Host Cell Factors Involved in Filovirus Infection

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Abstract Filoviruses (ebolaviruses and marburgviruses) cause severe hemorrhagic fever in humans and nonhuman primates with high mortality rates of up to 90 %. The latest epidemic of Ebola virus disease in Western African countries has underscored the urgent need for effective prophylactic and therapeutic interventions for this deadly infectious disease. However, neither approved prophylactics nor therapeutics are currently available for filovirus diseases. Recent studies have been unveiling the molecular mechanisms underlying the filovirus lifecycle, including cellular entry, egress, and the evasion from host immunity, suggesting possibilities to develop effective pan-filovirus drugs.

Keywords Filovirus · Marburgvirus · Ebolavirus · Viral hemorrhagic fever · Virus receptor · Antivirals · Virus entry · Immune evasion · Assembly · Budding

Introduction

Ebolaviruses and marburgviruses in the family *Filoviridae* are notorious zoonotic pathogens causing severe hemorrhagic fevers in humans and nonhuman primates with extremely high mortality rates. The genus *Marburgvirus* includes two viruses (Marburg and Ravn viruses) in a single species *Marburg marburgvirus*, whereas the genus *Ebolavirus* consists of five distinct species, *Zaire ebolavirus*, *Sudan ebolavirus*, *Tai Forest ebolavirus* (formerly *Cote d'Ivoire ebolavirus*), *Bundibugyo ebolavirus*, and *Reston ebolavirus*, represented by Ebola virus, Sudan virus, Tai forest virus, Bundibugyo virus, and Reston virus, respectively [1]. The species *Lloviu cuevavirus* in the genus *Cuevavirus*, which includes a newly found filovirus (Lloviu virus) detected from carcasses of Schreiber's bats in Europe [2], has been designated a novel member of this family [1].

Filoviruses have non-segmented, single-stranded, and negative-sense RNA as the viral genome that encodes at least seven structural proteins: nucleoprotein (NP), polymerase co-factor (VP35), matrix protein (VP40), glycoprotein (GP), transcription activator (VP30), minor matrix protein (VP24), and RNA-dependent RNA polymerase (L) (Fig. 1a, b). In addition to these structural proteins, ebolaviruses produce at least two forms of nonstructural soluble GPs, sGP and ssGP, translated from the GP gene [3, 4]. Filovirus particles are filamentous, enveloped by a lipid bilayer with surface GP, and contain a helical nucleocapsid composed of the viral RNA encapsidated by NPs and other viral proteins [5, 6] (Fig. 1a).

As proven by the latest epidemic of Ebola virus disease in the West African region, filovirus diseases pose a significant public health threat, largely owing to the lack of the available prophylactics and therapeutics. Several studies demonstrated that administration of antisense oligonucleotides [7, 8, 9•] or GP-specific antibodies [10•, 11–13, 14••] protected monkeys from lethal challenge with filoviruses. However, due to the

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Fig. 1 Filovirus genes and proteins. Schematic diagrams of a filovirus particle (**a**), negative-sense genome organization (**b**), and the primary structure of GP (**c**) are shown. **a**, **b** NP nucleoprotein, VP35 polymerase cofactor, VP40 matrix protein, GP glycoprotein, VP30 transcription activator, VP24 minor matrix protein, and L RNA-dependent RNA polymerase. **c** The furin-cleavage site and a disulphide bond between the GP1 and GP2 subunits are indicated by the arrows and lines, respectively. SP signal peptide, RBR putative receptor-binding region, MLR mucin-like region, IFL internal fusion loop, TM transmembrane domain

genomic diversity and antigenic differences among the filovirus species, these treatments and prophylactics are mostly virus species-specific. It is indeed difficult to rapidly implement such interventions, since the prediction of filovirus disease outbreaks (i.e., where and which filovirus species?) is almost impossible due to the lack of information on the ecology of filoviruses [15].

To develop pan-filovirus prophylactics and therapies, it is important to understand the detailed mechanisms, particularly virus-host protein interactions, underlying filovirus infection. It has been demonstrated that filoviruses utilize a variety of cellular proteins for their replication and immune evasion. In this review article, we summarize the interactions between viral and host molecules involved in the life cycle of filoviruses and discuss potential targets for the development of anti-filoviral drugs.

