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The Ebola virus journey into the host cell

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Abstract

Ebola and Marburg filoviruses are enveloped viruses causing severe hemorrhagic fevers in humans and non-human primates. To date, there is no vaccine or effective antiviral therapy against these highly pathogenic viruses. The development of new drugs highly depends on the elucidation of the entire life cycle of the virus. Virus entry in the host cell is the first step of infection as it allows the virus to deliver its genome into the cell cytoplasm and initiate replication. Although only one viral protein, the viral glycoprotein (GP), is responsible for tropism and fusion of the viral membrane with the cell membrane, virus entry is far more complex than it seems. Upon infection, GP interacts with a multitude of cellular proteins to adhere to the host cell surface and promote the internalization of the virus particle in intracellular vesicles. The virus is then transported and trafficked in order to gain access to specific cellular compartments containing proteins necessary for the activation of the GP fusion activity. The catalysis of the fusion of the viral membrane with the cellular membrane represents the final step in entry and the beginning of virus replication. In recent years, several research groups have greatly contributed to improve our understanding of the mechanisms of filovirus entry by the identification of cellular factors involved in infection. This review mostly focuses on these new findings by tracing the journey of the filovirus particle before and after it enters the host cell.

Introduction

The Ebola and Marburg viruses cause severe and often fatal hemorrhagic fever in human and non-human primates [1,2]. Along with the recently discovered Lloviu Cuevavirus, they belong to the *Filoviridae* family named for the virus' peculiar filamentous morphology [1,3]. They are endemic in many parts of Central Africa and the disease they cause is a typical zoonosis, the potential natural reservoir being bats [1,2,4-6]. Therefore, humans are accidental hosts and infection by Ebola and Marburg causes an exaggerated inflammatory response resulting in multiple organ failures and death between 6-9 days after onset of symptoms [1].

While there is only one species of Marburg virus, there are five species of Ebola virus: Zaire, Sudan, Thai Forest (formerly Côte d'Ivoire), Bundibugyo and Reston [1]. The first case of hemorrhagic fever caused by a filovirus was reported in 1967 in Marburg, Germany, when laboratory workers were infected while handling dead monkeys from Uganda. This led to the identification of the Marburg virus [7]. Epidemics of hemorrhagic fevers caused by Marburg virus are relatively common in Central Africa and have a fatality rate of around 70%-85% [8]. In 1976, two outbreaks of hemorrhagic fever in Zaire (now Democratic Republic of Congo) and Sudan led to the identification of Ebola Zaire and Sudan [9,10]. A third species of the Ebola virus was discovered in Ivory Coast (Côte d'Ivoire) in 1994. More recently, in 2004, a new species of Ebola virus, Bundibugyo, was identified during an outbreak in Uganda [1]. Each species of Ebola virus has a different degree of virulence in humans, Ebola Zaire being the most common and most deadly with a fatality rate of up to 90%. The exception is the Ebola Reston, which was identified in 1989 after isolation from cynomolgus macaques in the Philippines and does not appear to cause symptoms in humans [1]. This difference in virulence is still unclear.

The primary mode of transmission of Ebola virus is through direct contact with infected patients, animals, or cadavers [1]. The virus has a broad cell tropism with virtually all cells being permissive for infection and replication of the virus, with the notable exception of lymphocytes

which can be infected, albeit at lower levels [1,5,8]. Time-course experiments on non-human primates suggested that monocytes, macrophages, and dendritic cells are infected early [11]. Once infected, these cells circulate to several different tissues, thereby disseminating the infection all over the infected host [11].

Filoviruses are enveloped viruses containing a non-segmented single stranded RNA genome of negative polarity [8]. This 19-kb genome encodes seven viral genes: nucleoprotein, viral protein (VP) 35, VP40, glycoprotein (GP), VP30, VP24 and a RNA-dependent RNA polymerase (L). The viral particles have a uniform diameter of 80 nm and a variable length that can reach 14 µm [8]. In order to replicate, viral particles must recognize and penetrate the host cell, allowing the genetic material to enter the cytoplasm and initiate the replication of the virus. This entry step is performed by a single viral protein, the glycoprotein, which interacts with many cellular proteins during infection and catalyzes the fusion of the viral membrane with the cellular membrane [8].

