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Review

Filoviruses: Interactions with the host cell

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Abstract. The highly pathogenic filoviruses, Marburg and Ebola virus, are difficult to handle and knowledge of the interactions between filoviruses and their host cells remained enigmatic for many years. Two developments were crucial for the presented advances in our understanding of the cell biology of filoviruses, which is still fragmentary. On the one hand, the number of high containment laboratories increased where handling of the highly pathogenic filoviruses is possible. On the other hand, molecular biological tools have been developed that allow investigation of certain aspects of filoviral replication under normal laboratory conditions which considerably accelerated research on filoviruses. This review describes advances in understanding the interactions between host cells and filoviruses during viral attachment, entry, transcription, assembly and budding.

Keywords. Filoviruses, Ebola virus, Marburg virus, host cell interactions, virus entry, assembly, budding.

Introduction

Filoviruses are enveloped, non-segmented, negative-stranded RNA viruses with filamentous shape that constitute the family Filoviridae within the order Mononegavirales. The family comprises the genera Marburg virus (MARV) and Ebola virus (EBOV). The genus EBOV is further subdivided into the four species Zaire, Sudan, Ivory Coast and Reston [1]. Filoviruses were discovered in 1967 when an outbreak of viral hemorrhagic fever (HF) was reported among laboratory workers in Europe who had been exposed to tissue and blood from imported African green monkeys [2–5]. The causative agent was called Marburg virus. In 1976 Ebola virus was discovered as the cause of outbreaks of viral HFs in Sudan and the Democratic Republic of the Congo. The history of filovirus outbreaks is reviewed in details elsewhere [6–8].

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Filoviruses cause sporadic outbreaks of fulminant HF in human and non-human primates in Central Africa with case-fatality rates up to 90%. Filovirus HF is associated with high levels of inflammatory cytokines and coagulation disorders resulting in septic shock and multiorgan failure [9–12]. Poor immune response and lymphopenia are characteristic features of the disease [13–18]. The route of transmission involves direct contact with body fluids from either infected patients or animals as gorillas and chimpanzees or contact with a natural host [19]. Recently fruit bats were suggested to represent a natural reservoir of filoviruses [20, 21].

No vaccine or specific antiviral treatment is licensed for the use in humans. However, several experimental approaches showed promising results in recent years, which are reviewed elsewhere [22, 23].

Filovirus proteins are encoded by a 19 kb genome with the following gene order: 3′ leader, nucleoprotein (NP), virion protein (VP) 35, VP40, glycoprotein (GP), VP30, VP24, polymerase protein (L) and 5′

Figure 1. Filovirus genome organization. Non-transcribed regions (leader, trailer, and intergenic regions) are shown as black line. The genes are depicted as boxes: nucleoprotein (NP), viral protein (VP) 35, matrix protein VP40, glycoprotein (GP), VP30, VP24 and polymerase protein (L). Black stars indicate EBOV gene overlaps and the white star indicates MARV gene overlap.

trailer (Fig. 1). The viral genome is associated with NP, VP30, VP35 and L in the ribonucleoprotein complex [24]. NP encapsidates the viral genome and is necessary and sufficient for replication and transcription in an artificial MARV minigenome system together with L and VP35, which represents the polymerase cofactor [25]. For the replication of EBOV, NP, VP35 and L are sufficient for replication as well; however, viral transcription is dependent in addition on VP30, a filovirus-specific transcription factor [26]. Filovirus envelope consists of cell-derived membrane containing the viral GP. The matrix proteins VP40 and VP24 are positioned underneath the viral membrane and ensure the structural integrity of the particle [27, 28]. VP24 and VP35 are capable to antagonise the type I interferon (IFN) immune response [29–32].

Because of the limited genome sizes, viruses use host cell factors for many if not all steps of replication. Detailed understanding of specific virus – host cell interactions is substantial for understanding of virus replication and the development of antivirals. In this article, we review specific aspects of filovirus-host interactions during viral entry, replication, assembly and budding.

Host cell factors required for filovirus entry and establishment of infection

Initiation of infection by binding of surface GP to cellular receptors

The envelope GP of filoviruses is a type I transmembrane protein that forms homotrimers and constitutes the viral spikes mediating binding and entry into the target cell [33–35]. GP is synthesized as endoplasmic reticulum (ER) precursor and processed in the classical secretory pathway from ER via Golgi apparatus to the plasma membrane [35]. ER processing includes removal of the signal peptide, N-glycosylation and oligomerization [33, 36–38]. Passing the Golgi apparatus GP is processed by acylation, phosphorylation, O-glycosylation and maturation of N-glycans and finally by proteolytic cleavage by furin [34, 36–45]. Mature GP consists of two subunits, the large extracellular GP_1 and the membrane-anchored GP_2, which are linked by a disulfide bond [34, 46]. GP_1 is highly N- and O-glycosylated mainly in a variable serine-threonine-rich C-terminal region described as a mucin-like domain [33, 38, 39, 41, 45]. Recently, the receptor binding domain (RBD) of the filovirus GP has been mapped to the first 200 amino acids at the highly conserved N-terminus of GP_1 suggesting a common receptor of viral entry for MARV and EBOV [47–49].

To date, the search for the filovirus receptors has been elusive and implicated a set of distinct and functionally unrelated cell surface proteins in the entry process (Table 1). The first group of cellular receptors described to bind filoviruses is C-Type lectins containing carbohydrate recognition domains (CRDs) responsible for binding to specific glycans in a calcium-dependent manner [50]. Since filovirus GP is highly glycosylated displaying several types of sugar side chains, hijacking these cellular surface proteins for the infection process seemed to make sense.

Asialoglycoprotein receptor (ASGP-R), exclusively expressed in hepatocytes, was described as the first cellular factor enhancing MARV infection of a mouse fibroblast cell line expressing ASGP-R [51]. ASGP-R is a C-type lectin which binds and facilitates endocytosis of glycoproteins containing terminal galactose. Since both MARV and EBOV infect many ASGP-R-negative cell types, other factors must function in facilitating entry in different cellular contexts. Later three other type II receptors from the C-type lectin family with different carbohydrate specificities and distinct cell type tropisms were identified to enhance filovirus entry (Table 1).

The dendritic cell-specific ICAM-3-grabbing non-integrin (DC-SIGN) expressed on immature dendritic cells (DC) and macrophages and the homolog L-SIGN expressed on endothelial cells in liver and lymph nodes were shown to bind EBOV and MARV GP pseudotype lentiviruses and to enhance infection of non-permissive Jurkat cells [52–55]. It has been suggested that DC/L-SIGN, which recognize specifically high-mannose carbohydrate moieties on many different viruses and other pathogens, mediate attachment to the cell surface rather then function as a direct entry receptor [56–60]. DC/L-SIGN are suggested to capture and concentrate HIV/SHIV virions at the cell surface augmenting *cis* interaction with other host cell factors necessary for entry and infection [61, 62]. As

Table 1. Entry factors of filoviruses.

Cellular entry factors	Ligand specificity	Cell tropism	Filovirus	System	References
ASGP-R	Terminal galactose	Hepatocytes	MARV	Virus	[51]
DC/L-SIGN	High-mannose glycans	DC, macrophages / endothelial cells in liver, lymph nodes	EBOV	Virus, pseudotype*	[52, 53]
			MARV	Pseudotype*	[54]
hMGL	N-acetylgalactosamine	DC, macrophages	EBOV	Virus, pseudotype**	[66]
			MARV	Pseudotype**	
LSECtin/ CLEC4G	N-acetylglucosamine	Endothelial cells in liver, lymph nodes, DC, macrophages	EBOV	Virus, pseudotype*	[67]
			MARV	Pseudotype*	[68]
β1 integrins	Laminin, collagen, fibronectin	Wide range of cells	EBOV	Pseudotype**	[73]
FR-α	Folic acid	Wide range of cells	EBOV	Pseudotype*	[75]
			MARV	Virus, pseudotype*	[70, 78]
Tyro3 (Axl, Dtk, Mer)	Gas6	Wide range of cells	EBOV	Virus, pseudotype*	[79]
			MARV	Pseudotype*	

* lentiviral particles pseudotyped with EBOV or MARV GP
** VSV pseudotype with EBOV or MARV GP in the envelope

for HIV-1, it is suggested for EBOV that DC-SIGN can facilitate viral spread by the transfer of captured virions to neighboring permissive cells and enhance infection in *trans* [52, 56, 63–65].

