CHAPTER 5

FILOVIRUS ENTRY

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Abstract: A number of advances in recent years have significantly furthered our understanding of filovirus attachment and cellular tropism. For example, several cell-surface molecules have been identified as attachment factors with the potential to facilitate the in vivo targeting of particular cell types such as macrophages and hepatic cells. Furthermore, our knowledge of internalization and subsequent events during filovirus entry has also been widened, adding new variations to the paradigms for viral entry established for HIV and influenza. In particular, host cell factors such as endosomal proteases and the intracellular receptor Niemann-Pick C1 are now known to play a vital role in activating the membrane fusion potential of filovirus glycoproteins.

INTRODUCTION

The family filoviridae consists of just two members, Marburg virus (MARV) and Ebola virus, first identified in 1967 and 1976, respectively. The Ebola viruses comprise five distinct species: the prototypical Ebola virus (EBOV, Zaire ebolavirus); Sudan ebolavirus (SUDV); Bundibugyo ebolavirus (BDBV); Taï Forest ebolavirus (TAFV, formerly known as Côte d’Ivoire) and Reston ebolavirus (RESTV). Filoviruses are responsible for sporadic, highly lethal outbreaks of severe hemorrhagic fever in both humans and apes in sub-Saharan Africa. While human cases of filovirus infection are rare, with less than 1700 deaths and 2500 total cases between their discovery in 1967 and 2010,1 recent years have seen a more sustained level of outbreaks, including multiple introductions of filoviruses into the human population. Although the primary animal host for the filoviruses is still unclear, as with other tropical viral diseases, bats have been strongly implicated as a possible reservoir.2,3 In addition, the less pathogenic RESTV has been identified in populations of pigs in Asia.4
Filoviruses encode a single membrane-bound surface glycoprotein, termed GP, responsible for mediating viral attachment and entry into cells. While MARV encodes GP within a single open-reading frame, the equivalent open-reading frame for ebola virus encodes a truncated, non-structural protein, termed sGP, secreted by infected cells. However, in approximately 20% of GP transcripts, a cotranscriptional editing event introduces a frame-shifting adenine, leading to production of full-length GP. Given that sGP is easily detectable in the blood of infected individuals, a number of roles have been proposed, including sGP synthesis as a mechanism for controlling membrane-bound GP expression levels. The virus may be required to limit GP expression due to excessive levels of membrane-bound GP leading to a loss of cell adhesion, resulting from a downregulation of adhesion molecules. In addition, viral particles can tolerate very inefficient levels of GP incorporation without a resultant loss of infectivity, thus methods to reduce GP expression, in particular from the surface of infected cells, may help blunt the immune response mounted against EBOV.

As with many other viral fusion proteins, full-length GP forms a trimer on the virion surface, with each monomer proteolytically processed into two subunits during transit through the trans golgi by the proprotein convertase furin. The resulting proteins, a surface subunit, GP1, and a transmembrane-bound subunit, GP2, are linked by a single disulfide bond. Unlike most viral fusion proteins, proteolysis of EBOV-GP at the furin cleavage site is not absolutely required for GP to mediate membrane fusion and infection, both in tissue culture and in vivo. However, due to the generally conserved nature of the cleavage site between the different strains of EBOV and MARV, it seems likely that cleavage plays a role during natural infection.

GP contains many of the hallmarks of a class I viral fusion protein, most notably the presence of two heptad repeats within GP2 that associate to form an antiparallel six-helix bundle during membrane fusion. In addition, a highly hydrophobic fusion peptide is positioned close to the N terminus of GP2. As with a subset of other class I viral fusion proteins, the fusion peptide is not at the very amino terminus of GP2, but rather forms an internal loop created by two flanking cysteine residues linked by a disulfide-bond. This may explain why furin-mediated cleavage of GP is not absolutely required for function, as viruses without fusion peptides bounded by two cysteines require the cleavage event itself in order to free the fusion peptide.

