The Ebola Virus Interferon Antagonist VP24 Directly Binds STAT1 and Has a Novel, Pyramidal Fold

Adrianna P. P. Zhang1, Zachary A. Bornholdt1, Tong Liu2, Dafna M. Abelson1, David E. Lee2, Sheng Li2, Virgil L. Woods, Jr.2, Erica Ollmann Saphire1,3

1 Department of Immunology and Microbial Science, The Scripps Research Institute, La Jolla, California, United States of America, 2 Department of Medicine and Biomedical Sciences Graduate Program, University of California at San Diego, La Jolla, California, United States of America, 3 The Skaggs Institute for Chemical Biology, The Scripps Research Institute, La Jolla, California, United States of America

Abstract

Ebolaviruses cause hemorrhagic fever with up to 90% lethality and in fatal cases, are characterized by early suppression of the host innate immune system. One of the proteins likely responsible for this effect is VP24. VP24 is known to antagonize interferon signaling by binding host karyopherin α proteins, thereby preventing them from transporting the tyrosine-phosphorylated transcription factor STAT1 to the nucleus. Here, we report that VP24 binds STAT1 directly, suggesting that VP24 can suppress at least two distinct branches of the interferon pathway. Here, we also report the first crystal structures of VP24, derived from different species of ebolavirus that are pathogenic (Sudan) and nonpathogenic to humans (Reston). These structures reveal that VP24 has a novel, pyramidal fold. A site on a particular face of the pyramid exhibits reduced solvent exchange when in complex with STAT1. This site is above two highly conserved pockets in VP24 that contain key residues previously implicated in virulence. These crystal structures and accompanying biochemical analysis map differences between pathogenic and nonpathogenic viruses, offer templates for drug design, and provide the three-dimensional framework necessary for biological dissection of the many functions of VP24 in the virus life cycle.

Introduction

The ebolaviruses and marburgviruses are enveloped, non-segmented, negative-strand RNA viruses that belong to the family Filoviridae. There are five antigenically distinct ebolaviruses that have been identified: Zaire (now known simply as Ebola virus or EBOV), Sudan virus (SUDV), Tai Forest virus (TAFV), Reston virus (RESTV) and Bundibugyo virus (BDBV). Marburgviruses and most ebolaviruses cause severe hemorrhagic fever in both humans and nonhuman primates, with fatality up to 90%. The exception is RESTV, which appears to be non-pathogenic in humans, and nonhuman primates, with fatality up to 40% different in amino acid sequence, and are each named after the location of the outbreak during which they were first identified: Zaire (now known simply as Ebola virus or EBOV), Sudan virus (SUDV), Tai Forest virus (TAFV), Reston virus (RESTV) and Bundibugyo virus (BDBV). Marburgviruses and most ebolaviruses cause severe hemorrhagic fever in both humans and nonhuman primates, with fatality up to 90%. The exception is RESTV, which appears to be non-pathogenic in humans, although it remains pathogenic to non-human primates [1,2]. Reasons why RESTV has not caused disease in humans is unclear. However, microarray analyses have shown that RESTV has a reduced ability to suppress host immune responses [3].

For the pathogenic ebolaviruses, early suppression of host interferon (IFN) production and signaling plays a decisive factor in disease outcome [4,5]. Two proteins of the ebolaviruses are used in this strike. The protein VP35 blocks production of IFN-α/β [6] by binding dsRNA, a key hallmark of viral infection, and shielding it from recognition by host immune sensors such as RIG-I and MDA-5 [7,8]. By contrast, the protein VP24 inhibits signaling downstream of both IFN-α/β and IFN-γ by sequestering karyopherin α proteins (α1, α5 and α6) [9]. Binding to these proteins prevents them from shuttling otherwise activated, phosphorylated STAT1 to the nucleus [9–11].

STAT1 belongs to the STAT family of transcription factors, is a key mediator of the IFN response pathway [12–14] and plays an essential role in the immune response to viruses [15–17]. STAT1 is a transmembrane receptor whose intracellular domain is a key mediator of the IFN response pathway and plays an essential role in the immune response to viruses [15–17]. STAT1 is a transmembrane receptor whose intracellular domain is a key mediator of the IFN response pathway and plays an essential role in the immune response to viruses [15–17].

