone. Taken together, these data indicate that high levels of $GP_{1,2}$ expression significantly impair the production of infectious pseudovirions. Furthermore, the ability of sGP co-expression to rescue production of infectious pseudovirus results from reduction of $GP_{1,2}$ expression rather than sGP-mediated enhancement of infectivity.

$GP_{1,2}$ expression levels from different filovirus strains have a similar effect on virus production and infectivity

In order to determine if impairment of infectious virus production under conditions of high $GP_{1,2}$ expression is a common feature of all filovirus glycoproteins, we generated pseudoviruses with $GP_{1,2}$ from several different filovirus strains. Pseudoviruses generated with all filovirus glycoproteins tested exhibited a similar infectivity pattern, in which infectivity was maximized at an intermediate level of glycoprotein expression, while increasing or decreasing $GP_{1,2}$ DNA from this optimal level significantly decreased virus production (Fig 2A). Interestingly, the maximum infectivity titer varied among pseudoviruses generated with different glycoproteins, with Lloviu (LLOV) $GP_{1,2}$ exhibiting the lowest peak infectivity, and Marburg (MARV) $GP_{1,2}$ exhibiting the highest. These data indicate that expression levels of all filovirus glycoproteins exhibit a similar effect on virus production and infectivity.

We were interested to know if the infectivity pattern we observed with titration of filovirus $GP_{1,2}$ expression levels held true for glycoproteins from an unrelated virus. To address this, we generated pseudoviruses with HIV Env and with ZEBOV $GP_{1,2}$ in parallel, and compared their infectivity. Similarly to Figure 1, infectivity of ZEBOV $GP_{1,2}$-pseudotyped viruses was maximum using 16 ng of transfected $GP_{1,2}$ DNA (Fig 2B), while increasing $GP_{1,2}$ DNA 256-fold to 4ug, resulted in infectivity titers dropping by a factor of over 80. In marked contrast, Env-pseudotyped viruses yielded the highest titers with the highest levels of Env plasmid transfection, while decreasing amounts of Env-encoding DNA dramatically decreased infectious virus production.

High expression levels of EBOV $GP_{1,2}$ but not HIV Env impair synthesis as well as release of p24.

In order to elucidate the mechanism by which high EBOV $GP_{1,2}$ expression reduces production of infectious virus , we measured p24 expression by ELISA in the $GP_{1,2}$ and Env-pseudotyped viruses described in Figure 2B. Additionally, we also measured p24 in the lysate of producer cells. Interestingly, in $GP_{1,2}$-expressing cells, the maximum levels of p24 in both supernatant and cell lysate were from cells transfected with 16 ng of EBOV $GP_{1,2}$ DNA. Increasing the amount of EBOV $GP_{1,2}$ DNA to 4 ug resulted in a 10-fold decrease in supernatant p24 (Fig 3A) and a 5-fold decrease lysate p24 (Fig 3B). On the other hand, in HIV-Env expressing cells, a modest 2-fold decrease in supernatant p24 was seen only at the highest levels of Env DNA transfection, while little variation in lysate p24 was observed across all Env transfection levels.

We calculated the ratio of supernatant:lysate p24, and observed that high expression levels of both EBOV $GP_{1,2}$ and of HIV Env resulted in a roughly 2-fold decrease in supernatant:lysate p24 ratio as compared to low levels of $GP_{1,2}$ and Env expression (Fig 3C). This raised the possibility that high levels of glycoprotein expression impair either assembly or release of virions, and may result in increased retention of virus core proteins by producer cells. Additionally, we also calculated "specific infectivity" for both $GP_{1,2}$ and Env-pseudotyped viruses, which we defined as PFU per pg p24. Surprisingly, $GP_{1,2}$ pseudotyped viruses generated at high levels of $GP_{1,2}$ expression (4 ug and 1 ug of $GP_{1,2}$ DNA) demonstrated a 10-fold decrease in specific infectivity compared to viruses generated with 16 ng $GP_{1,2}$ DNA (Fig 3D). On the other hand, HIV Env-pseudotyped viruses displayed the highest specific infectivity under conditions of high Env expression. This finding indicates that even when correcting for impaired pseudovirus production and release under conditions of high EBOV $GP_{1,2}$ expression, pseudoviruses containing high levels of $GP_{1,2}$ are intrinsically less infectious than their low-$GP_{1,2}$ containing counterparts. Importantly, this pattern does not hold true for HIV Env.

High levels of EBOV $GP_{1,2}$ expression impair production and release of EBOV virus-like particles

While our data clearly demonstrate that high levels of EBOV $GP_{1,2}$ expression impair production of infectious pseudovirus, we wanted determine whether this effect held true for EBOV virus-like particles. We generated EBOV VLPs by co-transfecting 293T cells with plasmids encoding Zaire $GP_{1,2}$ as well as ZEBOV VP40, the primary EBOV matrix protein. We varied the amount of transfected $GP_{1,2}$ DNA over a 1000-fold range while keeping the amount of VP40 DNA constant. VLP's and cell lysates were collected at 48 hours and analyzed by western

blot. Consistent with the results of our pseudovirus experiments, high expression levels of $GP_{1,2}$ resulted in drastic attenuation of VLP release into the supernatant as well as VP40 present in the cell lysate (Fig 4A, B). Interestingly, moderate levels of $GP_{1,2}$ expression results in increased release of VP40 into the supernatant compared to very low levels of $GP_{1,2}$, a finding consistent with the previously published observation that $GP_{1,2}$ enhances EBOV virion budding[135]. Unexpectedly, the highest level of $GP_{1,2}$ expression also appeared to drastically decrease levels of $GP_{1,2}$ in the supernatant, indicating that $GP_{1,2}$ over-expression may impair proper release or processing of $GP_{1,2}$, or may otherwise interfere with virion budding. Under these conditions, large amounts of $GP_{1,2}$ accumulated in the cell lysate (Fig 4B).

In order to visualize co-expression of VP40 and $GP_{1,2}$ within VLP producer cells, we transfected cells as described above, except that we substituted wild-type VP40 with a VP40-GFP fusion protein that has previously been demonstrated to generate VLP's morphologically similar to those produced with wild-type VP40[76]. At 48 hours, cells were stained and visualized by confocal microscopy. We observed that high levels of $GP_{1,2}$ expression resulted in reduced expression VP40-GFP, as well as a more punctate cytoplasmic distribution of VP40-GFP (Fig 4C). On the other hand, lower levels of $GP_{1,2}$ expression resulted in significantly increased levels of VP40-GFP expression, which was localized to large densities near the periphery of the cell. Taken together, our findings indicate that high $GP_{1,2}$ expression results in both decreased virion release, as well as decreased expression and altered distribution of the matrix protein within cells. This suggests that at least part of the mechanism by which high $GP_{1,2}$ expression attenuates production of infectious virus is by impairing synthesis and assembly of other viral proteins.

Inhibition of protein synthesis by $GP_{1,2}$

It has been previously suggested that EBOV $GP_{1,2}$ is metabolically costly to synthesize and process, and that over-expression of $GP_{1,2}$ can overwhelm the protein processing machinery of cells[50]. Because high $GP_{1,2}$ expression reduces intracellular levels of other virus proteins, it is possible that impaired protein synthesis may be partly responsible for decreased release and infectivity of viruses produced in cells expressing high levels of $GP_{1,2}$. In order to test this hypothesis, we generated EBOV $GP_{1,2}$ pseudotyped viruses as described in Figure 1, except that we also co-transfected cells with fixed amounts of plasmid encoding either GFP, or Influenza PR8 NP. We chose foreign, non-exported proteins instead of endogenous housekeeping proteins in order to mimic the kinetics of protein synthesis during viral infection. GFP-transfected cells expressing high levels of $GP_{1,2}$ were dimmer than those expressing low $GP_{1,2}$ as observed by fluorescence microscopy (Fig 5A). Cells were collected at 48h post-transfection and stained for surface $GP_{1,2}$ expression and analyzed by flow cytometry. Consistent with previous results, surface $GP_{1,2}$ MFI increased with the amount of transfected $GP_{1,2}$ DNA, while GFP MFI decreased (Fig 5B). Similar to GFP-transfected cells, cells transfected with high amounts of $GP_{1,2}$ DNA expressed lower levels of influenza NP intracellularly, while cells transfected with low amounts of $GP_{1,2}$ expressed higher levels of NP (Fig 5C). These data indicate that EBOV $GP_{1,2}$ impairs synthesis of other proteins, likely contributing to decreased virus production and release under conditions of high $GP_{1,2}$ expression.

Cytotoxicity of EBOV $GP_{1,2}$ does not account for impaired infectivity of high-$GP_{1,2}$ containing pseudoviruses

In order to better characterize the effect of $GP_{1,2}$ expression levels on pseudovirus production and infectivity, we investigated the contribution of $GP_{1,2}$-mediated cytotoxicity. First, we examined the role of the $GP_{1,2}$ mucin domain on infectivity of pseudoviruses. The mucin domain has previously been implicated in many of the cytopathic effects attributed to high levels of $GP_{1,2}$ expression[46,49,60,62]. It has also been demonstrated that deletion of the mucin domain can increase incorporation of $GP_{1,2}$ into pseudoviruses, as well as enhance their infectivity[125,126,136]. We generated pseudoviruses with mucin-deleted $GP_{1,2}$ (GPΔMuc), varying the amount of transfected GPΔMuc DNA over a 1000-fold range. Unexpectedly, GPΔMuc pseudoviruses demonstrated a very similar infectivity pattern to $GP_{1,2}$ pseudoviruses, in which infectivity was maximum when we used 16 ng of GPΔMuc DNA, while infectivity dropped by over 30-fold when GPΔMuc DNA was increased to 4 ug (Fig 6A). Interestingly, GPΔMuc also demonstrated a similar pattern of specific infectivity as $GP_{1,2}$ pseudoviruses, with pseudoviruses generated with 4 ug of GPΔMuc DNA exhibiting over 3-fold lower PFU/pg p24 than those generated with lower amounts of GPΔMuc DNA (Fig 6B). These data indicate that impairment of virus production and infectivity at high $GP_{1,2}$ expression levels is independent of the mucin domain.

In addition to investigating the role of the mucin domain, we also examined the production of infectious pseudoviruses at $GP_{1,2}$ expression levels well below those required for $GP_{1,2}$-mediated cytotoxicity[137]. We generated pseudoviruses using $1/20^{th}$ the total DNA used in previous pseudovirus experiments (0.25 ug DNA/well), while keeping the ratios of SG3:$GP_{1,2}$ DNA the same. Consistent with previous results, pseudoviruses exhibited an infectivity pattern in which infectious titers were maximum at 3.2 ng of transfected $GP_{1,2}$ DNA, while increasing or decreasing transfected $GP_{1,2}$ DNA significantly decreased pseudovirus titers (Fig 6C). This

finding indicates that the ratio of $GP_{1,2}$:core expression levels has a greater impact on virus infectivity than do the absolute levels of $GP_{1,2}$ expression, further suggesting that $GP_{1,2}$ cytotoxicity is not the main factor for its observed inhibitory effect on virus particle release and infectivity.

