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Structural and functional characterization of Reston Ebola VP35 Interferon Inhibitory Domain

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Abstract

Ebola viruses (EBOV) are causative agents of lethal hemorrhagic fever in humans and non-human primates. Among the filoviruses characterized thus far, Reston Ebola virus (REBOV) is the only Ebola species that is non-pathogenic in humans despite the fact that REBOV can cause lethal disease in non-human primates. Previous studies also suggest that Reston EBOV is less effective at inhibiting host innate immune responses, compared with Zaire EBOV or Marburg virus. Virally encoded VP35 protein is critical for immune suppression, but an understanding of the relative contributions of VP35 proteins from REBOV and other filoviruses is currently lacking. In order to address this question, we characterized REBOV VP35 IFN inhibitory domain (IID) using structural, biochemical, and virological studies. These studies reveal differences in dsRNA binding and IFN inhibition between the two species. These observed differences are likely due to increased stability and loss of flexibility in REBOV VP35 IID as demonstrated by thermal-shift stability assays. Consistent with this finding, our 1.71 Å crystal structure of the REBOV VP35 IID reveal that the structure is highly similar to ZEBOV VP35 IID with an overall backbone r.m.s.d. of 0.64 Å, but contains an additional helical element at the linker between the two subdomains of VP35 IID. Mutations near the linker, including swapping sequences between REBOV and ZEBOV,

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Accession numbers: Coordinates and structure factors for Reston Ebola VP35 IID protein has been deposited in the Protein Data Bank under the PDB ID: 3L2A.

Note added during manuscript preparation: While this manuscript was in preparation, a structure of the Reston Ebola VP35 IID free and bound to 18 bp dsRNA has appeared online and based on the published images, the two structures appear similar⁴⁸. However, we were unable to compare as the coordinates for these structures were not released at the time of initial submission.

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reveal that the linker sequence has limited tolerance for variability. Together with the previously solved ligand free and dsRNA bound forms of ZEBOV VP35 IID structures, our current studies of REBOV VP35 IID reinforce the importance of VP35 in immune suppression. Functional differences observed between REBOV and ZEBOV VP35 proteins may contribute to observed differences in pathogenicity, but these are unlikely to be the major determinant. However, the high similarity in structure and the low tolerance of sequence variability, coupled with the multiple critical roles played by EBOV VP35 proteins, highlight the viability of VP35 as a potential target for therapeutic development.

Filoviruses, including Ebola virus (EBOV) and Marburg virus (MARV), are enveloped negative-sense RNA viruses associated with zoonotic infections in humans^{1; 2; 3; 4}. Only a single species of MARV (*Lake Victoria*) has been identified thus far, but currently five known species of EBOV have been reported, including Zaire, Sudan, Ivory Coast, Bundibugyo and Reston^{1; 2; 3; 4}. Reston Ebola virus (REBOV) is the only known non-pathogenic filovirus to humans. The ability to cause lethal disease in humans and non-human primates has resulted in outbreaks primarily due to Zaire EBOV (ZEBOV) with human fatality rates near 90 percent⁴. In contrast, only a few documented cases of human infection with REBOV have been documented. Moreover, these infections were not associated with illness or death, suggesting that REBOV may be attenuated and likely avirulent in humans. The recent discovery of the presence of REBOV in samples collected from domestic swine in the Philippines^{5; 6; 7} suggests that REBOV has a greater potential to enter the human food chain than previously recognized. Comparison of global cellular transcriptional responses to ZEBOV, REBOV, and MARV suggest that MARV is less efficient than other EBOVs in inhibition of host cell IFN responses⁸, although interpretation of these cellular responses is complicated by the fact that REBOV replicates more slowly in cell culture than ZEBOV⁹. However, the molecular basis for species variation among EBOVs is currently lacking. Therefore, studies directed at examining structural and functional comparisons may shed light on this highly lethal family of native-stranded RNA viruses and suggest novel antiviral strategies against EBOVs.

