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Hiding the evidence: two strategies for innate immune evasion by hemorrhagic fever viruses

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The innate immune system is one of the first lines of defense against invading pathogens. Pathogens have, in turn, evolved different strategies to counteract these responses. Recent studies have illuminated how the hemorrhagic fever viruses Ebola and Lassa fever prevent host sensing of double-stranded RNA (dsRNA), a key hallmark of viral infection. The ebolavirus protein VP35 adopts a unique bimodal configuration to mask key cellular recognition sites on dsRNA. Conversely, the Lassa fever virus nucleoprotein actually digests the dsRNA signature. Collectively, these structural and functional studies shed new light on the mechanisms of pathogenesis of these viruses and provide new targets for therapeutic intervention.

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Introduction

Pathogens and their hosts are locked in an evolutionary arms race, each trying to gain the upper hand over the other. As a result of this process, host organisms have developed a sophisticated, multi-tier defense against invading pathogens. The innate immune system provides the first line of defense, and plays a particularly important role in viral infections, as viruses are obligate intracellular pathogens, dependent on host cell machinery and resources for replication.

Early in infection, components of the innate immune system sense pathogen-associated molecular patterns (PAMPs). Among PAMPs, double-stranded RNA (dsRNA) is a key hallmark of infection by negative-sense viruses. dsRNA is detected in the cytoplasm of an infected cell by viral dsRNA receptors such as retinoic acid-inducible gene I (RIG-I) [1] and melanoma differentiation-associated gene 5 (MDA-5) [2]. Upon recognition of their ligand, these receptors initiate signaling pathways that result in the translocation of interferon regulatory factor 3 (IRF-3) and other transcription factors to the nucleus. Once in the nucleus, these transcription factors activate expression of interferons (IFN) α and β, which initiate the antiviral response in the infected cells and prime neighboring cells for a rapid response to viral invasion.

IFNs establish an antiviral state through both an autocrine and paracrine manner and trigger pathogen-specific, adaptive immune responses. Early responses of the innate immune system thus may be able to control viral replication and permit a subsequent, robust, and specific adaptive immune response to clear the virus and prevent establishment of reinfection. However, viruses have developed a myriad of countermeasures that allow them to block, circumvent, or mitigate host cell defenses.

Numerous viruses have developed multiple strategies for attenuating the initial innate immune response mounted by their hosts. Suppression of these responses allows the virus to gain an early foothold during infection, replicating to a high titer before an effective adaptive immune response can be mounted. This is particularly true for certain hemorrhagic fever viruses, such as the filoviruses Ebola and Marburg and several members of the arenavirus family (Lassa (LASV), Lujo, Junin, and Machupo).

Filoviruses and arenaviruses are enveloped, negative-sense, single-stranded RNA (ssRNA) viruses and as such, produce dsRNA during viral replication. The Ebola virus (EBOV) protein VP35 and the arenavirus nucleoprotein (NP) have been shown to inhibit IFN-α/β production [3–5]. In cases of human infection with EBOV and LASV, patients with hemorrhagic fever display very high viral titers (as high as 10^9 in some cases) and marked immune dysregulation [6–11]. Recent structural studies of EBOV VP35 and LASV NP have provided valuable insight into the mechanisms by which these key viral proteins subvert host immune defenses.

Ebola virus VP35

Significant immunosuppression is noted in natural ebolavirus infection [12,13]. Five species in the *ebolavirus*