Viral glycoprotein

All enveloped viruses require the fusion of the viral lipid membrane with that of the host cell [12]. This step is performed by a fusion protein found in a metastable state at the surface of the virus. Upon specific triggers, the glycoprotein will undergo a dramatic conformational change and adopt a stable post-fusion conformation [12]. The transition from a pre- to a low energy post-fusion conformation releases the energy required for the fusion of the viral membrane with the cell membrane. Despite differences in sequence, viral fusion proteins have striking similarities in structure and activation mechanisms that led to their classification into three main classes [12]. In the case of Ebola and Marburg viruses, the protein responsible for membrane fusion is the glycoprotein (GP), a class I viral fusion protein. The GP of filoviruses thus resembles other viral fusion proteins such as those of human immunodeficiency virus (HIV), influenza virus and severe acute respiratory syndrome coronavirus (SARS-CoV). The class I fusion proteins are normally synthesized as a transmembrane precursor that trimerizes in the endoplasmic reticulum. The precursor then transits in the Golgi apparatus where it is cleaved by a cellular protease into two subunits: a soluble subunit and a transmembrane subunit [12] (Figure 1A). For filoviruses, the precursor, GP0, is cleaved in the Golgi apparatus by a furin-like protease that generates the soluble subunit GP1 and the transmembrane subunit GP2 linked by a disulfide bridge [12-14] (Figure 1A). This proteolytic step is crucial for class I fusion proteins as it releases the fusion peptide located near the N-terminus of the transmembrane subunit [12]. Interestingly, as it will be discussed later, the Ebola virus glycoprotein is an

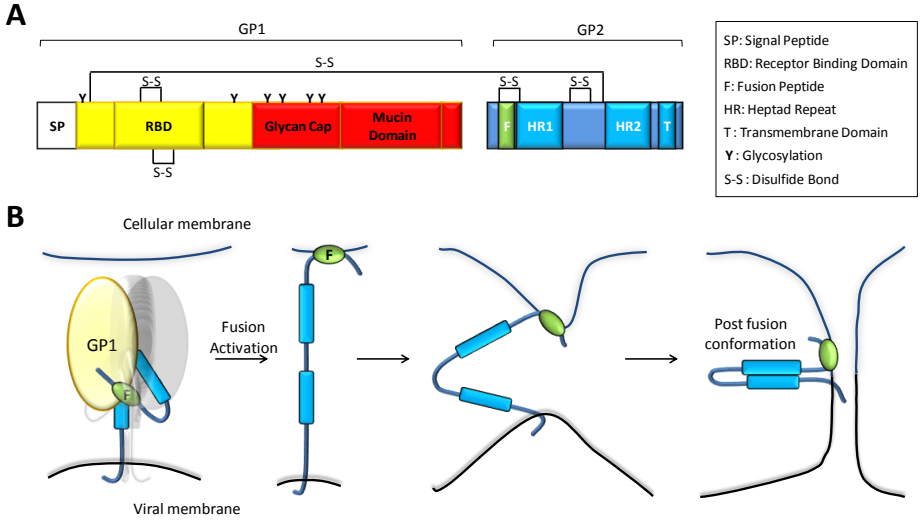

Figure 1. The viral glycoprotein. (A) Functional domains of GP1 and GP2. (B) Conformational rearrangement during membrane fusion activation. Trimers of GP1-GP2 dimers are anchored to the viral membrane via GP2 transmembrane domain. Upon activation, GP2 refolds into a rod-like structure allowing the insertion of the fusion peptide in the target membrane (For clarity, only one GP2 of the trimer is illustrated). GP2 then folds back into a hairpin to bring the two membranes in close proximity, leading to membrane fusion.