Human macrophage galactose- and acetylgalactosamine-specific C-type lectin (hMGL) specifically recognizing galactosyl residues is expressed on immature DCs and macrophages, and was next described as an attachment factor for EBOV and MARV [66]. Recently, the lymph node sinusoidal endothelial cell C-type lectin (LSECtin/CLEC4G), an additional member from the DC-SIGN/CD23 gene cluster, expressed on endothelial cells in lymph nodes and liver was shown to interact with filovirus glycoproteins and to enhance infection [67]. A recent report demonstrated that LSECtin expression can also be triggered by IL-4 on human peripheral blood monocytes and is expressed by *in vitro*-generated DCs, *ex vivo* thymic myeloid cells and activated macrophages [68]. Sugar binding studies indicated that LSECtin specifically recognizes N-acetylglucosamine and not mannose or N-acetyl-galactosamine [68].

Taken together, these studies suggest that filovirus cell tropism especially during early stages of infection is determined by C-Type lectin distribution in the tissue which explains the preferential infection of DCs, macrophages and hepatocytes by filoviruses. It also became evident that, although the mucin-like domain is not required for filoviral GP-pseudotyped viral entry, it is indispensable for the interactions with the described C-Type lectins in filovirus infection of specific cell types involved in pathogenesis [46, 47, 69–71]. It should be taken into consideration that the glycosylation of viral glycoproteins is dependent on the virus-producing cell type and may therefore affect the specificity for C-type lectins [72]. It can be suggested that C-Type lectins which belong to the pathogen pattern recognition system are hijacked by filoviruses to concentrate virus on the primary targets of infection and to facilitate interaction with another receptor(s) ultimately mediating internalization into the cells. Further studies are therefore necessary to show a direct correlation between lectin-tropism and filovirus pathogenicity.

A second group of cell surface molecules involved in adhesion, the β1 integrin adhesion receptors, was implicated in EBOV GP-mediated entry [73]. The β1 integrins constitute a family of heterodimeric cell-surface receptors responsible for a variety of cellular processes including extracellular matrix attachment, cell-cell adhesion, migration, proliferation, differentiation and apoptosis [74]. Down-regulation of β1 integrins from the cell surface of EBOV-infected cells, which caused cell rounding and detachment, suggested a role of these proteins in EBOV entry [73]. This suggestion was supported by the result that anti-β1 integrin antibodies and soluble α5β1 integrin complex inhibited entry of EBOV GP-pseudotyped vesicular stomatitis virus (VSV) [73]. However, evidence for direct binding of EBOV GP to β1 integrins or reconstitution of infection in non-permissive cells is missing.

Using genetic reconstitution of entry in a non-permissive cell line, folate receptor α (FR-α) has

been described as a significant cofactor for cellular entry of filoviruses [75]. FR-α is a glycosyl-phosphatidylinositol-linked (GPI-linked) protein that binds folic acid for transport into the cytosol via endocytosis [76]. Corresponding to the wide species and cell tropism of filoviruses, FR-α is highly conserved in many mammalian species and expressed in a variety of different cell types at variable expression levels [77]. Importantly, not all filovirus-permissive cell types express FR-α suggesting an additional alternative factor involved in virus entry [75]. Other groups later confirmed the occurrence of FR-α-independent entry pathways in different filovirus-susceptible cells including macrophages which play a crucial role in filovirus infection [70, 78].

Recently it was reported that three members of the Tyro3 family Axl, Dtk and Mer of receptor tyrosine kinases mediate entry of filoviruses [79]. These molecules are highly conserved among different species and are widely expressed in many cell types except lymphocytes and granulocytes consistent with the host and cell tropism of filoviruses [80–83]. However, Vero-E6 cells, commonly used for filovirus production, revealed a Tyro3 family-independent entry of GP-pseudotyped murine leukemia virus (MLV) demonstrating the complexity of filovirus entry mechanism [79]. Of course, further investigations into the role of lectins, integrins and Tyro3 family members are necessary to elucidate their specific roles in filovirus entry, host cell tropism and pathogenicity. Taken together, a model emerges in which filoviruses do not use a single common receptor to infect a broad range of cells. More likely, a combination of attachment and receptor molecules is employed to enhance and promote infection of different primary cell types. Recently it was demonstrated that infected primary cells releasing specific cytokines seem to be able to facilitate infection of secondary target cells like endothelial cells, which play a key role in filovirus pathogenesis [84].

Filovirus entry mediated by endocytosis

It is widely accepted that after binding to the receptor(s) filoviruses enter the cell through the endocytic pathway although direct evidence and detailed studies with replication competent viruses are rare due to the requirement to study filoviruses under BSL-4 conditions. Endocytosis from the plasma membrane occurs by different mechanisms: macropinocytosis, clathrin-mediated endocytosis, caveolin-mediated endocytosis and clathrin- and caveolin-independent endocytosis [85]. Caveolin-mediated endocytosis and clathrin- and caveolin-independent endocytosis are dependent on high amounts of lipid rafts which represent highly ordered domains of specific lipids, mainly glycosphingolipids and cholesterol [86, 87]. Dependence of EBOV entry on lipid rafts was suggested by reduced viral titers after preincubation of cells with raft-disrupting agents [88]. Studies using cholesterol-sequestering drugs and phorbol esters confirmed the involvement of lipid rafts in entry of filovirus GP MLV pseudotypes [84, 89]. Empig and Goldsmith showed by immunofluorescence and confocal microscopy that filovirus pseudotypes colocalized with the caveolae marker protein caveolin-1 suggesting caveolae-mediated endocytosis for entry [89]. Because of the difference in size between MLV (100×100 nm), VSV (70×180 nm) and filoviruses ($80 \times 650 - 1000$ nm) it remains to be shown that filoviruses use caveolae for entry, which have a diameter of about 60 nm.

It is possible that filoviruses use different entry mechanisms in different cell types. Lipid rafts are involved in clathrin-mediated endocytosis and in macropinocytosis, therefore it is conceivable that, dependent on cell type, both pathways participate in filovirus entry [90, 91]. In addition, filoviruses may infect specialized immune-defense cells as macrophages and DCs by phagocytosis and possibly also non-professional phagocytic cells via a recently described phagocytosis-like uptake [92].

Following endocytosis, the next step in the entry of enveloped viruses is the fusion of the viral membrane with endosomal membranes releasing the viral genome. Analogous to HIV gp120/41 and influenza virus HA, which are type I fusion proteins, as well, it was suggested that a specific trigger such as receptor binding or exposure to acidic pH is responsible for a conformational change of filovirus GP which leads to solvent accessibility of the viral fusion peptide. The fusion peptide is then inserted into the cellular membrane which ultimately mediates fusion [93, 94]. Mutation analysis demonstrated that the fusion peptide at the N-terminus of GP_2 is responsible for the virus-cell membrane fusion [95, 96]. Low pH requirement for filovirus entry was demonstrated by several groups using lysosomotropic agents affecting the acidic pH of the endosomal compartment [97–99]. However, attempts to induce acidic pH-dependent fusion of GP-expressing cells remained either unsuccessful or were only possible under particular experimental conditions with very low efficiency. These results suggested that an unidentified host cell factor is critical for filovirus entry which is sensitive to acidic pH [97, 100].

Proteolytic cleavage of the precursor of influenza virus HA and HIV gp160 as well as of other viral glycoproteins is indispensable for the activation of their fusogenic potential [101, 102]. The precursor of filovirus GP is cleaved by furin, a subtilisin-like

proprotein convertase present in the constitutive secretory pathway, into GP_1 and GP_2 [34, 44]. Elimination of the furin consensus cleavage site did not avoid infection of cell cultures and rhesus macaques with EBOV GP-pseudotyped VSV or recombinant EBOV questioning the contribution of furin cleavage for filovirus entry [42, 103–105]. Nevertheless, the role of furin cleavage of GP of different filovirus subtypes for viral entry in natural hosts remains open as the Reston strain which is presumed to be non-pathogenic for humans, exhibits reduced GP cleavability due to an amino acid sequence at the cleavage site which is suboptimal for furin activity [34]. Efficiency of GP processing-cleavage and its contribution to filovirus infection and pathogenicity may remain a potential target for antiviral development.

