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RODENT-ADAPTED FILOVIRUSES AND THE MOLECULAR BASIS OF PATHOGENESIS

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Abstract

Ebola, Marburg, and Ravn virus, all filoviruses, are the causative agents of severe hemorrhagic fever. Much of what we understand about the pathogenesis of filovirus disease is derived from work with animal models, including non-human primates (NHPs), which are considered the “gold standard” filovirus model since they faithfully recapitulate the clinical hallmarks of filovirus disease. However, rodent models, including the mouse, guinea pig, and hamster, also exist for Ebola, Marburg, and Ravn viruses, and although they may not reproduce all the clinical signs of filovirus disease, thanks to their relative ease-of-use and low cost they are often the first choice for initial descriptions of virus pathogenesis and evaluation of anti-viral prophylactics and therapeutics. Since filoviruses do not cause significant disease in adult, immunocompetent rodents, these models rely on “rodent-adapted” viruses that have been passaged several times through their host until virulence and lethality are achieved. In the process of adaptation, the viruses acquire numerous nucleotide/amino acid mutations that contribute to virulence in their rodent host. Interestingly, virus protein 24 (VP24) and nucleoprotein (NP) appear to be major virulence factors for ebolaviruses in rodents, whereas VP40 appears to be the major virulence factor for marburgviruses. By characterizing these mutations and understanding the molecular mechanisms that lead to the acquisition of virulence, we can gain better insight into the pathogenic processes that underlie filovirus disease in humans. These processes, and the viral and/or cellular proteins that contribute to them, will make attractive targets for the development of novel therapeutics and countermeasures.

Graphical abstract

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INTRODUCTION

Filoviruses

The family *Filoviridae* comprises several non-segmented, negative sense RNA viruses that are further subdivided into one of three genera: *Ebolavirus*, *Marburgvirus*, and *Cuevavirus* [1, 2]. Within the *Ebolavirus* genus are five species—*Zaire ebolavirus*, *Sudan ebolavirus*, *Bundibugyo ebolavirus*, *Tai Forest ebolavirus*, and *Reston ebolavirus*—each of which is typified by a single virus, Ebola virus (EBOV), Sudan virus (SUDV), Bundibugyo virus (BDBV), Tai Forest virus (TAFV), and Reston virus (RESTV), respectively. The genus *Marburgvirus* is composed of only a single species, *Marburg marburgvirus*, which contains two closely related viruses, Marburg virus (MARV) and Ravn virus (RAVV). The third genus, *Cuevavirus*, is also composed of a single species, *Lloviu cuevavirus*, to which Lloviu virus belongs, although this virus has yet to be isolated [3].

All filoviruses, with the exception of RESTV and LLOV, are known to cause disease in humans with a range of severity [4, 5]. Both EBOV and MARV appear to be the most devastating filoviruses, having caused several outbreaks of Ebola hemorrhagic fever (EHF; also referred to as Ebola virus disease) and Marburg hemorrhagic fever (MHF; also referred to as Marburg virus disease), respectively, throughout Africa, with high case fatality rates [4, 6]. SUDV, BDBV, and RAVV also cause severe disease but have so far been responsible for fewer and smaller outbreaks than MARV and EBOV. TAFV has only caused a single known case, which did not result in fatality [4, 6]. Notably, however, no filovirus other than EBOV has caused an epidemic on the scale of that seen in the Western African Ebola virus outbreak that began in 2013 and resulted in excess of 28 000 cases and over 11 000 deaths, mostly in Guinea, Liberia, and Sierra Leone [7]. Because of their extreme virulence and threat to public health, filoviruses are considered Category A Bioterrorism Agents by the Centers for Disease Control and Prevention, Tier 1 Select Agents by the United States Department of Health and Human Services, and priority pathogens needing urgent research by the World Health Organization. Accordingly, all work with filoviruses is performed under maximum containment conditions.

EHF and MHF are severe diseases characterized by systemic and robust virus replication that leads to a dysregulated immune response, coagulation abnormalities, and, in many cases, death [4, 6]. Disease typically begins with the rapid onset of influenza-like symptoms, followed by a variety of gastrointestinal, vascular, and neurological symptoms as the infection progresses. The liver and lymphoid organs, including the spleen, are primary target organs of filovirus infection, and damage to the liver in particular is thought to contribute significantly to pathogenesis. Much of the organ damage and resulting symptoms are thought to be caused by an uncontrolled pro-inflammatory cytokine response, which, in addition to causing organ damage, is ineffective at controlling viral replication or promoting an adaptive immune response. Hemorrhagic manifestations, including the characteristic maculopapular/petechial rash, occur in about two-thirds of the cases and derive from coagulation abnormalities, which are also linked to the dysregulated immune response as well as extensive virus replication. Death usually occurs within six to sixteen days post-infection as a result of multi-organ failure and hypovolemic shock (due to electrolyte imbalances and fluid loss).

Filovirus Molecular Virology

The genomes of all filoviruses are about 19 kilobases long and share a similar organization, with seven genes arrayed in order and encoding the viral nucleoprotein (NP), viral protein 35 (VP35), VP40, the glycoprotein (GP), VP30, VP24, and the RNA-dependent RNA polymerase (L) [8–13]. Because of an internal RNA editing site within the GP gene, ebolaviruses also encode a soluble glycoprotein (sGP), as well as a small soluble glycoprotein (ssGP), although these products have only been verified for EBOV [14–16]. Each gene is flanked by 3′ and 5′ untranslated regions (UTR) that contain conserved transcriptional start and stop signals [9, 11–13, 17]. Intergenic regions separate some of the genes, while other genes overlap, with the start site of one gene occurring prior to the stop site of the preceding gene [8–12]. The genome itself is flanked by a conserved 3′ leader and a 5′ trailer that contain genome replication promoters and packaging signals (Fig. 1a) [9–13, 18, 19].