The importance of STAT1 to the antiviral response is underlined by the fact that viruses (and other microbes) have evolved proteins that inhibit every step of STAT1 activation [14]. As examples, the V proteins of Nipah and Hendra viruses and the P protein of rabies virus directly bind to P-STAT1 to sequester it in the cytoplasm [34–36]. By contrast, the P protein of measles virus and an unidentified protein of human metapneumovirus...
Ebolaviruses cause severe hemorrhagic fever that is exacerbated by immediate suppression of host immune function. VP24, one of only eight proteins encoded by ebolaviruses, functions in virus replication and assembly, and is thought to contribute to immune suppression by binding to a certain class of molecules called karyopherins to prevent them from transporting a transcription factor termed STAT1. Here we report that VP24 is also able to directly bind STAT1 by itself, and thereby likely contributes to immune suppression by an additional mechanism. Analysis of these multiple roles of VP24 and design of drugs against them have been hindered by the lack of structural information on VP24 and its lack of homology to any other known protein. Hence, here we also present X-ray structures of VP24 derived from two different ebolavirus species that are pathogenic and nonpathogenic to humans. These structures and accompanying deuterium exchange mass spectrometry identify the likely binding site of STAT1 onto VP24, map sites that are conserved or differ between pathogenic and nonpathogenic species, and provide the critical 3D templates by which we may dissect and interpret the many roles that VP24 plays in the virus life cycle.

In addition to its role in interferon antagonism, ebolavirus VP24 has also been proposed to associate with membranes [42, 43], and is important for assembly and function of the viral ribonucleoprotein complex (RNP) [44–46], where VP24 binds to the viral nucleoprotein NP [45]. VP24 has no sequence homology to any known protein and the molecular mechanisms by which VP24 suppresses immune signaling and contributes to RNP assembly are poorly understood.

Here, we demonstrate that VP24 also binds directly to STAT1 itself, present the first X-ray crystal structures of VP24 from both Sudan and Reston viruses, and map a possible site of VP24 interaction on the VP24 crystal structure by deuterium exchange mass spectrometry (DXMS). The biochemical and structural analysis presented here identifies a new function by which VP24 may contribute to and/or prolong innate immunosuppression, and provides the necessary three-dimensional templates for understanding the multiple roles of VP24 in the ebolavirus life cycle and design of antiviral compounds against them.

Results

VP24 binds purified STAT1

Other negative-sense viruses encode proteins that suppress innate immune signaling by direct interaction with STAT1. VP24 was previously known to indirectly affect STAT1 by binding karyopherins to prevent them from transporting phosphorylated STAT1 (P-STAT1). However, we wondered if VP24 could play a more direct role as well. To answer this question, we performed an ELISA to test binding of purified Ebola virus VP24 or Sudan virus VP24 to purified STAT11–683 (truncated prior to its phosphorylation site at Tyr701). BSA was used as a negative control. Indeed, VP24 is able directly associate with STAT11–683 (Figure 1).

![VP24 binding to STAT1](image)

**Figure 1.** Purified truncated VP24s, SUDV1–233 and EBOV1–233, were determined to bind to purified STAT11–683 using an ELISA assay. Either SUDV1–233 or EBOV1–233 was coated onto the ELISA plate at 0.01 mg/ml as described in the Materials and methods section. Upon subsequent incubation with STAT11–683, binding was detected with HRP conjugated secondary antibody and O.D. was read at 450 nm. BSA was used as a negative control. doi:10.1371/journal.ppat.1002550.g001
binds STAT1–683 suggests an additional, unexplored way by
which VP24 might contribute to innate immune suppression.

VP24 adopts a novel “pyramidal” fold
In order to provide 3D templates for understanding VP24 and
its many roles in immune evasion, replication and assembly, we
crystallized VP24 from Sudan virus (two versions crystallized:
SUDV1–233 and SUDV11–233) and Reston virus (one version
crystallized: RESTV11–237) (Figure S1a). We determined the
structure of SUDV11–233 at 2.1 Å resolution by multiwavelength
anomalous diffraction (MAD) using selenomethionine-incorpo-
rated protein expressed recombinantly in E. coli. We subse-
quently determined structures of SUDV1–233 and RESTV11–237
by molecular replacement, both at 2.0 Å resolution (Table 1).

VP24 adopts a compact, single domain, α/β structure of novel
fold (DaliLite v.3 [47]). The overall shape of VP24 resembles a
triangular pyramid of dimensions 73 Å x 630 Å x 630 Å. The three
faces of the pyramid are numbered 1, 2 and 3 (Figures 2 and S1b).
A collection of α helices (α1 and α5-10) and a small, three-
stranded, antiparallel β sheet (β1-3) form the top of the pyramid
with the N-terminus at the apex. A five-stranded antiparallel
β sheet (β4-8) forms the center, while a second collection of α helices
(α2-4) forms the base. Portions of the C-terminal region resemble
prior de novo predictions: as predicted, helices 5–8 are indeed
observed, helix 8 is quite long, and a β sheet exists at the base of
the structure. Differences between the prediction and the
experimental structure are that a three-stranded sheet was
predicted, but a five-stranded sheet is observed [48] and that an
armadillo repeat-type domain structure was predicted, but no such
domain is observed in VP24.