Recently it was demonstrated that endosomal proteolysis of EBOV GP by cysteine proteases is necessary for infection [71, 106]. Several lines of evidence including selective inhibitors, protease-deficient cell lines and down-regulation by siRNA were used to confirm involvement of cathepsin B (CatB) and cathepsin L (CatL) in EBOV GP-mediated infection [71, 106]. Moreover, the authors propose a two-step model in which cleavage of GP_1 by CatB and CatL primes GP by generating a key 19-kDa GP_1 intermediate, while a third endosomal/lysosomal factor whose function is inhibited by cysteine-protease inhibitors and requires low pH, perhaps a lysosomal thiol reductase, triggers fusion [106]. The reduction of a critical disulfide bond in EBOV GP could thus allow conformational changes in GP_2 and induce fusion similar as shown recently for MLV envelope protein [107, 108]. Further work is necessary to identify all factors required to trigger filovirus GP-mediated fusion.

Taken together, while several cellular proteins have been identified which are involved in the filovirus entry process, several questions remain open: What is the nature of the endocytic pathways involved in filovirus entry and the cellular membrane compartments which fuse with the viral membrane, which cellular factors are involved in trafficking and sorting of the endocytosed virus particle and how do filoviruses escape from lysosomal degradation?

Immune system deregulation and impairment induced by filoviruses

Lymphocytes apoptosis

Since transmembrane-anchored GP of filoviruses is the single viral protein exposed at the surface of virions, it represents the main factor which mediates direct contacts to the environment. Interaction of virus-associated GP with host cells is presumed to contribute to the dramatic impact of filovirus infection on the immune response of the host which, amongst other features, is characterized by a drastic depletion of lymphocytes [109]. The GP_2 subunit of filoviruses contains a region of 26 amino acids near the transmembrane anchor with high homology to an immunosuppressive peptide present in the transmembrane domain of some retrovirus transmembrane glycoproteins. The immunosuppressive peptide was shown to inhibit lymphocyte activation and proliferation in vitro [45, 110–113]. Similar to the retroviral peptide, a synthetic peptide corresponding to the presumptive filovirus immunosuppressive motif inhibited lymphocyte blastogenesis and NK cell activity [114]. It was also shown that mitogen-stimulated proliferation of lymphocytes was inhibited by incubation with inactivated EBOV or a recombinant protein containing the N-terminal sequence of GP_1 suggesting an additional immunosuppressive domain [115]. Recently, presumptive filovirus immunosuppressive peptides were used to study their effects on peripheral blood mononuclear cells (PBMC) [116]. Exposure of human and rhesus PBMC to the peptides of MARV, EBOV Zaire or Sudan and to inactivated EBOV Zaire particles resulted in apoptosis, inhibition of CD4 and CD8 cell cycle progression and in decreased expression of CD4 and CD8 activation markers, of IL-2, IFN-γ and IL-12-p40. IL-10 expression of the peptide-treated PBMC was increased [116]. The authors suggest that dysfunction of T cells might be induced either by contact with the surface GP or indirect by inadequate stimulation of the antigen-presenting cells [116]. Interestingly, while rhesus macaques T cells were shown to be sensitive to the EBOV Reston peptide, human T cells were not, correlating with the observation that EBOV Reston is non-pathogenic in human in contrast to rhesus macaques [117]. The role of the immunosuppressive motif(s) in filoviral GP during infection of animals and the molecular mechanisms of the immunosuppressive effects on T cells remain to be analyzed further using recombinant filoviruses.

It has been shown that macrophages exposed to replication-competent or inactivated EBOV particles express tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) which can be released and, thus, by binding to lymphocytes, induce apoptosis [12]. In contrast, another report did not observe TRAIL expression on infected macrophages, but describes TRAIL mRNA up-regulation in T cells upon infection of PBMC with EBOV [118]. TRAIL has been shown to play an important role in the apoptosis of HIV-infected CD4 T cells and in primary small airway and

tracheal-bronchial cells infected by respiratory syncytial virus most likely by up-regulation of TRAIL death receptors [119–121]. The contribution of cellular and viral factors and molecular mechanisms leading to the extensive apoptosis of lymphocytes during filovirus infection are awaiting further investigations.

Impairment of DC by filoviruses

In contrast to lymphocytes which do not internalize filoviral particles, DCs take up filoviruses very efficiently. Filovirus infection of DCs results in their dysfunction and inability to induce T cell-mediated immunity compromising severely a proper immune response to filovirus infection [122, 123]. The same effect was observed with inactivated virus indicating that the uptake of filoviral particles without viral replication is sufficient to impair DC functions. In this case surface GP as well as all other structural viral proteins can interact with specific host factors to modulate the immune response. Indeed, it was shown that VP35 blocks IFN-α production and signaling by interference with interferon regulatory factor 3 (IRF3) phosphorylation [29–32, 123]. In addition, EBOV VP35 binds to double-stranded RNA (dsRNA) and may thus interfere with antiviral function of cellular dsRNA sensors [124]. Finally, VP35 has been suggested to be involved in the block of IRF3 activation upstream of IRF3 kinases [31]. Later, VP24 was found to be implicated in the block of IFN-α production as well. VP24 specifically interacts with karyopherin alpha1, the nuclear localization signal receptor for tyrosin-phosphorylated signal transducer and activator of transcription 1 (STAT1) and impairs nuclear accumulation of STAT1 which is required for IFN signaling [125]. IFN production plays a major role in the innate immune response to many viral infections (reviewed in [126]). The role of IFN antagonism during filovirus infection however remains uncertain. On the one hand it was shown that activation of IRF3 was significantly stronger in cell culture infected with recombinant EBOV in which IRF3 inhibitory domain of VP35 was mutated [127], on the other hand, high levels of IFNs were found in the blood of EBOV-infected humans and monkeys [10, 12, 128].

Activation of DCs by virus-like particles (VLP)

In contrast to replication-competent or inactivated viral particles, EBOV-VLP containing VP40 and GP activated macrophages and DCs which then were able to support T cell activation in allogenic reactions [129, 130]. Moreover, it was demonstrated recently, that the mucin-like domain of EBOV GP is required for activation of DCs by EBOV-VLP [131]. Therefore, filovirus-specific VLPs composed of VP40 and GP are very good candidate vaccines which were shown to protect mice from EBOV infection and guinea pigs from MARV infection [132, 133]. These data are also in line with the high immunogenic potential of GP which induced protective antibodies and cellular immune response [134–136]. The context in which filovirus GP is presented to specific immune cells is most likely to determine their response to the infection.

Activation of neutrophils and macrophages by filoviruses

Filoviruses do not productively replicate in neutrophils. However, uptake of replication-competent or of inactivated filoviral particles into neutrophils resulted in activation and release of a triggering receptor expressed in myeloid cells (TREM-1) [137]. TREM-1 activation by filoviruses induced secretion of proinflammatory cytokines and phenotypic changes similar to profiles described for sepsis induced by bacterial infection [137]. Interestingly, the authors did not observe differences in activation of TREM-1 on neutrophils by EBOV Zaire, Reston and MARV. Macrophages were activated by filoviral infection and incubation with inactivated filoviruses and released proinflammatory cytokines and chemokines [138, 139].

Role of soluble forms of filovirus glycoproteins

During filovirus infection several forms of GP are expressed and exposed to the host cells. In contrast to EBOV GP gene which encodes two open reading frames, the MARV GP gene codes only one open reading frame which is translated into the full-length transmembrane GP exposed at the surface of virions and infected cells [33, 140, 141]. The primary product of EBOV GP gene is a secreted non-structural glycoprotein sGP, which is released in soluble form from infected cells and was recently described to contain a rare post-translational modification, C-mannosylation [142–144]. During sGP processing a soluble Δ-peptide is generated by proteolytic cleavage and also released from cells [145]. The expression of the transmembrane anchored EBOV $GP_{1,2}$ requires transcriptional RNA editing which combines the two open reading frames of the GP gene [38, 143]. GP is efficiently shed from the surface of infected cells by the activity of TNF-α-converting enzyme (TACE). TACE chops off GP near the transmembrane anchor resulting in the release of $GP_{1,2\Delta}$ in a soluble form [146].