The filovirus genome is bound by NP, which, along with VP35, VP24, and VP30, forms the nucleocapsid that packages and protects the genome [20–23]. VP40, the matrix protein, forms the filamentous structure of the virion and connects the nucleocapsid to the host-derived lipid envelope [24–27]. Proteolytic processing of GP produces the GP_{1,2} heterodimer, which trimerizes to form the GP_{1,2} peplomer and resides in the viral envelope [28–33]. The GP_{1,2} peplomer facilitates recognition of, and entry into, target cells [34–37], while L, along with VP35 as a cofactor, forms the polymerase complex that replicates the genome and transcribes the genes (Fig. 1b) [19, 38–41]. In addition to playing a key structural role in condensing viral nucleocapsids, VP24 also acts as potent antagonist of the interferon (IFN) pathway—but only in ebolaviruses and cuevaviruses. Both EBOV and LLOV VP24 interact with the NPI-1 subfamily of karyopherin- α proteins and restrict their ability to translocate phosphorylated STAT1 homodimers or STAT1-STAT2 heterodimers into the nucleus [42–46]. In this way, VP24 prevents the induction of anti-viral IFN-stimulated genes (ISG) in response to type I IFN signaling. Marburgvirus VP24, on the other hand, has no such IFN antagonist ability—instead, this function lies with VP40 [47, 48].

Though the mechanism has yet to be completely defined, both MARV and RAVV VP40 inhibit JAK1 phosphorylation in response to IFN receptor activation. As a result, JAK1 is unable to phosphorylate STAT1 and 2, thereby preventing their dimerization and subsequent translocation into the nucleus to induce ISGs. Notably, VP35 from all filoviruses also functions to bind double-stranded RNA, sequestering it from recognition by RIG-I-like receptors and preventing the induction of a type I IFN response in the first place [49–55].

Filovirus Disease Modeling

Since the Western African Ebola virus outbreak began, several candidate vaccines and anti-viral therapies for preventing or treating EBOV infection have entered phase I, II, and III clinical trials, although to date no medically licensed countermeasures have been approved to treat any filovirus infection [56, 57]. The development of these countermeasures has relied, and will continue to rely, on our emerging understanding of filovirus molecular biology and how this results in the pathogenesis that characterizes filoviral hemorrhagic fevers. Much of what we currently understand about filovirus pathogenesis comes from extensive work with non-human primate (NHP) models, particularly the cynomolgus and rhesus macaques, which are considered “gold standard” models since they recapitulates many of the clinical hallmarks of fatal filovirus disease observed in humans, including high viremia, coagulation abnormalities, and an aberrant proinflammatory cytokine response (Fig. 2) [58–60]. Nevertheless, work with NHPs presents several practical and ethical concerns, especially with respect to studies that require large numbers of animals, so their use is typically reserved for the final evaluation of vaccines and anti-viral countermeasures. In place of NHP models, rodent models, which are convenient and relatively inexpensive, have been used for decades to study filoviruses, beginning shortly after MARV was first discovered in 1967 [61–66]. Today, several well characterized rodent models, including mice, guinea pigs, and hamsters, exist to study filoviral hemorrhagic fevers, particularly MHF and EHF (Fig. 2) [59, 60, 67–71].

Interestingly, although the currently developed mouse, guinea pig, and hamster filovirus models recapitulate many of the clinical features of filoviral hemorrhagic fever, the viruses must first be adapted to their rodent host before they are able to cause lethal disease. Adaptation is typically achieved by initially infecting young or immunocompromised rodents with wild-type (WT) virus, then harvesting and homogenizing the livers or spleens, using the homogenates to inoculate a second group of animals, and repeating this serial passage until the virus gains a virulent phenotype in adult, immunocompetent animals. On one hand, the requirement for rodent adaptation is a detriment to filovirus modeling since it involves non-WT viruses containing potentially several nucleotide mutations to be used in place of clinically relevant virus isolates. On the other hand, however, the genomic mutations acquired during rodent adaptation offer remarkable insight into some of the molecular mechanisms that contribute to, or restrict, filovirus pathogenesis. By understanding how these acquired mutations affect virus biology, we can identify key viral and host processes that affect filovirus pathogenesis, and by extension, develop anti-viral therapies that specifically counter these processes.

In recent years, much progress has been made in identifying and characterizing the mutations that confer virulence to filoviruses in rodents. Although filovirus rodent models have been discussed in depth elsewhere [59, 60, 72–74], we will briefly review the pathogenesis of filoviruses in three models—the mouse, guinea pig, and hamster—as it pertains to infection with rodent-adapted filoviruses. We will then examine the mutations that arise during rodent adaptation and the contribution these mutations make to filovirus pathogenesis.

FILOVIRUS RODENT MODELS

The Mouse

Adult, immunocompetent laboratory mice are resistant to disease caused by EBOV, MARV, or RAVV regardless of dose or route of infection [67, 68]. In order to generate an Ebola virus that caused lethal disease in mice, wild-type EBOV variant Mayinga had to be sequentially passaged nine times in progressively older suckling mice [75]. The resulting mouse-adapted EBOV (MA-EBOV) caused lethal disease in adult BALB/c, C57BL/6, and CD-1 mice, with death occurring between four and six days post-infection. As observed in NHPs infected with WT-EBOV, MA-EBOV replicates rapidly in mice and to extremely high titers. In conjunction with a robust pro-inflammatory cytokine response, this results in extensive and systemic organ damage, especially to the liver and spleen [75, 76]. MA-EBOV-infected mice also exhibit widespread lymphocyte apoptosis, which is considered a hallmark of EHF [77–79]. Unlike NHPs, all commonly used mouse models (such as BALB/c and C57BL/6 mice) exhibit little to no coagulopathy or hemorrhagic manifestations, such as tissue fibrin deposition, disseminated intravascular coagulation, or the characteristic maculopapular/petechial rash [75, 80]. Notably, however, some of the Collaborative Cross recombinant mice strains do exhibit coagulopathy, suggesting that the host's genetic background can significantly affect disease processes [81]. Mouse-adapted RAVV (MA-RAVV) as well as mouse-adapted MARV variant Ci67 (MA-MARV/Ci67) and variant Angola (MA-MARV/Ang) show a clinical picture in traditional mouse models similar to MA-EBOV, although MA-MARV/Ci67 appears to be less virulent in mice and is not 100% lethal [68, 82, 83]. Mouse adaptation of MARV/Ci67 and RAVV required 15 and 10 passages, respectively, in SCID mice, which lack T and B cells, followed by an additional 15 and 14 passages in BALB/c mice before the viruses became lethal [68, 82]. Similarly, MA-MARV/Ang required 24 passages in SCID mice before it was capable of causing lethal disease in mice [83]. Interestingly, none of the adapted filoviruses are capable of causing disease in mice when injected via the intramuscular or subcutaneous route; instead, intraperitoneal injection is required to achieve uniform disease and lethality [67, 68, 75, 83].