Among the seven structural proteins encoded by the negative single stranded RNA genome of Ebola virus, VP24 and VP35 display immune antagonism. In particular, VP24 functions through inhibition of the JAK/STAT pathway, while VP35 functions to inhibit IFN- α/β signaling^{10; 11; 12}. Additionally, VP35 is critical for RNA-dependent protein kinase (PKR) inhibition^{13; 14}, viral replication^{15; 16}, and RNA silencing suppression¹⁷. As shown in Fig. 1a, VP35 contains an N-terminal coiled-coil domain that is important for oligomerization^{18; 19} and a C-terminal interferon inhibitory domain (IID) that binds dsRNA^{15; 20; 21; 22}. The coiled-coil domain is required for several VP35-mediated functions, including viral replication and nucleocapsid formation^{18; 19; 23; 24; 25; 26} as well as immune suppression, as the EBOV VP35 IID is unable to suppress IFN induction to the same level as the full length protein^{19; 20}. However, we and others have shown that VP35 binds dsRNA through the C-terminal IID, which alone is sufficient for interferon (IFN) inhibition^{15; 20; 21; 22}. Our recent crystal structure of the ZEBOV VP35 IID revealed a cluster of conserved basic residues that are important for dsRNA binding²⁷. From this first structure of a VP35 IID and associated solution NMR experiments, we demonstrated that VP35 IID consists of two subdomains that form a single independently folded unit. The α -helical subdomain forms a four helix bundle whereas the β -sheet subdomain forms a 4 stranded β -sheet with a short helix. Examination of the structure identified two distinguishable charged patches, termed first and central basic patch. Interestingly, this region was previously identified as a critical element for IFN inhibition and many residues within these charged patches display high sequence similarity among members of the filoviral family (Fig. 1a)²¹. Mutation of R312 in ZEBOV VP35 IID (corresponding to R301 in REBOV VP35 IID), which is located at the center of the central

basic patch, prevents dsRNA binding and display attenuated IFN inhibition. Several studies have extensively characterized mutations from the ZEBOV central basic patch and demonstrated the functional importance of residues within this patch^{16; 21; 15; 22}. Consistent with these reports, recent mouse-²⁸ and guinea pig-adapted^{29; 30} ZEBOV viruses show that growth rates of R312A and K319A/R322A mutation were greatly attenuated compared to viruses with wildtype VP35³¹. Moreover, K319A/R322A mutant virus was avirulent in a lethal guinea pig model³⁰. High sequence similarity, including the presence of identical residues at key sites, within the central basic patch suggests that VP35 proteins in all EBOVs may function in a similar manner^{9; 20}.

Previous studies have shown that nearly all components from the REBOV and ZEBOV nucleocapsid (viral polymerase L, NP, VP30, and VP35) are interchangeable in a minigenome assay that mimics viral replication⁹. Only the combination of ZEBOV VP35 and REBOV viral polymerase L displayed diminished replication function⁹. Therefore, despite significant differences in their virulence in humans, limited information about functional comparisons between REBOV and ZEBOV gene products is currently available³². Our studies described below address these questions in the context of critical VP35 proteins through combined biochemical, structural, and virological studies by comparing the VP35 IID proteins from REBOV and ZEBOV. Biochemical and virological studies show that VP35 IID proteins from REBOV and ZEBOV function similarly, but display different structural stabilities. Consistent with these findings, our crystal structure of REBOV VP35 IID, solved to 1.71 Å resolution, reveal similar folds, with a single difference at the interface of the α -helical and β -sheet subdomains. While this difference is likely responsible for the higher structural stability observed for REBOV VP35 IID, structural and functional studies show that only sequence swapping between REBOV and ZEBOV near the linker are tolerated as additional mutations result in loss of structure and function. While the observed differences in structure, dsRNA binding, and IFN inhibition likely result from loss of conformational plasticity, our results suggest that VP35 proteins from REBOV and ZEBOV probably function in a similar manner. Therefore, while VP35 may contribute to observed pathogenic differences, it is unlikely to be the main determinant. Given the structural and functional similarities, coupled with a low tolerance for mutations at the subdomain interface, our results suggest that VP35 IID is viable candidate for antiviral development and the availability of a high resolution crystal structure from our study should facilitate these efforts.