It is anticipated that soluble forms of glycoproteins released during EBOV infection can modulate the host's immune response. It was suggested that sGP binds via the FcγRIIIB (CD16b) receptor to neutrophils and inhibit L-selectin down-regulation impor-

tant for neutrophils migration into tissue [147]. It was later suggested that the physical linkage between FcγRIIIB and CR3, which is necessary for inflammatory signaling, was disturbed by sGP binding to neutrophils and inhibited their functions [148]. Other groups could not detect direct sGP binding to neutrophils by CD16b [149, 150]. Since TREM-1 is also activated by MARV, which does not produce sGP, it is questionable whether the inhibition of neutrophil activation is induced by sGP [137]. Interestingly, recombinant sGP protected endothelial cells *in vitro* from damage induced by inflammatory cytokines [151], a result which proposes an anti-inflammatory function of sGP.

Shed $GP_{1,2\Delta}$ generated by TACE cleavage and purified from infected cells was shown to block virus neutralizing antibodies suggesting a role as decoy since it is found in high amounts in the serum of infected guinea pigs [146]. TACE up-regulation and release of shed $GP_{1,2\Delta}$ was recently demonstrated in EBOV-infected non-human primates as well [152].

Recombinantly expressed soluble EBOV $GP_{1,2}$ lacking the transmembrane anchor, soluble GP_1 ectodomain or sGP were not able to activate macrophages to release proinflammatory cytokines [130]. Therefore, inhibitory effects of soluble GP molecules as down-regulation of activation markers on macrophages and DCs and inhibition of lymphocyte proliferation remain to be investigated further.

Taken together, it can be suggested that GP of filoviruses contributes to the deregulation of the immune response in the infected host by several mechanisms including stimulation of proinflammatory responses and inhibition of lymphocyte activation.

Molecular mechanisms of filovirus GP-induced cytotoxicity

GP of filoviruses induce cytotoxic effects described as cell rounding and detachment. Different molecular mechanisms have been suggested to play a role in the cytotoxic effect. Vectorial expression of EBOV GP in endothelial cells *in vitro* and *in vivo* demonstrated damage and loss of endothelial cells from the blood vessels and increased permeability [69]. Expression of EBOV Reston GP resulted in cytotoxicity only in nonhuman primate blood vessels and not in human endothelial cells [69]. Other cell types displayed only reduced cytotoxic effect upon expression of EBOV Reston GP in comparison to EBOV Zaire GP [153]. Several reports demonstrated that removal of the C-terminus of GP_1 containing the mucin-like domain abrogated cytotoxicity of GP suggesting that this domain is responsible for cell rounding and detachment [69, 153–155]. Another group showed that expression of C-terminally truncated EBOV GP_2 mutants in HEK293 cells resulted in reduced cytotoxicity proposing that mainly an extracellular region of GP_2 is necessary for the morphological changes [156]. Moreover, detachment of cells could be blocked by the Ser/Thr kinase inhibitor 2-aminopurine suggesting involvement of a phosporylation-dependent signaling cascade [156]. The authors also report that in contrast to EBOV, MARV GP is not cytotoxic when expressed in cells [156]. In accordance with our own observations, another group reported that upon transient expression, MARV GP induced cytotoxicity with slightly lower efficiency and longer incubation time as EBOV GP [157].

Using chimeric proteins with either the ectodomain of influenza HA and the transmembrane domain of EBOV GP or vice versa, Takada et al. proposed that the GP ectodomain and its anchorage in the membrane are required for the cytotoxic effect induced by EBOV GP [73]. GP expression resulted in down-regulation of β1 integrins from the cell surface which could explain cell rounding and detachment [73].

Global down-regulation of cell surface molecules like major histocompatibility complex class I (MHC I), epidermal growth factor receptor (EGF) and β1 and αV integrins from different cell types including macrophages and endothelial cells expressing GP was suggested to facilitate immune escape during infection since cells stay viable after detachment and continued to produce virus [153]. Specialized types of endothelial cells as human cardiac microvascular endothelial cells were shown to be more sensitive to EBOV GP-induced cytotoxicity and to undergo apoptosis upon recombinant expression of GP [158]. Dependent on cell type the EBOV GP-induced cytotoxicity appeared as cell rounding and detachment leaving cells viable and able to re-adhere or as cell damage and death [69, 153, 158]. Recently it was observed that interaction of EBOV GP with the GTPase dynamin, which mediates recycling of lipid raft resident molecules like integrins, is responsible for the specific cell surface down-regulation of αVβ3 integrins and MHC I molecules in endothelial cells [154]. It is possible that GP could disturb the normal intracellular trafficking of cell surface proteins that are essential for cell attachment, viability and immune signaling by interaction with dynamin [154]. Interestingly, reduced intracellular signaling of the extracellular signal-regulated kinase type 1 and 2 (ERK1/2) was observed in HEK293 cells expressing EBOV GP [155]. Moreover, expression of the constitutive active form of ERK2 protected cells from GP-induced cytotoxicity [155]. However, in this case the effect of ERK activity on GP cytotoxicity could not be attributed to dynamin.

Importantly, it was demonstrated by the use of reverse genetics that cytotoxicity of EBOV was dependent on the expression level of GP in infected cells [159]. Overexpression of GP resulted in early cell rounding and detachment of cells suggesting that GP expression is controlled during infection by RNA editing. Only 20 % of GP transcripts can be translated into transmembrane-anchored GP, the rest is translated into sGP, which is secreted [159]. Further studies comparing GP expression levels from the strong CMV promoter, from Kunjin virus replicon and during EBOV infection indicated that moderate expression levels of GP found during Kunjin replicon expression or during early stages of EBOV infection did not result in surface molecule down-regulation and cytotoxicity [157]. Cell rounding and detachment was observed during late stages of EBOV infection proposing that GP-induced cytotoxicity is controlled by editing. Moreover, $GP_{1,2\Delta}$ shedding from the surface of infected cells may represent an additional mechanism controlling GP-induced cytotoxicity during infection since cytotoxicity was abrogated upon expression of GP mutants lacking the transmembrane anchor which are efficiently releasing GP from cells [73, 144, 146].

Cellular phosphatases and kinases modulating functions of filoviral proteins during the viral replication cycle

Protein phosphorylation plays an important role in many cellular processes. The reversible introduction of negative charges by phosphate groups is highly suitable to change the activation status of proteins which can be regulated by the activity of specific kinases and phosphatases. It has been recognized several years ago that filovirus structural proteins, like cellular proteins, are subject to cellular kinase and phosphatase activity [160, 161]. Some phosphorylation sites of filoviral proteins have been mapped in recent years and we are beginning to understand the biological function of phosphorylation of filoviral proteins.

The major phosphoprotein of filoviruses is NP [160, 161]. For MARV it has been demonstrated that phosphorylation of NP results in a modification of the apparent molecular mass of the protein in SDS gel. Nonphosphorylated NP of MARV migrates at 92 kDa and phosphorylated NP at 94 kDa. Only the phosphorylated NP is recruited into virions, while both forms are detected intracellularly [162]. This finding suggests that phosphorylation of NP is important for the formation and/or release of viral nucleocapsids. Phosphorylated serine and threonine residues are detected in NP of MARV which are located in consensus recognition sites for protein kinase CKII and for proline-dependent kinases. The ratio of phosphoserine to phosphothreonine is approximately 85:15 in both, the virion-associated and recombinant NP. Altogether, seven different regions in the C-terminus of NP are phosphorylated. Mutation of the proline-dependent phosphorylation sites did not result in changes of the function of NP during viral RNA synthesis. Although the available data suggest that phosphorylation of NP is necessary for recruitment of the protein, it remains to be shown which of the detected phosphorylation sites are responsible for this function [163].