Filovirus GP is extensively glycosylated, as simply demonstrated by a dramatic increase in mobility by SDS-PAGE following treatment with glycosidases such as PNGase F. Both N- and O-linked carbohydrate moieties are present, with a particularly high density situated within a serine and threonine-rich region in the C-terminal half of GP1. This region is highly variable between the four characterized strains of EBOV, but due to the high density of O-linked glycans it shows homology to mucins. This region is also markedly hydrophilic, and due to the presence of a disulfide bond linking the more hydrophobic N-terminal region of GP1 to GP2, the C-terminus of GP1 is thought to project out into aqueous milieu. Surprisingly, deletion of the mucin-like domain from GP1 does not impact infectivity mediated by EBOV-GP, but rather actually enhances infection. Thus, it is unlikely that the determinants for receptor binding lie within this region of the glycoprotein. However, due to its highly glycosylated nature, and its position distal to the viral membrane, the mucin-like domain likely does contribute to relatively nonspecific viral attachment to some cellular lectins (see below). Further
mutagenic mapping of GP, highlights the N-terminal 150-160 amino-acids as critical for both EBOV- and MARV-GP mediated entry, suggesting that in fact the amino terminal region may be the site of the receptor-binding domain. Phenylalanines at positions 88 and 159, in particular, appear to be critical for GP function.

**CELLULAR TROPISM**

Studies using filovirus GP pseudotyped onto retroviral or rhabdovirus cores reveal a very broad range of cell tropism in vitro. In addition to the transduction of a diverse set of cell types, cell lines from a range of mammalian and avian sources were found to be valid targets for filovirus GP mediated entry. The only consistent exception to the pan-tropism of filoviruses are cell lines of lymphoid origin, which are totally refractory to entry mediated by EBOV-GP. These in vitro findings mirror those seen in infected patients and experimentally challenged animals, where little or no viral RNA or protein has been observed in lymphocytes. Thus, the step of viral entry appears to be the major determinant for filovirus tropism. Cells of the mononuclear phagocytic system are sites of early and sustained viral replication (reviewed in ref. 30). It is likely that organ-specific macrophages act as initial targets for viral replication within specific organs, while blood monocyte/macrophages play a role in dissemination of the virus to tissues, although in vitro assays suggest monocytes may be less infectible than fully mature macrophages. Dendritic cells are also a target for filovirus replication, and may also disseminate virus. Later in infection, other cell types, in addition to macrophages, demonstrate high levels of viral replication, including hepatocytes and endothelial cells, as well as a range of other parenchymal cells. All organs participate in viral replication, with the liver, lungs and spleen appearing to be central sites for viral production and viral induced damage.

**RECEPTORS FOR FILOVIRUS ENTRY**

Generally, in order to gain entry into target cells, enveloped viruses such as the filoviruses must first engage specific cellular receptors. The presence of these receptors on target cells is thus a major determinant of cellular and tissue tropism. Detailed studies of entry mediated by EBOV and MARV glycoproteins, established that for EBOV at least, a proteinaceous component, with a requirement for glycosylation, is necessary for entry. Interestingly, neither treatment with pronase, nor disrupting carbohydrate addition, inhibited entry mediated by MARV GP, suggesting that either the two filoviruses use different receptors, or there are functional differences in the way the same receptor is utilized. The fact that pronase treatment did not negatively impact MARV entry does not exclude the role of proteins in infection as other viruses known to use proteinaceous receptors are also insensitive to pronase. In support of this, soluble versions of EBOV GP can block MARV infection, and vice versa, suggesting a shared receptor.