Sequence analysis of REBOV VP35 IID

One of the hallmarks of EBOV infections is the inhibition of host immune responses that impair innate and adaptive immunity. *In vivo* studies comparing REBOV and ZEBOV infections show differences, suggesting marked differences in the immune suppression capacities. Although REBOV and ZEBOV show very different disease outcomes in humans, examination of the corresponding VP35 IID sequences reveal that only 7 residues are different between the two proteins within the IID region (Fig. 1a). Since, VP35 is an important EBOV encoded virulence factor responsible for immune suppression and evasion, we examined the functional properties of REBOV VP35 IID. Protein samples were prepared by modifying previously published protocols for ZEBOV VP35 IID³³. Given the high sequence homology, we were able to identify constructs that were well behaved based on hydrodynamic characterization, including size exclusion chromatography and dynamic light scattering (data not shown). Based on these studies, we identified a REBOV VP35 IID construct containing residues 204–329, which corresponds to ZEBOV VP35 IID residues 215–340.

REBOV VP35 IID displays slightly diminished dsRNA binding and IFN β inhibition

We recently determined the dsRNA bound structure of ZEBOV VP35 IID, which revealed that the VP35 IID protein uses multiple strategies to engage in protein-protein and protein-RNA interactions in order to facilitate viral infection and replication²². We also demonstrated that dsRNA binding is important for VP35-mediated antagonism of host immune responses that originate from cytosolic RIG-I like receptors (RLRs). As an initial test of the REBOV VP35 characterization and comparison, we assessed the ability of REBOV VP35 IID to bind 8 base pair (bp) dsRNA by isothermal titration calorimetry (ITC) (Fig. 1b). Both VP35 IIDs bound 8 bp dsRNA with a stoichiometry of 1:4 ratio of dsRNA:VP35 IID. However, comparison of binding constants revealed that REBOV VP35 IID displayed approximately 2–3 fold lower dsRNA binding for the same dsRNA when compared to ZEBOV VP35 IID. Moreover, the relative heats released upon binding were ~25% lower for REBOV VP35 IID than ZEBOV VP35 IID titrations, indicating that dsRNA interactions with REBOV and ZEBOV VP35 IIDs may not be identical despite the highly similar primary sequence, particularly near the central basic patch.

We next tested the inhibition of Sendai virus mediated activation of the IFN- β promoter by REBOV and ZEBOV VP35 proteins. As shown in Fig. 1c, both REBOV and ZEBOV VP35 proteins can inhibit IFN- β promoter activation, but REBOV VP35 inhibits this activation to levels lower than that observed for ZEBOV VP35. Examination of corresponding western blots indicate that the expression levels of REBOV and ZEBOV VP35 proteins are similar, suggesting that the differences observed in dsRNA binding and IFN inhibition may result from the reduced ability of REBOV VP35 to sequester dsRNA leading to higher levels of IFN promoter activation.

REBOV VP35 IID is more stable than ZEBOV VP35 IID

To further examine the physical basis for the difference in REBOV and ZEBOV VP35 function, we conducted ThermoFluor assays^{34; 35} with REBOV and ZEBOV VP35 IID proteins. Using a temperature gradient from 30 to 90 °C, we monitored the changes in fluorescence upon SYPRO Orange binding to unfolded hydrophobic regions. Analysis of the derivative of the fluorescence change, shown in Fig. 1d, revealed that the average melting temperature (T_m) for REBOV VP35 IID is 63.4 ± 0.3 °C while ZEBOV VP35 IID displayed a T_m value of 57.0 ± 0.1 °C. Given that the reported values are an average of at least 15 independent measurements, our results suggest that the REBOV VP35 IID is thermally more stable than ZEBOV VP35 IID by at least 6 °C. However, the source of the difference in thermal stability is not immediately evident as these two proteins only differ at 7 residues in the region encoded for VP35 IID (Fig. 1a).

Crystal structure of REBOV VP35 IID

In an effort to structurally characterize the REBOV VP35 IID protein, we determined the crystal structure of REBOV VP35 IID containing residues 204–329. Initial crystallization conditions were obtained from a commercial crystal screen (Hampton Research) and these conditions were subsequently optimized to obtain single crystals that were suitable for diffraction data collection at the synchrotron. Our data collection statistics are shown in Table 1. Data reduction and indexing revealed that the REBOV VP35 IID crystals belong to the $P4_1$ space group, with unit cell dimensions of $a=b=55.55$, $c=50.87$ and $\alpha=\beta=\gamma=90$. The asymmetric unit contained one molecule of REBOV VP35 IID and the Matthews coefficient was calculated at 2.55 with a solvent content of 51.72%.