Another heavily phosphorylated protein of filoviruses is VP30, which represents a filovirus-specific transcription factor [160, 161]. VP30 of MARV and EBOV are phosphorylated at the N-terminus. In MARV VP30, phosphorylation is concentrated in an N-terminal cluster of seven serine residues between amino acids 40 to 51 [164]. The phosphoserines in amino acid region 40 to 51 are only partly located in consensus recognition sites of known protein kinases. Phosphorylated serine 43, for example, is located in a conserved protein kinase C site, serine 46 in a protein kinase CKII site, and serine 51 might be suitable for phosphorylation by a proline-dependent kinase similar to the mitogen-activated protein kinases [165]. Exchange of these three serines to alanines decreased the amount of incorporated phosphates in VP30 by 30 %. Thus, data point to additional unidentified protein kinases with different recognition motifs which are involved in VP30 phosphorylation [164]. VP30 is colocalized with NP in MARV-infected cells and also accumulates in NP-induced inclusion bodies upon recombinant expression of the two proteins. When serine residues 40 and 42 of VP30 were exchanged by alanine, VP30 lost its ability to accumulate in the NP-induced inclusions and showed a homogeneous cytoplasmic distribution. Mutations of the other phosphorylated residues did not change the phenotype of VP30. The cellular kinases involved in phosphorylation of serines 40 and 42 are not yet identified [164].

EBOV VP30 is also heavily phosphorylated at N-terminal serine residues and one threonine residue (amino acids 29 to 51) [166]. Replacement of the phosphoserines by alanines resulted in an only slightly phosphorylated VP30 ($VP30_{6A}$) that is still able to activate EBOV-specific transcription in a plasmid-based minigenome system. $VP30_{6A}$, however, did not accumulate in inclusions that are induced by NP. While the acting kinase(s) is not yet identified, three intracellular phosphatases (PP1, PP2A, and PP2C) have been determined to dephosphorylate VP30. The presence of okadaic acid (OA), an inhibitor of PP1

and PP2A, had the same negative effect on transcription activation by VP30 as the substitution of the six phosphoserines for aspartate residues. However, OA did not impair transcription when VP30 was replaced by $VP30_{6A}$. In EBOV-infected cells, OA blocked virus growth dose-dependently. The block was mediated by the extensive phosphorylation of VP30, which is evidenced by the result that expression of $VP30_{6A}$, in *trans*, led to the progression of EBOV infection in the presence of OA [166].

Taken together, phosphorylation inversely influenced the two known functions of VP30. A completely phosphorylated form of VP30 was capable of interacting with NP inclusions, but was restricted in mediating viral transcription. In contrast, a phosphorylation-deficient VP30 was inhibited in its interaction with the NP inclusions but supported EBOV-specific transcription. An intermediately phosphorylated VP30 enabled both viral transcription and assembly. Phosphorylation of VP30 is therefore presumed to represent a molecular switch for the different functions of the protein. These results clearly show that the dynamic equilibrium of phosphorylation and dephosphorylation, which determines the steady state phosphorylation state, is important for the function of EBOV VP30 in the viral replication cycle.

Phosphorylation has also been detected in MARV GP [43]. In contrast to phosphorylation of the nucleocapsid protein NP and VP30, phosphorylation of GP takes place in the lumen of the Golgi apparatus. Target for cellular kinases are serine residues between amino acid 260 and 273 in GP_1. The respective serines are located in consensus recognition sites for luminal protein kinases (protein kinase CKII and Golgi casein kinase). Consistent with this data, it was found that GP was phosphorylated in the Golgi apparatus before cleavage of the molecule into GP_1 and GP_2 [43]. GP is one of the rare examples of glycoproteins with a phosphorylated ectodomain. The function of ectodomain phosphorylation has not yet been elucidated.

Exploitation of cellular protein transport and sorting machineries during assembly and budding of filoviruses

VP40, the matrix protein of filoviruses

VP40 of filoviruses is thought to be the functional homologue of the matrix (M) proteins of other nonsegmented negative-sense RNA viruses. EBOV and MARV VP40 consist of 326 and 303 amino acids, respectively. There is a 29% sequence homology between the two proteins, and no sequence or structural homology exists to the matrix proteins of other negative-strand RNA viruses [167]. The assumption that VP40 represents the viral matrix protein is based on its position in the genome, its hydrophobicity, and its abundance within the virion [168]. It is currently believed that matrix proteins of Mononegavirales are the driving force of assembly and budding of viral particles [169, 170]. In the course of their life cycle, matrix proteins should interact with and polymerize at cellular membranes, link other viral components to the matrix-membrane complex inducing the characteristic shape and integrity of the viral particle. Matrix proteins are initially soluble cytoplasmic proteins which are later bound to membranes and viral nucleocapsids. It is suggested that the ability to interact with different partners is dependent on conformational changes of the matrix proteins [170].

The elucidation of the crystal structure of EBOV VP40 showed that it consists of two domains with unique folds (an N-terminal oligomerization and a C-terminal membrane-binding domain), connected by a flexible linker [171]. It has been shown that this conformation of VP40 is metastable, which allows an easy transition into oligomeric ring-like structures *in vitro* [172, 173]. The ring-like structures are either octamers or hexamers [174]. In both cases, the N-terminal domain of VP40 constitutes the oligomerization domain, which forms an anti-parallel dimer, which is the building block for oligomerization [173, 175]. The C-terminal domains are flexibly attached to the rings and mediate membrane association *in vitro* and *in vivo* [172, 173, 176]. A recent study shows that three amino acids in the C-terminal region (212KLR214) are important for oligomerization of EBOV VP40 [177]. Octamerization of EBOV VP40 requires binding of a sequence-specific single-stranded RNA triplet UAG [175]. Presence of octamers was confirmed in infected cells [175], in virus particles and VLPs [178]. The N-terminal domain of MARV VP40 (residues 1 to 186) can also form ring-like structures, similar to EBOV VP40, however these oligomers are most likely free of nucleic acids [174].

Oligomerization is a key factor in the regulation of proteins such as enzymes, ion channels, receptors and transcription factors; it can confer several advantages to proteins, like improved stability, control over the accessibility and specificity of active sites, and increased complexity [179]. Although the cellular mechanisms to control VP40 oligomerization are not well defined, it is currently suggested that the oligomerization status of the VP40 might determine its association with different viral and cellular partners and therefore its biochemical functions.

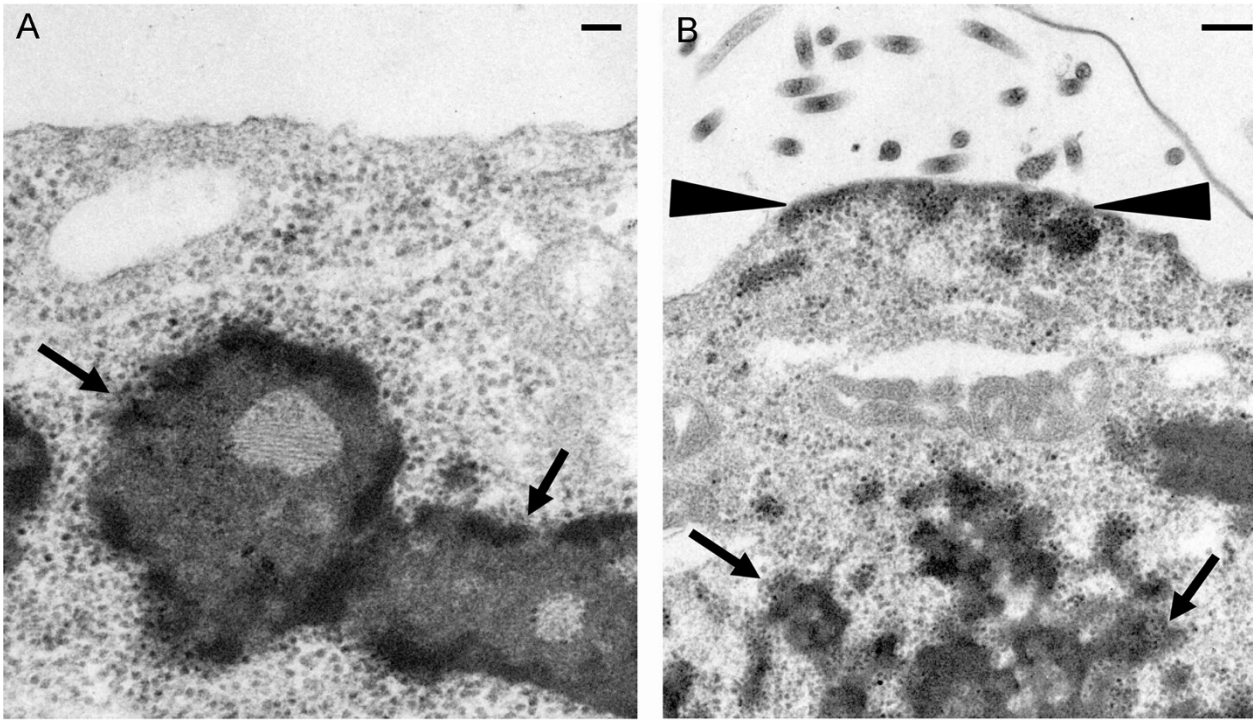

Figure 2. VP40 induces redistribution of nucleocapsid-like structures to the cell surface. HUH-7 cells were transfected with plasmids encoding MARV NP, VP35, VP30 and VP24 (*A*); or with plasmids mentioned above and VP40 (*B*). At one day post-transfection, cells were fixed, dehydrated and embedded in Epon. Ultrathin sections were analyzed by electron microscope. Expression of MARV NP, VP35, VP30 and VP24 results in formation of electron dense inclusions located far from plasma membrane (A, arrows). Coexpression of proteins mentioned above and VP40 induces partial redistribution of viral proteins to the plasma membrane (B; arrowheads). Bar, A, 100 nm, B, 200 nm.