High $GP_{1,2}$ expression levels do not restrict virus release

In order to elucidate the mechanism of impairment of virus production under conditions of high $GP_{1,2}$ expression, we investigated whether high $GP_{1,2}$ expression levels impaired release of virus into the supernatant. We previously demonstrated that high levels of $GP_{1,2}$ expression result in a reduced ratio of supernatant:lysate p24, suggesting that over-expression of $GP_{1,2}$ results in retention of p24 by producer cells (Fig 2D). It is known that $GP_{1,2}$ mediates initial virus attachment to host cells by binding lectins such as DC-SIGN. Thus, we hypothesized that nascent virions with higher $GP_{1,2}$ content might be trapped at the cell surface by these interactions instead of escaping into the supernatant. To test this hypothesis, we generated pseudoviruses as previously described, except that after collecting the supernatant, cells were washed with EDTA, trypsinized, and then lysed. Analysis of p24 in each of these fractions revealed that high $GP_{1,2}$ expression resulted in very little retention of p24 at the cell surface, while p24 was most efficiently retained under conditions of low $GP_{1,2}$ expression (Fig 7A). A similar pattern was observed with EBOV VLP's, in which higher levels of VP40 were liberated by trypsinization from low-$GP_{1,2}$ expressing cells, while increasing $GP_{1,2}$ expression resulted in less release of VP40 in the trypsinized fraction (Fig 7B). Taken together, these data indicate not only that high $GP_{1,2}$ expression does not restrict virus release, but that $GP_{1,2}$ actually reduces retention of viruses at the cell surface. This finding is consistent with previously published

studies in which EBOV GP was demonstrated to aid in budding of virus-like particles while antagonizing host factors that restrict release of virions[76,135].

Infectivity of viruses containing high levels of $GP_{1,2}$ can be rescued by proteolysis

As demonstrated above, high-$GP_{1,2}$ containing pseudoviruses exhibited 10-fold lower specific infectivity (PFU/pg p24) than pseudoviruses containing lower levels of $GP_{1,2}$. It has previously been determined that proteolysis of EBOV $GP_{1,2}$ can enhance binding and infectivity of pseudotyped viruses by removing regions of the glycoprotein that may interfere with optimal attachment to host cells[136]. Thus, we hypothesized that the density of $GP_{1,2}$ expression also has an effect on optimal binding to host cells, and that proteolysis would differentially enhance infectivity of high versus low-$GP_{1,2}$ containing viruses. To test this hypothesis, ZEBOV $GP_{1,2}$-pseudotyped viruses were digested with thermolysin, a protease that has previously been demonstrated to cleave GP_1 and enhance infectivity in a similar manner as Cathepsins B and L [19,136,138]. Viruses were digested for 5 minutes and analyzed by SDS-PAGE, which revealed proteolysis of GP_1 from 150 kD to a 37 kD intermediate (Fig 8A). Digested viruses were then titrated for infectivity alongside untreated viruses. Thermolysin treatment enhanced virus infectivity for all viruses regardless of $GP_{1,2}$ expression level (Fig 8B). However, the infectivity of high-$GP_{1,2}$ expressing viruses was enhanced by a factor of 10, while that of low-$GP_{1,2}$ expressing viruses was only enhanced by a factor of 2 (Fig 8C). Importantly, specific infectivity as measured by PFU/pg p24, appeared to plateau for proteolyzed viruses, suggesting a theoretical limit for pseudovirus infectivity(Fig 8D).

Because $GP_{1,2}$ mediates virus attachment via constitutively expressed host surface factors, and because enveloped viruses derive their envelope from the host cell membrane, we

also examined whether viruses expressing high levels of $GP_{1,2}$ aggregate. Aggregation could explain low infectivity of high-$GP_{1,2}$ containing viruses, as well as proteolytic enhancement of infectivity in these viruses. $GP_{1,2}$-pseudotyped viruses were sonicated and then titered, as sonication has been previously demonstrated to break up virus aggregates[139,140]. No change in infectivity was observed in sonicated viruses, compared to untreated viruses (Fig S1), suggesting that aggregation of virus is not responsible for decreased infectivity under conditions of high $GP_{1,2}$ expression. Taken together, our data indicate that high $GP_{1,2}$ content interferes with infectivity of viruses, and that proteolysis of $GP_{1,2}$ can rescue infectivity.

Discussion

In this study, we investigated the effect that EBOV $GP_{1,2}$ expression levels have on the production and infectivity of EBOV VLP's and pseudoviruses. We demonstrate here that there is an an optimal level of $GP_{1,2}$ expression at which virus release and infectivity are maximized. Importantly, we observed that high levels of $GP_{1,2}$ expression can profoundly impair both the production of VLP's and the infectivity of pseudoviruses, indicating that tight regulation of $GP_{1,2}$ expression is of critical importance to the EBOV life cycle. We further demonstrate that high $GP_{1,2}$ expression impairs production of other proteins required for viral assembly, possibly due to the metabolic cost associated with producing and processing $GP_{1,2.}$ Additionally, we observed that viruses containing high amounts of $GP_{1,2}$ are intrinsically less infectious. Importantly, these $GP_{1,2}$-mediated effects are not dependent on the GP_1 mucin domain, long associated with putative cytotoxicity of $GP_{1,2}$, nor are they dependent on high absolute expression levels of $GP_{1,2}$, but rather on the ratio of $GP_{1,2}$ to the virus core. Finally, we observed that proteolysis of high-$GP_{1,2}$ containing viruses with thermolysin can rescue infectivity, possibly by relieving steric shielding resulting from dense packing of $GP_{1,2}$ on the virus surface.

It has long been hypothesized that RNA editing and the production of sGP by EBOV-infected cells was a mechanism of host immune evasion. Indeed, sGP has been implicated in modulation of host immunity and in interference with the host antibody response [25-28]. However, our findings indicate that RNA editing may also be a mechanism of moderating $GP_{1,2}$ expression levels to optimize virus production and infectivity. A recent study found that when serially passaged *in vitro* in Vero E6 cells, EBOV rapidly mutates the editing site of the GP gene to adopt a predominantly $GP_{1,2}$-forming phenotype[48]. Mutant virus rapidly reverted back to a predominantly sGP-forming phenotype when reintroduced to a naïve guinea pig host. The fact

that reversion occurred within 6 days of infection suggests that adaptive immunity had little to do with this phenotypic transformation. Instead, it is possible that different host environments select for high-$GP_{1,2}$ vs low-$GP_{1,2}$ expressing viruses, depending factors within that environment that restrict virus budding and dissemination. These factors may include tetherin expression (which restricts virus budding, but is antagonized by $GP_{1,2}$), as well as other interferon-inducible antiviral factors[40,49,50]. It would be revealing to examine how passaging in different cell types, as well as treatment with interferons and other inflammatory cytokines, affects emergence of editing-site mutants.

$GP_{1,2}$ has a variety of toxic effects on cells, including induction of cell rounding, detachment, and loss of detection of surface factors[18,44]. The exact mechanism of these effects has been debated. Some studies have indicated that $GP_{1,2}$ induces active internalization of surface molecules required for attachment and communication with the immune system, and also promotes cell death[41,42]. Other studies have suggested that $GP_{1,2}$ sterically occludes surface factors without actually reducing cell viability[20,51]. In one study, it was reported that over-expression of $GP_{1,2,}$ through mutation of the GP editing site, resulted in enhanced cytopathicity, with the recombinant virus growing to lower infectivity titers [19]. The authors noted accumulation of incompletely processed $GP_{1,2}$ in the ER and early Golgi, and hypothesized that toxicity of high-$GP_{1,2}$ expression was at least partly due to saturation of the protein processing machinery. This hypothesis is consistent with our finding that high expression levels of $GP_{1,2}$ results in reduced synthesis of other proteins required to assemble VLP's or pseudoviruses. Furthermore, $GP_{1,2}$-mediated reduction in protein expression is not specific to exported viral proteins, since high $GP_{1,2}$ expression levels also impaired production of non-exported proteins such as influenza NP and GFP. As illustrated in Figure 9A, impaired protein

synthesis represents one mechanism by which high levels of $GP_{1,2}$ expression impair production of infectious virus. Future studies will further elucidate the exact mechanisms of impaired protein production, as well as the specific features of $GP_{1,2}$ required to mediate this effect.

A significant finding of this study is that, even when normalized for p24 content, high-$GP_{1,2}$-containing viruses are less infectious than their low-$GP_{1,2}$ counterparts. Because $GP_{1,2}$ mediates viral attachment and fusion, it would seem that higher $GP_{1,2}$ expression levels should translate into higher efficiency of infection. Indeed, HIV Env-pseudotyped viruses with high Env content displayed much higher specific infectivity than those with low Env content, a finding consistent with previously published studies[38]. However, $GP_{1,2}$-pseudotyped viruses were maximally infectious at intermediate levels of $GP_{1,2}$ expression, while increasing or decreasing $GP_{1,2}$ expression levels from the optimal level resulted in drastic attenuation of infectivity. It is possible that a high density of $GP_{1,2}$ interferes with proper $GP_{1,2}$ function through steric hindrance, as illustrated in Figure 9B and C. Packing of $GP_{1,2}$ trimers at high density at the virion surface may result in enhanced shielding of $GP_{1,2}$ receptor binding motifs, while a lower density of $GP_{1,2}$ results in increased exposure of receptor binding motifs (Fig 9B). Alternatively, steric shielding due to high $GP_{1,2}$ density may also interfere with endosomal processing required for the final steps of infection (Fig 9C). Both of these mechanisms are consistent with our observation that proteolytic processing of $GP_{1,2}$ was able to rescue infectivity of high-$GP_{1,2}$ containing viruses.

Proteolysis likely relieves steric hindrance resulting from $GP_{1,2}$ packing, promoting more efficient receptor binding and obviating the need for endosomal proteolysis. This is similar to the way in which removal of the GP_1 mucin domain also enhances infectivity by alleviating shielding of receptor binding epitopes and by enhancing attachment to host cells[43]. Once

proteolyzed, high $GP_{1,2}$-containing viruses are actually more infectious than their low-$GP_{1,2}$ counterparts, indicating that $GP_{1,2}$ generated under conditions of high expression is still intrinsically functional, and that it is likely an effect of high density of $GP_{1,2}$ on the virion surface that impairs proper function. Interestingly, it has been demonstrated that $GP_{1,2}$ lacking a transmembrane domain ($GP_{1,2}\Delta TM$) is efficiently shed by host cells through cleavage by TNFα-converting enzyme (TACE) at the membrane-proximal external region[52]. Similarly to the production of sGP, TACE-mediated secretion of $GP_{1,2}\Delta TM$ has also been hypothesized to allow for absorption of host anti-GP antibodies. In light of our findings in this study, it is also possible that gradual TACE-mediated cleavage of $GP_{1,2}$ may allow the virus to modulate its infectivity by decreasing the surface density of $GP_{1,2}$ on virions. Further, the proteolytic cleavage of $GP_{1,2}$ may function as a timing mechanism to ensure that virions are less infectious when they first bud to allow for efficient release, but have increased infectivity after they have spread to more distal locations.