In terms of their convenience, low cost, and the vast availability of reagents, mice represent attractive animal models for filovirus research, despite the fact that they do not completely recapitulate all clinical hallmarks [72]. Accordingly, the mouse model can be used for studies that are simply not possible with other filovirus animal models. For example, the aforementioned demonstration that disease caused by MA-EBOV differs dramatically depending on the genetic background of the mouse was accomplished with a large panel of

recombinant inbred mice from the Collaborative Cross, and such a detailed study could not have been completed with any other animal model [81].

The Guinea Pig

Similar to mice, guinea pigs (*Cavia porcellus*) do not succumb to infection with WT filoviruses, although they do develop a transient febrile illness [61, 62]. Several different guinea pig-adapted filoviruses have been generated over the last two decades, and with the exception of guinea pig-adapted SUDV (GPA-SUDV), all of them required only a maximum of eight passages in adult guinea pigs before a lethal phenotype was achieved [69, 70, 74, 84–90]. GPA-SUDV, on the other hand, required 25 passages in guinea pigs before becoming lethal [88]. Disease caused by all guinea pig-adapted filoviruses is similar to EHF and MHF in humans and NHPs: The viruses replicate systemically and to high titers (albeit not as high as observed in mice or NHP models), causing significant pathology in multiple organs, especially the liver and spleen [69, 70, 85, 87–89]. Compared to older models of GPA-EBOV and MARV in inbred strain 13 guinea pigs, recently developed models of GPA-EBOV, MARV, and RAVV in outbred Hartley strain guinea pigs show evidence of bystander lymphocyte apoptosis as well as a marked pro-inflammatory response, although the observation of the latter may simply be a function of the increasing availability of reagents [87, 89]. Moreover, although both inbred and outbred guinea pig models display coagulopathy, including fibrin deposition in tissues and evidence of disseminated intravascular coagulation, it appears to be more significant in the outbred model [70, 80, 87, 89]. However, like the mouse model, no maculopapular/petechial rash is observed. Unlike the mouse model, guinea pigs are susceptible to inoculation by subcutaneous and intramuscular routes, in addition to the intraperitoneal route [70, 80, 87, 89].

Historically, few reagents have been available for working with guinea pigs, and, along with their larger size, this has made guinea pigs a difficult model system to work with. Nevertheless, the recently developed outbred guinea pig models of filovirus infection seem to accurately recapitulate many of the clinical markers of filovirus disease, and they have contributed to a growing arsenal of research tools [87, 89].

The Hamster

Syrian golden hamsters (*Mesocricetus auratus*) have been used extensively as model systems for hemorrhagic fever viruses [73], and they were used in some of the original MARV pathogenesis studies [63]. To date, however, the hamster model has only been thoroughly characterized for EBOV [71]. Like the mouse model, WT-EBOV does not cause observable illness in hamsters, despite a small degree of replication. Instead, the hamster model of EBOV relies on MA-EBOV, which, unlike in mice, causes disease in hamsters that almost perfectly recapitulates the clinical hallmark features observed in humans and NHPs. MA-EBOV replicates systemically and to high titers in hamsters, causing significant organ damage, especially in the liver and spleen. Extensive lymphocyte apoptosis is also observed, along with a robust, uncontrolled pro-inflammatory cytokine response. Similar to the outbred guinea pig models, MA-EBOV also produces severe coagulopathy, reminiscent of disseminated intravascular coagulation. Notably, like in mice, disease in hamsters results only when MA-EBOV is inoculated intraperitoneally [71].

Of all the filovirus rodent models, the hamster seems to most accurately recapitulate EHF as it is observed in humans and NHPs. Along with its convenience and low cost, the advent of several molecular research tools—including qRT-PCR assays for examining host gene expression [91]; microarray, transcriptome, and kinome platforms [92–94]; and knock-out animals [95]—make the hamster a useful system for modeling filovirus disease.

RODENT-ADAPTED FILOVIRUSES AND THEIR MUTATIONS

Mouse-adapted EBOV

The single MA-EBOV virus described to date differs from WT-EBOV (variant Mayinga) by thirteen nucleotide mutations, although four of these mutations were present prior to mouse adaptation (Fig. 3 and Supplemental Fig. 1) [96]. Five of the nine mouse-adapted mutations resulted in non-synonymous amino acid substitutions, and, of these, only two were required to confer virulence in mice: S72G in NP and T50I in VP24. Interestingly, neither of these mutations alone had any effect on virulence, suggesting that the function of NP and VP24 in this case is linked.

The precise molecular function of these two mutations remains to be determined, but some data suggest they may play a role in the evasion of the mouse IFN system. Indeed, recombinant WT-EBOV possessing mouse-adapted NP (NP_{MA}) and VP24 (VP24_{MA}) is resistant to exogenous IFN treatment in mouse macrophages compared to WT-EBOV and WT-EBOV possessing either NP_{MA} or VP24_{MA} [96]. These results make sense considering the established function of EBOV VP24 as an IFN antagonist [42–44], and they fit well with previous studies that identified the mouse IFN response as a major barrier to WT-EBOV infection [67]. Mice that lack the IFN- α/β receptor (IFNAR)—and are therefore unable to respond to IFN—succumb to infection with WT-EBOV variant Mayinga, as do mice that have been treated with antibodies that target IFNAR. Moreover, IFNAR^{-/-} mice, unlike WT mice, are also susceptible to subcutaneous inoculation of either WT- or MA-EBOV, highlighting the key role that the IFN system plays in WT-EBOV restriction.