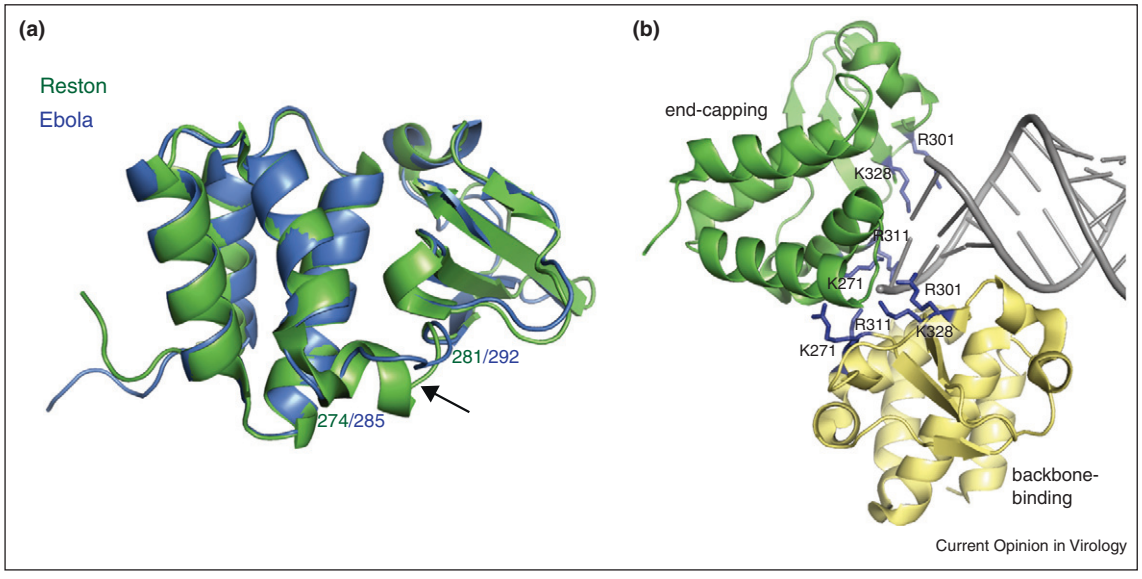
genus have thus far been described: Ebola (formerly known as Zaire), Sudan, Taï Forest, Bundibugyo and Reston viruses [14,15]. The VP35 protein of these viruses appears to play a key role in blocking critical immune signaling events early in infection. Indeed, VP35 has been shown to prevent phosphorylation and dimerization of IRF-3 [16], block induction of IFN α/β expression [3,16], inhibit activation of protein kinase R [17,18], and serve as a suppressor of RNA silencing [19]. This ability of VP35 to block IFNα/β expression has been mapped to its C-terminal dsRNA-binding domain [20,21]. Recent structural analysis of the pathogenic EBOV [22,23••] and the nonpathogenic (to humans) Reston virus (RESTV) [24••,25•] identified key residues critical for IFN inhibition and suggested a mechanism by which VP35 sequesters dsRNA and prevents it from being recognized by immune sentry proteins. Interestingly, the VP35 structures are largely similar between EBOV and RESTV; reasons why RESTV is nonpathogenic to humans remain unclear.

The crystal structures reveal that VP35 RBD contains two subdomains: an α helical subdomain, which consists of a four-helix bundle, and a β sheet subdomain, which is formed by four antiparallel β strands, an α helix and a polyproline II helix (Figure 1a) [22,24••]. Electrostatic surface calculations demonstrate the presence of two highly conserved basic patches, one in each subdomain. The first conserved basic patch lies in the α helical subdomain, contains residues K222/K211, R225/K214, K248/K237, and K251/K240 (numbering is EBOV/RESTV henceforth), and is not critical for binding dsRNA [22]. The second, 'central basic patch' lies in the β sheet subdomain and contains K282/K271, R305/R294, K309/K298, R312*/R301, K319/K308, R322*/R311, and K339*/K328. Many of these residues were previously identified in EBOV as critical for immune suppression [21,26], and residues marked by * have been shown to be critical for dsRNA binding [23••].

Structures of EBOV VP35 in complex with 8 bp of dsRNA [23••] and RESTV VP35 in complex with 18 bp of dsRNA [24••] revealed the VP35 of both viruses binds to dsRNA using a unique bimodal strategy. In both structures, VP35 forms an asymmetric dimer that wraps about the end of the dsRNA. Within each dimer, one VP35 RBD monomer binds terminal nucleotides of the dsRNA as well as the backbone ('end-capping'). The other VP35 RBD monomer binds only the phosphate backbone of the dsRNA ('backbone-binding') (Figure 1b). The two monomers together assemble a continuous, positively charged pocket for receiving dsRNA.