Entry

Filovirus particles possess a single surface GP that is responsible for virus entry into cells (i.e., attachment to cell surface receptors and membrane fusion between the viral envelope and cellular membrane) [16] (Figs. 1a and 2). Thus, GP is deemed to be an important factor for the pathogenicity, tissue

tropism, and host range of filoviruses [17, 18]. It is well documented that precursor GP is posttranslationally modified during trafficking to cell surfaces. The ubiquitous host proprotein convertase, furin, and its family members proteolytically cleave the precursor GP into covalently linked GP1 and GP2 subunits, through the trans-Golgi network [19, 20] (Figs. 1c and 2b). Intriguingly, the furin recognition motif is highly conserved among GPs of all known filoviruses, including the newly found cuevavirus [3, 19–21]. However, the biological significance of the furin-mediated GP cleavage remains unknown since the cleavage has been shown to be nonessential for viral replication in vitro and pathogenicity in monkeys [22, 23]. It is also known that GP becomes heavily decorated with sugar chains through glycosylation in the endoplasmic reticulum and Golgi apparatus [24, 25]. O-linked glycans are concentrated on the mucin-like region in the middle one-third of the GP amino acid sequence (Fig. 1c). Three GP1/GP2 heterodimers form a trimer, anchored to the viral envelope in type I orientation [26].

Filovirus infection is initiated by the interaction with attachment factors expressed on target cell surfaces (Fig. 2a). Following the attachment to cells, filoviruses are internalized into host cells by micropinocytosis-like mechanisms [27] (Fig. 2b). Although several cell surface molecules have been proposed to be involved in GP-mediated attachment, none of

a

GP

Sugar chain

C-type lectin

PtdSer

TIM-1

Antibody

Fc receptor

C1q/C1q ligand

Ubiquitous attachment factor?

Cell

b

Axl, Dtk, Mer → Enhance → Macropinocytosis

Attachment

a

Late endosome/lysosome

Cathepsin B/L

Entry

Assembly

Filopodia

Budding

Membrane fusion

NPC1

Translation

Furin

Tsg101

Nedd4

VP40

Multivesicular body

VP40

Actin

Transcription

VP35

L

RNA (-)

Replication

Topoisomerase

mRNA

Nucleocapsid

Anti-genomic RNA (+)

Genomic RNA (-)

Inclusion body

Nucleus

Kondratowicz et al. identified T cell immunoglobulin and mucin domain 1 (TIM-1) as a candidate filovirus receptor by using a bioinformatics approach in which cell susceptibility to

C-type lectins, calcium-dependent carbohydrate-binding proteins, are also known to contribute to filovirus infection by binding to glycans on GP [35–40] (Fig. 2a). However, several studies showed that interaction of GP with C-type lectins did not trigger membrane fusion [37, 41, 42],

indicating that C-type lectins are not functional receptors mediating entire steps of filovirus entry (i.e., both attachment and membrane fusion) but strongly enhance virus infection by facilitating attachment to cell surfaces. Importantly, C-type lectins likely play important roles in the filovirus pathogenicity and tissue tropism by promoting attachment to the preferred target cells, including macrophages, dendritic cells, hepatocytes, and endothelial cells. Indeed, asialoglycoprotein receptors on hepatocytes [35], DC-SIGN and DC-SIGN(R) on dendritic cells [36, 37], L-SIGN and LSECtin on endothelial cells [36, 39], and hMGL on macrophages [38, 40] were shown to enhance filovirus infections. Interestingly, the difference in the relative pathogenicity among filoviruses is correlated with the ability to utilize C-type lectins for cellular entry in vitro [38, 40]. Administration of soluble mannose-binding C-type lectins showed protective efficacy against lethal ebolavirus challenge in mice [43], suggesting that the interaction between GP and C-type lectins could be a potential target of treatments. As well as C-type lectins, some anti-GP antibodies recognizing the mucin-like region also enhance GP-mediated filovirus infection through interaction with the cellular Fc receptor or complement component C1q and its receptor, probably due to increased virus attachment to target cells [44–46] (Fig. 2a). However, the significance of antibody-dependent enhancement of infectivity in filovirus pathogenicity remains elusive.