exception, since other cellular proteases can perform this function during virus entry [15-17].
The role of the transmembrane subunit is to catalyze the fusion of the viral membrane with the cell membrane. In order to induce membrane fusion, four areas of GP2 are essential: the fusion peptide (F), the N-terminal and C-terminal heptad repeats (HR1 and HR2), and the transmembrane domain (Figure 1A). The fusion peptide is a short sequence of hydrophobic amino acids, while the heptad repeats form alpha helices that strongly interact in the post-fusion conformation. For membrane fusion, the first step consists of a conformational change of GP2 into an elongated structure, which allows the insertion of the fusion peptide in the cell membrane (Figure 1B). Subsequently, GP2 folds on itself and forms a hairpin conformation through strong interaction between HR1 and HR2. This refolding causes the fusion peptide and the transmembrane domain to be brought in close proximity, thereby bringing both membranes together and inducing fusion (Figure 1B) [11]. Importantly, the conformational rearrangement of GP2 must be tightly regulated since the reaction is irreversible. Therefore, premature activation causes inactivation of the fusion protein and consequently inactivation of the virus. The regulation of GP fusion activity is provided by the GP1 subunit, which keeps GP2 in the pre-fusion conformation. Thus, an important function of GP1 is to restrict the fusion activity of GP2. During viral entry, GP1 interacts with a multitude of cellular proteins. Some of the cellular proteins get activated and promote internalization of the virus, while other(s), the receptor / co-receptors, cause a change in GP1 leading to a loosening of the restriction on GP2 and ultimately trigger the fusion of the cell membrane with the viral membrane [11].

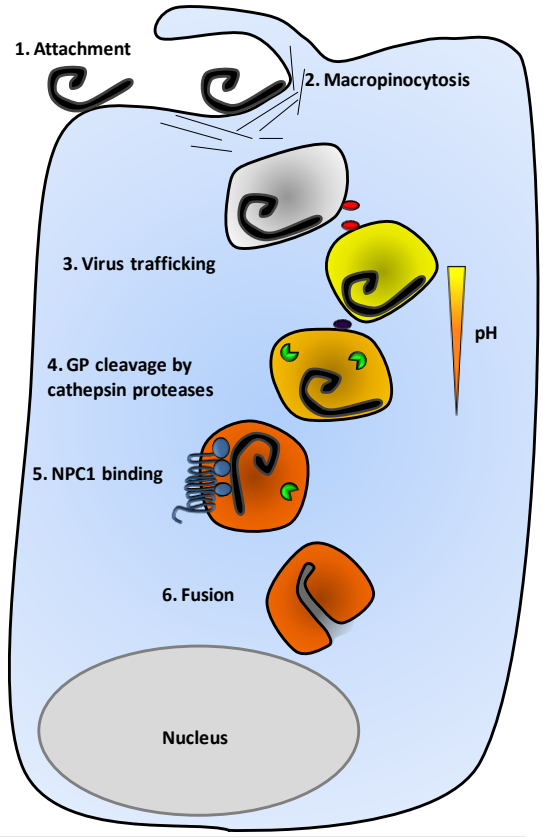

Figure 2. Ebola virus entry into the host cell.

Attachment factors

The first step of infection involves the adsorption of the virus to the host cell which is favored by the presence of attachment factors found at the cell surface (Figure 2). The expression of attachment factors in permissive cells generally increases the infectivity, although they are not essential for infection. A peculiarity of attachment factors is that they interact with the viral GP but do not activate the fusion activity of GP. In fact, the viral glycoprotein can activate these cellular proteins and initiate a cascade of intracellular signaling leading to virus internalization by endocytosis. Ebola and Marburg viruses use multiple attachment factors during infection such as DC-SIGN, DC-SIGNR, β-integrins and TIM-1 [18-21].

DC-SIGN is expressed in certain types of macrophages and dendritic cells while DC-SIGNR is expressed in most endothelial cells. The expression of DC-SIGN and DC-SIGNR facilitates infection by the Ebola virus [18,19]. The degree of glycosylation of the viral glycoprotein is important since DC-SIGN and DC-SIGNR interact

with proteins glycosylated with mannose residues. Changes in glycosylation of the viral glycoprotein depend on the virus producer cell and could have an impact on disease progression in infected hosts. Intriguingly, DC-SIGN and DC-SIGNR not only increase the infection of cells that express them, but also promote the infection of surrounding cells in *trans* [19].

β-integrins and T-cell Ig domain and mucin 1 (TIM-1) are surface proteins that bind the viral glycoprotein [20,21]. They are not essential, but significantly increase the Ebola virus infection. An important property of these cellular proteins is that they possibly play a role not only in virus adsorption to the surface of the host cell, but also in the process of endocytosis by macropinocytosis [20-22].