The end-capping RBD contains several conserved hydrophobic residues, which form a nonpolar face that packs against the terminal bases of the dsRNA. The C-terminal carboxyl group of the end-capping VP35 polypeptide hydrogen bonds to the terminal base of the dsRNA oligo. Also, residues in the central basic patch of the end-capping molecule (K282/K271, R312/R301, and R322/R311) make hydrogen bonds to the phosphate backbone, of the dsRNA.

Figure 1



The structure of Ebola and Reston virus VP35. **(a)** RESTV (green, PDB codes 3KS4 and 3L2A) and EBOV (blue, PDB code 3FKE) VP35 RBDs are highly similar aside from a single helix in the linker between the two subdomains (residues 274–281 in RESTV and 285–292 in EBOV, indicated by an arrow). **(b)** Structure of RESTV VP35 in complex with dsRNA. Ribbon models of the end-capping VP35 and backbone-binding VP35 are colored green and yellow, respectively. Residues comprising the central basic patch in both molecules are illustrated in blue.

Interestingly, in the backbone-binding VP35 monomer, two of those central basic patch residues (R312/R301 and R322/R311), as well as an additional central basic patch residue (K339/K328) do not bind RNA, but instead form the dimer interface to the end-capping molecule. Instead, it is the residues I340/I329 (C-terminal carboxyl group), S272/S261, R305/R294, and Q274/Q263 that make hydrogen bonds to dsRNA in the backbone-binding monomer. The structural information suggests two roles for the critical residues in the central basic patch for EBOV/RESTV VP35: first, binding the backbone of dsRNA, as seen in the end-capping subunit and second, forming the dimer, as seen in the backbone-binding subunit (Figure 1b).

Alignment of the VP35 RBDs of RESTV [24••] and EBOV [22] shows a high degree of similarity between the two species with an overall RMSD of 0.7 Å among backbone atoms. Some flexibility is evident in residues 274–281 (RESTV numbering), which forms a helix in the linker between the two subdomains of RESTV, but adopts a distinct loop structure in EBOV. The helical nature of the linker in RESTV could limit the flexibility required for multiple interactions between the VP35 RBD and the dsRNA. However, the conservation of structures and binding mechanism between pathogenic (EBOV) and nonpathogenic (RESTV) viruses suggests that the virulence determinant lies elsewhere: in a contact of the VP35 RBD with a non-dsRNA factor, in N-terminal regions of VP35, or in another component of the virus.

One of the most unusual findings from the structural studies of VP35 is the unique mode of dsRNA recognition. Each, otherwise identical monomer makes a different interaction with the dsRNA ligand. Together, the complete RNA-binding surface assembled by VP35 molecules masks the terminus and backbone of dsRNA from recognition by host dsRNA sensors such as RIG-I and MDA-5. Indeed, recent structural analysis of RIG-I in complex with dsRNA demonstrates that the different domains of the protein coat the phosphate backbone and also the terminal bases of the dsRNA [27•,28•,29•,30•]. Hence, while other viral proteins, such as influenza NS1, appear to prevent dsRNA recognition by solely coating the dsRNA backbone [31,32], ebolavirus VP35 caps the ends of the dsRNA and the specific recognition site of RIG-I. Whether VP35 also coats expanses of dsRNA backbone between the ends is currently unknown.