Filovirus particles internalized through micropinocytosis are trafficked to the late endosomes/lysosomes, where GP undergoes proteolytic processing by host cysteine-proteases such as cathepsins B and L, to be primed for membrane fusion [47] (Fig. 2b). Cathepsins B and L are activated in the acidic environment of these vesicles and remove the C-terminus of GP1 subunit including a mucin-like region, leading to exposure of the putative receptor-binding region [48]. Of note, $\alpha 5 \beta 1$ integrin is required for cathepsins B and L to be catalytically active for GP priming [49], supporting an earlier study showing the importance of integrins for filovirus entry [50]. However, since each filovirus species has a different dependency on cathepsin-processing [51, 52] and mouse-adapted ebolavirus retains full pathogenicity in mice deficient in either cathepsin B or L [53], other important host proteases are likely involved in proteolytic processing of GPs.

The exposed receptor-binding region in the primed GP interacts with a ubiquitous host endosomal/lysosomal cholesterol transporter, Niemann-Pick C1 (NPC1), which is believed to be required for triggering membrane fusion [54•, 55•] (Fig. 2b). Expression of human NPC1 rendered even a non-permissive reptile cell susceptible to filovirus GP-mediated infection in vitro [56•]. Pseudotyped viruses bearing ebolavirus GP failed to access the cytoplasm in NPC1-deficient cells and accumulated in late endosome-like vesicles [54•]. These findings indicate that NPC1 is an essential endosomal receptor (i.e., fusion factor) for filovirus infection.

Since the function of NPC1 in filovirus entry is independent of its physiological function as a cholesterol transporter [54•, 55•, 56•], the interaction between primed GP and NPC1 was hypothesized to be a promising target of treatments. It was accordingly shown that heterozygous mice with reduced NPC1 expression in the late endosomes/lysosomes were significantly resistant to filovirus infection [54•], and several small compounds that disrupt the GP-NPC1 interaction inhibited filovirus infection in vitro [55•, 57].

While previous studies have demonstrated that filoviruses utilize multiple entry pathways (i.e., receptors and coreceptors) depending on the cell type, the passive immunization of nonhuman primates with filovirus-specific antibody cocktails has been shown to be protective against lethal filovirus infection [10•, 11–13, 14•], highlighting the importance of the virus entry step as a target of antivirals. A comprehensive understanding of the mechanism underlying the entry of filoviruses into cells may accelerate the development of effective prophylactic and therapeutic interventions for filovirus infection.

Replication

Following GP-mediated membrane fusion, the nucleocapsid is released into the cytoplasm of cells (Fig. 2b). Each filovirus gene, which is flanked by conserved transcriptional start and stop signals, is transcribed into mRNA by the viral RNA polymerase, L protein [58]. Subsequently, viral mRNAs are translated by the host cell machinery. The promoter at the 3' end of the genome RNA also drives the synthesis of full-length complementary and antigenomic RNAs, which in turn serve as templates for synthesizing genomic RNA. Marburgvirus requires NP, VP35, and L for transcription and genome replication [59], whereas ebolavirus requires VP30 in addition to these viral proteins [22, 60, 61]. L protein provides the catalytic activity of the RNA-dependent RNA-polymerase together with VP35.

It was recently shown that a host nuclear protein, topoisomerase 1, interacted with the ebolavirus L protein and enhanced the viral polymerase activity [62]. It has been also suggested that topoisomerase 1 directly interacts with the stem-loop RNA structure and regulates the transcription and replication of the retrovirus genome [63]. Under normal physiological conditions, topoisomerase 1 binds to double-stranded DNA to unwind the DNA helical structure for transcription and replication. While the phosphodiester bridge-cleaving and recombination activities of topoisomerase 1 are required for the ebolavirus polymerase activity [62], the precise mechanism whereby this host protein contributes to the filovirus transcription and replication remains unclear. The polymerase cofactor VP35 is also essential for transcription and replication of the filovirus genome [60]. The dynein light chain, a component of

the microtubule transport system, is reported to interact with ebolavirus VP35 [64]. Since disruption of the interaction between this protein and VP35 does not affect the antagonistic activity of VP35 against type I interferon (IFN) production (see the section “**Immune Evasion**”), the dynein light chain might play a role in facilitating the viral polymerase activity [64]. Although ebolavirus VP40 and VP24 are also known to be involved in viral genome transcription and replication, it remains to be clarified how these viral proteins regulate viral RNA synthesis [65].