The structure of REBOV VP35 IID was determined by molecular replacement using our previously determined structure of ZEBOV VP35 IID as a search model (PDB: 3FKE)²⁷ (Fig. 2a). REBOV VP35 IID shows remarkable similarity to ZEBOV VP35 IID (backbone r.m.s.d. value of 0.64 Å) (Fig. 2b). Overall, the REBOV VP35 IID structure retained the α -helical subdomain and the β -sheet subdomains present in the ZEBOV VP35 IID structure, which aligned with r.m.s.d. values of 0.3 Å and 0.5 Å for the α -helical and β -sheet subdomains, respectively²⁷. These results suggest that other filoviral VP35 IID proteins are likely to contain a similar fold and, more importantly, that the VP35 IID fold is substantially different from the canonical dsRBD fold of $\alpha\beta\beta\alpha$. Comparisons using the DALI server³⁵ reveal that the α -helical subdomain has a topology similar to many functionally unrelated proteins (DALI z-score >2). However, like the ZEBOV IID structure, we were unable to identify other known structurally or functionally related proteins, confirming the uniqueness of the REBOV VP35 IID fold.

Previous studies have shown that mutation of individual residues within the central basic patch results in loss of dsRNA binding and a concomitant loss of IFN inhibition. Initial solution NMR analysis revealed that mutant VP35 proteins retain their overall fold. In contrast, recent crystal structures of three ZEBOV VP35 IID central basic patch mutants, R312A (PDB: 3L27), K319A/R322A (PDB: 3L29), and K339A (PDB: 3L28), showed that substantial changes to the electrostatic surface are evident as a result of mutating charged residues. However, in these mutant structures, only subtle changes to the overall protein conformation are observed. Comparison of the electrostatic surface of the REBOV and ZEBOV IID structures, shown in Fig. 2c, confirm our predictions that residues previously known to be important for dsRNA binding in ZEBOV VP35 IID are forming a similar charged surface in REBOV VP35 IID.

Structural comparison of Reston vs Zaire VP35 proteins

Despite the remarkable similarities at the level of global structure, we observe one notable difference between the REBOV and ZEBOV VP35 IID structure at the subdomain interface. In the REBOV VP35 IID structure, the linker between the α -helical and β -sheet subdomains forms an additional short 5 residue alpha helix in the REBOV VP35 IID structure (α 4b in Fig. 2a). This helix is absent in the ZEBOV VP35 IID structure and is replaced by a loop. We have previously demonstrated that the subdomain interface, formed by highly conserved residues, is critical for folding and stability of ZEBOV VP35 IID²⁷. It is likely then that the presence of the α 4b helix provides additional stabilization of the REBOV VP35 IID structure near the subdomain interface and contributes to the increased thermal stability observed in the Thermfluor assays. As a consequence of this helix, the relative orientation between the α -helical and β -sheet subdomains is rotated by 1.0–4.0° when the structures of dsRNA-VP35 complex (PDB: 3L25, 3L26) as well mutant structures are compared by aligning the α -helical subdomain (or the β -sheet subdomain) (Fig. 3a–b). Moreover, we observed that the ZEBOV VP35 IID was able to bind both dsRNA backbone and the blunt dsRNA ends forming an “end cap” and this interaction also depends on the ability of the penultimate Lysine residue (Lys 328 in REBOV or Lys339 in ZEBOV) to coordinate the terminal carboxylate group. As shown in Fig. 3c, charge neutralization of the terminal carboxylate is less efficient in REBOV VP35 IID due to the relative orientation (Fig. 3c). Thus, the helical nature of the linker in REBOV VP35 IID likely limits the conformational flexibility required for multiple interactions between VP35 IID and dsRNA, such as those observed in the ZEBOV IID/dsRNA complex²².

In order to further assess the differences between the REBOV and ZEBOV VP35 IID linker regions and to identify structural characteristics that may be functionally relevant, we generated a series of mutations for REBOV and ZEBOV VP35 IID near the linker region.