How regulation of $GP_{1,2}$ expression contributes to overall viral fitness remains a complex question. Viruses, including Ebola virus, do not necessarily evolve to optimize infectivity. Indeed, it has been previously demonstrated that single amino acid mutations, as well as deletions of regions of $GP_{1,2}$, can significantly enhance infectivity of $GP_{1,2}$-peudotyped viruses[30]. While it may seem intuitive that enhanced infectivity is synonymous with optimizing viral fitness, this is probably not the case, given that EBOV would have likely adopted these simple infectivity-enhancing mutations had they conferred a selective advantage. Instead, it is possible that EBOV has evolved mechanisms such as steric shielding of receptor binding regions, as well as regulation of glycoprotein expression levels, to actively modulate infectivity. There are several possible arguments to support this hypothesis. First, moderation of

infectivity may be necessary to facilitate effective dissemination of virus within the host. While viruses which exhibit too low infectivity may have trouble finding and infecting host cells, viruses that are overly infectious may not be efficiently released from producer cells, or may be unable to spread to distal locations. This is particularly important for viruses such as EBOV, which infect a wide range of host cell types and whose life cycle involves rapidly establishing a systemic infection. A second reason why infectivity may be sacrificed for increased viral fitness is due to host immune evasion. HIV expresses very low levels of Env to avoid immune detection[36]. On the other hand, when expressed at sufficiently high levels, EBOV $GP_{1,2}$ can shield cell surface molecules involved in immune recognition, as well as sensitive epitopes on $GP_{1,2}$itself [20,51]. The density of $GP_{1,2}$ required to mediate these effects is unknown, but it likely requires close packing of $GP_{1,2}$ trimers to form a continuous glycan canopy. Finally, $GP_{1,2}$ has been demonstrated to directly modulate host immunity through a variety of mechanisms. TheGP_2 subunit contains an immunosuppressive sequence similar to that of many retroviruses, which causes lymphocytes to downregulate markers of activation, inhibits elaboration of inflammatory cytokines, and even induces lymphocyte apoptosis[53]. Additionally, $GP_{1,2}$ can antagonize the activity of tetherin (CD317), an interferon-induced molecule that normally prevents budding of enveloped viruses by "tethering" them to the cell surface[40]. It appears that these immunomodulatory effects are dose-dependent, though it is unclear what expression levels of $GP_{1,2}$ are required *in vivo* to aid the virus.

Our findings, combined with previous studies of the role of EBOV $GP_{1,2}$ in host immune evasion, argue for a model in which expression levels of $GP_{1.2}$ represent a finely negotiated balance between optimizing virus production and infectivity, while also evading innate and adaptive host immune responses. It has been hypothesized that HIV regulates Env expression

levels to be high enough that viruses can mediate infection, but low enough that viruses can avoid host neutralizing antibodies[54]. Thus, HIV balances the pressure to increase Env expression to enhance infectivity, with the pressure to decrease Env expression to minimize visibility to the immune system. Our data, on the other hand, suggest that EBOV may face the inverse problem because high levels of $GP_{1,2}$ expression actually impair infectivity and virus production. It is possible that EBOV must maintain $GP_{1,2}$ expression at low enough levels that it can produce large quantities of infectious virus, while maintaining sufficient $GP_{1,2}$ expression to effectively modulate the host immune response. Future studies using recombinant EBOV, in which expression levels of $GP_{1,2}$ are altered, will allow us to better understand how EBOV negotiates trade-offs in infectivity versus host immune evasion to optimize fitness. Nevertheless, our findings represent the first demonstration, to our knowledge, that high levels of glycoprotein expression suppress virus infectivity. Our data add yet another dimension to our understanding of the complex balance viruses must strike as they evolve to survive in ever changing host environments.

Figures

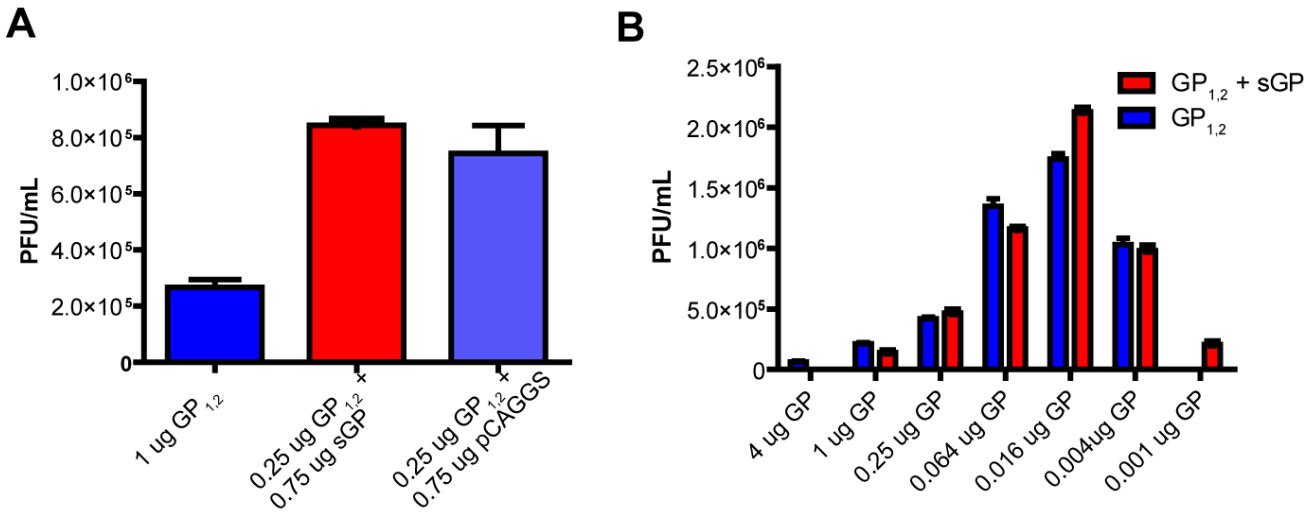


Figure 3-1– Coexpression of sGP with $GP_{1,2}$ can enhance production of infectious pseudovirus

(A) Pseudovirus infectivity titers. 293T cells were transfected with 500 ng of plasmid encoding HIVΔEnv (SG3) backbone and either 1 ug of $GP_{1,2}$ DNA or 0.25 ug $GP_{1,2}$ DNA + 0.75 ug sGP DNA. A control well was transfected with 0.25 ug $GP_{1,2}$ DNA + 0.75 ug empty pCAGGS vector. Infectivity titers were measured at 48 hours post-transfection by luciferase assay and converted to PFU/mL as described in methods. Results reported are representative of three separate experiments, and are the means and standard deviations for samples run in triplicate. (B) Effect of $GP_{1,2}$ titration on pseudovirus infectivity. Pseudoviruses were generated as described in (A), except transfected GP DNA was varied from 4 ug to 4 ng. $GP_{1,2}$+sGP pseudoviruses (red) were generated with a 3:1 ratio of sGP:$GP_{1,2}$ DNA, while $GP_{1,2}$ pseudoviruses (blue) were generated with only $GP_{1,2}$ DNA. Infectivity titers were measured at 48 hours post-transfection.

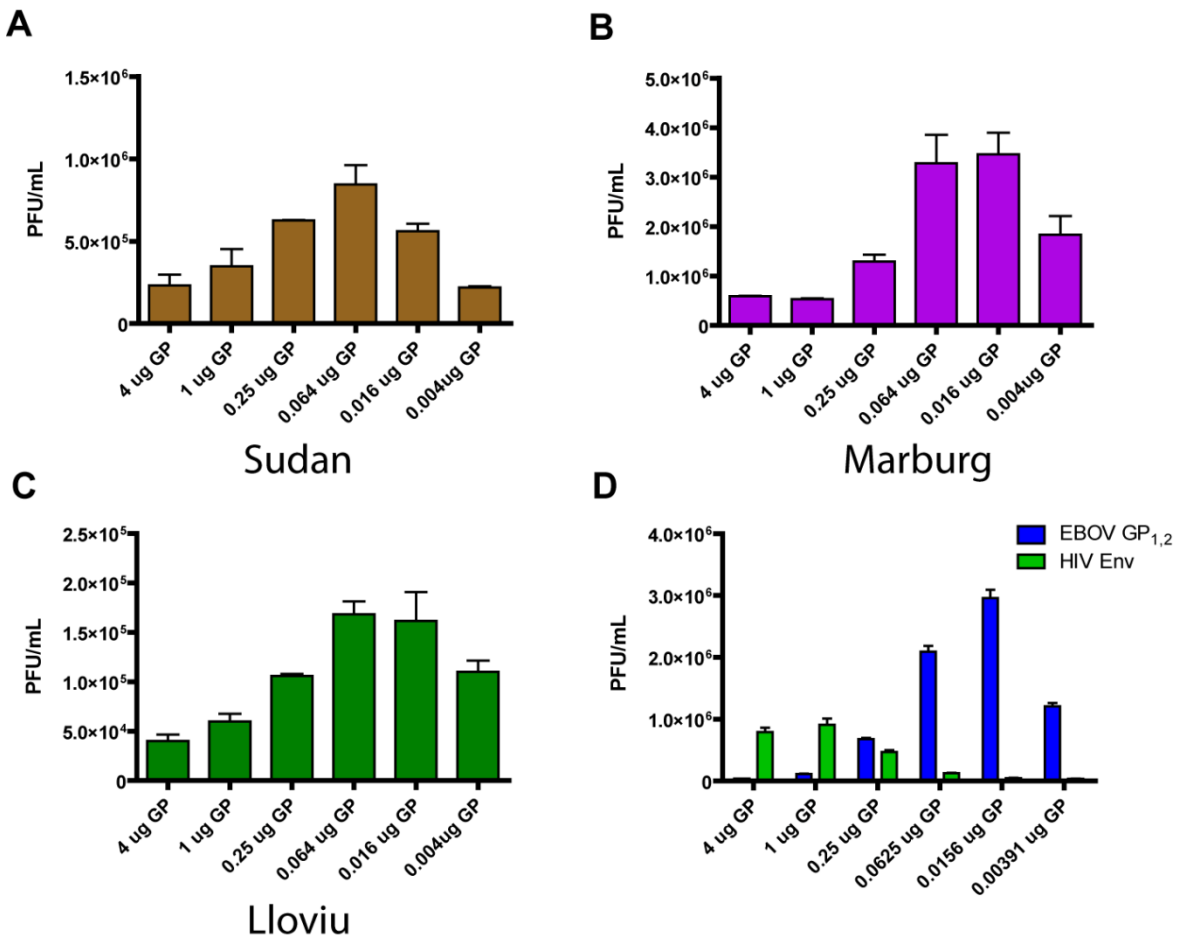


Figure 3-2 – Effect on infectivity of glycoprotein expression levels for different filovirus strains, as well as for HIV Env