Recently, a bioinformatics approach was used to identify T-cell immunoglobulin and mucin-domain containing protein-1 (TIM-1) as a potential receptor for EBOV on mucosal epithelia. It remains to be seen whether TIM-1 acts as a true receptor, or merely as a tissue-specific attachment factor, particularly as TIM-1 is also expressed on subsets of activated T cells which are refractory to infection. Either way, use of TIM-1 could explain the high levels of filovirus replication seen in lung tissue. Three members of
the Tyro3 receptor tyrosine kinase family have been demonstrated to facilitate filovirus infection. Stable expression of any of Axl, Dtk or Mer in refractory lymphocyte cell lines permitted infection with either pseudovirions or live virus. In turn, antibodies, Gas6 ligand and soluble versions of the ectodomains from each Tyro3 family member were able to prevent the enhancement of infection seen on Tyro3-expressing lymphocytes. Axl, but not Dtk or Mer, is readily detectable on many EBOV sensitive cell lines, such as Vero, HT1080 and HeLa cells. Antibodies and RNAi directed against Axl potently inhibited infection on some of these lines. However, in Vero cells no inhibition was noted, suggesting the existence of alternate factors for EBOV infection in these cells. Furthermore, RNAi knockout of Axl did not alter binding to cells, nor was direct binding between the extracellular domains of Axl and EBOV GP noted, strongly suggesting Axl does not operate directly as a receptor for the virus, but rather acts downstream.

Folate receptor alpha (FRα) has also been implicated as a receptor for filoviruses. Transcripts encoding FRα, that allowed MARV GP-mediated infection of a normally refractory T-cell line, were identified in a cDNA library screen. Furthermore, expression of FRα on T cells reconstituted infection by EBOV GP-bearing pseudotypes, as well as live MARV and EBOV. Surprisingly, the initial cDNA clone of FRα isolated in these studies was not full length, but rather was truncated at the 5’ end and hence failed to encode a functional signal peptide for FRα. It is thus unclear how such a protein would be expressed on the cell surface and hence act as a receptor for viral entry. In other studies, transfection of refractory cell lines with plasmids expressing functional FRα was unable to reconstitute EBOV GP-mediated infection. Regardless of the role of FRα in entry, other molecules must be capable of functioning as receptors for filoviruses, as GP can mediate efficient infection of primary and established cell lines that are negative for both FRα mRNA and protein. In addition, various ligands to FRα, such as folate and antibodies, were unable to consistently inhibit EBOV GP mediated infection of a range of cell types. A number of other molecules, such as α1 integrins, have been postulated to be involved in filovirus entry, however, none of these potential receptors has passed the acid test of making refractory cells permissive to filoviruses. Thus, these molecules may only play minor, if any, roles in filovirus attachment and entry into target cells. Given the novel mechanisms required for triggering EBOV mediated fusion described below, it may well in fact be that specific cell surface receptors are not required by this virus, but rather any of a multitude of less specific attachment factors or random events leading to internalization are sufficient to lead to entry. Lack of specific receptors for entry has also be hypothesized for highly pH-dependent viruses such as many of the flaviviruses. One could also speculate that with their large, filamentous shape, filoviruses could rely on numerous low-affinity surface interactions for effective initiation of attachment.

ATTACHMENT FACTORS

For many viruses, receptor engagement is a relatively inefficient process. Thus, additional cellular factors that enhance viral attachment to the cell surface can dramatically alter infectivity and cellular tropism without being absolutely required for viral infection (see Chapter 1). These so-called attachment factors often recognize and bind to viruses in a relatively nonspecific manner, for example through carbohydrate modifications on the viral envelope proteins. Calcium-dependent (C-type) lectins represent one of a number of families of molecules termed pattern recognition receptors that are responsible for
identifying, and inducing responses to, unique pathogen signatures. Ironically, many of these molecules have been subverted by pathogens in order to either gain entry into antigen-presenting cells such as macrophages, or to subvert immune responses. The prototypical C-type lectin involved in pattern recognition is CD209 (formerly known as DC-SIGN). CD209 maps to chromosome 19p13.3, together with a cluster of related C-type lectins including CD23, CD209L (also called DC-SIGNR or L-SIGN) and LSECtin.42,43 CD209 and the highly related CD209L (henceforth collectively referred to as CD209(L)) are tetrameric, membrane-anchored lectins reported to act as ligands for the intercellular adhesion molecules (ICAM) -2 and -3, through recognition of N-linked high-mannose carbohydrate moieties.42,44,45 Transcripts corresponding to CD209 were originally identified in screens of human placental cDNA libraries for molecules capable of binding HIV gp120.46 Subsequent analysis demonstrated the ability of CD209(L) to interact with glycoproteins from a variety of pathogens, including HIV, hepatitis C virus, dengue, Leishmania and Mycobacterium tuberculosis.47-52 However, CD209(L) are not universal attachment factors, as glycoproteins from many viruses, including vesicular stomatitis virus, herpes simplex virus and lassa virus, are not enhanced by CD209(L).53 Some viral glycoproteins that do not interact with CD209(L) efficiently can be engineered to contain high-mannose carbohydrate moieties by treating producer cells with mannosidase I inhibitors.54 This leads to a marked increase in binding to, and usage of, CD209(L), suggesting that the presence or absence of high mannose is the major determinant of viral glycoprotein interactions with CD209(L).55 However, it has also been suggested that the spatial arrangement of high-mannose moieties either within a single glycoprotein, or between multiple glycoproteins on the viral surface, is also important in order to allow optimal interactions with these tetrameric calcium-dependent lectins.56,57