These include mutation of the linker sequence to a flexible linker (linker 1, Table 2), swapping of the linker region between ZEBOV and REBOV (linker 2, Table 2), and insertion of a flexible linker (linker 3, Table 2). As shown in Fig. 4, swapping the linker sequence between REBOV and ZEBOV IID did not affect overall thermal stability (Fig. 4a), structure (Fig. 4b, left), or function (Fig. 4c). However, substitution of the linker sequences (linker 1) or insertion (linker 3) with a linker composed of Gly and Ser residues resulted in highly destabilized VP35 IID domains. All linker 1 constructs were highly unstable when expressed in *E. coli* under conditions similar to wildtype protein. Although we were able to purify the linker 3 proteins, they show diminished stability (Fig. 4a, compare black (wildtype) with blue (linker 3)) and a large number of structural changes, highlighted for REBOV linker 3 (Fig. 4b, right). Consistent with these observations, expression of both linker 1 and linker 3 in the context of full length REBOV and ZEBOV proteins displayed diminished IFN inhibitory activity in the Sendai virus infection assay (Fig. 4c). In contrast, even at lower plasmid concentrations than those in Fig. 4d, linker 2 constructs show wildtype behavior. Together, the results described above highlight the importance of the linker sequence connecting the two subdomains in VP35 IID to activity and demonstrate that EBOV VP35 sequences have very low sequence tolerance at the subdomain interface.

The critical role played by VP35 in immune suppression and the importance to viral replication and pathogenesis of Ebola viruses is well established. We have recently provided additional experimental evidence that links the ability of ZEBOV VP35 to bind dsRNA and its ability to antagonize host immune responses²². Consistent with these observations, recent reports show that antiviral pathways initiated by cytosolic RIG-I-like helicases are important for EBOV infections^{36; 37; 38; 39; 40; 41; 42} as preactivation of RIG-I alone can reduce EBOV infections up to ~1000-fold⁴³. Therefore, inhibition RIG-I like receptor (RLR) function, which detect viral nucleic acids such as dsRNA during infection and replication, is likely important for EBOV propagation. Comparison of EBOV VP35 IID structures with those of other viruses suggests that VP35 is significantly different from other viral and cellular proteins, but these properties are highly conserved among filoviral VP35 proteins. Therefore, the availability of multiple structures of Ebola VP35 IID proteins, including the REBOV VP35 IID structure described here, coupled with the high sequence similarity among VP35 from different filoviruses and the low tolerance for sequence variation provides unique information that will facilitate new opportunities for therapeutic and diagnostic drug design.

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Fig. 1. REBOV VP35 is not as potent an IFN-antagonist as ZEBOV VP35

a. Domain organization of REBOV VP35 based on previous biochemical and structural studies²⁷. VP35 sequences for the IID region from Reston *ebolavirus* (REBOV residues 204–329; accession number AAN04449.1) and Zaire *ebolavirus* (ZEBOV residues 215–340; accession number AF086833) were aligned using CLUSTALW version 1.81⁴⁴. Identical residues (black) and similar residues (gray) are highlighted. **b.** Representative ITC data showing raw heat absorbance (top) and integrated heats per injection (bottom) for REBOV VP35 IID (left) and ZEBOV VP35 IID (right) binding to 8 base pair dsRNA corresponding to the following palindromic sequence, 5'-CGCAUGCG-3'. A vector containing the coding region for REBOV and ZEBOV²⁷ were used as a template to generate PCR products for subcloning VP35 IID constructs into a modified pET15b vector (Novagen). All VP35 IID constructs were grown and overexpressed as MBP fusion proteins in BL21(DE3) cells (Novagen) in either LB or minimal media. Protein expression in *E. coli* was induced at optical density of 0.8 at 600 nm with 0.5 mM IPTG and grown overnight at 18 °C. Cells were harvested, resuspended in lysis buffer (25 mM Na-phosphate pH 7.0, 1M NaCl, 20 mM imidazole, and 5 mM β-mecaptoethanol), lysed using an EmulsiFlex-C5 homogenizer (Avestin), and clarified by centrifugation at 30,000 × *g* at 4 °C for 30 minutes. The supernatant was purified by affinity and ion exchange chromatographies. Fusion tags were removed prior to final purification by size exclusion chromatography. The purity of the samples was assessed by SDS-PAGE. Average dissociation constants are 1200 nM and 500 nM, for REBOV and ZEBOV VP35 IID, respectively. **c.** To measure VP35 IFN-antagonist function, 293T cells were transfected with empty vector (EV), increasing amounts of HA-tagged VP35 expression plasmid, IFN-β promoter-firefly luciferase reporter plasmid, and a constitutively-expressed *Renilla* luciferase reporter plasmid. 18 hours post-transfection, the cells were infected with Sendai virus (SeV). 24 hours post-infection, cells were harvested and lysates prepared. Reporter gene assays were performed using a dual luciferase reporter assay (Promega). Firefly luciferase activity was normalized to *Renilla* luciferase activity, and the results were expressed as percent induction relative to the positive control. Error bars represent the standard deviation for 3 independent experiments. **d.** First derivative of normalized fluorescence emission data for ThermoFluor assays of REBOV and ZEBOV VP35 IID proteins reveal differences in T_m values for mutant proteins. The melting temperature (T_m) of VP35 IID variants were measured by following established ThermoFluor assay protocols^{34; 35}. Experiments were carried out in a MiniOpticon real-time PCR instrument (BioRad). Measurements were performed using λ_{ex} = 470–505 nm and λ_{em} = 540–700 nm. Data were acquired using a temperature gradient from 30–90 °C in 0.5 °C increments. Samples contained 20 μM VP35 IID protein, 1× SYPRO Orange (Invitrogen), 10 mM HEPES pH 7, 150 mM NaCl, and 2 mM TCEP. Fluorescence increases due to the association of SYPRO Orange to exposed hydrophobic residues as the protein unfolds with