Lassa virus NP

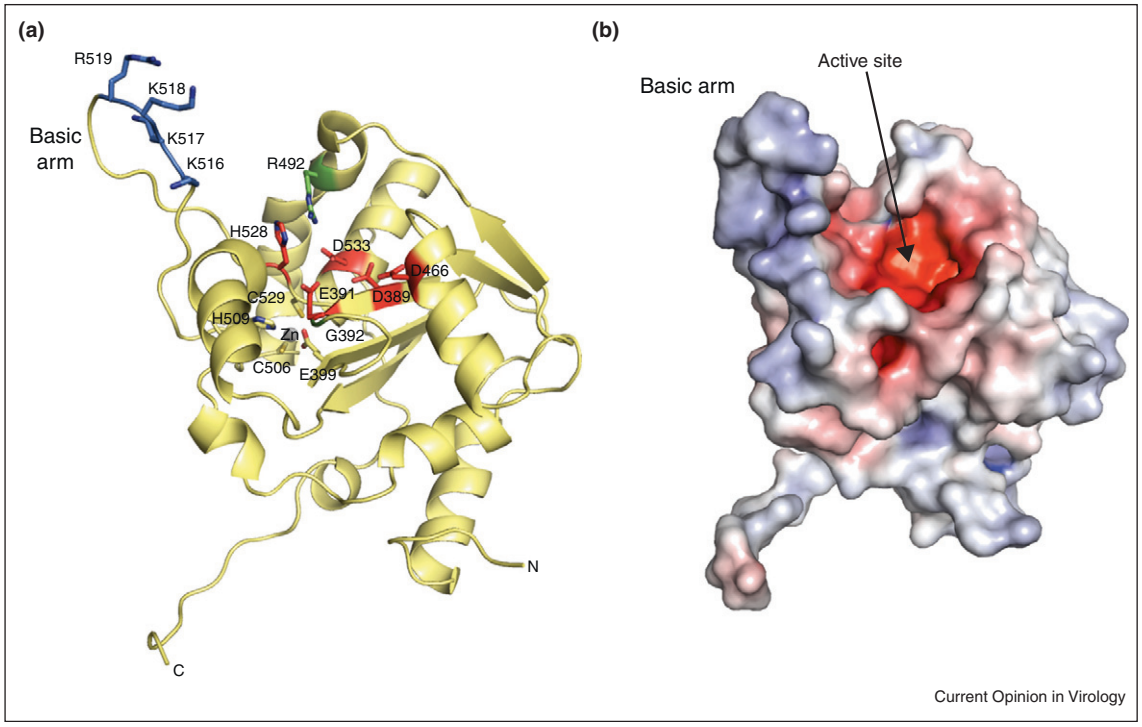
The arenavirus NP is one of the only four proteins encoded by the virus. NP is multifunctional, playing roles in viral replication and transcription and also in suppression of the host innate IFN response, by inhibiting nuclear translocation of IRF-3 [4]. Arenavirus infection often results in unchecked viral replication, failure to initiate an adaptive immune response and increased morbidity and mortality [33–38]. Mutagenesis throughout LCMV NP, before the availability of a structure, identified key residues within the C-terminal half of NP critical for such immunosuppressive function [5,39]. However, the role of these residues, and the mechanism by which arenavirus NPs cause immunosuppression remained unknown. Recent structural analysis of LASV NP has shed light onto these questions [40••,41••].

Crystal structures reveal that the C-terminal domain of LASV NP consists of a mixed, five-stranded β sheet with one antiparallel strand (β2) and six α helices connected by a series of loops. One particularly long loop between α5 and α6 forms a basic 'arm' off one side (Figure 2a,b). A zinc atom at the center of the structure is coordinated by E399, C506, H509, and C529, which are located at the bases of β2, the protruding basic arm and α6, respectively.

Primary sequence analysis of LASV NP showed no similarity to other proteins outside the arenavirus family. Although no particular enzymatic activity was previously known for LASV NP, structural studies unexpectedly revealed that the 3D fold was extremely similar to members of the DEDDh family of exonucleases [40••,41••]. The members of this family are so named because they contain strictly conserved Asp-Glu-Asp-Asp and His catalytic residues in the active site [42]. LASV NP similarly encodes D389, E391, D466, D533, and H528. Not only does the overall fold align with known DEDDh enzymes, such as IFN-stimulated gene-20 [43] and the ε subunit of *Escherichia coli* DNA polymerase III (DNA pol IIIε) [44], it also appears to have the same active site as well. The positions of D389, E391, D466, D533, and H528 in LASV NP align beautifully with equivalent residues in the active sites of known exonucleases.