Two adjacent, conserved pockets
VP24 is 63% identical among ebolaviruses and ~30% identical
between ebola- and marburgviruses. Regions of high sequence
conservation congregate on Faces 1 and 3 (Figures 3 and S2a). The
conserved center of Face 1 is formed by α5, β3, β5 and β8. The
conserved center of Face 3 is formed by α5, α6, β5 and β6. The base
of each of Faces 1 and 3 also contains a conserved cavity, and the two
cavities are located adjacent to each other on the protein surface.

The Face 1 cavity is hydrophobic and is 14 x 14 x 12 Å in size
(Figure 4a). The interior of the cavity is lined with five absolutely
conserved leucine residues: L57, L75, L79, L198, and L221. The
entrance to the hydrophobic cavity (11 Å wide) appears to be
gated by two residues (Y172 and M71) that point away from each
other in RESTV VP24 but toward each other in SUDV VP24,
appearing to block the hydrophobic cavity (Figure S3a–S3b).

Table 1. Data collection and refinement statistics.

| Crystals         | SUDV11–233 (Native) | SUDV11–233 (Sepeak) | SUDV11–233 (Seinflection) | SUDV1–233 (Native) | RESTV11–237 (Native) |
|------------------|---------------------|---------------------|---------------------------|-------------------|---------------------|
| **Data collection** |                     |                     |                           |                   |                     |
| Space group      | P3,21               | P3,21               | P3,21                     | P3,21             | P12,1               |
| Cell dimensions: |                     |                     |                           |                   |                     |
| a, b, c (Å)      | 61.1, 61.1, 126.8   | 61.2, 61.2, 106.9   | 61.3, 61.3, 106.7         | 61.1, 61.1, 130.4 | 38.4, 103.9, 59.8   |
| α, β, γ (°)      | 90, 90, 120         | 90, 90, 120         | 90, 90, 120               | 90, 90, 120       | 90, 94, 90          |
| Resolution (Å)   | 50–2.1              | 50–2.2              | 50–2.3                    | 50–2.0            | 50–2.0              |
| Solvent content (%) | 63                  | 63                  | 63                        | 63                | 63                  |
| Rmerge (%)       | 0.075               | 0.065               | 0.095                     | 0.068             | 0.046               |
| (I/σI)b         | 8.3 (1.6)           | 9.5 (2.3)           | 6.0 (1.8)                 | 8.4 (1.3)         | 21 (1.9)            |
| Completeness (%) | 95.8                | 99.6                | 100.0                     | 99.6              | 98.7                |
| Redundancy       | 8.3                 | 5.7                 | 5.7                       | 10.5              | 3.0                 |
| **Refinement**   |                     |                     |                           |                   |                     |
| Resolution (Å)   | 33–2.1              | 34–2.0              | 31–2.0                    |                   |                     |
| No. reflections  | 16550               | 18099               | 29379                     |                   |                     |
| Rmerge/Rfree (%) | 23.4/27.0           | 22.0/26.3           | 18.6/22.7                 |                   |                     |
| No. of atoms:    | Protein: 1592       | 1727                | 3166                      |                   |                     |
|                  | Water: 46           | 78                  | 110                       |                   |                     |
| R.m.s deviations | Bond lengths (Å)    | 0.014               | 0.015                     | 0.013             |                     |
|                  | Bond angles (°)     | 1.102               | 1.106                     | 1.047             |                     |
| Ramachandran plotc | Most Favored        | 93.1                | 92.7                      | 92.3              |                     |
|                  | Additionally Allowed| 6.9                 | 7.3                       | 7.4               |                     |
|                  | Generously Allowed  | 0.0                 | 0.0                       | 0.3               |                     |
|                  | Disallowed          | 0.0                 | 0.0                       | 0.0               |                     |

*R linear = ∑(|I−<I>|/I) ∑(I).
1Values in parentheses refer to the last shell.
2As defined in MolProbity.

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The Face 3 cavity is shallower than that of Face 1 (18 × 14 × 5 Å), and is hydrophilic rather than hydrophobic (Figure 4b). Five residues that are conserved across all filoviruses (S178, E180, I189, T191, and E200) populate the base of the cavity. Six conserved residues circle the rim (P77, T193, K206, and M209 are conserved across all ebolaviruses; H78 and N82 are conserved across all

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**Figure 2. Alternate views of VP24 secondary structure.** The overall shape of VP24 resembles a three-sided pyramid with Faces 1 (a), 2 (b), and 3 (c) as illustrated. Only SUDV1–233 is shown for clarity. Arrows indicate conserved pockets on Faces 1 and 3.