Despite the circumstantial evidence supporting a role for NP_{MA} and VP24_{MA} in evading the mouse IFN system, there remains no direct, molecular evidence to support this hypothesis. In fact, the only study to directly address the ability of VP24_{MA} to inhibit the IFN response showed no difference between VP24_{MA} and VP24_{WT} [43]. Human epithelial cells (293T), human liver cells (Huh7), and mouse fibroblast cells (NIH-3T3 and MEF) were transfected with either VP24_{MA} or VP24_{WT} and treated with IFN. The response to IFN was measured using an ISG reporter plasmid, and the results indicated that both VP24_{MA} and VP24_{WT} were equally as effective at inhibiting the IFN response, regardless of cell type or species. There were also no obvious differences between the ability of VP24_{MA} and VP24_{WT} to interact with karyopherin- α or inhibit STAT1 phosphorylation [43]. These results suggest that the T50I mutation in VP24_{MA} does not affect the ability of VP24 to inhibit the IFN response in mice, and they also demonstrate that VP24_{WT} is capable, in any case, of strongly inhibiting the IFN response in both human and mouse cells.

Nevertheless, more work is required to understand the molecular basis that underlies EBOV mouse adaptation and the mutations that were acquired in the process. For example, it is

clear that during virus infection of macrophages VP24_{MA} and NP_{MA} are both critical for promoting virus replication in spite of IFN treatment [96]. Since cells of the mononuclear phagocyte system are initial and major target cells of filovirus replication [97–99], it would be of interest to examine whether VP24_{MA} and VP24_{WT} show a differential ability to regulate the IFN response in mouse primary or transformed macrophages, rather than fibroblast cells. In primary human macrophages, BDBV, which is thought to be less virulent than EBOV, at least in NHPs [100, 101], replicates more slowly and results in the production of fewer cytokines than EBOV, raising the idea that filovirus pathogenesis is at least in part dictated by replication in macrophages [102]. Interestingly, gene expression profile analysis revealed that mice infected with WT-EBOV possessing NP_{MA} and VP24_{MA} exhibited a dramatic upregulation of pro-inflammatory genes compared to mice infected with WT-EBOV [103]. Much of this host transcriptional response was driven by VP24_{MA} alone, but in the presence of both VP24_{MA} and NP_{MA}, genes encoding metalloproteinases were highly upregulated. How the induction of metalloproteinases is related to virulence or lethality remains to be determined, but it represents an intriguing avenue of investigation. The specific role that NP_{MA} plays in MA-EBOV virulence also remains to be determined. NP_{MA}, in conjunction with VP24_{MA}, is clearly required to confer virulence to MA-EBOV, but beyond that, its function is subject to conjecture. VP24 and NP do interact with each other [104, 105], and it is possible that NP may somehow augment or enhance the ability of VP24 to inhibit the IFN response. Alternatively, NP may have a completely different—and so far uncharacterized—role in modifying host response pathways, or its connection to mouse adaptation may be linked to its participation in virus replication and assembly [20–23, 104]. Beyond the mutations in NP and VP24, the contribution that the other seven mouse-adapted mutations make to virulence has not been examined in detail. It is known, however, that the presence of all nine mouse-adapted mutations in MA-EBOV, including the two mutations in the untranslated region between VP30 and VP24, plus the four pre-existing mutations broadens the range of virus dose that is lethal to mice, indicating that all mutations are required for complete virulence and suggesting at least some minor function for one or more of the other mutations [96].

Guinea Pig-adapted EBOV

Several different GPA-EBOV (variant Mayinga) strains have been developed, and all of those that have been sequenced possess one or more critical mutations in VP24, arguing strongly that this protein is singularly important for EBOV adaptation in guinea pigs [74, 84–86, 89]. Interestingly, however, the mechanism of adaptation appears to be structural in nature and unrelated to evasion of the IFN response. In molecular terms, the best characterized GPA-EBOV is GPA-EBOV/8mc, which contains ten nucleotide mutations, six of which result in a non-synonymous amino acid substitution and one of which results in a frame shift (Fig. 3 and Supplemental Fig. 1) [84]. Analysis of recombinant viruses possessing various combinations of these mutations revealed that only the three guinea pig adapted-mutations in VP24 (M71I, L147P, and T187I) were required to produce lethal disease in guinea pigs [86]. The single mutation in NP (F648L) had no effect on pathogenicity. WT-EBOV possessing only the three 8mc guinea pig-adapted mutations in VP24 (WT-EBOV-VP24_{GPA/8mc}) replicated robustly in guinea pig macrophages, and electron microscopic analysis of liver tissue from infected guinea pigs and primary guinea

pig macrophages revealed typical viral inclusion bodies containing typical nucleocapsids. In contrast, although WT-EBOV was capable of infecting guinea pig macrophages, replication in these cells was drastically impaired and inclusion bodies appeared abnormal, containing atypically condensed and aggregated nucleocapsids. Moreover, in liver tissue, no free WT-EBOV virions were observed in the extracellular space and no other cell types became infected. Notably, both VP24_{WT} and VP24_{GPA/8mc} were equally as effective at inhibiting the IFN response in human (293T) and guinea pig (GPC-16) epithelial cells treated with IFN- β . Together, these observations imply that EBOV adaptation to guinea pigs is not linked to evasion of the IFN response, but, instead, to a structural role fulfilled by VP24 [86]. Interestingly, the three guinea pig-adapted VP24 mutations decreased the mobility of VP24 on SDS-PAGE, suggesting that at least one of the mutations resulted in a significant change to the physical properties of the molecule [84]. Accordingly, L147P was predicted to alter the secondary structure of the protein, and M71I was predicted to increase the protein's thermostability [85]; however, none of these predictions have been validated since the crystal structure of VP24 was determined [106]. VP24 is thought to play a key role in condensing the viral nucleocapsid such that it is competent for packaging and egress from the cell [22, 23, 86, 104, 107–112]. It is therefore possible that the guinea pig-adapted mutations in VP24 somehow facilitate this process in guinea pig cells, although the molecular details have yet to be elucidated.