(A-D) Filovirus glycoproteins. 293T cells were transfected with 500 ng of plasmid encoding HIVΔEnv (SG3) backbone plus $GP_{1,2}$ from Sudan Ebolavirus (SEBOV, Brown), Marburg Marburgvirus (MARV, Purple), or Lloviu Cuevavirus (LLOV, Dark Green). $GP_{1,2}$ DNA was varied from 4 ug to 4 ng and infectivity was measured at 48 hours post-transfection by luciferase assay and converted to PFU/mL as described in methods. Results reported are representative of three separate experiments, and are the means and standard deviations for samples run in triplicate. (E) EBOV $GP_{1,2}$ vs HIV Env. 293T cells were transfected with 500 ng of plasmid

encoding HIVΔEnv (SG3) backbone plus either ZEBOV $GP_{1,2}$ (blue) or HIV Env (green). Surface glycoprotein DNA was varied from 4 ug to 4 ng and infectivity was measured at 48 hours post-transfection by luciferase assay and converted to PFU/mL as described in methods. Results reported are representative of three separate experiments, and are the means and standard deviations for samples run in triplicate.

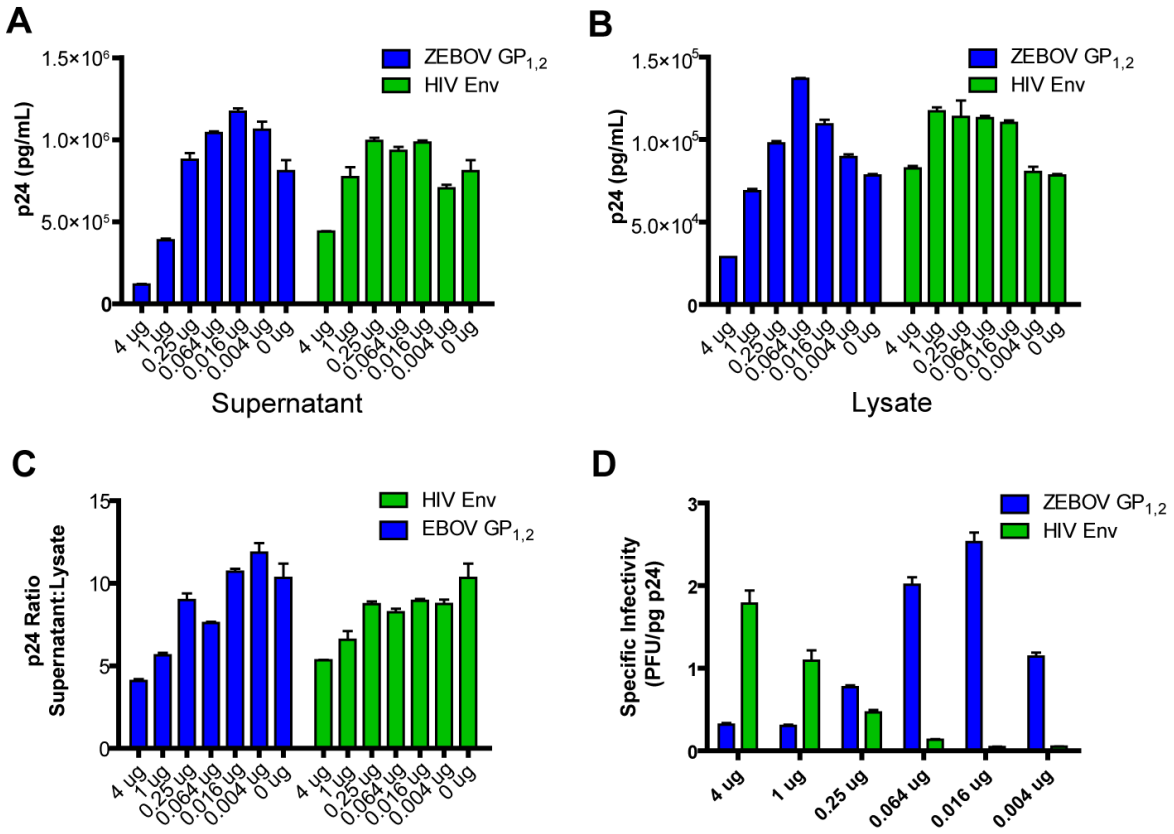


Figure 3-3 – Effect of glycoprotein expression levels on production of EBOV $GP_{1,2}$ and HIV Env-pseudotyped viruses

(A) Supernatant and (B) producer cell lysate p24 levels were measured by sandwich ELISA from cells producing EBOV $GP_{1,2}$ pseudoviruses (blue) and HIV Env pseudoviruses (green). Lysate p24 levels reported were normalized for β-actin content. Results are the representative of two separate experiments, and are the means and standard deviations for samples run in triplicate. (C) Ratio of supernatant:lysate p24. Based on results reported in (A) and (B), the ratio of p24 in supernatant to lysate was calculated and plotted versus amounts of transfected surface glycoprotein DNA. (D) Specific infectivity of EBOV $GP_{1,2}$ versus HIV Env-pseudoviruses. Based on results reported in (Fig 2E) and (A), PFU/pg p24 was calculated and plotted versus amounts of transfected $GP_{1,2}$ DNA.

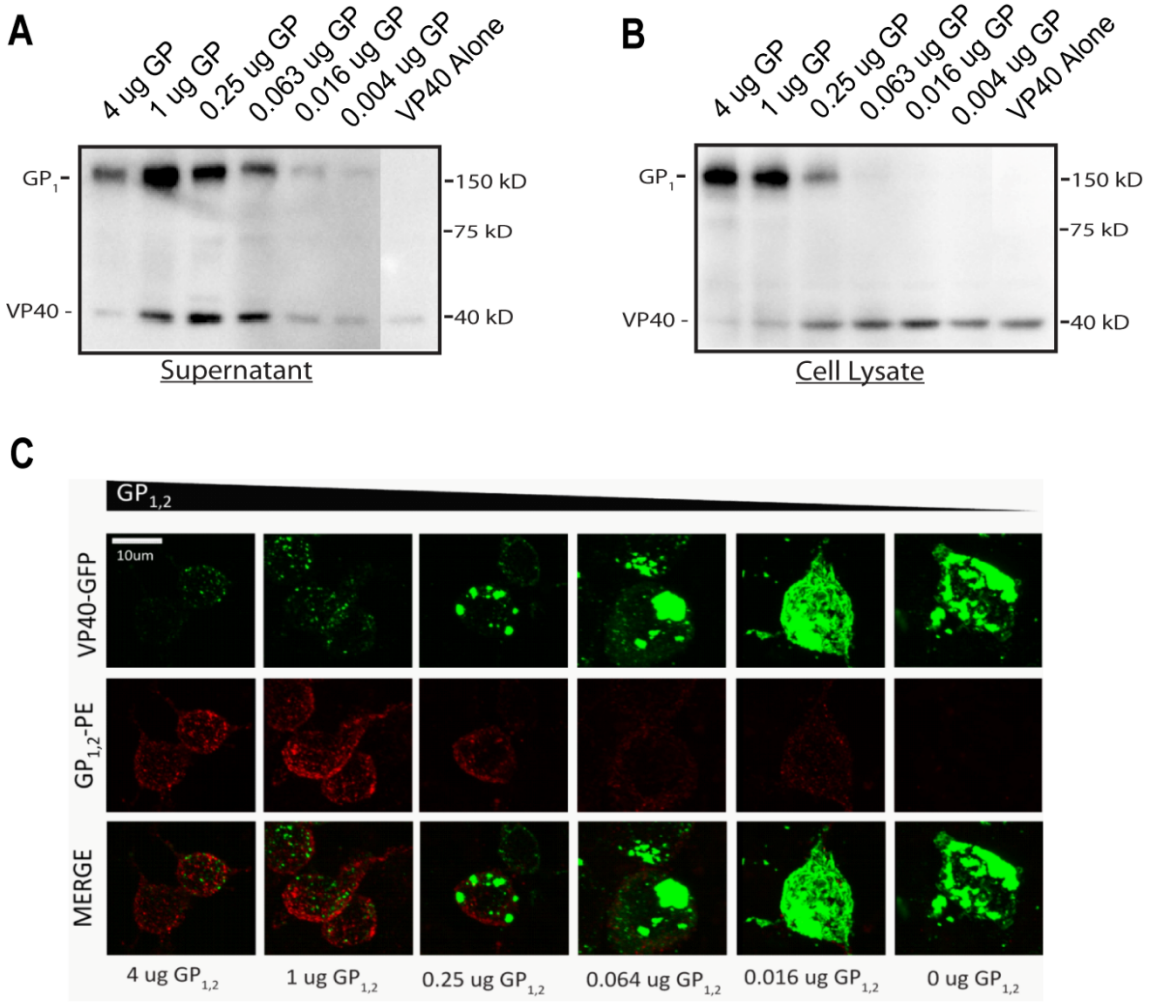


Figure 3-4 – High levels of $GP_{1,2}$ expression impair EBOV VLP production

(A, B) Production of EBOV VLP's with varying $GP_{1,2}$ expression levels. 293T cells were transfected with 100 ng of EBOV VP40 DNA, plus varying amounts of $GP_{1,2}$ DNA (4 ug to 4 ng). (A) Supernatant and (B) cell lysate were assayed by Western blot at 48 h post-transfection using polyclonal rabbit anti EBOV antiserum specific for all Zaire EBOV proteins. (C) Fluorescence microscopy of EBOV VLP-producing cells*. 293T cells were transfected with 100 ng of VP40-GFP fusion protein DNA, plus varying amounts of $GP_{1,2}$ DNA (4 ug to 16 ng). At 48 h post-transfection, cells were fixed and stained for $GP_{1,2}$ using mouse anti-$GP_{1,2}$ immune

sera, followed by a PE-conjugated anti-mouse antibody. Cells were then visualized by fluorescence microscopy for $GP_{1,2}$ (PE – Red) and VP40 (GFP-Green). Scale bar = 10 um.

* The experiment described in Figure 3-4(C) was performed by Gopi Mohan and Ana Monteiro, and the figure itself was generated primarily by Ana Monteiro.