Cellular partners of VP40 in the course of its transport to the site of budding

VP40 of EBOV and MARV were detected in viral inclusions [180] and close to the plasma membrane of infected cells [24], indicating that the matrix proteins are able to interact with nucleocapsid structures and with cellular membranes. By using immunogold staining, VP40 was detected in association with MARV nucleocapsids and EBOV nucleocapsid-like structures [181, 182]. In addition, ultrastructural analyses of MARV-infected cells identified VP40 in foci of virus-induced membrane proliferation and in intracellular membrane clusters which had the appearance of multi-vesicular bodies (MVBs). VP40-containing MVBs were free of NP and nucleocapsids [181].

Facing the different localization of VP40 (inclusions, nucleocapsids, MVBs) it is tempting to presume that VP40 can use several independent transport routes to the site of budding: (i) in association with nucleocapsid structures, and (ii) in association with cellular membranes.

The association of VP40 with nucleocapsid structures most likely represents a step in virus assembly. Recent studies showed that VP40 plays a leading role in the redistribution of nucleocapsid-like structures from perinuclear region to the plasma membrane [182]. Our studies showed the same redistribution of nucleocapsid-like structures when MARV nucleocapsid proteins were coexpressed with VP40 (Fig. 2, Kolesnikova, unpublished). These data suggest that both EBOV and MARV VP40 induce the relocalization of nucleocapsids from the perinuclearly located viral inclusions to the plasma membrane. It is not clear yet in which form (monomeric or oligomeric, membrane-bound or soluble) VP40 interacts with nucleocapsids. Based on the intensity of the immunogold staining of VP40 in ultrathin sections of MARV-infected cells it has been calculated that the amount of Marburg VP40 that is transported to the plasma membrane independent of nucleocapsids and in association with cellular membranes, is six times higher than the amount that comes along with nucleocapsids [181].

Although the membrane-binding capability of EBOV and MARV VP40 is well established [173, 176, 181], the exact pathway how the proteins are targeted to the plasma membrane is still unclear. Pulse-chase analysis of EBOV VP40 showed that 20 min after chase 50% of protein was already present in membrane-bound form (Dolnik, unpublished). However, the origin of target membranes remains uncertain. Recent observation showed that Rab9 GTPase is required for the

replication of EBOV and MARV suggesting that a vesicular transport from the late endosome to the plasma membrane might be important in viral assembly [183]. Indeed, the study of the transport of recombinant MARV VP40 showed that the accumulation of VP40 in the late endosomal compartment seems to represent an intermediate step in the delivery of protein to the plasma membrane [184]. Using a combination of time-course studies and ultrastructural analyses it has been shown that VP40 was bound to cellular membranes rapidly after synthesis, and approximately 80% of the protein was membrane-associated at steady state levels [184]. VP40 was associated with several types of cellular membranes in the course of its transport to the plasma membrane [184]. Initially, VP40 bound to small vesicles, then it was found associated with MVBs and finally VP40 could be detected in patches at the plasma membrane [184]. Apart from the small vesicles, all other VP40-positive membranes contain marker proteins of the late/recycling endosomal compartment, like Lamp-1, Rab11 and CD63 [184, 185]. VP40 is accumulated in the late endosome to high amounts, suggesting that the environment of the endosomal compartment is necessary for oligomerization and formation of the regular arrays of VP40 detected in the virion [184]. Moreover, coexpression of MARV VP40 with viral surface glycoprotein GP induced redistribution of GP to the VP40-enriched MVBs, indicating that MVBs are sites of intersection between the transport of GP through the classical secretory pathway and the transport of VP40 through the retrograde late endosomal pathway [186, 187].

In polarized cells, in particular in MDCK cells, budding of MARV occurs from the basolateral domain [188]. Upon recombinant expression of VP40 in polarized cells, VP40-enriched MVBs were detected at the basolateral surface as well [189]. Only a diffuse VP40-positive signal was detected at the apical surface (http://www.uni-marburg.de/fb20/virologie/forschung/researchviro/beckerfold/3D_VP40). It remains to be clarified whether the diffuse and clustered forms of VP40 represent different post-translationally processed forms of VP40 which have two independent targets, or whether they reflect successive steps of VP40 transport. It cannot be ruled out that VP40 is first targeted to the apical surface and is then delivered to the basolateral domain via 'apical-to-basolateral transcytosis'. If this is the case, VP40-enriched MVBs might correspond to a subclass of late recycling endosomes, which can also serve as an intermediate step in the transport from the Golgi to the plasma membrane [190]. Upon coexpression with VP40, the exclusively apical located GP is partly retargeted to the basolateral membrane which explains the results gained with infected cells and suggests that VP40 determines the site of budding.

Possible mechanisms of targeting of VP40 to the late endosome: ubiquitination, late domain motifs, lipid rafts

Interaction of VP40 with the late endosomal compartment seems to serve not only transportation of viral matrix protein to the site of budding, but also to provide the virus with contact to the cellular machinery which drives inward budding of cellular vesicles. This budding machinery is used in the late endosomal compartment (MVBs) for the formation of intraluminal vesicles by invagination of endosomal limiting membrane, for review see [191, 192]. It is currently assumed that MVB formation and virus budding are highly analogous processes, for review see [193, 194]. Mechanisms of targeting VP40 to the late endosomal compartment are not well understood. Among the potential mechanisms, which might be involved in targeting VP40 to the endosomal compartment are (i) ubiquitination of VP40, (ii) interaction of VP40 late-domain motifs with proteins of endosomal sorting complexes required for transport (ESCRTs), and (iii) VP40 binding to specific lipids.

Ubiquitination

Previous studies have shown that integral membrane protein cargo is marked for delivery into the intraluminal vesicles of MVBs by monoubiquitination of their cytosolic domains [195–197], for review see [198]. Recognition and sorting of monoubiquitinated MVB cargo is carried out by a subset of class E vacular protein sorting (Vps) proteins that assemble into several *e*ndosomal *s*orting *c*omplexes *r*equired for *t*ransport (ESCRTs), for review see [199]. Loss of cargo ubiquitination via mutations of the cytosolic Lys residues within such proteins precludes their entry into MVBs and results in their accumulation on the limiting membrane of the vacuole [195]. Thus, monoubiquitin represents an internalization signal that can be attached to proteins in a regulated manner to target them for rapid entry into the endocytic pathway [200]. While EBOV VP40 can be ubiquitinated *in vitro* [201, 202], it remains unclear whether MARV VP40 is monoubiquitinated as well. In the future it will be necessary to investigate whether mutation of sites of ubiquitination will impair proper trafficking of this protein to the site of budding.