All other guinea pig-adapted EBOV isolates possess mutations in VP24, and many of these mutations are the same as or similar to those observed in GPA-EBOV/8mc. The independently derived GPA-EBOV/P7 isolate, which was generated by seven passages in guinea pigs, acquired a total of fifteen nucleotide mutations, seven of which resulted in non-synonymous amino acid substitutions (Fig. 3 and Supplemental Fig. 1) [85]. Three of these amino acid mutations occurred in VP24 (M71I, L147P, and R154L), and two of them, M71I and L147P, were identical to those identified in GPA-EBOV/8mc [84]. Another guinea pig-adapted EBOV variant, GPA-EBOV/K5, which was generated by three passages in monkeys, followed by multiple passages in Vero cells and L68 human embryonic lung cells prior to five passages in guinea pigs, also acquired a single mutation in VP24 during guinea pig adaptation (Fig. 3 and Supplemental Fig. 1) [84]. This mutation, H186Y, arose during virus passage at the same time as virulence in guinea pigs increased, and it occurs adjacent to the T187I mutation in VP24 of GPA-EBOV/8mc (Fig. 5a). Together, these data argue that the VP24 regions around M71, L147, and H186/T187 are critical to the virulence of guinea pig-adapted EBOV isolates, possibly by somehow modulating the structural assembly of EBOV virions [84–86]. Intriguingly, another mutation in VP24, L26F, was identified during experiments with recombinant EBOV. WT-EBOV-NP_{8mc}, which contains only the F648L mutation found in NP of GPA-EBOV/8mc, did not cause disease in five out of six guinea pigs [86]. However, the sixth guinea pig did succumb to infection, and sequence analysis identified the L26F mutation in VP24. When this single mutation was inserted into recombinant WT-EBOV and then used to infect guinea pigs, all animals succumbed to disease. Since this mutation had no effect on the ability of VP24 to inhibit the IFN response in either human or guinea pig cells, the study's authors concluded that, like the other guinea pig-adapted mutations identified in VP24, L26F affected the ability of VP24 to promote virion assembly and release. Recently, another guinea pig-adapted EBOV, GPA-EBOV/

UTMB, was generated by four passages of WT-EBOV in guinea pigs [89]. This virus possesses eight nucleotide mutations that result in five non-synonymous amino acid substitutions (Fig. 3 and Supplemental Fig. 1). Remarkably, this virus possesses the L26F mutation in VP24, as well as a K142E mutation of uncertain functional importance.

The circumstances surrounding the appearance of the L26F mutation in the WT-EBOV-NP_{8mc} virus may hint at a more complex guinea pig-adaptation process than simply acquiring mutations in VP24. Since the L26F mutation arose during infection with WT-EBOV-NP_{8mc}, the authors of that study suggest that the F648L mutation in NP may have facilitated the appearance of the L26F mutation in VP24 [86]. Whether this NP mutation predated and enabled the appearance of the three VP24_{8mc} mutations during the course of GPA-EBOV/8mc adaptation is not known since sequence data are not available for the viruses after each passage. However, during GPA-EBOV/P7 adaptation, two mutations in NP (L544P and S598P) appeared within the first two passages and a third mutation (N566S) arose during passages three or four and became fixed by passage five [85]. NP L544P and S598P predate the appearance of the first VP24 mutation, L147P, which arose during passages three or four and became fixed by passage five, concomitant with NP N566S. The other two VP24 mutations, M71I and R154L, appeared in the sixth and seventh passages, respectively. Remarkably, as each VP24 mutation became fixed in the virus genome, the virulence of the virus increased: Whereas passage four virus was 0% lethal in guinea pigs, passage five, six, and seven virus was 40%, 80%, and 100% lethal, respectively, reflecting the appearance of VP24 mutations L147P, M71I, and R154L [85]. Indeed, GPA-EBOV/UTMB also possesses two mutations in NP, A575T and S647Y, the latter of which is adjacent to the F648L mutation from GPA-EBOV/8mc [89]. Although these are correlative data, given the known functional relationship between NP and VP24 and its presumed role in virion assembly, it is tempting to speculate that the appearance of mutations in VP24 is facilitated by prior mutations in NP.

Guinea pig-adapted SUDV

The recently generated GPA-SUDV (variant Boneface) possesses sixteen nucleotide mutations, six of which result in non-synonymous amino acid substitutions (Fig. 3 and Supplemental Fig. 1) [88]. Three of these mutations occur in GP, and one each occurs in VP35, VP40, and VP24. Notably, no amino acid mutations in NP were acquired. The relevance of the mutation identified in VP24, P204L, is unclear; however, it is interesting to note that, on the structural model of VP24, P204L occurs next to H186 and T187, both residues that are presumed to be important for the virulence of GPA-EBOV/8mc and GPA-EBOV/K5 (Fig. 5a). The fact that each rodent-adapted EBOV acquires at least one mutation in VP24 suggests that this protein may be a universal virulence factor for rodent adaptation, and it will therefore be of interest to determine whether guinea pig adaptation of SUDV is driven by the same forces that appear to drive EBOV adaptation. It is also interesting to note that GPA-SUDV is the only rodent-adapted ebolavirus to acquire a mutation in VP40 [88]. Since VP40 mutations are critical to MARV rodent adaption (as discussed below), it will be of interest to determine whether this mutation in GPA-SUDV is a critical one.

Rodent-adapted RAVV and MARV

Whereas NP and VP24 seem to be critical for ebolavirus adaptation to rodents, it is VP40 that likely plays the most significant role in marburgvirus rodent adaptation. RAVV and several variants of MARV have been adapted to both mice and guinea pigs, and although mutations are acquired throughout the genome, the majority of the amino acid mutations occur in VP40 (Fig. 4 and Supplemental Fig. 2) [68, 82, 83, 87, 90]. Many of these mutations are shared among different rodent-adapted marburgviruses, and evidence suggests that some of them affect virus replication and assembly, while others contribute to IFN response inhibition.

To date, there have been sequence reports of three independently derived mouse-adapted marburgviruses—MA-RAVV, MA-MARV/Ci67, and MA-MARV/Ang—each with seven, five, and six amino acid mutations, respectively, in VP40 (Fig. 4 and Supplemental Fig. 2) [68, 82, 83]. Of all these mutations in VP40, each virus shares a common one: D184N. Moreover, both MA-RAVV and MA-MARV/Ci67 share the Y19H mutation and both MA-MARV/Ci67 and MA-MARV/Ang share the G79S mutation. Conversely, MA-RAVV and MA-MARV/Ci67 have only a single amino acid mutation in NP, and MA-MARV/Ang has only a single amino acid mutation in VP24. The preponderance of mutations in VP40 implies that it, as opposed to NP and VP24, is a key virulence factor in marburgvirus adaptation to mice.