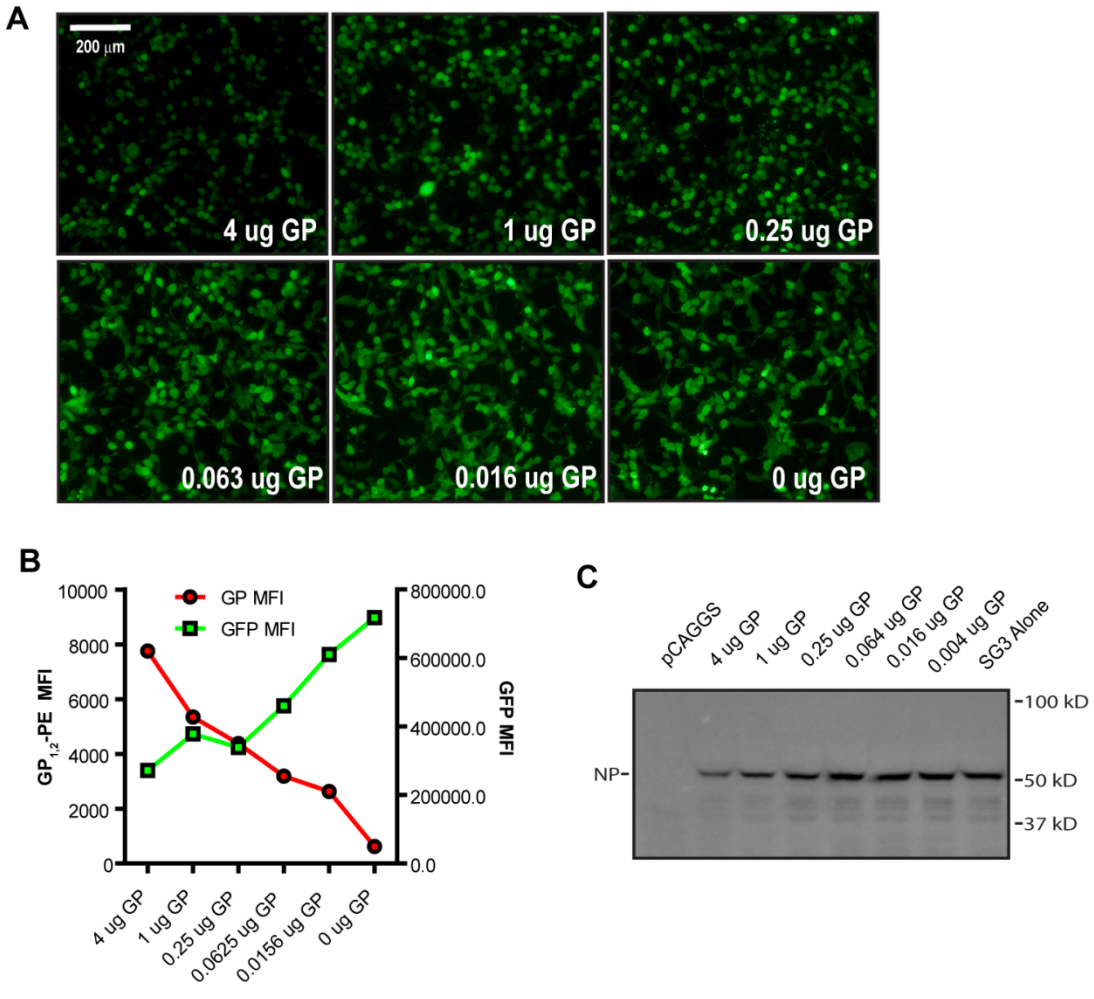


Figure 3-5 – High levels of $GP_{1,2}$ expression impair protein production

(A, B) Decreased GFP expression correlates with increased $GP_{1,2}$ expression levels. 293T cells were transfected with 500 ng of a plasmid encoding HIVΔEnv (SG3) backbone and 100 ng of a plasmid encoding GFP, as well as varying amounts of $GP_{1,2}$ DNA (4 ug to 4 ng). (A) At 48 h post-transfection, cells were imaged by fluorescence microscopy to visualize GFP expression. Results are representative of three separate experiments. (B) Cells were harvested by EDTA treatment and stained for surface $GP_{1,2}$ expression using mouse anti-$GP_{1,2}$ immune sera, followed by a PE-conjugated anti-mouse antibody. Cells were then analyze by flow cytometry, and MFI

was plotted for $GP_{1,2}$ (red) and GFP (green) versus amount of transfected $GP_{1,2}$ plasmid. (C) 293T cells were transfected as described for (A), except instead of GFP, cells were cotransfected with 100 ng of plasmid expressing PR8 Influenza A nucleoprotein (NP). Cell lysate was harvested at 48 h post-transfection, normalized for β-actin, and analyzed by western blot using a mouse monoclonal anti-NP antibody.

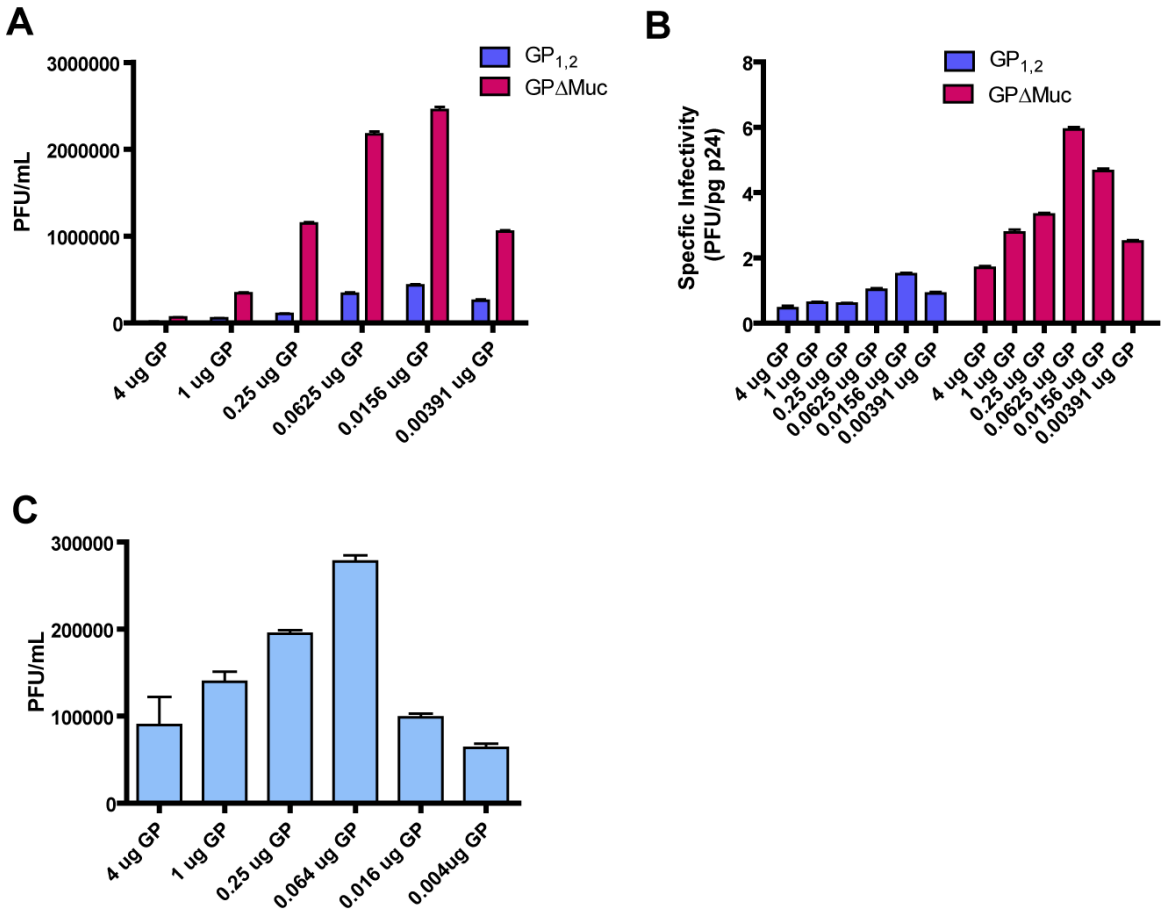


Figure 3-6 – Putative $GP_{1,2}$ cytotoxicity not responsible for impaired virus production

(A) $GP_{1,2}$ΔMuc pseudovirus infectivity titers. 293T cells were transfected with 500 ng of plasmid encoding HIVΔEnv (SG3) backbone plus either wild-type ZEBOV $GP_{1,2}$ (blue) or $GP_{1,2}$ΔMuc (purple) DNA. $GP_{1,2}$ DNA was varied from 4 ug to 4 ng and infectivity was measured at 48 hours post-transfection by luciferase assay and converted to PFU/mL as described in methods. Results reported are representative of three separate experiments, and are the means and standard deviations for samples run in triplicate. (B) Specific infectivity of EBOV $GP_{1,2}$ versus $GP_{1,2}$ΔMuc pseudoviruses. Based on results reported in (A) and supernatant p24 ELISA (not shown), PFU/pg p24 was calculated and plotted versus amounts of transfected $GP_{1,2}$ DNA. (C) Reduction of total transfected DNA. Pseudoviruses were generated by

transfecting 293T cells with 25 ng of plasmid encoding HIVΔEnv (SG3) backbone plus wild-type ZEBOV $GP_{1,2}$ DNA. $GP_{1,2}$ DNA was varied from 200 ng to 0.2 ng and infectivity was measured at 48 hours post-transfection by luciferase assay and converted to PFU/mL as described in methods.