Late-domain motifs

Another mechanism which could serve as an entry ticket for VP40 into MVBs is interaction with ESCRTs proteins via so-called late-domain motifs, for review see [203, 204]. Late-domain motifs consist of the amino acid sequences P[T/S]AP, PPxY, or YxxL, where 'x' stands for any amino acid. Late-domain motifs are highly conserved and have been shown to mediate interaction with components of the ESCRTs [203, 204]. For example, the P[T/S]AP motif interacts with tumor susceptibility gene 101 (Tsg101), which is part of ESCRT-I [205–207]. The PPxY motif interacts with WW domains of Nedd4-like ubiquitin ligases [201, 208], and the YxxL motif interacts with AIP1/Alix, which in turn interacts with Tsg101 and CHMP4 proteins (ESCRT-III), thus providing a link between ESCRT-I and ESCRT-III [209–211]. Recently a novel late-domain motif, FPIV, has been identified in paramyxovirus SV5 [212]. It is considered that there remain more late-domain motifs to be discovered, for review see [203]. EBOV VP40 contains two overlapping PTAP and PPxY motifs (PTAPPEY, amino acids 7 to 13). MARV VP40 contains only a PPPY motif at amino acids 16 to 19. Several studies have assessed the role of recombinantly expressed VP40 in VLP production and found that these motifs are critical for the efficiency of this process both for EBOV VP40 [176, 201, 206, 213, 214] and for MARV VP40 [215]. However, a study of recombinant EBOV containing mutations in either one or both of the late-domains showed that these motifs enhance virus release but are not absolutely required for virus assembly and replication in cell culture [216]. These data indicate that the known late-domain motifs of VP40 are not crucial for the viral assembly and their role might be compensated by other viral proteins or by other mechanisms.

Lipid rafts

VP40 might possibly bind to membranes with some special lipid composition, and further transport of VP40 might depend on the transport route of these lipids. Several studies showed that the EBOV VP40 is associated with special micro-compartments of the plasma membrane, so-called lipid rafts [178, 213]. Moreover, it has been suggested that lipid rafts are sites for the filoviral entry and exit [88]. Despite this large body of work, doubts persist, in particular, because the methods which are commonly used for the detection of lipid rafts, like resistance to solubilisation by the non-ionic detergent Triton X-100 and sensitivity to cholesterol depletion, are indirect and potentially open to alternative interpretations, for review see [217], and [218–220]. Other data concerning colocalization of VP40 or GP with a marker of lipid rafts, ganglioside GM1 [88, 186] might allow alternative interpretation as well. Although GM1 is the major surface receptor for cholera toxin B (CT-B) [221, 222], the toxin can also bind to other sugar structures with terminal galactose residues [223]. Thus, it is possible that some of the surface bound CT-B is attached to glycoproteins. Indeed, expression of MARV GP resulted in diffuse distribution of the protein at the cell surface and diffuse distribution of CT-B. However, the signal was slightly stronger than in cells which did not express GP (Fig. 3, Kolesnikova, unpublished). Coexpression of MARV VP40 and GP induced clustering of GP [186]. VP40 and GP containing clusters were colocalized with CT-B suggesting targeting of GM1 and GP to lipid rafts. Scanning electron microscopy showed that sites of colocalization of VP40, GP and CT-B represent foci of filamentous protrusions where amounts of membrane per unit of cell surface were much higher than in an area outside of these foci (Fig. 3, Kolesnikova, unpublished). Thus, a CT-B-positive signal at the site of VP40-induced membrane protrusions might be induced by higher amounts of membrane, and not by higher concentration of GM1 per unit of membrane. Further analyses using new methodological approaches are necessary to show whether sorting of VP40 is associated with specific lipid composition of membrane.

Interaction of VP40 with the cytoskeleton

The role of the cytoskeleton for filoviruses remains mostly enigmatic in comparison with other viruses for which the importance of the host cell cytoskeleton for viral entry, replication, intracellular transport and budding has already been well-established [224–229]. As mentioned above, both EBOV and MARV VP40 induce redistribution of nucleocapsid-like structures from the perinuclear area to the plasma membrane (Fig. 2 and [182]). These results suggest that VP40 represents a connection between viral nucleocapsids and the cytoskeleton. Recent studies showed that the release of EBOV nucleocapsid-containing VLPs was not dependent on actin, but dependent on microtubules [182]. Moreover, it has been shown that EBOV VP40 can directly associate with microtubules [230]. In contrast, budding of EBOV-VLPs induced by coexpression of VP40 and GP was decreased by an actin-depolymerizing agent [231]. Release of MARV proteins from virus-infected cells, as well as release of MARV VP40-induced VLPs, were significantly inhibited by depolymerization of actin filaments, and were not sensitive to the depolymerization of microtubules [185]. It is unclear whether these discrepancies

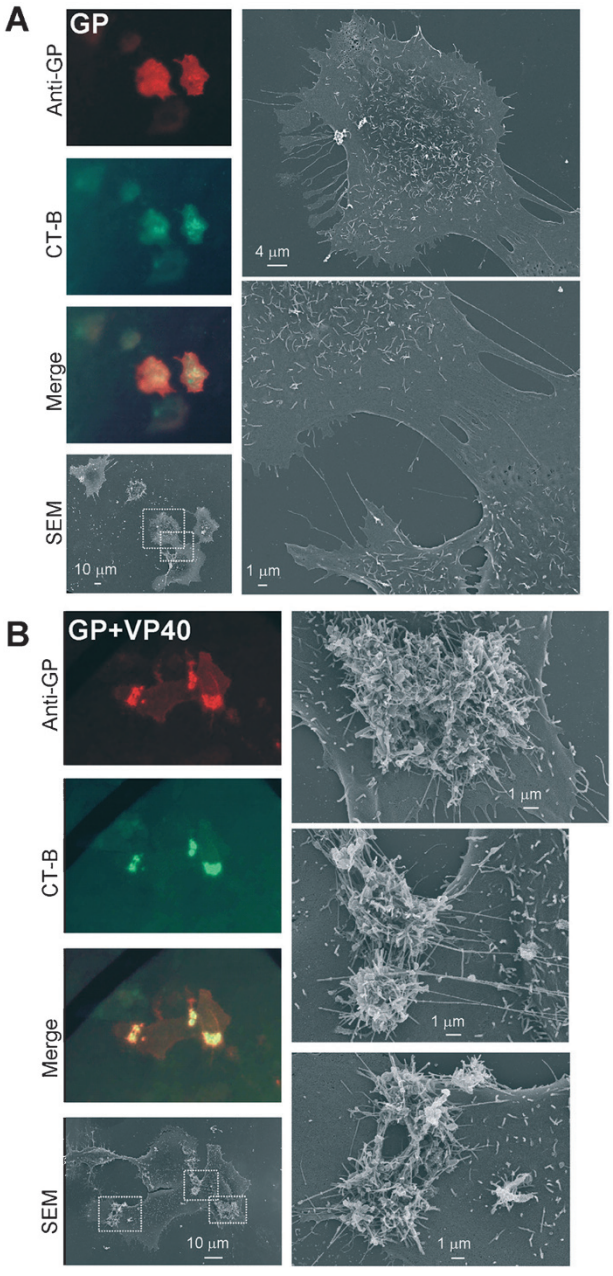

Figure 3. Colocalization of GP and marker of lipid rafts, GM1. HUH-7 cells were transfected with plasmids encoding MARV GP (*A*), or GP and VP40 (*B*). At one day post-transfection non-permeabilized HUH-7 cells were stained with a goat anti-GP antibody which was detected with a rhodamine-labelled donkey anti-goat antibody (red) and Cholera toxin B FITC-conjugated (green). After immunofluorescence analysis, cells were processed for scanning electron microscopy (SEM). Left hand panels. The same cells were subjected first to immunofluorescence and then to SEM analysis. Right hand panels. High power magnification of the framed areas of the SEM image.

reflect differences in the experimental methods used, differences among nucleocapsid- and membrane-bound VP40 in their interaction with cytoskeleton components, or differences among EBOV and MARV in their requirements for host cell factors.

Site of filoviral budding

A commonly used method to harvest cells for the subsequent electron microscopical analysis is scraping the cells off the dish. However, this method destroys delicate cellular protrusions and makes identification of sites of viral budding almost impossible. When the cellular margins were preserved by special methods, budding of more than 80 % of MARV particles was found to be associated with long thin cellular protrusions – filopodia [185]. MARV particles which were partially or completely enveloped were found either at the side or at the tip of filopodia. Association of MARV budding with filopodia suggests that one or several viral proteins interact with cellular proteins involved in filopodia function or formation. Indeed, it has been shown that localization of VP40 and release of VP40-induced VLPs are influenced by Myosin 10, an unconventional myosin which is involved in intra-filopodia motility [185, 232, 233]. Although coimmunoprecipitation assays failed to show direct interaction between MARV VP40 and Myosin 10, significant decrease of VP40-induced VLPs from cells coexpressing dominant-negative mutants of Myosin 10 was detected and points into the direction that intra-filopodia motility represents an important step in MARV release [185].