Unlike ebolavirus VP24, MARV VP24 does not inhibit the IFN response. Instead, this function is carried out in marburgviruses by VP40 [47]. Notably, however, neither WT-MARV nor WT-RAVV VP40 is capable of inhibiting the IFN response in mouse cells following IFN- α/β treatment [48]. In contrast, VP40 from MA-RAVV is able to effectively abrogate the IFN response in both human and mouse cells by preventing JAK1 phosphorylation and, therefore, STAT1/STAT2 phosphorylation. Mutational analyses revealed this function to be dependent on only two mutations in VP40: V57A and T165A [48]. V57A, in particular, prevents VP40 oligomerization, and while it is not clear how that affects IFN response inhibition, it is known to abolish resistance to human tetherin [113]. Importantly, when these mutations were introduced into WT-MARV VP40, it too was capable of inhibiting the IFN response in mouse cells [48]. Thus these data indicate that the IFN response comprises at least one barrier to marburgvirus mouse adaptation, and they suggest a mutational path for overcoming it. Interestingly, VP40 from MA-MARV/Ci67 does not possess either the V57A or T165A mutation, yet it is still able to counter the IFN response in mouse cells, an ability that was narrowed down to the G79S mutation [114]. Like MA-MARV/Ci67, MA-MARV/Ang possesses the G79S mutation and not the V57A or T165A mutations, suggesting that both of these viruses adapted to evade the IFN response in the same manner [83]. These data were affirmed by the recent structural determination of MARV VP40, which revealed that an N-terminal portion of the molecule—composed of amino acids 36 to 173—is necessary and sufficient for inhibition of the IFN response [115]. Moreover, G79 and T165 occur adjacent to each other in the structure (Fig. 5b), perhaps implying that mutation of either residue confers the same function.

Guinea pig-adapted MARV variant Musoke (GPA-MARV/Mus) contains eleven nucleotide mutations, but only four that result in non-synonymous amino acid substitutions (Fig. 4 and

Supplemental Fig. 2) [90]. Three of these amino acid mutations occur in L and one occurs in VP40. It is likely no coincidence that the single VP40 mutation, D184N, is also shared by all three mouse-adapted marburgviruses. This mutation, which occurs in the flexible linker region between the VP40 N- and C-terminal domains [90], has no effect on the ability of VP40 to inhibit the IFN response in guinea pig or human cells. Instead, D184N contributes to VP40's role as the viral matrix protein [116]. In guinea pig but not human cells, the D184N mutation improves the ability of VP40 to attract nucleocapsids to the site of virion budding, and it improves budding, itself. The mutation also reduces the suppressive effect that VP40 has on viral RNA synthesis in guinea pig cells, thereby improving replication and transcription. Accordingly, recombinant MARV containing only the D184N mutation in VP40 outgrows WT-MARV in guinea pig cells, although whether the mutation is solely responsible for the increase in GPA-MARV/Mus virulence remains to be determined [116]. Two other guinea pig-adapted marburgviruses have been generated, GPA-MARV variant Angola (GPA-MARV/Ang) and GPA-RAVV; however, neither of these viruses possesses the D184N mutation (Fig. 4 and Supplemental Fig. 2) [87]. GPA-MARV/Ang has only a single mutation in VP40, N56K, and given the proximity of this mutation to V57 (Fig. 5b), which is important for IFN response inhibition, it is tempting to speculate that N56K also plays a role in IFN inhibition [87]. GPA-RAVV has three mutations in VP40, two of which—L27S and L33P—are shared with MA-MARV/Ang and the third of which—F53S—is novel [87]. While it is unclear what the former two mutations in VP40 may be doing, it is again interesting to speculate that F53S, which is close to V57, may be involved in the inhibition of IFN responses. Besides the mutations in VP40, GPA-MARV/Ang has two additional mutations in VP24, and GPA-RAVV has a single mutation in both NP and GP [87].

Although it has not been directly demonstrated, it seems reasonable to assume that the D184N mutation in VP40 has the same function in mice as it does in guinea pigs. Interestingly, during the course of MA-RAVV and MA-MARV/Ci67 adaptation, the D184N mutation arises after the first and third passage, respectively, suggesting that this mutation—and, by extension, the replicative/structural advantages it confers—are fundamentally critical to mouse adaptation [82]. The first mice do not succumb to MA-RAVV infection until around passage twenty-five, coincident with the appearance of numerous mutations, including V57A and T165A in VP40, suggesting that evasion of the IFN response may be the ultimate barrier to overcome, at least for MA-RAVV [82]. During MA-MARV/Ci67 adaptation, the G79S mutation arises after passage 14, but the first animals do not succumb until after passage twenty-nine, coincident with the appearance of the Y536H mutation in NP and the F445P mutation in GP [82]. Thus, in addition to altering the structural properties of VP40 (D184N) and adapting to the murine IFN system (G79S), the as yet uncharacterized NP and GP mutations in MA-MARV/Ci67 may hint at yet another barrier to adaptation in mouse cells.

Indeed, even though the rodent-adapted marburgviruses acquire a preponderance of mutations in VP40, many of which have been demonstrated to have significant functional consequences in rodent cells, there are still numerous other rodent-adapted mutations that may be important to adaptation. For example, both MA-RAVV and MA-MARV/Ci67 possess the Y19H mutation in the late domain of VP40, PPPY [68, 82]. This late domain facilitates interaction with Tsg101, a component of the ESCRT-I complex, and promotes

budding of viral particles, yet the Y19H mutation appears to only modestly inhibit this process [48, 117]. In any case, since MARV NP, unlike EBOV NP, also plays a significant role in virus budding [118], disruption of the VP40 late domain alone may not critically impact virus viability in rodents, although this remains to be experimentally determined. Additionally, MA-RAVV and MA-MARV/Ang both contain amino acid mutations in VP35 [68, 83]. Since VP35 functions to antagonize the induction of an IFN response, it is possible that some of these mutations affect the ability of VP35 to inhibit IFN induction in the first place. Intriguingly, many of the rodent-adapted marburgviruses acquire numerous U to C mutations in the 3' and 5' untranslated regions of various genes, including NP, VP35, and GP; however, the functional consequences of these mutations remain unclear [68, 82, 83]. Amino acid mutations in L may also affect virus adaptation, but these mutations have only been observed in GPA-MARV/Mus [90]. Interestingly, MARV/Mus has been shown to replicate less efficiently than MARV/Ang, so it is possible that these mutations in GPA-MARV/Mus L may affect the efficiency of replication/transcription [119]. Further work will be required to conclusively determine which mutations contribute to virus virulence and to what extent they do so.