Internalization via macropinocytosis

Many viruses use the cellular mechanisms of endocytosis during infection. Several enveloped viruses use this strategy allowing them access to compartments containing specific cellular factors and / or environmental conditions that activate the viral fusion protein. There are various types of endocytosis pathways and viruses have developed means to trigger distinct mechanisms of endocytosis. This is especially important since the endocytic pathway used can direct the virus to intracellular compartments containing the essential host factors leading to a productive infection, or to intracellular degradative compartments resulting in a dead-end for the virus. Two common mechanisms of endocytosis used by viruses are clathrin-coated vesicles and caveolin-dependent endocytosis. However, the resulting vesicles are about 120 nm and 60-80 nm respectively which would not accommodate the very large size of filoviral particles [8,22]. Some initial studies with pseudotypes containing the GP of Ebola suggest that virus internalization can occur via clathrin-mediated endocytosis or caveolae [23,24]. However, other studies using filoviral particles have demonstrated that the Ebola virus uses macropinocytosis [25-27]. The latter internalization route is most likely the type used by native Ebola and Marburg viruses, since macropinocytosis generates vesicles from 200 to 10 000 nm that can accommodate the filoparticle size [22].

Apart from the dendritic cells that are capable of constitutive macropinocytosis, macropinocytosis must normally be stimulated. It is a complex process that requires a reorganization of the actin cytoskeleton and ruffling of the plasma membrane [22]. The attachment of the Ebola virus at the cell surface activates the PI3K signaling pathway, a phenomenon that is dependent on the viral glycoprotein [28]. The PI3K pathway is known to be involved in the stimulation of macropinocytosis [22]. The activation of this signaling pathway seems important since PI3K inhibitors block Ebola virus infection. A few years ago, a study indicated that the receptor tyrosine kinase Axl facilitated the entry by Ebola virus [29]. However, no direct interaction between the viral glycoprotein and the cellular protein had been demonstrated [30]. Instead, the presence of Axl helped to accentuate the macropinocytosis in the target cell, thereby promoting Ebola virus infection [31].

Unlike clathrin-mediated endocytosis, the formation of macropinosomes and entry by Ebola virus does not require the cellular protein dynamin [26,28]. However, it is possible that the virus uses dynamin in some cell types via atypical macropinocytosis [27]. The infection is sensitive to inhibitors of the reorganization of the actin cytoskeleton or Na(+)/H(+) exchangers required for macropinocytosis [25]. Other cellular factors are also important such as p53-activated kinase 1 (Pak1) and Arp2 involved in the reorganization of the actin cytoskeleton [25].

Intracellular trafficking

Other viruses, such as smallpox virus and adenovirus serotype 3, also use macropinocytosis for internalization [22,32]. Since these viruses require specific cellular factors and a specific environment, they must

use the vesicular trafficking system from the host cell to be routed to the proper compartment. In recent years, several studies have contributed to extending our knowledge of the intracellular pathway and proteins needed for Ebola virus infection. For transport, the Ebola virus requires small GTPases Rab5 and Rab7 proteins and the HOPS complex [25-27,33].

The transition from early endosomes to late endosomes requires Rab5 and Rab7 proteins. Rab proteins are small GTPase proteins that act as organizers of a large number of cellular factors and thereby regulate vesicle transport [34]. Their effects on the destination or maturation of a vesicle depend on their association with GTP or GDP whose levels are tightly regulated by GEFs (guanine-exchange factors) and GAPs (GTPase-activating proteins). Studies have shown that the Ebola virus traffics from early endosomes to late endosomes [25,26]. The Rab5 protein localizes to early endosomes, while the Rab7 protein is localized to late endosomes. The maturation of these vesicles requires a coordinated exchange of proteins Rab5 by Rab7 [34]. The use of small interfering RNA or dominant negative forms of Rab5 and Rab7 inhibit infection by the Ebola virus [25].

