The ebolavirus VP24 interferon antagonist
Know your enemy

Adrianna P.P. Zhang,1 Dafna M. Abelson,1 Zachary A. Bornholdt,1 Tong Liu,2 Virgil L. Woods, Jr2 and Erica Ollmann Saphire1,3,*

1Department of Immunology and Microbial Science; The Scripps Research Institute; La Jolla, CA USA; 2Department of Medicine; University of California, San Diego; San Diego, CA USA; 3The Skaggs Institute for Chemical Biology; The Scripps Research Institute; La Jolla, CA USA

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*Correspondence to: Erica Ollmann Saphire; Email: erica@scripps.edu

Suppression during the early phases of the immune system often correlates directly with a fatal outcome for the host. The ebolaviruses, some of the most lethal viruses known, appear to cripple initial stages of the host defense network via multiple distinct paths. Two of the eight viral proteins are critical for immunosuppression. One of these proteins is VP35, which binds double-stranded RNA and antagonizes several antiviral signaling pathways. The other protein is VP24, which binds transporter molecules to prevent STAT1 translocation. A more recent discovery is that VP24 also binds STAT1 directly, suggesting that VP24 may operate in at least two separate branches of the interferon pathway. New crystal structures of VP24 derived from pathogenic and nonpathogenic ebolaviruses reveal its novel, pyramidal fold, upon which can be mapped sites required for virulence and for STAT1 binding. These structures of VP24, and new information about its direct binding to STAT1, provide avenues by which we may explore its many roles in the viral life cycle, and reasons for differences in pathogenesis among the ebolaviruses.

In The Art of War (6th century BCE), Sun Tzu wrote, "It is the rule in war: if ten times the enemy’s strength, surround them; if five times, attack them; if double, be able to divide them; if equal, engage them; if fewer, be able to evade them; if weaker, be able to avoid them." The ebolaviruses are among the most lethal viruses known. Recent studies are revealing how these viruses encode multiple strategies to surround, attack, evade and/or avoid human immune defenses. Of course, the relative "strengths" of a virus and the host immune system vary by viral strain, host and environmental factors alike, and as a result, the interplay between these factors is multifactorial and complex.

The ebolaviruses and their cousins, the marburgviruses, are members of the filovirus family. These viruses are enveloped, non-segmented, negative-strand RNA viruses that cause severe hemorrhagic fever in both humans and nonhuman primates. Of the five antigenically distinct ebolaviruses, Ebola virus (EBOV; formerly known as Zaire ebolavirus) and Sudan virus (SUDV; formerly known as Sudan ebolavirus) are the most pathogenic, causing 50–90% lethality. Also among them, Reston virus (RESTV; formerly known as Reston ebolavirus) curiously is uniquely non-pathogenic to humans, although it is highly lethal to nonhuman primates. Microarray analyses performed on human cells suggest that RESTV has a reduced ability to suppress host cellular interferon (IFN) α/β and interferon (IFN) γ responses. IFNα/β and IFNγ responses play key roles in protection of host cells against foreign invaders. Early suppression of cellular IFN production and signaling by key viral proteins serves as a critical turning point in the course of disease. VP24 is one of two proteins of the ebolaviruses known to antagonize IFN responses; the other is VP35. VP24 inhibits signaling downstream of both IFNα/β and IFNγ, by trapping karyopherin α proteins (α1, α5 and α6) in the
cytoplasm. Binding to these proteins prevents them from shuttling otherwise activated, phosphorylated STAT1 to the nucleus. VP24 also prevents phosphorylation of p38 mitogen-activated protein (MAP) kinase, which thwarts the heterogonous nuclear ribonuclear protein complex C1/C2 (hnRNPs C1/C2, a complex primarily involved in host mRNA transcription) from relocating into the nucleus by karyopherin α proteins, and is required for assembly of the viral ribonucleoprotein complex. The second protein, VP35, blocks production of IFNα/β by binding dsRNA, a trademark of viral infection, and shielding it from recognition by host immune sensors such as RIG-I and MDA-5. VP35 also inhibits phosphorylation of IRF3/7 by interacting with the kinase domain of TBK-1/IKKε, suppresses secretion of tumor necrosis factor α, suppresses RNA silencing, and is similarly required for assembly and function of the viral ribonucleoprotein complex.