LASV NP not only resembles an exonuclease, it also functions like an exonuclease. Biochemical analysis demonstrates that NP readily hydrolyzes an 18 bp dsRNA oligonucleotide in the 3′–5′ direction, but has no effect on ssRNA, ssDNA, or dsDNA [40••] (although dsRNA specificity was not observed in a separate study that used different assay conditions and substrates [41••]). Mutational analysis confirms the importance of the active site DEDDh residues [40••,41••] and those that coordinate the zinc ion [40••] for exonuclease activity. Additional residues within the active site, such as G392 and R492, were also identified as critical to the exonuclease function of NP [40••]. The position of these amino acids in the active site suggests they may play a role in binding to the terminal RNA residue. The stretch of four basic residues within the basic arm of NP was also shown to be important, but not critical, for exonuclease activity [40••]. The *E. coli* DNA polymerase I Klenow fragment uses a basic arm to make contact with the primer strand of the duplex DNA. Perhaps the basic arm of LASV NP contacts the undigested strand of the dsRNA in a similar fashion.

Figure 2



The structure of the exonuclease domain of Lassa virus NP. **(a)** Cartoon representation of the C-terminal domain of Lassa virus NP (PDB code 3Q7B). Residues within the active site are colored red, and residues proximal to the active site and known to disrupt exonuclease activity (G392 and R492) are colored green. The basic arm includes residues K516, K517, K518, and R519. A single zinc (gray sphere) is coordinated by E399, H506, C509, and C529. **(b)** Electrostatic surface potential calculated using APBS [49] shows that NP has an acidic active site and highlights the basic arm. Positive surface is colored blue; negative surface is colored red with limits ±10 kT/e.

Importantly, mutational analysis indicates that those residues important for exonuclease activity are also important for immunosuppression. Wild-type NP blocks translocation of IRF-3 into the nucleus. Mutants that knock out exonuclease activity fail to block IRF-3 translocation [40••,41••] (proper fold and expression of these mutants was verified by use of ELISA with conformational antibodies). These results indicate that the exonuclease activity is essential for the observed immunosuppressive activities of NP *in vitro*.

The high sequence similarity among the arenavirus NPs, conservation of the exonuclease active site, and the demonstrated importance of the same D, E, D, D, and H residues in LCMV NP [39] suggests the exonuclease activity is a shared feature of arenavirus NPs.

This is the first time a virus has been shown to have dsRNA-specific exonuclease activity, and the first time a virus has been shown to counteract IFN responses by actually digesting that PAMP. As previously discussed, the ebolaviruses suppress dsRNA-mediated responses by encoding proteins that bind to and physically shield dsRNA from recognition by immune sensors like RIG-I and MDA-5 and by RNA interference machinery [19,20,23••,24••,25•]. Tombusvirus, Flock house virus, and influenza virus also encode proteins that bind to and shield dsRNA from immune recognition, although these proteins bind the central backbone and not the ends of the dsRNA oligos [32,45,46]. By contrast, rather than coating dsRNA generated during replication and transcription, the arenavirus NP instead digests the dsRNA.

Only one other mammalian RNA virus is known to encode a protein with exonuclease activity. Nsp14 from SARS coronavirus is also a DEDDh exonuclease, but appears to have activity toward both ssRNA and dsRNA [47]. Furthermore, nsp14 has been implicated in the control of RNA synthesis and genome fidelity [48] and has no known IFN suppressive activity. Thus, the exonuclease activity of the arenavirus NP provides the first example of a virus digesting dsRNA to evade host innate immune responses.

Discussion and future directions

The importance of filovirus VP35 and arenavirus NP to both the viral life cycle and the immunosuppression of the host cell, and the availability of high-resolution

structures of these proteins should facilitate design of antiviral drugs that target VP35 and NP. The RNA-binding basic patch and the dimerization site of the VP35 RBD offer two targets for binding of small molecules that inhibit dsRNA binding. Compounds that target the exonuclease active site or the zinc-coordination site of NP may also prove to be useful antiviral therapies. In this endeavor, it will be important to develop compounds that are specific for arenavirus NP and do not cross-react to other, structurally similar host exonuclease active sites. A structure of NP bound to its dsRNA substrate would provide additional and perhaps more specific targets for small molecules.

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- •• of outstanding interest

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