The composition of the glycans decorating filovirus GP is highly heterologous, consisting of both high-mannose and complex carbohydrate structures.55,58,59 Thus, it is not surprising that mammalian lectins, such as CD209(L), are able to interact with filovirus GP. The expression of CD209(L) on primary macrophages led to an almost ten-fold enhancement of infection by live, replication-competent EBOV, despite these cells already being a highly competent cell type for EBOV replication.53 Similarly, transduction of infectible cells by retroviral pseudovirions bearing either EBOV or MARV GP is dramatically enhanced by transient expression of CD209(L), as well as another C-type lectin clustered with CD209(L) on chromosome 19, LSECtin.53,60-62 Whether expression of CD209(L) on nonpermissive lymphocyte cell lines can make them permissive to filovirus infection is controversial.53,60 While CD209(L) may inefficiently directly mediate viral infection, this may be a moot point as the majority of CD209(L) positive cells likely also express other receptors for filovirus entry. It is more likely that CD209(L) predominately act to concentrate virus at the cell surface of target cells, and hence increase the likelihood of GP interactions with its cognate receptor. Thus, CD209(L) may act in vivo to target particular cell types for enhanced infection, as well as promoting infection despite very low levels of infectious viral particles, for example during transmission.

Ex-vivo, CD209 is very highly expressed on monocyte-derived dendritic cells but not monocyte-derived macrophages.45 However, somewhat lower expression is observed on a variety of both dendritic cells and tissue macrophages in vivo, as well as liver sinusoidal endothelium.45,63-66 CD209L, together with LSECtin, are found predominantly on microvascular endothelial cells in lymph nodes and liver sinusoids.43,67 Many of these cell types may be important for the establishment and spread of filovirus throughout its target organs.
Hepatic asialoglycoprotein receptor (ASGP-R) was the first attachment factor to be identified for filoviruses. ASGP-R binds galactose on asialylated carbohydrate structures, such as the serum constituent, asialofetuin. Asialofetuin, together with antisera to ASGP-R, were able to block MARV infection of ASGP-R positive HepG2 cells, while transient expression of ASGP-R in mouse cells led to enhanced infection. Similarly, ASGP-R is also able to enhance infection mediated by EBOV GP on already infectible cell lines by over 30-fold. Given that the liver is an important target organ for filovirus infection, ASGP-R may play a role in targeting filovirus infection of liver cells, due to its presence on hepatocytes. Another calcium dependent lectin with specificity for galactose, human macrophage galactose- and N-acetylgalactosamine-specific C-type lectin (hMGL), has also been shown to significantly enhance infection mediated by filovirus glycoproteins. As its name suggests, hMGL is highly expressed on macrophages, as well as dendritic cells, important cell types for filovirus infection in vivo, particularly early during infection. For both galactose-specific lectins, enhancement of infection mediated by EBOV GP lacking the mucin-like domain is greatly reduced (Unpublished observations—G. Simmons). Thus, the determinants for binding likely predominantly lie within this highly glycosylated region.

Given that lectins are expressed on a range of cell types that could function as “doorways” to viral infection within mucosal and epithelial surfaces, as well as target organs, it is tempting to speculate that lectins play an important role both in transmission of filovirus infections, and the dissemination of virus throughout the body.