CONCLUSIONS AND PERSPECTIVES

Animal models that accurately recapitulate the clinical hallmarks of Ebola and Marburg hemorrhagic fevers are invaluable to the development of vaccines and anti-viral therapeutics. Compared to NHP models, rodent models have the added advantage of being relatively inexpensive and easy to handle. As such, they are well suited to large and thorough experiments aimed at dissecting the molecular bases of filovirus pathogenesis. The fact that filoviruses must first be adapted to their rodent hosts is occasionally seen as a disadvantage to disease modeling, but in many cases it can provide a distinct advantage in the pursuit to understand filoviral hemorrhagic fevers at a molecular level. Many, if not all, adaptive mutations that arise in filoviruses directly highlight pathogenic processes that are important for causing serious disease, and by analyzing these mutations we can not only better understand these processes, but also potentially develop therapeutics that target them.

Research over the last several years has identified two major barriers that must be overcome during rodent adaptation of filoviruses: the IFN response and the structural assembly of virions. Of these, the rationale behind evading the IFN response is perhaps the most straightforward to understand. During virus infection, type I IFN production incites a signaling cascade that results in the upregulation of numerous IFN-stimulated genes, ultimately producing an anti-viral state that constrains virus infection [120]. In mice, the IFN response is critical to restricting infection by EBOV, MARV, and RAHV [67], so it is therefore not surprising that each of these viruses would acquire mutations during the course of mouse adaptation that allow them to effectively avert this anti-viral pathway (Fig. 6). These mutations have been mapped to VP40 G79S in mouse-adapted MARV and VP40 V57A/T165A in mouse-adapted RAHV [48, 114], yet it remains unclear exactly how MA-EBOV evades the IFN response. This work will undoubtedly aid in identifying precisely how MARV VP40 counteracts the IFN response, and continued work should be aimed at understanding how MA-EBOV is able to replicate better than WT-EBOV in the face of IFN- α/β . Notably, EBOV variant Kikwit, which is ~99% identical to the variant Mayinga used

for all EBOV rodent adaptation, was unable to cause disease in mice, even in the absence of a functional IFN response [67, 121]. While the molecular basis for this discrepancy is still unclear, it may hint at other important barriers to filovirus pathogenesis in rodents. Indeed, although IFN evasion appears to be critical to mouse adaptation, evidence suggests that it may not be sufficient.

Mutations affecting virus replication and virion assembly also appear to be important during rodent adaptation, although the cellular mechanisms that underlie this role have yet to be completely elucidated (Fig. 6). In guinea pigs, mutations in EBOV VP24 are critical to the formation of normal viral inclusion bodies and nucleocapsids [86]. This necessarily implies the existence of some host-specific factor or condition that is required for proper structural assembly of virions; however, the identity of this host factor is unknown. Likewise, mouse- and guinea pig-adaptation of marburgviruses produces in most, but not all, cases the D184N mutation in VP40 that affects virion assembly and budding [68, 82, 83, 90, 116]. The question of why this step in the viral life-cycle should be a restricting factor remains unanswered, but it presumably depends on elements or conditions in rodent cells that differ from human cells. Continued work with the rodent-adapted mutations in EBOV VP24 and MARV VP40 may help us identify what these factors are. Moreover, why should structural changes in VP24 be selected for in rodent-adapted EBOV and not MARV? And why should the budding efficiency of VP40 be improved in rodent-adapted MARV but not EBOV? The answers to these questions must be linked to the idiosyncrasies of EBOV and MARV biology, for which more research is required.

It is also worth considering the virulence of rodent-adapted filoviruses in hosts to which they were not adapted. MA-EBOV, for example, is lethal in hamsters and guinea pigs, as well as mice, indicating that the mouse-adapted mutations also represent virulence factors in other rodents [71, 80]. Indeed, the observation that the IFN response is delayed in hamsters infected with MA-EBOV but not in hamsters infected with WT-EBOV indicates that MA-EBOV counters the hamster IFN response and implies that this response represents a barrier to EBOV adaptation [71]. In contrast, however, it appears as if structural and/or replicative changes—and not evasion of the IFN response—are required to confer virulence in guinea pigs. Perhaps filovirus virulence in mice (and possibly hamsters) requires mutations that facilitate IFN response evasion in addition to mutations that supply replicative and structural advantages, whereas virulence in guinea pigs requires only the latter. This hypothesis seems to be supported by the available evidence, and it would be consistent with the unpublished observation that guinea pig-adapted EBOV is avirulent in mice [80]—perhaps because it lacks a mechanism to evade the IFN response. Indeed, at least for GPA-MARV/Mus, no mutation that promotes IFN response evasion has been identified [116]. Additional “host-swapping” experiments among rodent-adapted filoviruses would be required to draw meaningful conclusions, but it is interesting to speculate that such experiments could facilitate the development of a pan-virulent filovirus capable of causing disease in multiple hosts. It is naïve to assume that the IFN response and virion assembly are the only two factors driving filovirus adaptation in rodents, however, and the continued investigation of other rodent-adapted mutations may lead to the identification of additional processes that contribute to pathogenesis. Interestingly, EBOV passaged up to three times in guinea pigs remains lethal in NHPs [122, 123] and GPA-EBOV/8mc was responsible for a fatal human

infection resulting from a laboratory accident [5, 124]. Similarly, MA-EBOV causes significant, albeit slightly attenuated, disease in NHPs [80]. These data suggest that rodent adaptation may not completely compromise virulence in NHPs and humans, adding further intrigue to the interpretation of mutant function.