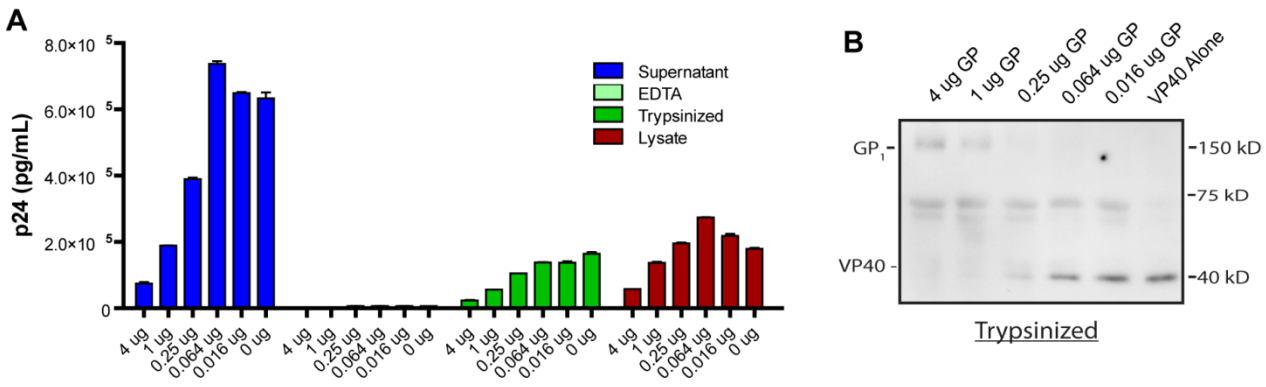


Figure 3-7 – High levels of $GP_{1,2}$ expression do not restrict virus release from producer cells

(A) Levels of p24 liberated by EDTA treatment and by trypsinization. 293T cells were transfected with 500 ng of HIVDEnv (SG3) backbone DNA and varying amounts of $GP_{1,2}$ DNA (4 ug to 0 ng). At 48 h post-transfection, supernatants were collected, at which points cells were washed and treated with 20mM EDTA in PBS for 10 minutes at 37°C, followed by trypsinization for 5 minutes at 37°C. Trypsinized cells were then lysed and cell lysate was collected. Supernatant (blue), EDTA wash (teal), trypsin fraction (green), and cell lysate (red) were all analyzed in parallel for p24 content by ELISA. Lysate p24 levels reported were normalized for β-actin. Results are the representative of two separate experiments, and are the means and standard deviations for samples run in triplicate. (B) Levels of VP40 liberated by trypsinization of EBOV VLP-producing cells. 293T cells were transfected with 100 ng of EBOV VP40 DNA, plus varying amounts of $GP_{1,2}$ DNA (4 ug to 4 ng). At 48 h post-transfection, producer cells were trypsinized and the trypsinized fraction was analyzed by Western blot using polyclonal rabbit anti-EBOV antiserum specific for all Zaire EBOV proteins.

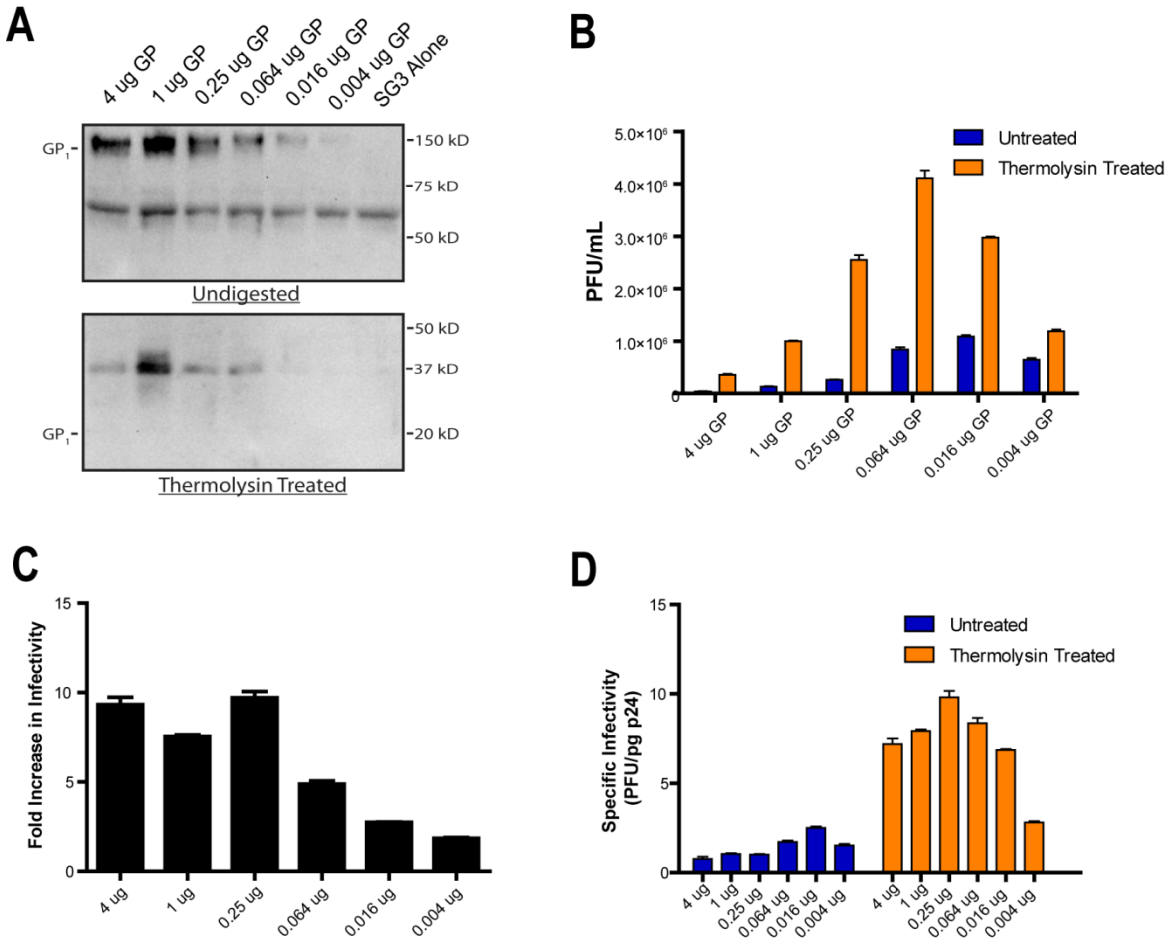


Figure 3-8 – Proteolysis of high $GP_{1,2}$-containing pseudoviruses rescues infectivity

(A) Thermolysin digestion of $GP_{1,2}$. Pseudoviruses were treated for 5 minutes at 37°C with 0.5 mg/mL thermolysin. Reactions were then terminated with protease inhibitor cocktail and samples were analyzed by Western blot using polyclonal rabbit anti-EBOV antiserum. Untreated pseudoviruses were also analyzed as a control. (B) Thermolysin treated pseudoviruses were normalized for p24 content and then titrated on JC53 cells by luciferase assay, followed by conversion to PFU/mL as described in methods. Treated viruses were titrated alongside untreated viruses as a control. Results reported are representative of three separate experiments, and are the means and standard deviations for samples run in triplicate. (C) The

fold-increase in infectivity of pseudoviruses with thermolysin treatment was calculated from the data reported in (B) and plotted versus amounts of transfected $GP_{1,2}$ DNA. (D) Specific infectivity of untreated versus thermolysin-treated pseudoviruses. Based on results reported in (B) and supernatant p24 ELISA (data not shown), specifc infectivity, defined as PFU/pg p24, was calculated for untreated versus thermolysin-treated pseudoviruses and plotted versus amounts of transfected $GP_{1,2}$ DNA.

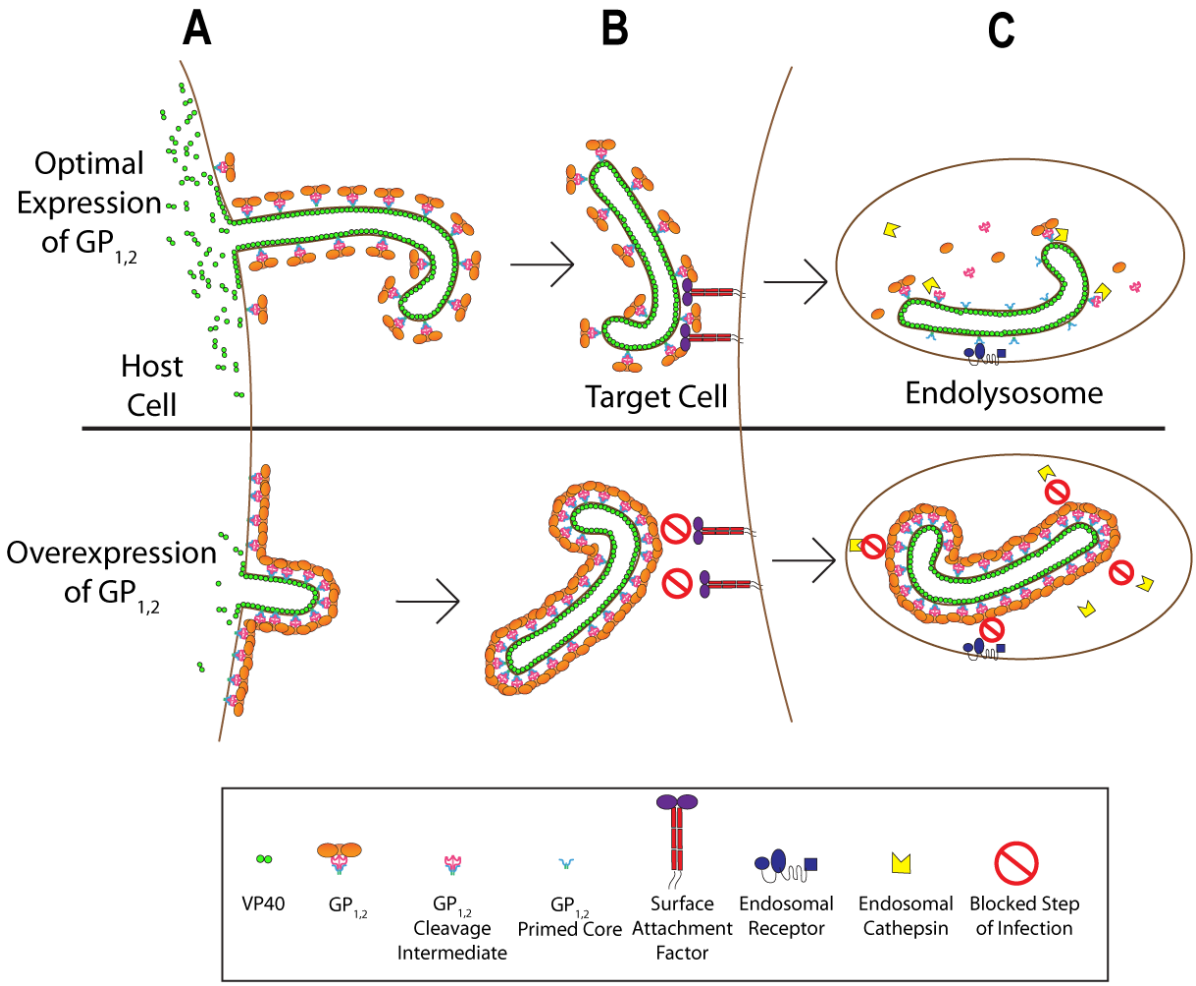
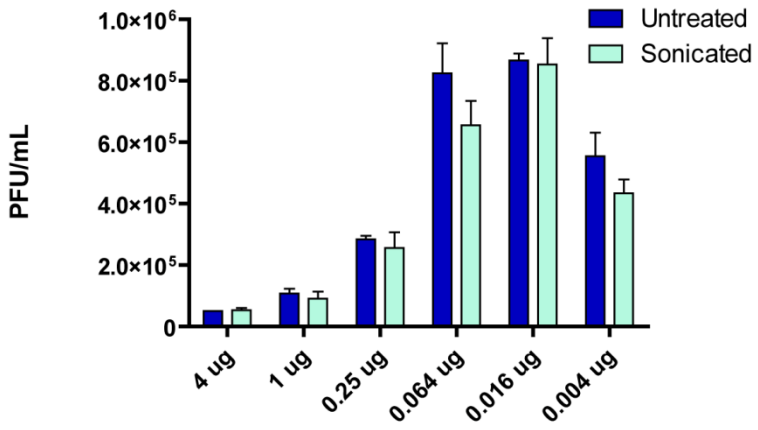


Figure 3-9 – Proposed model for impairment of virus production and infectivity at high levels of $GP_{1,2}$ expression.