**ROUTES OF ENTRY**

A major component of filovirus GP-mediated membrane fusion is an acidic pH-dependent step (see section on mechanisms of membrane fusion below). This implies that the virus requires internalization and trafficking to the low pH environment of an acidified endosome, as demonstrated by a necessity for an intact microtubule network within target cells. Several potential routes of internalization are present within cells and have been hijacked by various viruses for the purpose of entry or trafficking to specific subcellular compartments. These include classical clathrin-mediated endocytosis, lipid raft-associated caveolae, macropinocytosis and less defined nonclathrin, noncaveolae routes of entry. Several groups have determined that cholesterol and lipid rafts are required for EBOV GP-mediated infection, both using pseudovirions, and live virus. Given that caveolae invaginate from cholesterol-rich lipid rafts, Empig et al looked at the partitioning of GP-bearing pseudovirions following exposure of target cells. Indeed, following internalization, pseudotypes incorporating either EBOV or MARV GP, colocalized with markers of caveolae to a large extent, suggesting a role for caveolae in filovirus entry. However, cells lacking functional components for caveolae formation, such as caveolin-1, remain fully infectious to pseudovirions bearing EBOV GP. Utilizing pseudoparticles, and inhibition of live virus with specific inhibitors, other groups have also implicated clathrin-mediated endocytosis as an efficient mode of entry. However, it appears likely that in this instance pseudoparticles do not adequately mimic authentic virus particles which are typically filamentous and up to 1-2 μm in length. Studies with virus-like particles (VLPs) and replication-competent virus have largely coalesced opinion around the idea that macropinocytosis is the predominant route of entry for filoviruses,
with clathrin-dependent endocytosis playing a lesser role.\textsuperscript{75-77} Furthermore, it appears that Axl, which had previously been suggested as a receptor, is involved in mediating early events in internalization through macropinocytosis.\textsuperscript{78}

**MECHANISMS OF MEMBRANE FUSION**

Class I viral fusion protein induced membrane fusion, such as that mediated by filovirus GP, occurs through a complex cascade of conformational rearrangements within the glycoprotein. Mature, native class I glycoproteins generally exist as trimers of heterodimers held in a so-called metastable state, primed for fusion.\textsuperscript{79} The metastable protein is destabilized during attachment and entry, leading to the exposure of the hydrophobic fusion peptide and subsequently membrane fusion. Three distinct triggers of these conformational rearrangements have been elucidated for the induction of membrane fusion by class I viral glycoproteins; interactions with receptor(s) as seen with HIV, exposure to low pH as is the case with influenza virus, or a two-step process requiring specific interactions with receptor followed by acidic pH treatment.\textsuperscript{80}

The exact triggers necessary for filovirus GP conformational change have yet to be elucidated, but it appears that filoviruses present a new paradigm whereby additional non-cell surface cellular factors are required. Low pH clearly plays an important role in filovirus entry as infection by GP-bearing pseudovirions can be inhibited by agents such as bafilomycin A that prevent acidification of endosomes.\textsuperscript{25-27} Interestingly, however, in cell-to-cell fusion assays, while acidic pH is required in order to prime the membrane fusion potential of EBOV GP expressing effector cells, treatment of target cells with low pH inhibits fusion.\textsuperscript{81} Also, unlike many pH-dependent viruses, acid treatment of virus bound to cells does not induce fusion at the plasma membrane and hence viral entry.\textsuperscript{82} Likewise, preincubation at pH 5 does not inactivate EBOV GP, suggesting that low pH does not act as a trigger of irreversible conformational rearrangements within the glycoprotein. These findings suggest, as with other viruses inhibited by Bafilomycin A but not directly sensitive to low pH,\textsuperscript{83} that rather than acting purely as a direct trigger, the requirement for low pH indicates the necessity for the action of a cellular factor that is itself sensitive to endosomal pH. Indeed, inhibitors of acid-dependent endosomal cysteine proteases specifically inhibit EBOV GP-mediated entry.\textsuperscript{84,85} In particular, a specific inhibitor of the ubiquitous endosomal protease, cathepsin B (CTSB) inhibits both EBOV GP bearing pseudovirions and live EBOV infection.\textsuperscript{84} The requirement for CTSB was confirmed by an 80-90% loss of infectivity on both CTSB deficient mouse cells and Vero cells treated with RNAi duplexes capable of reducing CTSB activity by 85%.\textsuperscript{84,85} A second cathepsin, cathepsin L (CTSL) was also demonstrated to play a possibly more minor role. Specific inhibitors and loss of function experiments suggest that CTSL has a synergistic effect together with CTSB, but is not sufficient for entry by itself. More recent data suggests that while EBOV, TAFV and BDBV are strongly dependent on CTSB, SUDV, RESTV and MARV have a requirement for as yet unidentified proteases.\textsuperscript{86,87}

