

Mechanisms and consequences of gp- mediated cytopathology



**ASSIGN
BUSTER**

EBOV is a member of the family *Filoviridae* in the order *Mononegavirales*, and so encodes its genome in single-stranded linear RNA in the negative orientation. The genome is approximately 19 kb long, and encodes 7 open reading frames (ORFs) [29].

The different subtypes of EBOV are approximately 35-45% divergent at the nucleotide level but are considered highly genomically stable over time [28, 72]. The EBOV genome contains 3' and 5' (leader, trailer) extragenic sequences that can form secondary structures and serve to initiate transcription and genome replication [73, 74]. Individual genes are separated by conserved transcriptional signals. Figure 1-3 (A) shows the organization of ORFs and intragenic features of the EBOV genome.

The EBOV genome encodes 8 major gene products, 7 of which are incorporated into viral particles. The viral genomic RNA is encapsulated in a ribonuclear protein (RNP) complex (nucleocapsid) consisting of the nucleoprotein (NP), minor nucleoprotein (VP30), VP35, and polymerase (L) protein [75]. This complex is then coated in a matrix layer consisting of the major matrix protein (VP40) and minor matrix protein (VP24).

This is further enveloped by a lipid bilayer studded with the main viral glycoprotein (GP) (Figure 1-3 B). The eighth viral protein is the major product of the GP ORF, but is a smaller, secreted glycoprotein, sGP [76]. The full-length membrane-spanning form found in the viral envelope, GP, originates from the addition of an extra adenosine residue in the glycoprotein transcript by the polymerase during transcription [76]. During infection, the ratio between sGP and GP transcripts is approximately 80% / 20% [77].

The processing and function of the different glycoprotein forms are described in the following sections.

EBOV particles are filamentous in shape but are often branched or in circular conformations; they have a diameter of approximately 80 nm and a variable length that can exceed 1000 nm [78, 79].

Glycoprotein processing, function and structure

Transcription and translation of the glycoprotein gene gives rise to several major and minor products during infection. The major product of transcription is the pre- sGP transcript. This is translated into pre- sGP protein, which is then cleaved by furin into sGP and a small C-terminal fragment, the ω peptide [80]. However, during transcription the viral polymerase occasionally adds an extra adenosine residue while reading through an editing region of 7 uridine residues. The resulting -1 ORF reads through the remaining portion of the gene and encodes for the membrane-bound form of the EBOV glycoprotein, GP [76, 77]. Similar editing by the polymerase into a -2 ORF produces a small secreted glycoprotein product (ssGP); however, this product has not been demonstrated in the context of EBOV infection [81].

The GP transcript is initially translated as a precursor (GP0), which is then cleaved by furin in the Golgi into two subunits: a surface subunit, GP1, and a membrane-spanning subunit, GP2 [82]. These subunits remain covalently connected through a single intermolecular cysteine bond [83]. This

heterodimer associates non-covalently with two other heterodimers in a

higher-order trimeric complex to produce the GP spike that incorporates into
<https://assignbuster.com/mechanisms-and-consequences-of-gp-mediated-cytopathology/>

budding virions [84]. Figure 1-4 (A) shows the domains and features of GP. In addition to anchoring the glycoprotein to the membrane, GP2 houses the fusion machinery, which allows the RNP complex to be delivered to the cytosol [85]. Fusion is accomplished by the formation of a 6-helix bundle between the alpha-helical heptad repeat (HR) 1 and 2 regions after an internal fusion loop becomes anchored in the host membrane [86, 87, 88]. The GP1 subunit is responsible for mediating attachment and entry of the virus. This subunit houses a putative receptor-binding domain (RBD), which binds tightly to the surface of susceptible cells [89, 90, 91]. Interestingly, at the C-terminus of GP1 there is a large, highly glycosylated domain, called the mucin domain. This domain plays a role in entry and GP-mediated cytopathology, both of which are described in the following sections.

Glycosylation is a prominent feature of GP and composes about half the mass of GP1 [83, 92]. The Zaire GP contains 17 predicted N-linked oligosaccharides, 8 of which are located in the mucin domain. The mucin domain may contain up to 80 O-linked sites, with at least 17 highly-predicted, clustered sites, which confer mucin-like properties to this domain. The level of N- and O- linked glycosylation in the mucin domain is maintained across the subtypes of EBOV, despite extremely low sequence conservation.