The formation of inclusion bodies that contain NP, VP35, VP30, and L [66–68] in the cytoplasm of infected cells is a prominent feature of filovirus infection (Fig. 2b). The formation of inclusion bodies corresponds to the onset of filovirus genome replication but that transcription occurs prior to the inclusion body formation [67]. It has been shown that phosphorylation of VP30 suppresses the transcriptional activity of the ebolavirus polymerase but increases genome replication activity [69, 70, 71•]. Importantly, phosphorylated VP30 is localized in the inclusion bodies and dissociated from the L/VP35 polymerase complex [69, 71•]. Contrastingly, dephosphorylation of VP30 restores the transcriptional activity of the polymerase [71•, 72]. These data suggest that the VP30 phosphorylation governs the mode of ebolavirus RNA synthesis [71•, 72]. Presumably, the viral polymerase complex predominantly synthesizes anti-genomic and genomic RNAs when VP30 is phosphorylated [71•]. However, the polymerase complex may function as a transcriptase when VP30 is not phosphorylated [71•]. Although there are no reports indicating that marburgvirus VP30 regulates viral polymerase activity, it was implied that phosphorylation of NP modulated the transcription and/or replication of the marburgvirus genome [73].

Thus far, phosphatase 1 is the only host protein found to control dephosphorylation of ebolavirus VP30 [72], and the host kinases that phosphorylate ebolavirus VP30 or marburgvirus NP are totally unknown. It is noteworthy that small compounds binding to phosphatase 1 inhibit ebolavirus growth in vitro without showing cytotoxicity [72], supporting the idea that the virus replication step may also be a target for antivirals. Indeed, it was recently demonstrated that nucleotide analogues such as favipiravir (T-705) and BCX4430 provided significant protection against filovirus infection in mice [74, 75••]. BCX4430 completely protected nonhuman primates from lethal marburgvirus infection [75••].

Assembly/Egress

The filovirus matrix protein VP40 is the key driving force for virion formation and budding. It has been suggested that VP40 is retrogradely trafficked via late endosomes to assembly sites such as multivesicular bodies and the plasma

membrane by hijacking the host vacuolar protein sorting pathway including the ESCRT machinery [76] (Fig. 2b). Filovirus VP40 contains the motif (i.e., late domain) [77] that mediates interactions of VP40 with components of the VPS pathway such as Nedd4 and Tsg101 and facilitates intracellular trafficking of VP40 and budding of virus particles [76]. However, it was shown that mutations in the late domain motif did not dramatically reduce the virus replication [78], suggesting that filoviruses might employ multiple pathways to transport viral proteins. Indeed, GBF1 and ARF in COPI and Sec24C in the COPII vesicular transport system were subsequently shown to be involved in ebolavirus particle formation [79, 80]. VP40 is targeted to the plasma membrane and has high affinity for lipid bilayers containing a high level of PtdSer [81]. This property may support the GP-independent entry mediated by TIM-1.

NP, the primary component of the nucleocapsid, is trafficked to inclusion bodies and encapsidates genomic RNA to form the helical nucleocapsid together with VP24, VP30, VP35, and L [5, 6, 67, 68] (Fig. 2b). Subsequently, the nucleocapsid is targeted to the assembly site and incorporated into virus particles. Interestingly, marburgvirus NP also contains the late domain motif and interacts with Tsg101 [82], suggesting that NP may share transport pathways with VP40. It was indeed demonstrated that both VP40 and nucleocapsid were associated with cytoskeletal proteins such as actins in cytoplasmic transport [83–86]. Since VP40 is also detected in inclusion bodies along with NP and VP35 [68], it is of interest to clarify whether the nucleocapsid is trafficked to the assembly site alone or with VP40.