The vacuolar protein sorting (VPS) family comprises about 80 genes that encode proteins involved in endo-lysosomal trafficking [35]. The mutation of four of these proteins in yeast, Vps11, Vps16, Vps33, and Vps18, results in a lack of vacuolar lysosome. These proteins form the core of a protein complex, Vps-C. This complex can then associate with the accessory subunits Vps39 and Vps41 to form the homotypic fusion and vacuole protein sorting (HOPS) complex or with Vps3 and Vps8 to form the Class-C core vacuole/endosome tethering (CORVET) complex [35]. The HOPS complex regulates membrane fusion at the lysosome and interacts with several proteins including Rab and SNARES. In fact, it was shown that the HOPS complex functions as a GEF for Rab7 and interacts with Rab5, suggesting that this protein complex is involved in the transition or maturation of endosomes [36]. Using a genetic approach, Carette *et al.* reported that Ebola virus requires the expression of each Vps forming the HOPS complex [33]. However, it is interesting to note that Marburg virus infection does not seem to absolutely require the HOPS complex. It remains to be determined whether vesicular trafficking by Marburg virus is distinct from the one used by the Ebola virus.

Cathepsins B and L

A peculiarity of the Ebola virus glycoprotein is that it does not require cleavage by the furin protease in the virus producer cells to form functional viruses [15,16]. The reason for the optional role of "priming" is that during infection, the virus is internalized by endocytosis and trafficked to endosomes where the glycoprotein is digested by endosomal proteases, particularly cathepsins B and L. Pretreatment of cells with the cysteine protease inhibitor E64 dramatically inhibits infection by Ebola and Marburg viruses [37-40].

Interestingly, there are differences in cathepsin usage that depend on the species of filovirus. The Ebola Zaire and Thai Forest are extremely dependent on cathepsin B, Sudan slightly less, while the Reston and Marburg viruses do not require the activity of cathepsin B [40,41]. However, although the activity of cathepsin L is dispensable, cat L increases infection by Ebola Zaire, Thai Forest, Reston and Marburg [39,40].

Cathepsins do not cleave at specific amino acid sequences. According to the structure of the Ebola glycoprotein, it is proposed that cathepsins cleave a disordered loop in GP1 (Figure 3) [42].

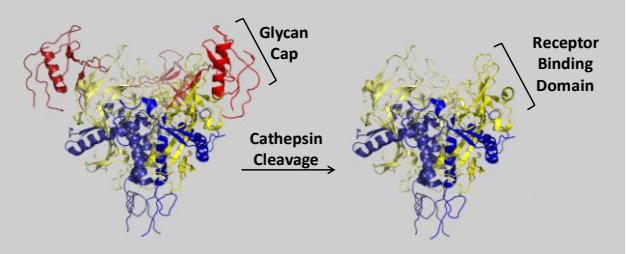

Figure 3. Ebola glycoprotein cleavage by cathepsin proteases.

The *in vitro* cleavage of the Ebola Zaire glycoprotein with cathepsin L results in a cleavage product of about 20 kDa, while incubation with cathepsin B gives a slightly smaller GP1 fragment of 17 kDa [37,38]. Mass spectrometry studies confirmed the hypothesis that the proteases target the loop, leaving the N-terminal domain of GP1 linked to GP2 by a disulfide bond (Figure 1A and 3) [43]. Digestion by cathepsins removes the variable regions, the glycosylated area and mucin cap, and exposes the N-terminal domain of GP1, which is a region that is more conserved and corresponds to the receptor binding domain (Figure 3).

The Niemann-Pick C1 protein

Although the activity of cathepsin B is required for infection, it is not sufficient to induce the fusion activity of GP, suggesting that at least one additional cellular factor is required [44,45]. Several groups have hypothesized that cathepsin cleavage of GP1 exposes a binding domain for an unknown receptor [46,47]. Indeed, the 17 kDa fragment of GP1 had binding activity to an unidentified cellular protein. Competition experiments revealed that this cellular protein would also be used by all filoviruses [48]. Recently, studies have shown that the Niemann-Pick C1 (NPC1) protein is essential for infection by the Ebola and Marburg viruses and that NPC1 binds the 17 kDa fragment of GP1 suggesting that NPC1 is the virus receptor [33,49,50].