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**Figure 3. Conservation map of VP24.** (a) Sequence conservation in VP24 among Ebola (Zaire), Sudan, Reston, Tai Forest, and Bundibugyo viruses mapped onto the structure of SUDV1–233. (b) Sequence conservation in VP24 between ebola- and marburgviruses. Sequence conservation is mapped as navy (completely conserved) to red (least conserved). A hydrophobic cavity in Face 1 and a polar cavity in Face 3 are indicated by arrows. Least conserved regions are clustered around Face 2. Sequence identity is calculated using Homolmapper [83]. Figures are illustrated using SUDV1–233.

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filoviruses). Also, three residues conserved across the filoviruses (L75, F76, and L198) line the edge between the Face 1 and Face 3 cavities and are accessible from either side.

Serial passage studies to confer lethality of EBOV to rodents resulted in five mutations in VP24 (H186Y, T187I, M71I, L147P [49], and T50I [50]), four of which lie in or near these cavities. H186 (Y186 in SUDV), T187 (A187 in SUDV) and T50 (N50 in SUDV and S50 in RESTV) lie on the rim of the Face 3 cavity (Figure 4c). M71 forms the “gate” to the Face 1 cavity. The fifth residue, L147 (M147 in SUDV), is located toward the top of the pyramid in helix α6, and is accessible from Face 3. L147 is thought to be involved in karyopherin α1 binding (Figure 4c).

Residues implicated in karyopherin α1 binding

In infected cells, EBOV VP24 binds to karyopherin α1, α5, and α6 to prevent translocation of P-STAT1 into the nucleus [10]. Previous mutagenesis studies have shown VP24 residues W42 and 142–146 to be critical for karyopherin α1 binding [11]. W42 is buried in the interior of the single globular VP24 fold. Hence, mutagenesis of W42 most likely compromised the VP24 structure and affected karyopherin α1 affinity indirectly (Figure 4c). By contrast, residues 142–146 are exposed to solvent and would be available to directly bind karyopherin α1. As previously described, an L147P mutation (β6, adjacent to residues 142–146) in EBOV VP24 increases virulence in guinea pigs [49].

Unlike the ebolaviruses, the VP24 protein of Marburg virus does not block interaction of P-STAT1 with karyopherin α1 [51]. W42 is conserved between ebola- and marburgviruses, but residues 142–147 are not. Residues 142–147 are K_{D/DQLS} in the ebolaviruses but are GIYLT8 in the marburgviruses (Figure S2a).

Interaction with STAT1_{1–683}

Deuterium exchange mass spectrometry (DXMS) is able to rapidly map footprints of protein-protein binding sites and offers a broader picture than analysis of point mutants alone [52–56]. In this method the ability of peptide amide hydrogens to freely and reversibly exchange with solvent deuterium is measured. Hydrogens for which mobility is restricted (by conformational anchoring and/or ligand binding) exchange more slowly. Hydrogens for which mobility is unrestricted (conformational mobility) exchange more rapidly. We performed comparative DXMS studies on VP24 alone and VP24 in complex with STAT1_{1–683}. The resulting exchange maps identify some peptidic regions of VP24 that exchange with solvent less rapidly when in complex with STAT1 (possible binding sites), and other regions of VP24 that exchange with solvent more rapidly when in complex with STAT1, perhaps due to conformational change and increased mobility.

In the presence of purified STAT1_{1–683}, VP24 peptidic regions 96–98 and 106–121 demonstrate slower H/D exchange kinetics, suggesting a site of protein-protein interaction (Figure 5). By contrast, VP24 peptidic regions 71–79 and 181–198 demonstrated increased H/D exchange in the presence of STAT1_{1–683}, suggesting possible conformational change with enhanced flexibility. The faster exchanging peptides 71–79 and 181–198 map to helix α3–4 and strands β5–7, respectively. Both of these secondary structure elements exist in the polar cavity at the bottom of Face 3 and are highly conserved across the filoviruses. The slower-exchanging peptides 96–98 and 106–121 map to helices α5 and α6, also on the conserved portion of Face 3. Helices α5 and α6 are amphipathic in nature: the hydrophobic side of each coil points into the core of VP24. Hydrophilic residues extend to the surface of Face 3. The C-terminal region of α5 is negatively charged and the N-terminal portion of α6 is positively charged.

Sites in VP24 that differ between ebolaviruses that are pathogenic and nonpathogenic to humans

Mapping of sequence differences between RESTV and the major pathogenic ebolaviruses (SUDV and EBOV) onto the RESTV_{11–237} structure indicates that RESTV VP24 differs in ~30 sites (Figure S4). One of these sites, a cluster of residues L136, R139 and S140 (in RESTV), is next to the 142–146 loop, which is important for binding karyopherin α1 [11] (Figure S4 and Figure...
### Structures of the Ebolavirus Protein VP24

#### Figure a

[Image of protein structure]

#### Figure b

[Image of protein structure]