Continued work with rodent-adapted filoviruses will be aided by a variety of experimental techniques and methodologies that are now becoming routine. The availability of reverse genetics systems for both EBOV and MARV [41, 125–128] will permit the systematic evaluation of rodent-adapted mutations on virus phenotype, and the various virus life-cycle modeling systems, including minigenome assays available for EBOV and MARV [129, 130], as well as the transcription/replication-competent virus like particle (trVLP) systems [107, 111, 131–133], will permit evaluation of these mutations outside of maximum containment [134]. Moreover, techniques such as immunoprecipitation combined with mass spectrometry analysis will allow for greater elucidation of the virus interactome [135, 136], and next-generation sequencing platforms will permit an even more detailed analysis of the genomes of rodent-adapted filoviruses. Indeed, in combination with the many powerful molecular biological techniques that now exist, rodent-adapted filoviruses represent excellent tools for dissecting cellular processes relevant to pathogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Research Highlights

- Ebola and Marburg virus, both filoviruses, are the causative agents of severe hemorrhagic fever
- Rodent models for Ebola and Marburg viruses are often the first choice for initial descriptions of virus pathogenesis and evaluation of anti-viral prophylactics and therapeutics
- Rodent models rely on “rodent-adapted” viruses that have been passaged several times through their host until virulence and lethality are achieved
- Rodent-adapted Ebola and Marburg acquire numerous nucleotide/ amino acid mutations that contribute to virulence in their rodent host
- Virus protein 24 (VP24) and nucleoprotein (NP) appear to be major virulence factors for ebolaviruses in rodents, whereas VP40 appears to be the major virulence factor for marburgviruses

Fig. 1.

The genomic structure and morphology of filoviruses. (a) The genomic structure of Marburg and Ebola virus is depicted, with the protein-coding sequences labeled and colored and the untranslated regions in grey. The 3' leader, intergenic regions (IR), gene overlaps, and 5' trailer are indicated with arrows. Functional descriptions of the viral proteins are provided in word bubbles. (b) The single-stranded, negative sense RNA genome (RNA) is encapsidated by the nucleocapsid, which is composed of NP, VP30, VP35, and VP24. The matrix protein (VP40) connects the nucleocapsid to the host-derived lipid envelope, in which GP_{1,2} resides. L is the RNA-dependent RNA polymerase.

	HUMAN	NHP	GUINEA PIG	HAMSTER	MOUSE
Virus Adaptation	No	No	Yes	Yes	Yes
CLINICAL SIGNS					
Viremia	+++	+++	+++	+++	+++
Coagulopathy	+++	+++	+++*	+++	-/+
Cytokine Response	+++	+++	+	+++	++
Rash	++	++	-	-	-
USE IN RESEARCH					
Availability	N/A	+	+++	+++	+++
Ease of Handling	N/A	+	++	+++	+++
Cost	N/A	+++	+	+	+
Tools & Reagents	N/A	++	-/+	+/+	+++
Large N	N/A	-	++	+++	+++

Fig. 2.

Comparison of filovirus hosts and model systems. Clinical signs of filovirus disease as well as factors influencing research are compared among humans, non-human primates (NHP), guinea pigs, hamsters, and mice. *, indicates coagulopathy for outbred guinea pig model only.

Fig. 3.

Depiction of non-synonymous mutations in rodent-adapted ebolaviruses. Non-synonymous mutations only are indicated with arrows along the length of the ebolavirus genome. Corresponding amino acid mutations are given using the one-letter amino acid code and the number of the residue mutated in each particular protein. Arrows and text in red indicate mutations that existed in the virus prior to rodent adaptation. Note that only the VP24 coding sequence was sequenced for GPA-EBOV/K5.

Fig. 4.

Depiction of non-synonymous mutations in rodent-adapted marburgviruses. Non-synonymous mutations only are indicated with arrows along the length of the marburgvirus genome. Corresponding amino acid mutations are labeled using the one-letter amino acid code and the number of the residue mutated in each particular protein.

Fig. 5. Depiction of the location of amino acid mutations on ebolavirus VP24 and marburgvirus VP40. (a) All residues found to be mutated in rodent-adapted ebolavirus VP24 are highlighted in blue on the structure of EBOV VP24 (PDB ID 4M0Q) [137]. Residue L26 is not surface exposed on this structure, so it is not visible. (b) All residues found to be mutated in rodent-adapted marburgvirus VP40 are highlighted in blue on the structure of MARV VP40 (PDB ID 5B0V) [115]. Residues Y7, Y19, L27, and L33 were not included in the crystal structure; residue I187 is not visible. Mutations are indicated with an arrow and labeled using the one-letter amino acid code and the number of the residue mutated. Arrows and text in red indicate mutations that existed in the virus prior to rodent adaptation. The structures were rotated on the y-axis approximately 90° to depict all mutations.

Fig. 6.

The IFN- α/β response and viral assembly/packaging are key pathogenic processes in rodents. Ligation of the IFN- α/β receptor (IFNAR) with IFN- α/β results in the phosphorylation of Jak1 and Tyk2, followed by the phosphorylation of STAT1 and STAT2, and the formation of STAT1 homodimers and/or STAT1-STAT2 heterodimers. Karyopherin- α facilitates the transfer of these dimers into the nucleus, where, along with IRF9, the complex binds to IFN-stimulated response elements (ISRE) in the genome and drives the expression of IFN-stimulated genes (ISG). MARV VP40 inhibits the IFN response by preventing Jak1 phosphorylation, and the V57A, G79S, and T165A mutations acquired during rodent adaptation permit this process in rodent cells. MARV VP40 also plays a critical role in virion assembly and budding, and the D184N mutation acquired during rodent adaptation affects this process in rodent cells. EBOV VP24 also inhibits the response to IFN- α/β by interacting with KPN1 and preventing STAT1 homodimer or STAT1-STAT2 heterodimer translocation into the nucleus. Mutations acquired in EBOV VP24 (T50I) and NP (S72G) during mouse adaptation may be critical to evading the IFN response, but more research is required. EBOV VP24 also plays a critical role in nucleocapsid assembly. The L26F, M71I, L147P, H186Y, and T187I mutations acquired in EBOV VP24 during guinea pig-adaptation appear to be critical to nucleocapsid formation, and mutations acquired in NP may also be involved in this process. Whether and what host factors are involved in viral assembly is unclear.