increasing temperature. Fluorescence data were analyzed and the derivative of the curve represents the melting temperature.

Fig 2. Crystal structure of REBOV IID

a. Ribbon representation of the 1.71 Å REBOV VP35 IID (PDB: 3L2A) contains six alpha helices, including the same 4 α -helices in the α -helical subdomain and four beta strands in the β -sheet subdomain of ZEBOV VP35 IID27. The linker between the two subdomains of REBOV VP35 IID contains a short α -helix (α 4b). **b.** Alignment between REBOV (blue) and ZEBOV (green; PDB: 3FKE) VP35 IID structures. Highlighted by a box is the α 4b helix observed in the REBOV VP35 IID structure. **c.** Comparison of electrostatic surface representation in REBOV (left) and ZEBOV (right) VP35 IIDs (scale of -10 to $+10$ kT e $^{-1}$), highlighting the central basic patch residues. Structure figures were prepared using PyMOL⁴⁵.

Fig. 3. Structural comparison of REBOV and ZEBOV VP35 IID proteins

a. Structural alignment of the α -helical subdomains of REBOV (blue; PDB: 3L2A) and ZEBOV (green; PDB: 3FKE) show the structural and orientation differences between the β -sheet subdomains relative to the α -helical subdomains. **b.** Same alignment as in **a** rotated 180° in the y-axis. **c.** Comparison of critical residues in VP35 IID that form the dsRNA endcap structure that is important for dsRNA blunt end recognition. Evaluation of the interactions between REBOV VP35 IID residue K328 sidechain and oxygen atoms (OXT1 and OXT2) of terminal residue I329 reveal important differences between REBOV VP35 IID and ZEBOV VP35 IID that may affect the properties of the dsRNA endcap. REBOV VP35 IID residue K328 is located ~ 5 Å away from I329 OXT1, whereas ZEBOV VP35 IID residue K339 N ϵ is within 4 Å of OXT1 and OXT2 of the terminal I340 residue (two black

dotted lines for ZEBOV VP35 IID vs. only 1 black dotted line for REBOV VP35 IID. Structure figures were prepared using PyMOL⁴⁵.