#### Figure c

| SUDV<sub>1-233</sub> |
|------------------|
| 1 21 61          |
| MACATGRYNLVDFKRELQGTVFSDLNLFVTVPQ@WKNVYVGLEFIVNQKGTLLNLKVNDAPAMAMTRNLFPFLF |
| 81 101 121 141   |
| KNQQSEVQTPILRALRILAGILDMGSLSEPLGALNNILADMLLTSTNHFNMRTQRVLDQSMRMLSLIRSN1NIF |
| 161 181 201 221   |
| INKLETLHVLVNYKLSVEEQTPSYATIIIRTRTNMYGLVEVQEPDKSAMDIRHPGVFSSLLHSTLKPVATS |

#### Figure d

| SUDV<sub>1-233</sub> in complex with STAT1<sub>1-463</sub> |
|------------------|
| 1 21 61          |
| MACATGRYNLVDFKRELQGTVFSDLNLFVTVPQ@WKNVYVGLEFIVNQKGTLLNLKVNDAPAMAMTRNLFPFLF |
| 81 101 121 141   |
| KNQQSEVQTPILRALRILAGILDMGSLSEPLGALNNILADMLLTSTNHFNMRTQRVLDQSMRMLSLIRSN1NIF |
| 161 181 201 221   |
| INKLETLHVLVNYKLSVEEQTPSYATIIIRTRTNMYGLVEVQEPDKSAMDIRHPGVFSSLLHSTLKPVATS |

#### Deuteration level

- 10%
- 50%
- 90%
S2a for sequence alignment). A second site is the cluster of residues L107, H109, T116 and G120 that exists within the 106–121 polypeptide that exhibits decreased H/D exchange in complex with STAT11–683 and may serve as a STAT1 binding site. A third site, the cluster of residues S184, T185, H186, T187 and F197, lies in the 181–198 polypeptide that undergoes enhanced H/D exchange in the presence of STAT11–683. A fourth site, V201, lies next to this region. A fifth site, residue S50, was previously implicated in a serial passage study to confer lethality to mice [50].

Purified VP24 is monomeric

Previous studies analyzing VP24 in the context of whole cell lysate found that the majority of VP24 was monomeric. A smaller portion appeared as a high molecular weight aggregate and a smaller oligomer, likely a tetramer [42,43]. We performed gel filtration analysis of purified, full-length SUDV, RESTV and EBOV VP24 [whether produced in E. coli or 293T cells] and find that purified VP24 from all three viruses is monomeric in solution (Figure S5). The significance of the multimodified portion observed in cell lysate is unclear. Perhaps a portion of VP24 homooligomerizes in cells, or perhaps factors present in whole-cell lysate are needed for VP24 to form oligomers.

Crystal packing can sometimes illustrate biologically relevant assemblies, but no tetrameric or other higher oligomeric interactions are observed in crystals of SUDV or RESTV VP24. One pairwise VP24–VP24 interaction is observed in the crystal packing between the SUDV (P321) and RESTV VP24 (P121) structures, although it is currently unclear if it is biologically relevant (Figure S3c). This interaction involves z1, b1–3 and the N-terminal region of z6, and buries residue L147 that was previously implicated for virulence [49], although 142–146 remain solvent exposed. Another crystal lattice interaction observed in both RESTV and SUDV structures involves packing of the hydrophobic N-terminal regions of VP24 into the Face 3 pocket of a neighboring molecule (Figure S3d–S3c).

Discussion

The crystal structures presented here illustrate the novel, pyramidal fold of ebolavirus VP24. In this work, we have also identified STAT1 as a new binding partner of VP24 and have used DXMS to suggest that residues 96–98 and 106–121 are contained in a putative binding site for STAT11–683.

Although VP24 differs by 37% in protein sequence among the ebolaviruses, there are large patches of complete conservation on Faces 1 and 3 including the two pockets in these faces at the base of the pyramid. Several residues, found in serial passage studies to increase virulence of Ebola virus, map to these sites, although the precise role of the conserved pockets remains unclear. Another residue identified in these studies maps to a site thought to be involved in binding host karyopherin z1 proteins. The putative STAT1-binding site identified by DXMS lies in the conserved region of Face 3 and is distinct from the site proposed to interact with karyopherin z1.

Crystal structures presented here include VP24 from an ebolavirus that is pathogenic to humans (Sudan virus; SUDV) and VP24 from an ebolavirus that thus far, appears nonpathogenic to humans (Reston virus; RESTV), although it is lethal to nonhuman primates. The overall folds of SUDV and RESTV VP24 are similar, as expected (r.m.s.d. of 0.81 Å; also see Figure S2b for structural alignment). Specific viral or host factors responsible for the differences in pathogenicity between these viruses have not yet been identified, but it has been proposed that RESTV has a diminished ability to suppress cellular IFN-α/β and IFN-γ responses [3]. Residues in VP24 that are unique to RESTV often colocalize with residues that appear to be important for karyopherin and STAT1 binding, or are important for virulence in rodents through an unknown mechanism. The location of these RESTV-specific amino acids invites speculation that RESTV VP24 and EBOV/SUDV VP24 could potentially bind immune factors like karyopherins and STAT1 with differing affinity.