STAT1 belongs to the STAT family of transcription factors, plays key roles in immune signaling, and as a result, is a common target of viral proteins (for an excellent review, please see ref. 26). In a healthy cell, STAT1 predominately exists in an unphosphorylated form (U-STAT1). During viral infection, production of interferons and cytokines such as IFNα, IFNγ, tumor necrosis factor (TNF) α, IL-6 and IL-10 cause STAT1 to be phosphorylated (P-STAT1) by the Janus family kinases (JAKs). After activation by IFNα/β or IFNγ, P-STAT1 either forms a complex with STAT2 and IRF-9 or else P-STAT1 homodimerizes. Subsequently, these STAT1-containing complexes are transported to the nucleus by karyopherin α proteins where they regulate genes involved in the immune response. Certain proteins of the Nipah and Hendra viruses, measles virus, vaccinia virus, Japanese encephalitis virus and mumps virus each antagonize the STAT1 complex by directly interacting with STAT1 at one or multiple points along its signaling pathway. In contrast, the ebolavirus VP24 protein was shown to employ an alternative method and indirectly affect STAT1, by binding to karyopherin α1 and preventing it from translocating P-STAT1 to the nucleus.

Since multiple other viral proteins interact directly with STAT1 at several points along the STAT1 pathway, we wanted to know if VP24 could also bind STAT1 directly. We recently found that indeed, it does. By ELISA, we note that purified VP24 from EBOV and SUDV binds directly to purified STAT1 protein that is truncated prior to its phosphorylation site (STAT1Δ683). Here, in this manuscript, we show that purified VP24 from Reston virus (RESTV) also binds to STAT1 at levels roughly equivalent to VP24 of EBOV and SUDV (Fig. 1A).

VP24 has no significant sequence homology to any other known protein and the molecular structure(s) and mechanism(s) by which it suppresses immune signaling and contributes to RNP assembly were not well understood. In order to illuminate the structural basis for its action, we determined X-ray crystal structures of VP24 from two different species within the Ebolavirus genus: Sudan virus, which is pathogenic to humans, and Reston virus, which is not pathogenic to humans. Two versions of VP24 from SUDV were crystallized: SUDVΔ1-233 (which contains a ten-amino acid truncation from the N-terminus and a 18 amino acid truncation from the C-terminus to improve solubility) and subsequently SUDVΔ1-233 (which contains only the C-terminal 18 amino acid truncation) (Fig. 1B and C). One version of VP24 from RESTV was crystallized, RESTVΔ1-237. VP24 from EBOV did not produce diffraction quality crystals, but was used in accompanying functional studies.

VP24 from all three constructs is a single domain, αβ structure with an overall shape resembling a triangular pyramid (Fig. 1B and C). The three sides of the pyramid are termed Faces 1, 2 and 3. At the bottom of Faces 1 and 3 are located two pockets that are highly conserved across the filoviruses. The narrower of the two pockets is on Face 1 and is lined with a series of hydrophobic residues. The wider of the two pockets is on Face 3, is lined with polar residues and is shallower in depth. Although the functions of these conserved pockets remain elusive, they constitute tantalizing possibilities for anchor sites of the many viral and host binding partners of VP24.

Deuterium exchange mass spectrometry (DXMS), which measures the ability of polypeptide main-chain amide hydrogens to exchange hydrogen for solvent deuterium, allows mapping of protein footprints by identifying regions of a protein that exchange more slowly when complexed to a binding partner than when free. Using DXMS, we identified a putative area of interaction between VP24 and STAT1Δ683. Amino acids 96–98 and 106–121 of VP24 demonstrate slower H/D exchange kinetics when in complex with STAT-1, suggesting a site of protein–protein interaction. By contrast, amino acids 71–79 and 181–198 of VP24 demonstrate increased H/D exchange in the presence of STAT1Δ683, suggesting possible conformational change upon binding that results in enhanced flexibility of those regions. The slower-exchanging peptides, 96–98 and 106–121, are located at the top of the conserved portion of Face 3 (Fig. 2A and B). The faster exchanging peptides, 71–79 and 181–198, map to the polar cavity at the bottom of Face 3. All of the faster- and slower-exchanging peptides, but 113–121 and 182–187, are conserved among both the ebolaviruses and marburgviruses. Interestingly, these two sites are conserved among the ebolaviruses, but are different in the marburgviruses. They may be important sites as only VP24 from the ebolaviruses is immunosuppressive, not VP24 from the marburgviruses.

It is not yet clear if VP24 binds both phosphorylated STAT1 (P-STAT1) and unphosphorylated STAT1 (U-STAT1) in the context of viral infection (note that the purified STAT1Δ683 is unphosphorylated). However, binding and inhibition of either are likely important in immunosuppression. The significance of P-STAT1 to the immune response has been well established. U-STAT1 is also important in regulation of the immune response as well, but it functions in different ways than its phosphorylated counterpart. For example, U-STAT1 is transported into the nucleus by direct interaction with nucleoporins and does not require transporter proteins as does P-STAT1. Once inside the nucleus, U-STAT1 activates and prolongs the expression of a number of IFN-induced immune regulatory genes like IFI27, IFI44, OAS and BST2.
Since U-STAT1 functions independently from P-STAT1, the set of genes on which U-STAT1 operates can be distinct from those of P-STAT1. Further, U-STAT1 and P-STAT1 also differ temporally: the phosphorylation of STAT1 lasts for several hours but the presence of U-STAT1 persists for several days. In this way, U-STAT1 is likely to be able to prolong an antiviral state.