Cleavage of EBOV GP by CTSB and CTSL can be performed in vitro, however the findings of different laboratories have not been consistent, perhaps due to separate preparations of proteases. Chandran et al demonstrate CTSL can digest GP1 to leave an 18kDa N-terminal fragment associated with GP2.\textsuperscript{84} CTSB can also perform this digest, although somewhat less efficiently. Interestingly, the 18kDa form is still infectious
further supporting the findings that the receptor binding domain of GP1 lies within its amino terminus.\textsuperscript{22} Infection mediated by pseudovirions bearing the 18kDa form remains inhibitable by high concentrations of specific CTSB inhibitors and these viruses do not infect cells from CTSB knockout mice. However, infection by the 18kDa form is significantly enhanced compared to untreated virus on cells undergoing partial inhibition of CTSB (i.e., likely to prevent the inefficient production of the 18kDa form mediated by CTSB) and full inhibition of CTSL. This supports a two-step process whereby CTSL efficiently digests GP1 to leave an 18 kDa fragment, which is then further digested by CTSB. Indeed incubation of the 18 kDa form with CTSB, but not CTSL, leads to a total loss of GP1 from particles. The fact that CTSB can perform both steps, albeit less efficiently, explains why CTSB inhibitors are more effective than those directed against CTSL, but not as potent as both together.

In contrast to the findings of Chandran et al, Schornberg and colleagues\textsuperscript{85} report that CTSB treatment reduces GP1 (approximately 130 kDa) to a 50 kDa species—consistent with the loss of the mucin-like domain from GP1. A second minor species at 19 kDa was also noted. CTSB treatment predominantly leads to a 20 kDa form, while combined cleavage gives a doublet of the 20 and 19 kDa fragments.\textsuperscript{85} These partially digested pseudovirions demonstrate enhanced infectivity, and while they are no longer sensitive to CTSB inhibition or ablation, they retain a requirement for acidified endosomes.

Cathepsin-mediated proteolysis also plays a role in the entry of several other viruses, including the corona- and reoviruses.\textsuperscript{88-90} In the case of the coronavirus, SARS-CoV, temperature-dependent interactions with receptor are required prior to cathepsin-mediated cleavage—an interesting modification of a two-step trigger mechanism for induction of membrane fusion.\textsuperscript{89} It appears that almost the reverse situation occurs for filoviruses. Proteolysis proceeds receptor engagement—which uniquely occurs on internal membranes. Following proteolysis, exposure of the receptor binding domain (RBD) allows EBOV GP to interact with Niemann-Pick C1 (NPC1), a cholesterol transporter present in late endosomal membranes.\textsuperscript{91,92} NPC1 can be demonstrated to directly bind to EBOV-GP and to allow infection of refractory cell types.\textsuperscript{93} Thus, the long search for a true filovirus receptor has finally yielded results in an unexpected place—inside the cell.

CONCLUSION

The recent advances in understanding filovirus entry highlight the requirement by enveloped viruses for a range of host factors other than classical receptors in order to achieve efficient entry. External membrane-bound molecules such as DC-SIGN and glycosaminoglycans are able to concentrate filovirus at the cell surface in a fairly nonspecific manner, while a more specific requirement for proteolysis by individual endosomal proteases is observed post-internalization. The recently identified lysosomal membrane located molecule, NPC-1, can then function as the true receptor, driving membrane fusion and entry.

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