Filoviral budding has been detected not only at the plasma membrane, but at intracellular membranes which contained markers of late endosomes [186]. Similar release into MVBs was detected for retroviruses [234, 235], suggesting that this mode of budding represents a common mechanism of viral assembly. Intracellular viral particles might serve as a source of infectious units that can be delivered in a signal-dependent manner, e.g. upon cell-to-cell contact. However, recent studies using staining with ruthenium red showed that retrovirus-containing intracellular structures are not endosomes, but an internally sequestered plasma membrane domain [236, 237]. Whether the same is true for the filoviruses remains to be investigated.

Conclusions

Considerable efforts have been undertaken in recent years to understand the interaction of filoviruses with the infected target cells in the different phases of the viral replication cycle (Fig. 4). Several cellular receptor candidates have been identified and a concept is

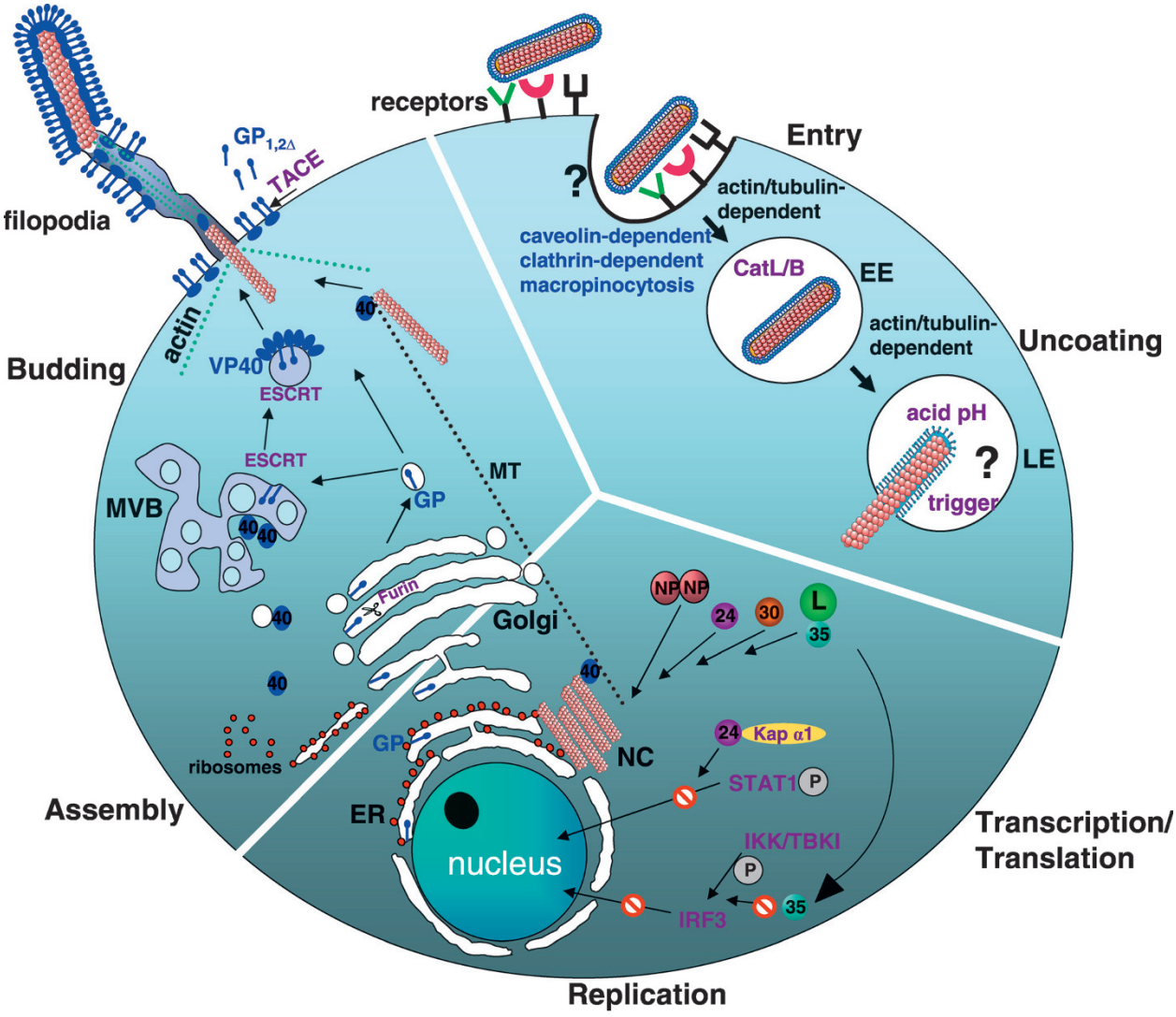

Figure 4. Filovirus replication cycle. *Entry:* The filovirus replication cycle begins with binding to cellular receptors inducing still undefined cellular uptake mechanisms. *Uncoating:* Following virus entry into endosomal compartments, acid pH, proteolytic cleavage and an unknown trigger induce fusion of viral and cellular membranes and uncoating of nucleocapsids, which are released into the cytosol. *Transcription/translation/replication:* Transcription of viral mRNA by the viral polymerase is followed by translation of viral proteins by the host cell. Replication of the viral genome by the viral polymerase is dependent on the presence of viral nucleocapsid proteins which encapsidate the nascent genome. The viral proteins VP35 and VP24 have the capacity to antagonize the interferon pathway of the infected cell. *Assembly/Budding:* Assembly of viral particles starts with formation of nucleocapsids which accumulate in inclusions in the perinuclear region and are transported to the sites of budding most likely by the activity of the cytoskeleton. Nucleocapsids are cotransported with the matrix protein VP40 to the sites of budding at the plasma membrane. The surface protein GP is transported along the classical secretory pathway and partly recruited to the late endosome to meet VP40. The late endosome probably plays a key role in the formation of the viral envelope. Budding takes place at long actin-containing protrusions, filopodia, where nucleocapsids are enwrapped with the viral envelope in which GP is incorporated. Abbreviations: CatL/B: Cathepsin L /B, EE: early endosomes, MT: microtubule, LE: late endosomes, Kap α1: Karyopherin alpha 1, STAT1: signal transducer and activator of transcription 1, P: phosphate group, IKK: IkappaB kinase, TBKI: TANK-binding kinase-1, IRF3: interferon regulatory facator 3, NC: nucleocapsids, ER: endoplasmic reticulum, MVB: multivesicular body, ESCRT: endosomal sorting complex required for transport, TACE: tumor necrosis factor-alpha-converting enzyme.

emerging that filoviruses might use different receptors on different cell types. During entry of filoviruses, the surface glycoprotein GP is subjected to proteolytic activation in the endosomal compartment which is mediated by cathepsins. Transcription of the viral mRNAs is activated by VP30, a phosphoprotein whose dynamic phosphorylation and dephosphorylation by cellular kinases and phosphatases is essential. The assembly of the filovirus envelope is dependent on the transport of the surface glycoprotein GP through the classical secretory pathway which leads to extensive posttranslational modifications of GP by acyltransferases, glycosyltranferases, proteases and kinases, which enable or modulate the function of

the protein. A portion of GP is shed from the plasma membrane by the activity of TNF-α-converting enzyme TACE and shed GP might play an important role as decoy factor. The transport of the MARV matrix protein VP40, which enables budding of progeny virions, is dependent on the interaction of VP40 with the late endosomal compartment. In the presence of VP40, GP is recruited to the late endosomal compartment, which possibly plays an important role for the formation of the viral envelope. Finally, virions bud at the plasma membrane. Long actin-containing protrusions of the plasma membrane, which represent filopodia, serve as budding points for MARV. The replication of filoviruses in target cells is supported by multiple interactions of different viral proteins with the cellular interferon response, which prevent the development of an antiviral state in response to the viral infection. Moreover, filovirus proteins interact with proteins of immune cells, like DCs, macrophages, lymphocytes and neutrophils, resulting in deregulation of their specific functions.

While recent investigations increased our understanding of virus host interactions in filovirus-infected cells, there are many questions left that need to be addressed in the future to understand the role of cellular factors in filoviral replication and to develop effective antivirals against filoviruses.

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