The composition and structure of the glycans found on GP have been studied by mass spectrometry [93, 94]. GP was found to contain bi-, tri-, and tetra-antennary branched Nlinked glycans bearing reduced amounts of galactose, some high-mannose residues, and very low amounts of sialic acid compared to sGP. Although glycosylation is heterogeneous, these analyses indicate

that, in general, glycans on sGP undergo more processing and modification
<https://assignbuster.com/mechanisms-and-consequences-of-gp-mediated-cytopathology/>

than do those on GP. O-linked glycosylation in the mucin domain of GP was also examined and found to be composed of mostly core 2 glycosylation structures, with variable amounts of sialic acid.

The molecular structure of GP was solved to 3.4 Å resolution by Lee and colleagues (Figure 1-4 B) [95]. This crystallographic structure reveals that the three GP1 subunits form a chalice-like globular structure. In the GP2 subunit, the fusion loop and HR1 regions wrap around the outside of the globular GP1 domains, and are thought to help stabilize the structure. The RBD sits on top of the base domain with residues critical for binding facing up. Positioned on top of and blocking access to the RBD is a small glycosylated domain, termed the glycan cap. The mucin domain, which was genetically deleted for crystallography, extends up and away from the viral membrane and globular GP domains. The entire trimeric complex is approximately 35 Å wide at the chalice base, 140 Å tall, and has a radius of approximately 125 Å from the center of the chalice to the distal end of the mucin domain (Lee, J. and Saphire, E. O., unpublished data)

Viral entry

EBOV, like all viruses, is an obligate intracellular pathogen, and so must enter a host cell to replicate. GP is the only viral protein found on the virion surface and so is responsible for mediating entry and fusion of the virus. The first step of entry is attachment. Because the mucin domain is the prominent feature on the virion surface, it seems likely that initial attachment steps occur through interactions of this domain with host cell surface factors. This hypothesis is supported by several studies, which have demonstrated that C-

type lectins such as DC-SIGN or L-SIGN enhance GP-mediated entry through interactions with the mucin domain [96, 97, 98]. This is significant because residues in the RBD that mediate binding to the cell surface are not exposed on fulllength GP, but buried under the glycan cap and mucin domains [95]. In the context of virus-like particles, the mucin domain has also been shown to induce intracellular signaling in dendritic cells, which may aid in downstream entry or replication steps [99].

EBOV must traffic to a low pH compartment for fusion, however the mechanism of endocytosis is poorly understood [100, 101]. Clathrin has been implicated in the endocytosis of EBOV, although the large size of EBOV particles would seem to exclude this pathway [102]. Several signaling molecules, such as phosphoinositide-3 kinase, Rho GTPases, and tyro3 family members have also been implicated in endocytic steps [103, 104, 105]. Recent, but as yet unpublished studies have also implicated macropinocytosis as an entry pathway [106, 107].

The most well-characterized step in EBOV entry is the post-endocytic, pre-fusion stage. After endocytosis, an EBOV-containing endosome matures into a late endosome with a low pH, around 5.5. It is in this low pH compartment that resident endosomal cathepsin proteases process GP1 into an activated form. Both cathepsin B and cathepsin L have been shown to endoproteolytically cleave GP1, a process that is required for entry [108]. Cathepsin processing takes place at residues 201 and 222, both of which are located in a disordered and solution-exposed loop between the RBD and the glycan cap [91, 95, 109]. This cleavage serves to remove the glycan cap and mucin domain from its position over the RBD, potentially exposing the

<https://assignbuster.com/mechanisms-and-consequences-of-gp-mediated-cytopathology/>

RBD for interactions with a receptor (Figure 1-5) [91, 95, 110, 111]. Studies describing these processing steps are further supported by the fact that cathepsin processing of GP increases the binding and infectivity of GP-bearing pseudovirions [110, 111]. Interestingly, this cleaved GP, termed primed GP, is still required for a second step during entry [108, 110]. Cathepsins could be additionally required to act on a cellular receptor, or could further process GP after receptor-induced structural rearrangements are triggered. After cathepsin processing, fusion occurs and the contents of the viral particle are introduced into the cytoplasm.