(A) Optimal levels of $GP_{1,2}$ expression result in efficient production, trafficking, and assembly of other viral proteins such as VP40. Nascent virions bud efficiently from the host cell membrane, with optimal incorporation of $GP_{1,2}$ peplomers. On the other hand, overexpression of $GP_{1,2}$ results in impaired synthesis of other viral proteins. Thus, viruses do not efficiently assemble, and those viruses that do bud from the host cell contain very high levels of $GP_{1,2}$. (B) Optimal levels of $GP_{1,2}$ incorporation into virions allows for proper engagement with target cell attachment factors, resulting in efficient virion internalization. High density of $GP_{1,2}$ on the

virion envelope results in steric shielding of domains that normally bind host cell surface factors, thus interfering with attachment and internalization of virus. (C) Optimal levels of $GP_{1,2}$ incorporation allow endosomal cathepsins to properly process $GP_{1,2}$ peplomers, generating the primed core capable of binding the endosomal receptor and mediating fusion with the host membrane. On the other hand, high levels of $GP_{1,2}$ incorporation result in impaired endosomal processing. Unprocessed $GP_{1,2}$ cannot engage with the endosomal receptor, and thus cannot mediate fusion with the host membrane. This prevents viral egress from the acidified endosome.



Supplemental Figure 3-S1 – Sonication of $GP_{1,2}$ pseudoviruses

$GP_{1,2}$ pseudoviruses described in (Fig 8A) were either sonicated for 30 seconds (light blue), or were untreated. Pseudoviruses were then titrated for infectivity on JC53 cells at 48 hours post-transfection. Results reported are representative of two separate experiments, and are the means and standard deviations for samples run in triplicate.

Chapter 4: Discussion and Future Directions

RNA editing is employed by many negative-strand RNA viruses for regulation of gene expression, as well as for generation of multiple gene products from a single gene. However, regulation of GP gene expression by the Ebola virus is unique, because the un-edited GP gene contains a premature stop codon. The primary GP gene product is sGP, which is a nonstructural protein secreted by infected cells. It is solely through editing of the GP gene, which only occurs 20% of the time, that full-length, membrane-bound $GP_{1,2}$ is produced. The fact that this RNA editing mechanism is conserved among all ebolaviruses indicates that tight control of $GP_{1,2}$ expression is of critical importance to viral fitness, and further suggests that sGP must play an important role in helping the virus survive within its host.

In this dissertation, we have provided evidence that regulation of the GP gene expression may play two important roles in the ebolavirus life cycle. First, production of sGP subverts the host immune response by eliciting antibodies that preferentially bind to sGP. Furthermore, those antibodies that do bind to $GP_{1,2}$ are likely to be cross-reactive with sGP, thereby allowing sGP to function as a "decoy". Second, we found that high levels of $GP_{1,2}$ expression and incorporation impair production and infectivity of viruses, suggesting that RNA editing may be a mechanism of modulating $GP_{1,2}$ expression levels to optimize these components of virus fitness.

While the data reported here provide a basic model for the roles of GP gene regulation in production, infectivity, and immune evasion of ebolaviruses, a great deal of work remains to be done. In particular, the studies reported here must be extended to live-virus systems, as well as to animal models of infection, in order to elucidate the relevance of our findings to actual EBOV infection, EHF pathogenesis, and filovirus vaccinology. Additionally, it is of great interest to more finely dissect the exact mechanisms by which sGP mediates antigenic subversion, and by

which high levels of $GP_{1,2}$ expression impair virus infectivity. The following sections describe some of the important questions that we hope to answer in the near future.

Defining the Relevance of Antigenic Subversion to EHF Pathogenesis

Chapter 2 describes studies in which we identified sGP-mediated "antigenic subversion" as a possible novel mechanism of host immune evasion. In these studies, we immunized mice with DNA constructs encoding $GP_{1,2}$ and sGP, and analyzed the resulting antibody responses by ELISA and Western blot against recombinant $GP_{1,2}$ and sGP. We then examined the ability of sGP to compete for anti-$GP_{1,2}$ antibodies through competition ELISA, through competition immunoprecipitation experiments, and through a competition neutralization assay. We found that sGP redirects the host antibody response towards epitopes that are either not present, or are inaccessible in $GP_{1,2,}$ or epitopes that are shared between the two GP isoforms, thereby allowing sGP to effectively compete for anti-$GP_{1,2}$ antibodies.

We coined the term "antigenic subversion" to describe this phenomenon, because the ability of sGP to compete for anti-$GP_{1,2}$ antibodies depends on active misdirection of the host immune response by sGP. This is distinct from previously proposed mechanisms in which which sGP passively absorbs anti-glycoprotein antibodies. We also propose a model for the mechanism of antigenic subversion in which, during natural infection, the host is exposed to much higher levels of sGP than $GP_{1,2}$, resulting in preferential activation and proliferation of sGP-reactive B-cells. While we posit that sGP-cross reactivity allows the virus to evade host immunity and avoid clearance, the significance of our findings to live EBOV infection relies on future live virus studies using animal models of EBOV infection.

There are many cases of humans and nonhuman primates clearing ebola virus infection without pre-existing immunity. While there is anecdotal evidence that EHF survivors develop

anti-glycoprotein antibodies (and that these antibodies preferentially react with sGP [32]), it is unclear what role antibodies play in virus clearance. It is of great interest to study how infection with EBOV in unvaccinated animals affects the generation of anti-glycoprotein antibodies, and whether theses antibodies preferentially react against sGP or $GP_{1,2}$. These studies would allow us to determine correlates of clearance in unvaccinated hosts, and whether the magnitude of the anti-GP response, as well tendency of these antibodies to react with sGP vs $GP_{1,2}$, have an affect on the ability of a naïve host to clear infection. Considering the rapidness of EHF progression, as well as the associated profound lymphopenia, it is possible that infected hosts do not have sufficient time to mount an effective antibody reponse in the first place. If naïve hosts are indeed able to clear the virus without inducing anti-GP antibodies, it may be that antigenic subversion does not play a major role in human EHF pathogenesis, and may instead have evolved to allow the virus to maintain chronic infection in its natural reservoir.

Choosing a Target: How Antigenic Subversion May Impact Ebola Vaccine Design

Related to its role in EHF pathogenesis, antigenic subversion may also be a primary concern for ebola vaccinology. In our study, we found that boosting $GP_{1,2}$-immunized mice with sGP could subvert the anti-$GP_{1,2}$ response and render it susceptible to competition by sGP. We extended our model of antigenic subversion to account for this observation by hypothesizing that while the majority of B-cells activated in mice immunized against $GP_{1,2}$ are directed against epitopes not shared with sGP, there may be a small population that is sGP cross-reactive. When these mice are boosted with sGP, sGP-crossreactive memory B-cells expand while the remaining $GP_{1,2}$-specific B-cells do not. This shifts the antibody response from $GP_{1,2}$-specific to sGP-cross reactive. The practical consequence of this finding is that an individual may be vaccinated against $GP_{1,2}$ and have circulating anti-$GP_{1,2}$ antibodies. However, if this individual is infected

with EBOV and unable to clear the virus before it establishes infection, the virus may be able generate sufficient quantities of sGP to subvert host immunity, despite the existence of baseline antibody titers and an anamnestic response. Thus, it may be necessary for a successful EBOV vaccine to induce high enough baseline titers of anti-$GP_{1,2}$ antibodies that incoming virus can be cleared before it is able to establish infection. Furthermore, our findings may be related to the failure of many vaccine strategies to fully to protect nonhuman primates, since survival in these studies was most closely correlated with maintenance of anti-$GP_{1,2}$ antibody titers above a threshold level, while lower antibody titers only delayed the time to death [108].

The first step in elucidating the relevance of antigenic subversion to EBOV vaccine design is to determine if there is a difference in the protective efficacy of sGP-induced antibodies versus $GP_{1,2}$-induced antibodies. This can be accomplished by immunizing mice with DNA constructs encoding sGP or $GP_{1,2}$ and then subjecting them to lethal challenge with mouse-adapted EBOV. However, immunization with sGP tends to induce lower titers of $GP_{1,2}$-binding antibodies than does immunization with $GP_{1,2}$. Thus, in addition to examining absolute survival rates between these groups, it would also be of interest to compare survival rates of animals between these groups that have similar titers of anti-$GP_{1,2}$ antibodies, in order to define the relevance of sGP cross-reactivity to morbidity and mortality. Furthermore, measuring anti-GP antibodies after challenge experiments would allow us to identify changes in antibody titers and in reactivity patterns with sGP versus $GP_{1,2}$, as well as the correlation of these changes to rapid clearance versus morbidity and mortality. The results of these experiments will hopefully lay the groundwork for similar nonhuman primate studies, and will clarify the correlates of protection in order to better define the requirements of promising vaccine candidates.

The identification of antigenic subversion as a possible immune evasion mechanism adds yet another wrinkle to the ongoing debate regarding which regions of $GP_{1,2}$ should be targeted by candidate vaccines. It has been proposed that a pan-filovirus vaccine could be generated by targeting conserved epitopes in the N-terminal region of GP_1, as well as in GP_2. Toward this aim, immunization strategies have been explored using mucin domain-deleted $GP_{1,2}$ ($GP_{1,2}\Delta$Muc), as well as proteolytically treated $GP_{1,2}$, in which the highly variable regions like the mucin domain have been removed (Fig 4-1A) [55,58,142]. However, because the most conserved sequences of GP_1 are shared with sGP, it is likely that antibodies induced against these epitopes are susceptible to competition by sGP. Indeed, unpublished data from our lab demonstrate that antibodies induced by $GP_{1,2}\Delta$Muc are more susceptible to sGP competition than those induced by wild-type $GP_{1,2}$ (Fig 4-1B). Furthermore, because the mucin domain forms a bulky glycan canopy that shields the rest of $GP_{1,2}$, it is likely that antibodies raised against conserved, buried regions of $GP_{1,2}$ may not be able to access their cognate epitopes during infection. This is also supported by unpublished data from our lab, which indicate that antibodies raised against sGP bind $GP_{1,2}\Delta$Muc better than they bind to $GP_{1,2}$(Fig 4-1C). Taken together, our preliminary data suggest that vaccines that target conserved regions of $GP_{1,2}$ may inadvertently play into antigenic subversion. Indeed, this may be why animals immunized against $GP_{1,2}\Delta$Muc are less well-protected against lethal EBOV challenge than those immunized against wild-type $GP_{1,2}$ [57]. It may be necessary to focus targeted vaccines even more finely, a strategy that will require identifying regions of $GP_{1,2}$ that are conserved, accessible to antibody binding, and not susceptible to sGP-mediated subversion.