The Niemann-Pick C1 protein contains 13 transmembrane domains and resides in late endosomes and lysosomes of all cell types. Its function is to transport the LDL-derived cholesterol from the late endosomes / lysosomes to the cell. NPC1 performs its role in conjunction with the Niemann-Pick C2 (NPC2) protein, a soluble lysosomal protein that transfers cholesterol to NPC1. Mutation of these proteins causes Niemann-Pick Disease, a rare but fatal disease in humans. It is characterized by an excessive accumulation of cholesterol in intracellular vesicles, causing severe mental retardation and death at a young age [51].

The NPC1 protein has been identified as necessary for infection by Ebola and Marburg viruses using two different approaches. Using a genetic approach that involves a haploid cell line and the random mutation of genes using retroviral vectors, Carette *et al.* have identified several critical factors including proteins forming the HOPS complex and NPC1 [33]. Using high-throughput screening of small molecules, we have discovered a molecule that specifically inhibits infection by the Ebola virus [49]. This molecule and its active derivatives caused a build-up of cholesterol typical of Niemann-Pick Disease and targeted the NPC1 protein [49]. The absence of NPC1 confers resistance to infection by all species of Ebola and Marburg viruses, indicating that the protein is essential for all filoviruses [33,49]. Although NPC1 expression is required, its cellular function is not, since cells expressing various NPC1 mutants that are unable to transport cholesterol still supported infection [49]. This latter observation and the fact that NPC1 is required by all filoviruses suggested that NPC1 is the virus receptor.

Using binding assays to membranes derived from endosomes and lysosomes, our group has demonstrated that GP binds these membranes only when NPC1 is present and when GP is first cleaved to expose the receptor binding domain [49] (Figure 4). This binding is inhibited in the presence of small molecule inhibitors of Ebola virus infection and targeting NPC1. The interaction between GP and NPC1 is direct since NPC1 co-immunoprecipitated with GP [49,50].

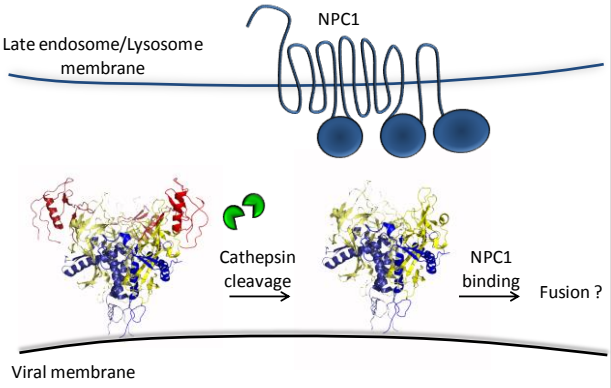

Figure 4. Schematic of Ebola glycoprotein activation.

The Niemann-Pick C1 protein contains three large areas located in the lumen of endosomes and lysosomes [51]. Recently, Miller *et al* reported that the second domain is sufficient to confer infection and bind cleaved GP [50].

Conclusion and perspectives

Despite recent advances, several questions remain unanswered. Is NPC1 the last factor that activates GP1 and causes the fusion of the viral membrane with the cell membrane? The domain of NPC1 that binds GP can block viral infection, but is unable to allow infection of cells that do not express NPC1 [50], indicating that other factors beside cathepsins and NPC1 are possibly required. Some groups have proposed that a rearrangement of the disulfide bond between GP1 and GP2 may be necessary [45]. This type of mechanism is required for the activation of the fusion protein of murine retroviruses. In this case, an intrinsic isomerase activity allows a rearrangement of the disulfide bond between the two subunits and consequently leading to their dissociation. This releases the restriction by the surface subunit on the fusion subunit and triggers the fusion of the viral and cellular membranes. Since the Ebola and Marburg glycoproteins do not possess an isomerase activity, a cellular protein residing in the lysosome reducing the disulfide bridge may be required.

Moreover, it is unclear what are the respective role and importance of cleavage by cathepsin B and L in binding to NPC1 and the fusion mechanism. A recent study shows that a cleavage product of GP1, comparable to that resulting from digestion by cathepsin L, can bind NPC1 [40]. However, the virus still requires cathepsin B activity for infection. This observation suggests that cleavage by cathepsin B has possibly an additional role to play in the activation of GP after binding to NPC1.

Since the use of cathepsins B and L varies among filoviruses, NPC1 is so far the only essential factor shared by all filoviruses. Thus, NPC1 is an excellent target candidate for the development of antiviral therapies.

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