Here we have found that purified VP24 binds directly to purified STAT1 truncated prior to its phosphorylation site. In a healthy cell, STAT1 exists in an unphosphorylated form. During viral infection, production of interferons and cytokines leads to phosphorylation and homodimerization of STAT1 or heterodimerization of STAT1 with its β isofrom. The resulting P-STAT1 dimer is then transported by karyopherin z proteins into the nucleus where it controls transcription-regulated genes. Interestingly, this P-STAT1 may have a different oligomeric structure than U-STAT1 [57,58].

U-STAT1 is not inactive, but rather, is also important in regulation of the immune response. Interestingly, U-STAT1 functions in different ways than its phosphorylated counterpart. U-STAT1 is transported into the nucleus [59,60] by direct involvement with nucleoporins [61], and does not need transport by karyopherins. In the nucleus, U-STAT1 activates and prolongs the expression of a number of IFN-induced immune regulatory genes like IFIT2, IFIH4, OAS, and BST2 [62]. U-STAT1 functions independently of P-STAT1 and the set of genes on which it operates can be distinct from those of P-STAT1 [59]. 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 [62,63]. 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 STAT11–683), VP24 could prevent or dampen antiviral responses through multiple routes. The combination of both ebolavirus VP24 and ebolavirus VP55 (which acts upon virally induced dsRNA) in the infected cell offers greater coverage of the different pathways by which antiviral responses occur. Interestingly, plasmacytoid dendritic cells (pDCs), which are major producers of type I interferon [64],...
are insensitive to VP35 inhibition [65]. Perhaps VP35 and VP24 exert a synergistic effect, and/or VP24 functions in cells where VP35 does not.

Although VP24 is key to the virulence of ebolaviruses, little is known about it due, in part, to the lack of any structural information on VP24 and the lack of any homology to other known proteins. We have shown that purified VP24 and purified STAT1 interact. The functional manifestation of this interaction remains to be determined. Does VP24 target STAT1 for degradation, sequester it in the cytoplasm or in high-molecular weight complexes, or prevent its phosphorylation? Does VP24 bind P-STAT1, and does it exhibit a preference for one form over the other? Does VP24 bind other STATs in addition to STAT1? Intriguingly, STAT3 shares about 72% sequence homology with STAT1 [66], and operates in intestinal epithelia where it regulates mucosal wound healing [67]. Inactivation of STAT3 may contribute to colitis and clinical manifestations of Ebola virus infection like abdominal pain and bloody stools [68,69]. Another question is if any of the mapped differences between RESTV and EBOV/SUDV VP24 are linked to or are responsible for the differences in pathogenicity in humans. The structures presented here provide a framework for answering these and other questions about the multiple roles of VP24 in the viral lifecycle. These structures also provide the much-needed templates for design of antiviral drugs to inhibit key functions of VP24 in transcription, replication, and immunosuppression.

**Materials and Methods**

**VP24 expression and purification**

VP24 from Sudan virus (SUDV1-233 and SUDV11-235 in pET46 Ek/LIC vector) was expressed in *E. coli* Rosetta-gami 2(DE3)pLysS cells. Truncation of the C terminus permitted enhanced solubility, and a Superdex 200 column was used for size exclusion. Addition of 2.5 mM CHAPS throughout the purification was then centrifuged for 50 minutes at 16,000 r.p.m. in a JA-17 rotor (Beckman Coulter). The supernatant was loaded on a HisTrap FF crude column (GE Healthcare) with a gradient of 0.1 M HEPES, pH 7.0, 6% MPD, and 14% (w/v) D-(+)-sacrose. All SUDV VP24 proteins contain a valine to alanine substitution at position 22 from the GenBank deposited sequence.

**STAT1 expression and purification**

Truncated, unphosphorylated STAT11-683 (human) in a pET46 Ek/LIC vector was expressed in *E. coli* Rosetta-gami 2(DE3)pLysS cells. Cultures were grown in LB medium supplemented with ampicillin (100 µg ml⁻¹), and expression was induced by the addition of 0.5 mM IPTG at 16°C. Harvested cells from overnight induction were resuspended in lysis buffer (50 mM NaH₂PO₄, pH 8.0, 0.3 M NaCl, 10 mM imidazole) for lysis at 25,000 psi using a Microfluidizer processor. The lysed mixture was then centrifuged for 50 minutes at 16,000 r.p.m. in a JA-17 rotor (Beckman Coulter). The supernatant was loaded on a HisTrap FF crude column (GE Healthcare) with a step gradient of 30 mM and 500 mM imidazole in lysis buffer. SUDV1-233 and SUDV11-235 VP24 were further purified by size exclusion on a HiLoad 16/60 Superdex 75 prep grade column (Amersham Pharmaica) in 10 mM Tris-HCl, pH 8.0, 0.3 M NaCl.