In the budding process, membrane-associated VP40 extruded from the cells accompanying surface GP and the nucleocapsid, and infectious virions are released by pinching off the plasma membrane [87] (Fig. 2b). It is assumed that majority of marburgvirus particles are released from the tips or sides of cellular filopodia [83]. It has been shown that actin-dependent molecular motor protein Myo10, which mediates intrafilopodial movement of proteins, is important for VP40-mediated vesicle release [83]. Similarly, IQGAP1, which is involved in cytoskeletal remodeling during cell migration and the formation of filopodia, interacts with ebolavirus VP40 and facilitates the release of virus-like particles [88]. Recently, phosphorylation of VP40 was shown to be essential for efficient assembly and budding [89, 90]. In addition, it has been shown that NP and VP40 detected in virus particles are phosphorylated [91, 92]. These data support the notion that other unidentified cellular kinases/phosphatases may also regulate filovirus assembly and budding through phosphorylation of NP and VP40.

It has been demonstrated that several small molecules that interfere with the late domain-mediated interactions between VP40 and host factors efficiently inhibit replication of a broad range of RNA viruses, including filoviruses in vitro [93, 94].

Importantly, one of these molecules protected mice from lethal ebolavirus challenge [93]. As evidenced by the well-known efficacy of influenza virus neuraminidase inhibitors, the viral budding process should also be a promising target for antiviral development. We accordingly reported that monoclonal antibodies recognizing particular epitopes on the marburgvirus GP molecule dramatically inhibited the marburgvirus budding in vitro [95], leading to the idea that budding-inhibiting antibodies play a role in protective immunity and may also be available for immunotherapy in combination with conventional neutralizing antibodies.

Immune Evasion

As with other RNA viruses, the ability to evade host immune defense mechanisms seems to be essential for the pathogenesis of filovirus infections. RIG-I-like receptors (RIG-I, MDA5, and LGP2) are important cytoplasmic pattern recognition receptors that trigger type I IFN signaling in response to binding of viral dsRNA (Fig. 3). The signal is transmitted via interferon regulatory transcription factors IRF-3 and IRF-7, which are activated through phosphorylation by host kinases such as TBK-1 and IKK- ϵ . Phosphorylated IRF-3 and/or IRF-7 are translocated to the nucleus and activate transcription of IFN- α/β and other IFN-induced genes. Filovirus VP35 has been well described as an antagonist of RIG-I-mediated host

innate immunity. VP35 binds to RNA and sequesters viral RNA from recognition by RIG-I and/or MDA-5 [96, 97]. It also serves as an alternative substrate for TBK-1 and IKK- and abrogates phosphorylation of IRF-3 and IRF-7, resulting in the reduction of type I IFN production [98–100]. Transcription of the IFN- β gene is also negatively regulated by VP35-promoted SUMOylation of IRF-3 and IRF-7 [101]. Recently, it has been reported that ebolavirus VP35 interacts with the RIG-I activator PACT and interferes with RIG-I activation [102]. Interestingly, interaction of PACT with VP35 prevents formation of a functional polymerase complex and suppresses viral RNA synthesis [102]. In addition to antagonism of RIG-I signaling, VP35 was shown to inhibit RNA silencing [103, 104] and the PKR-mediated cellular anti-stress response [105]. Since PACT is involved in both RNA silencing and PKR activation, it might be possible that the VP35-PACT interaction contributes to the suppression of RNA silencing and PKR-mediated antiviral response.

Ebolavirus VP24 is known to function as an antagonist of IFN signaling pathways. Upon virus infection, induced type I and II IFNs bind to membrane receptors and promote autophosphorylation of Janus kinases, JAK1/TYK2 and JAK1/JAK2, respectively (Fig. 3). Activated Janus kinases phosphorylate STATs that form hetero- and homodimers STAT1/STAT2 and STAT1/STAT1. Phosphorylated STATs are translocated into the nucleus and activate the transcription of IFN-stimulated genes. Ebolavirus VP24 interacts with

Fig. 3 Filovirus proteins and RIG-I/IFN signaling. Filoviruses counteract host immune responses through multiple mechanisms. Diagrams of RIG-I signaling (*left panel*) and type I/II IFN signaling (*right panel*) pathways are shown

Karyopherin alpha 1, which regulates nuclear translocation of STAT complexes containing STAT1, and inhibits accumulation of STATs in the nucleus [106–108]. Furthermore, ebolavirus VP24 directly interacts with STAT1, suggesting an additional molecular mechanism to block STAT1-mediated signal transduction [109•].