How do $GP_{1,2}$ Expression Levels Regulate Virus Fitness?

In Chapter 3, we demonstrate that high levels of $GP_{1,2}$ expression impair production of EBOV virus-like particles, and impair infectivity of $GP_{1,2}$ pseudotyped viruses. We further demonstrate that this effect is mediated primarily by two mechanisms. First, high levels of $GP_{1,2}$ expression impair synthesis of other viral proteins. Second, viruses containing high levels of $GP_{1,2}$ are intrinsically less infectious. We hypothesize that this second effect is due to steric shielding resulting from close packing of $GP_{1,2}$ peplomers on the virus surface, which interferes with either host receptor engagement, or with endosomal processing events required for the final steps of infection. This hypothesis is supported by our observation that exogenous proteolytic treatment of high-$GP_{1,2}$-containing viruses preferentially enhances their infectivity compared to low-$GP_{1,2}$-containing viruses.

While the results of our study indicate that $GP_{1,2}$ expression levels may have a profound impact on viral fitness and replicative capacity, it is important to extend these studies to live virus systems. First, it would be of great interest to generate recombinant viruses in which the GP gene is either codon-optimized or codon-deoptimized, and to examine the role of $GP_{1,2}$ expression levels on *in vitro* fitness. This could either be done with recombinant EBOV, or with other viruses such as recombinant Newcastle Disease Virus engineered to express EBOV $GP_{1,2}$, an option that would relieve the requirement for high level biological containment facilities. Following up i*n vitro* studies with animal challenge experiments would allow us to determine how virus survival and disease pathogenesis are correlated with *in vitro* replicative fitness.

It is also of interest to more finely dissect the mechanism of impairment of infectivity by high levels of $GP_{1,2}$ expression. First, at what steps do high levels of $GP_{1,2}$ expression impair infection? Measuring the trapping of $GP_{1,2}$-pseudoviruses and EBOV VLP's, incubated with

host cells, could reveal if high levels of $GP_{1,2}$ interfere with receptor engagement and host cell attachment. Furthermore, it would be of interest to determine if proteolysis changes the rates at which pseudoviruses and VLP's attach to host cells. In addition to attachment, it is possible that high-$GP_{1,2}$ viruses are unable to efficiently escape from the acidified endosome due to incomplete processing. It would be interesting to load target cells with VLP's made with GFP-VP40 fusion protein. Using fluorescence microscopy to observe the rates at which VLP's escape the acidified endosome may reveal differences between high-$GP_{1,2}$ and low-$GP_{1,2}$ viruses.

We observed a stark difference in the effects of glycoprotein expression levels on the infectivity patterns of pseudoviruses generated with EBOV $GP_{1,2}$ DNA vs. HIV Env DNA. However, without being able to actually compare precise levels of glycoprotein incorporation, it is impossible to state whether this effect is due to some fundamental structural difference between EBOV $GP_{1,2}$ and HIV Env, or to a difference in the rates at which EBOV $GP_{1,2}$ and HIV Env are loaded onto nascent virions. By engineering epitope tags into HIV Env and EBOV $GP_{1,2}$, it should be possible to more accurately track and quantify incorporation levels of different glycoproteins in viruses. Indeed, it is known that HIV maintains tight control over Env expression, and limits the number of Env trimers to around 10 per virion[143]. Furthermore, in our studies, it was difficult to observe Env incorporation into pseudoviruses by Western blot without first concentrating them, while EBOV $GP_{1,2}$ could easily be observed in low volumes of unconcentrated virus. Thus, it is entirely possible that the difference we observed between EBOV $GP_{1,2}$ and HIV Env pseudotyped viruses is due to rates of glycoprotein incorporation. If this is the case, it would be informative to identify the which features of Env versus $GP_{1,2}$ are responsible for different levels of incorporation. HIV Env has a very long cytoplasmic tail that has previously been associated with low levels of virus incorporation[144]. On the other hand,

$GP_{1,2}$ has a very short cytoplasmic tail, which may allow for high levels of incorporation. Examining the rates of glycoprotein incorporation of cytoplasmic tail mutants, as well as comparing the infectivity of pseudotyped viruses generated with cytoplasmic tail-swapped $GP_{1,2}$/Env may reveal the role of glycoprotein incorporation levels in regulating infectivity.

Perhaps the most interesting question that arises out of our studies on $GP_{1,2}$ expression levels is how antibody-mediated neutralization, as well as antibody-dependent cell-mediated cytotoxicity (ADCC), are affected by $GP_{1.2}$ expression. Because the regulation of $GP_{1,2}$ expression likely represents a balance between virus production, infectivity, and host immune evasion, and because $GP_{1,2}$ is the sole target for neutralizing antibodies, the ability of EBOV to avoid these antibodies is likely affected by levels of $GP_{1,2}$ content. It would be fairly straightforward to examine a panel of monoclonal anti-$GP_{1.2}$ antibodies for their neutralizing activities against high-$GP_{1,2}$ versus low-$GP_{1,2}$ viruses. These same antibodies could be employed in ADCC and ADVCI assays with GP-codon optimized/deoptimized viruses. These data would help paint a fuller picture of the trade-offs EBOV must make in regulating $GP_{1,2}$ expression levels, as well as the relative importance of immune evasion versus *in vitro* replicative fitness to overall viral fitness.

Figures

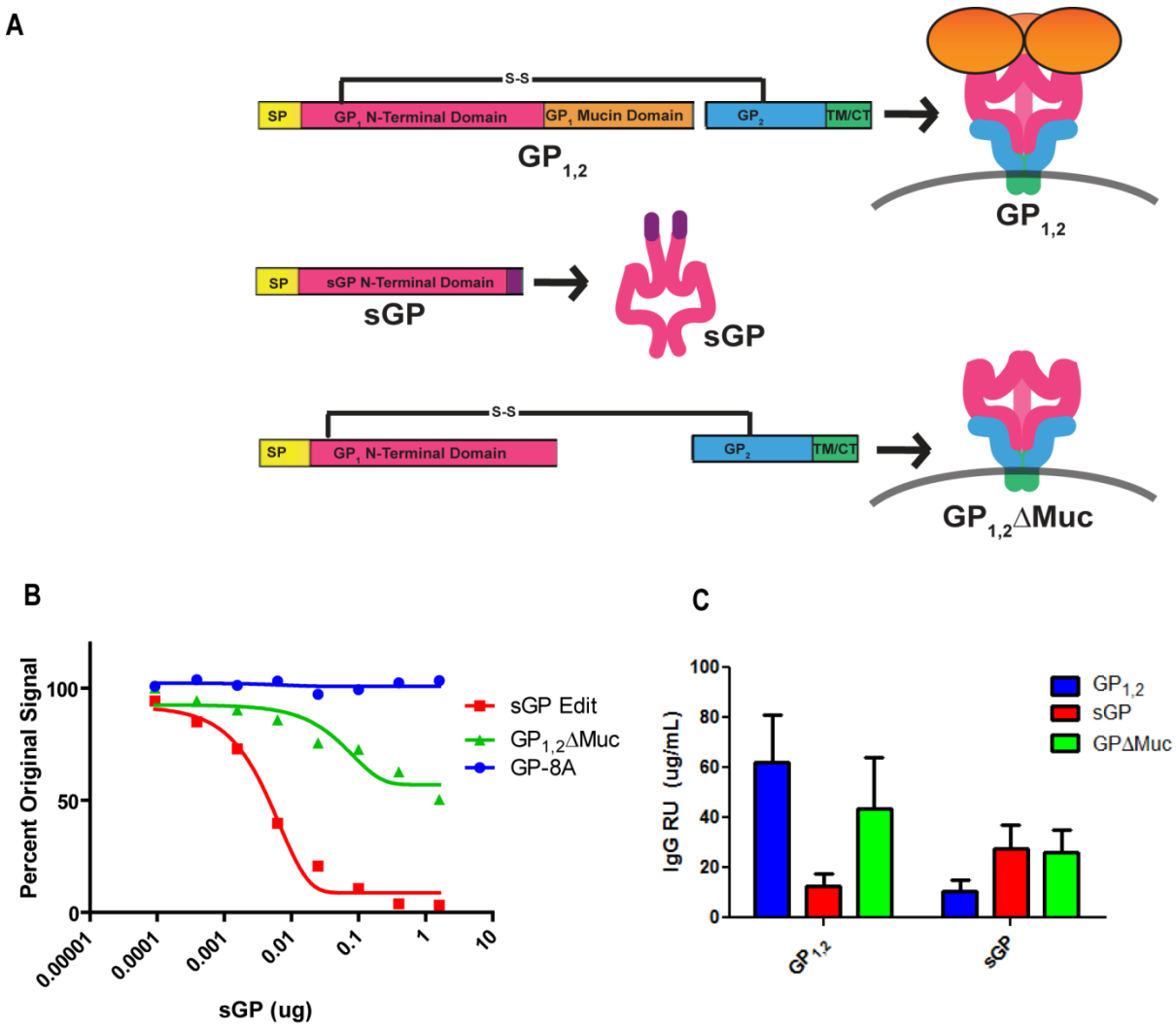


Figure 4-1. Reactivity profile of antibodies elicited by GP1,2ΔMuc

(A) Schematic diagram of $GP_{1,2}$, sGP, and $GP_{1,2}$ΔMuc. $GP_{1,2}$ΔMuc, like $GP_{1,2}$, is a transmembrane protein that can form trimeric spikes on the membrane of host cells and viruses, and is also capable of mediating infection. It contains the most highly conserved regions of $GP_{1,2}$, including the N-terminal region of GP_1 and all of GP_2. (B) Competition ELISA. Antisera from mice immunized against sGP (red), $GP_{1,2}$ (blue), or $GP_{1,2}$ΔMuc (green) were diluted to give similar anti-$GP_{1,2}$ signal. Diluted antiserum was mixed with increasing quantities of purified

His-sGP and incubated in His-$GP_{1,2}$ coated wells. Experiments were performed in duplicate and repeated at least three times, with representative results shown (C) Comparison of antibody levels against different GP isoforms induced by immunizing mice with $GP_{1,2}$ (left) and sGP (right). Average titers of anti-$GP_{1,2}$ (blue), anti-sGP (red), and anti-$GP_{1,2}\Delta$Muc (green) antibodies within immunization groups are shown.

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