Genome replication and viral budding

As with all RNA viruses with a negative sense genome, EBOV virus must package its own RNA-dependent RNA polymerase (RDRP) so that it can initiate transcription and replication. The L protein is the viral RDRP responsible for protein transcription and full-length genome and anti-genome transcripts [73]. Transcription and replication occurs in the cytoplasm where L works in complex with VP30, VP35 and NP [112]. After transcription of the negative sense viral genome from a positive sense intermediate, nascent genomes are packaged by nucleocapsid proteins.

Newly made RNP complexes must then associate with the matrix proteins for packaging into budding particles. VP40 is the main matrix protein, which drives budding at the plasma membrane and produces virus-like particles in the absence of other viral proteins [113, 114]. EBOV VP40 contains two overlapping late domains, which recruit members of the endosomal sorting complexes required for transport (ESCRT) pathway [115, 116, 117]. ESCRT

complexes contain members of the vacuolar protein sorting system, used by the cell to sort multivesicular body (MVB) cargo [118]. This same machinery is usurped by the virus to create the membrane invagination necessary to bud from the plasma membrane [117, 119]. Lipid rafts at the plasma membrane may serve as sensitive to inhibitors of cathepsins, suggesting that cathepsins are the site of viral assembly and budding, as VP40 has been shown to target to these microdomains [117, 120]. Other potential sites of budding include MVBs and filopodia, as have been proposed for MARV [121, 122].

Glycoprotein-mediated cytopathology

In the past few decades, the use of recombinant DNA technology became a common method to study individual viral gene products. The cloning of the EBOV GP gene allowed the independent expression of GP in cells for the study of processes related to this protein, such as viral entry. From these studies, a phenomenon was observed: GP appeared to induce toxicity in cells in which it was expressed. This observation is the focus of the studies described in this dissertation and is described in detail below.

EBOV GP expression, in the absence of other viral gene products, disrupts cell adhesion causing a loss of cell-cell contacts and of attachment to the culture substrate, resulting in rounded or floating cells [123, 124, 125]. This phenomenon is termed GP-mediated cytopathology and is displayed in Figure 1-6. Such cytopathology can be observed in a variety of cell lines, including human lines: 293T, 293H, HeLa, OV79, HT1080, U87, and PMA-pretreated U937 cell; other mammalian lines: Vero, CCC, BHK, and MC57 cells; and

primary human cell types: umbilical vein endothelial, pulmonary artery endothelial, coronary artery smooth muscle, cardiac microvascular endothelial, and blood monocyte-derived macrophages (unpublished observations and [124, 126]).

Interestingly, transient GP expression in the transformed human embryonic kidney cell line, 293T, does not cause death, as these cells will regain their adhesive properties after GP expression wanes if maintained in culture [124]. In contrast, primary human cardiac microvascular endothelial cells have been reported to undergo anoikis, or detachment-mediated apoptosis, upon transduction of GP [126].

The loss of cell adhesion associated with GP expression does not occur in *trans*, meaning that in culture, non-expressing cells adjacent to a GP-expressing cell will not undergo detachment. Similarly, when sGP or soluble, full-length, trimeric GP is secreted from cells, neither these nor neighboring cells are affected [123]. This indicates that GP must be expressed in a particular cell to induce cytopathology.

Because cell adhesion was so dramatically affected by GP, the integrin family members were examined in several studies related to this topic. By flow cytometry, cells expressing GP display significantly reduced surface levels of $\alpha_1\beta_1$, $\alpha_1\beta_2$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$ and $\alpha_V\beta_3$ integrins [123, 124, 127]. Other surface proteins such as major histocompatibility complex class I (MHC1) and platelet endothelial cell adhesion molecule 1 (PECAM-1) are similarly effected; however, the exact complement of surface proteins affected by GP appears to differ by cell type [124]. This apparent down-modulation of

surface proteins, in particular the various cell adhesion molecules (CAMs), provided an initial explanation for the cell rounding phenotype.

The GPs from 4 of the 5 subtypes of EBOV (Zaire, Sudan, Côte d'Ivoire, and Reston) have been examined for their ability to cause cytopathology. All 4 subtypes are able to induce some degree of cell rounding, although Reston GP seems less able to do so. Additionally, Reston GP induces only a modest down-modulation of surface integrins by flow cytometry [124]. Interestingly, MARV GP also contains a mucin domain, which does not appear to cause cytopathology [128].