Hence, both P-STAT1 and U-STAT1 play multiple roles in antiviral defense and may play somewhat different roles in different cell types. By affecting both P-STAT1 (by binding karyopherins and/or possibly by forming a karyopherin-STAT1-VP24 tertiary complex) and U-STAT1 (if it binds full-length U-STAT1 as well as unphosphorylated STAT1), VP24 could prevent or dampen antiviral responses through multiple routes (Fig. 3).

The combination of both ebolavirus VP24 and VP35 in the viral armamentarium offers greater coverage of the different pathways by which antiviral responses occur. It is interesting to note that plasmocytoid dendritic cells (pDCs), which are major producers of type I interferon, are insensitive to VP35 inhibition. It is conceivable that VP35 and VP24 exert a synergistic effect and/or VP24 functions in cells like pDCs where VP35 does not. Nevertheless, in tandem, VP24 and VP35 offer better coverage of immune signaling pathways that would be achievable by either of the proteins alone (Fig. 3).

A remaining, fascinating question is why RESTV is nonpathogenic toward humans, when the remaining ebolavirus species are pathogenic. The answer may be complex, as multiple host and viral factors (such as VP24 and VP35) are likely involved in modulating pathogenicity and that RESTV itself exhibits a reduced ability to suppress host immune responses.

The importance of VP24 to virulence suggests that some clues to the Reston-pathogenicity puzzle might lie in key amino acid differences between Reston and the pathogenic ebolaviruses (Fig. 2C–E). In VP24, these sequence differences tend to either cluster around likely binding sites for karyopherin α1 or STAT1, or else, they lie in positions previously implicated in passage studies.

Figure 1. VP24 key features. (A) Purified EBOV1–233 VP24, SUDV1–233 VP24 and RESTV11–237 VP24 bind to purified STAT11–683 by ELISA. VP24s were coated onto the ELISA plate at 0.02 mg/ml for overnight incubation at room temperature. STAT11–683 was incubated on the plates, binding was detected with HRP-conjugated secondary antibody and the resulting O.D. was read at 450 nm. BSA was used as a negative control. (B and C) The overall shape of VP24 resembles a three-sided pyramid with Faces 1 and 3 as illustrated. A hydrophobic cavity in Face 1 and a polar cavity in Face 3 are indicated by arrows. A ribbon model of SUDV1–233 VP24 illustrates the underlying secondary structural elements, while a surface representation of SUDV1–233 VP24 indicates the sequence conservation. Navy indicates residues that are absolutely conserved among the ebola- and marburgviruses, while red indicates residues that are least conserved among these viruses. Face 2 is the least conserved of the three faces and is not illustrated. Sequence identity is calculated using Homolmapper. The accession codes for SUDV1–233, SUDV11–233 and RESTV11–237 are 3VNE, 3VNF and 4D9O, respectively.
that conferred EBOV lethality to rodents. For example, one cluster of Reston-specific residues in VP24 is L136, R139 and S140. This cluster is located next to the 142–146 loop, thought to be important for binding karyopherin α1 and to residue L147, for which mutation confers virulence in guinea pig models of Ebola virus infection. A second cluster of Reston-specific residues (L107 and T116) exists within the 106–121 polypeptide that exhibits decreased H/D exchange in the presence of STAT1–683. A third cluster of Reston-specific residues (S184 and T185) lies in the 181–198 polypeptide that exhibits increased H/D exchange in the presence of STAT1–683. Note that this cluster includes residues H186 and T187 for which mutation confers virulence of EBOV in guinea pig models. A fourth site is the Reston-specific residue S50, which was also previously implicated in a different serial passage study to confer lethality of Ebola virus to mice. It is not known which site(s) is/are responsible for the unique Reston virus phenotype, or even if these sites lie in VP24 or in VP24...
alone. Note, however, that VP24 is a critical and common site for lethality-conferring mutations found in passage studies, and that as few as two or three amino acid changes is enough to confer lethality of Ebola virus to rodent models. Hence, these structures of RESTV and SUDV VP24 provide a three-dimensional framework by which we may begin to dissect differences between them.

New discoveries on the structure and function of VP24 have now illuminated a possible additional mechanism by which this protein suppresses the innate immune system in virus infection. This pathway is added to the list of other known anti-immune functions of VP24 and VP35, and suggests that these proteins likely work as a team to coordinate parallel schemes of immune silencing. Sun Tzu also wrote, “When the enemy has made a plan of attack against us, we must anticipate him by delivering our own attack first.” It is clear that pathogens like the ebolaviruses have developed a coordinated, multipronged strategy to defeat host immune molecules as or before they launch.

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