Thus far, there have been no reports showing that marburgvirus VP24 interacts with either Karyopherin alpha 1 or STAT1. However, it has been shown that marburgvirus blocks IFN signaling by a mechanism distinct from that of ebolavirus. Marburgvirus matrix protein VP40 has the capacity to inhibit phosphorylation of JAK1 and Tyk2 and abrogates transcriptional activation of interferon-stimulated genes [110] (Fig. 3). Interestingly, mutations in rodent-adapted ebolavirus and marburgvirus were found in VP24 [111, 112] and VP40 [113, 114], respectively, indicating that the ability to block IFN responses might be an important determinant of the filovirus host range. It should be noted that marburgvirus VP24 interacts with host Keap1 and activates a cytoprotective antioxidant response pathway via Nrf2 activation [115, 116]. While the underlying mechanisms remain elusive, the activation of this pathway might prolong survival of infected cells and allow efficient virus replication. Since Nrf2-deficient mice are more resistant to mouse-adapted marburgvirus than wild-type mice [116], the interaction between marburgvirus VP24 and Keap 1 may be partially involved in the outcome of marburgvirus infection.

Tetherin, a type I IFN-inducible host protein, has been shown to inhibit release of a variety of enveloped viruses, including filoviruses [117]. Since both the N- and C-terminus of tetherin are linked to the membrane through a single transmembrane domain and putative glycosylphosphatidylinositol anchor, respectively, this molecule is thought to directly tether virions to cells by bridging cellular and viral membranes [117]. Although tetherin significantly reduces the release of virus-like particles composed of VP40 alone, coexpression of GP efficiently restores release of the particles in the presence of tetherin, indicating that filovirus GP is antagonistic to tetherin [118, 119]. Accordingly, tetherin expression has little effect on filovirus growth in vitro [120]. Although the transmembrane domain of the GP2 subunit contributes to tetherin antagonism [121], the precise mechanisms underlying GP-mediated antagonism of tetherin remain to be elucidated.

It has also been demonstrated that clustered glycans on the mucin-like region of filovirus GP sterically cover host surface proteins such as the Fas receptor and MHC class I [122, 123] and that this steric shielding effect prevents the transduction of apoptotic and immune signaling pathways in vitro [122, 123]. The efficacy of GP-mediated steric shielding is correlated with the difference in the relative pathogenicities among filoviruses [124]. Nonstructural soluble forms of GPs (e.g., sGP), which are abundantly released into the bloodstream, have been

thought to act as decoys to absorb neutralizing antibodies targeting envelope GP [125]. It is also suggested that sGP redirects the host immune response (i.e., B cell memory) towards epitopes shared with the envelope GP, allowing efficient absorption of antibodies to GP [126].

Conclusions

Filovirus diseases are global public health threats even in previously unaffected areas. Importantly, the latest epidemic of Ebola virus disease in West Africa emphasizes that the development of prophylactic and therapeutic interventions for filovirus infections is urgently needed. Although several promising vaccine and treatment strategies for filovirus diseases have been proposed, most of them exert their protective effects in a filovirus species- or strain-specific manner. Hence, there is a pressing need to develop pan-filovirus therapies enabling quick responses to unexpected outbreaks of filovirus diseases. Recently reported nucleoside analogues may shed light on the possibility of developing pan-filovirus therapeutics. A detailed understanding of the mechanisms underlying the replication and immune evasion of filoviruses and deciphering of the structures of the intermolecular interfaces between virus-host protein interactions will provide essential information for developing effective prophylactic and therapeutic interventions for filovirus diseases.

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Compliance with Ethics Guidelines

Conflict of Interest Masahiro Kajihara and Ayato Takada declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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