Full-length SUDV VP24 was expressed and purified essentially as above. Addition of 2.5 mM CHAPS throughout the purification enhanced solubility, and a Superdex 200 column was used for size exclusion.

Selenomethionine-incorporated SUDV11-235 was expressed and purified as follows: 2 ml of an overnight culture in LB broth was transferred into 20 mL LB containing 0.4% glycerol and 100 µg ml⁻¹ ampicillin. After a one-hour incubation, the cells were harvested by centrifugation at 3000 r.p.m. and resuspended in 20 mL M9 minimal media, then transferred into 1 L M9 media containing ampicillin. At OD₆₀₀ 0.4, L-isoleucine, L-leucine, L-lysine, L-phenylalanine, L-threonine, and L-valine were added to final concentrations of 100 mg/L each, prior to addition of L-selenomethionine (to 60 mg/L). The culture was induced with IPTG after 15 min. Cells were harvested after 4 hr and purified as described above.

Full-length SUDV (in pTriEx 5 vector (Novagen)) was also transiently expressed in mammalian HEK293T cells in a five-layer CellStack (corning). The cells were transfected at 60% confluency with 420 µg of DNA and 1.2 mg of PEI diluted in 42 ml of PBS. The PEI mixture was incubated at room temperature for 20 min, before adding to the cells. After 48 hours, cells were freeze-thawed three times and lysed in 10 mM Tris-HCl, pH 8.0, 0.3 M NaCl. Protein was affinity purified with strep-tactin superflow beads (Qagen), then further purified by size exclusion on a Superdex 200 10/300 GL (GE Healthcare). 10 µl of the peak fraction was run on SDS-PAGE, and probed by Western blot with an anti-strep antibody.

All SUDV VP24 proteins contain a valine to alanine substitution at position 22 from the GenBank deposited sequence. Residue 22 is on helix z1 and is buried within the structure (Figure S6). The Sudan virus (strain Boniface) replicon was a gift of Dr. John M. Dye (USAMRIID). Oligonucleotides were purchased from Valuegene Inc.

Full-length Ebola virus (Zaire, EBOV) VP24 and both full-length and N- and C-terminally truncated Reston (RESTV) virus VP24 were expressed and purified in *E. coli* as previously described. Full-length EBOV VP24 was cloned into the pET46 Ek/LIC vector from cDNA that was a gift from Dr. Viktor Volchkov (Claude Bernard Université de Lyon 1). cDNA for RESTV VP24 was synthesized by GenScript (Piscataway, NJ). Both full length RESTV and RESTV11-237 were subcloned into pET46 Ek/LIC for expression.
Laboratory), and were processed either with HKL2000 [70] or d*Trek [71] (Table 1).

Structure determination and refinement
Experimental phases for SUDV1-233 were generated by MAD (multiwavelength anomalous diffraction) using Auto-Rickshaw [72]. Five of the seven internal SeMet residues were located and their locations were verified by hand through a difference Fourier anomalous electron-density map. Using the experimentally phased map, the orientations of two helices were determined in the initial partial model and the rest of the model was built using the MRSAD (molecular replacement with single-wavelength anomalous diffraction [73]) method in Auto-Rickshaw [72]. The structure of SUDV1-233 was determined by molecular replacement (also Auto-Rickshaw [72]) using SUDV11-233 as the search model. Refinement of both structures was performed with Phenix.refine [74] in PHENIX [75] and rebuilding was carried out in COOT [76]. Final rounds of refinement included TLS parameters [77] for SUDV1-233 and SUDV11-233. The quality of the structures was validated with MolProbity [78] and Procheck [79]. 92.7% (SUDV1-233) and 93.1% (SUDV11-233) of residues are in the most favored region of Ramachandran plots, with no residues in the disallowed regions. The final model of SUDV1-233 contains residues 9-106 and 113-232 with residues 115 and 210-213 replaced with alanines. The final model of SUDV11-233 contains residues 13-61, 71-107, 114-209, and 212-228. Residue 209 was replaced with alanine.

RESTV11-237 was determined by molecular replacement in Auto-Rickshaw [72] using SUDV11-233 as the initial search model. The structure was refined with Phenix.refine [74] in PHENIX [75] and rebuilt in COOT [76]. Separate NCS restraints and TLS parameters [77] over the two molecules of VP24 in the asymmetric unit were used during initial refinement. The quality of the structure was validated with MolProbity [78] and Procheck [79]. 92.3% of residues are in the most favored region of Ramachandran plots and no residues are in the disallowed regions. The final model of SUDV11-233 contains residues 11-62, 70-203, and 212-228 in molecule A and residues 15-61, 70-108, 113-203, and 216-231 in molecule B. Two residues (203 and 216) in molecule B were replaced with alanines. Figures were created using PyMol [80] (Delano Scientific).