EBOV GP-mediated cytopathology is known to be dependent on the highly glycosylated mucin domain within GP1. Constructs expressing GP with a genetic deletion of this domain, GP_{if}, muc, do not cause cell rounding or detachment and do not show down-modulation of surface proteins by flow cytometry [124, 125, 127]. Indeed, sequential deletions of the mucin domain result in a progressive loss of cell detachment, indicating the overall size or level of glycosylation of the domain is important for the disruption of cell adhesion [124].

The role that GP-mediated cytopathology plays during viral pathogenesis is largely unknown. It is important to note that EBOV infection of 293T cells was observed to cause similar disruption of adhesion and a reduction of $\alpha_1\beta_1$ and $\alpha_5\beta_1$ integrin and MHC1 staining by flow cytometry by 48 hours post infection, suggesting that observations from transient GP expression are not simply artifacts of overexpression [129]. However, it has been suggested that the balance between sGP and GP transcription, which

produces approximately 80% sGP and 20% GP, is a deliberate mechanism used by the virus to limit GP cytopathology. To directly address this hypothesis, Volchkov and colleagues used a reverse genetics system to rescue EBOV bearing an extra adenosine residue in the GP gene RNA edit site [77]. This mutant virus produced significantly more GP and less sGP and caused more cytopathic effects (CPE) in infected 293T cells than wt EBOV, for which the authors report minimal CPE [77]. Because the extent of cytopathology may differ between cell types, its effect on viral pathogenesis is difficult to gauge. In an initial study of GP-mediated cytopathology, the authors proposed that GP-induced loss of cell contacts could help explain the loss of vascular barrier integrity and resulting leakage often seen during infection, though this remains controversial [48, 125]. The disruption of integrins and other CAMs such as PECAM-1 suggests that trafficking and diapedesis of antigen presenting cells (APCs) could be disrupted upon infection [124]. Additionally, the apparent down-modulation of $\alpha 1$ integrin was suggested to be indicative of its role in viral entry, as viruses are known to down-modulate their receptors to aid in egress and prevent superinfection; however, integrins have not been directly implicated as receptors for the virus [123, 130, 131]. Additional mechanisms by which GP-mediated cytopathology may contribute to pathogenesis are proposed in this dissertation and discussed below.

Hypotheses addressed in this dissertation

The goal of this dissertation is to utilize cell biological and biochemical techniques to explore the mechanism and consequences of GP-mediated cytopathology.

<https://assignbuster.com/mechanisms-and-consequences-of-gp-mediated-cytopathology/>

The domain requirements for GP had initially been investigated by our group and others.

As previously detailed, the mucin domain is a known requirement for the disruption of adhesion and surface staining. However, it was not known whether the mucin domain was also sufficient to induce cytopathology. This hypothesis is addressed in Chapter 2, wherein we provide evidence that this domain is fully sufficient cause cytopathology. Of critical focus in this dissertation is the cellular and molecular mechanism by which GP-mediated cytopathology occurs. Before the present study was undertaken, there had been very few investigations into the mechanism by which Ebola GP disrupts adhesion and causes surface protein down-modulation. Sullivan and colleagues had reported that this process requires the cellular GTPase dynamin, which is an active regulator of several endocytic pathways [127]. Additionally, it had also been reported that the extracellular signal-regulated kinases (ERK 1/2) play a role in down-modulation, suggesting that active signaling helped to drive these effects [132]. Therefore, we first undertook studies to examine the role of dynamin in the process of cytopathology (also described in Chapter 2) but found results that contrasted with Sullivan's previous report. We then developed a hypothesis based on the structure of GP and queried whether the heavily glycosylated mucin domain of GP might be causing cytopathology by sterically inhibiting the function of cell surface proteins. Chapter 3 details this study, which supports a model of steric occlusion as the explanation for both the disruption of adhesion and the appearance of surface protein down-modulation by flow cytometry. Interestingly, we also found that GP could

sterically shield its own epitopes from antibody recognition at the cell surface, including a well-studied neutralizing epitope, bound by the KZ52 antibody.

We next wanted to address the potential consequences that GP-mediated cytopathology might have on the immune response during virus infection. First, we hypothesized that the occlusion of surface MHC1 molecules would have the functional consequence of blocking antigen presentation to CD8 T cells, thereby preventing their activation. We have found this to be the case, and have described these experiments in Chapter 3. In Chapter 4, we explore the possibility that the heavily glycosylated domains