Atomic coordinates and structure factors have been deposited in the Protein Data Bank under the accession codes 3VNE, 3VNF, 4D9O for SUDV1-233, SUDV11-233, and RESTV11-237, respectively.

ELISA
50 μl of each VP24 (SUDV1-233 and EBOV1-233) was bound to ELISA plates (Corning Costar 3690) at 0.01 mg/ml in 10 mM Tris-HCl, pH 8.0, 0.3 M NaCl, overnight at 4°C. Plates were then blocked for one hour at room temperature with 3% BSA. After washing with PBS containing 0.05% Tween 20, 50 μl of STAT1-6xHis with a C-terminal HA-tag was added at 0.03 mg/ml and allowed to bind for two hours at room temperature. Plates were then washed, 50 μl of anti-HA (Covance) were incubated for 2.7 and cooling to 0°C or below, conditions that dramatically slow further exchange and loss of deuterium label from the protein even when the protein structure is subsequently disrupted. The site and amount of deuterium that exchanged onto the protein are quantified (under continued quench conditions) by rapid denaturation, optional disulfide-reduction and digestion by solid-phase pepsin into overlapping fragments of ~3–15 amino acids. The perturbed masses of the resulting peptides, and therefore their deuterium content, are quantified by liquid chromatography-mass spectrometry.

Prior to the deuteration studies, quench conditions that produced an optimal pepsin fragmentation pattern were established as previously described [52,53,55,56]. For SUDV VP24 (10 mg/ml stock solution) and SUDV VP24-STAT1 (12 mg/ml stock solution), functional deamination of proteins was performed by mixing 1 μl of stock solution with 1 μl of H2O buffer (8.3 mM Tris, 150 mM NaCl, in H2O, pH 7.2) and then diluted into 6 μl of D2O buffer (8.3 mM Tris, 150 mM NaCl, in D2O, pH 7.2) at 0°C. At 10 s, 100 s and 1000 s, the deuterium exchange was quenched by adding 12 μl of optimized quench (1.6 M GuHCl, 0.8% formic acid, 16.6% glycerol) and then samples were frozen at −80°C. In addition, nondeuterated samples (incubated in H2O buffer mentioned above) and equilibrium-deuterated samples (incubated in D2O buffer containing 0.5% formic acid overnight at 25°C) were prepared. The samples were later thawed at 5°C and passed over an AL-20-pepsin column (16 μM bed volume (Sigma)) at a flow rate of 20 μl/min [81]. The resulting peptides were collected on a C18 trap (Michrom MAGIC C18AQ 0.2 x 2) and separated using a C18 reversed phase column (Michrom MAGIC C18AQ 0.2 x 50) running a linear gradient of 8–48% solvent B (30% acetonitrile and 0.01% TFA) over 30 minutes with column eluent directed into an LCQ mass spectrometer (Thermo Finnigan LCQ Classic). Data were acquired in both data-dependent MS1:MS2 mode and MS1 profile mode.

SEQUEST software (Thermo Finnigan Inc.) was used to identify the sequence of the peptide ions. The centroids of the isotopic envelopes of nondeuterated, functionally deuterated and equilibrium-deuterated peptides were measured using DXMS Explorer (Sierra Analytics Inc., Modesto, CA) and then converted to corresponding deuteration levels [82].

Supporting Information
Figure S1 Addition details of VP24 structures. (a) Crystallized constructs of VP24 from SUDV and RESTV. The first five residues of both the N- and C-termini are indicated respectively. The N-terminal 6xHis-tag was retained throughout purification and crystallization. (b) Stereo view of SUDV VP24 Faces 1, 2, and 3 in rainbow with blue indicating the N-terminus and red indicating the C-terminus. (TIF)
Figure S2 Sequence and structural alignment of VP24. (a) Sequence alignment of ebola- and marburgviruses. Secondary structures are assigned according to the crystal structures. Mostly conserved residues are in white boxes (red characters) while absolutely conserved residues are in red boxes (white characters). Grey stars indicate residues with alternate side-chain conformations observed in electron density maps. (b) Structural alignment of SUDV1–233 (pink), SUDV11–233 (green), and RESTV1–237 (blue) VP24. SUDV1–233 and SUDV11–233 align with an r.m.s.d. of 0.67 Å, and SUDV1–233 and RESTV1–237 align with an r.m.s.d. of 0.81 Å (CPC4: LSQKAB [85]). Loop residues 63-69 and 210–211 are visible in their entirety only in SUDV1–233. Loop residues 108–112 are only visible in their entirety in RESTV11–237.

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