Fig. 4. REBOV VP35 IID is more stable compared to ZEBOV VP35 IID

a. First derivative of normalized fluorescence emission data for Thermofluor assays of REBOV VP35 IID proteins reveal differences in T_m values for REBOV proteins with different linkers between the α -helical and β -sheet subdomains. A vector containing the coding region for REBOV was used as a template to generate PCR products containing alanine mutations at V279, P280, and I283 (REBOV VP35 linker 2) or a 10-residue stretch of glycine and serine residues between P281 and P282 (REBOV VP35 linker 3). Experiments were carried out as described in Fig. 1d legend. The T_m values are 62.9 °C, 62.4 °C, and 53.4 °C for wildtype REBOV VP35 IID (black), REBOV VP35 IID linker 2 (red), and REBOV VP35 IID linker 3 (blue), respectively. **b.** $^1\text{H}/^{15}\text{N}$ -HSQC spectra of REBOV VP35 IID wildtype (black) overlaid with REBOV VP35 linker 2 (red, left) or REBOV VP35 linker 3 (blue, right). Samples were generated using previously described methods^{22; 27; 33}. Data were acquired on a Bruker Avance II spectrometer operating at 700.13 MHz for ^1H at 25 °C. Data were processed with nmrPipe/NMRDraw⁴⁶ and nmrView⁴⁷. **c, d.** IFN inhibition function of REBOV and ZEBOV VP35 constructs were tested as described above using varying VP35 vector concentrations as indicated in the figure. Representative western blots and antibodies are indicated below each graph.

Table 1

Data collection and refinement statistics

Crystals were grown at 25 °C by hanging drop vapor diffusion method with 20 mg/mL protein solutions diluted with 100 mM sodium acetate pH 3.0, 14% Tacsimate, and 16% (w/v) PEG 3500. Crystals grew within 2–4 days to dimensions of 10 × 20 × 200 μm. Crystals were soaked in reservoir solution containing a final glycerol concentration of 25% (w/v) and frozen in a nitrogen stream. Data was collected from a single crystal at the Advanced Photon Source, SBC beamline 19ID at 100 K. Complete dataset of 180°, with 1° oscillations were collected at 0.979 Å wavelength. Data was processed using HKL2000⁴⁹ and intensities were converted to structure factors using the CCP4 program⁵⁰ TRUNCATE. Phases were determined using molecular replacement with the native wildtype structure of ZEBOV VP35 IID (PDB code: 3FKE³³) using either MOLREP⁵¹ or PHASER⁵². Refinement was performed with REFMAC5 interspaced with manual rebuilding with Coot⁵³. Water molecules were initially added using ARP/wARP^{54; 55} if a peak greater than 3.0σ was present in Fourier maps with coefficients $(F_{obs} - F_{calc})e^{i\alpha_{calc}}$ and later manually inspected with Coot⁵⁶. The model was further refined using REFMAC5 with the MLKF residual function, bulk solvent scaling, and individual isotropic B-factors. TLS parameters were determined with TLMDS⁵⁷. The quality of the refined model was validated with MOLPROBITY⁵⁸. Final refinement statistics for all structures are shown above.

	REBOV IID
Data collection	
Space group	$P4_1$
Cell dimensions	
a, b, c (Å)	51.55, 55.55, 50.87
$\alpha\beta\gamma$ (°)	90, 90, 90
Resolution (Å)	50.00-1.71 (1.74-1.71)*
R_{sym} or R_{merge} (%)	7.0 (60.3)
$I/\sigma I$	39.0 (2.9)
Completeness (%)	99.9 (100.0)
Redundancy	7.4 (7.3)
Refinement	
Resolution (Å)	22.99-1.71
No. reflections	14489
$R_{\text{work}}/R_{\text{free}}$	17.26/22.53
No. atoms	
Protein	1065
Water	93
B-factors	
Protein	24.4
Water	35.7
R.m.s. deviations	
Bond lengths (Å)	0.014
Bond angles (°)	1.31

Each dataset above was collected using one crystal.

* Values in parentheses are for highest-resolution shell.

Table 2

Mutant ZEBOV and REBOV linker construct sequences

Construct	Sequence
REBOV linker 1	²⁶⁸ QITKRVSGSGSGSGSIHIRSRGDIPRAC
REBOV linker 2	²⁶⁸ QITKRVPIFQDAAPPVIHIRSRGDIPRAC
REBOV linker 3	²⁶⁸ QITKRVPIFQDVPPSGSGSGSGSPIIHIRSRGDIPRAC
ZEBOV linker 1	²⁷⁹ QITKRVSGSGSGSGSIHIRSRGDIPRAC
ZEBOV linker 2	²⁷⁹ QITKRVPIFQDVPPPIHIRSRGDIPRAC
ZEBOV linker 3	²⁷⁹ QITKRVPIFQDAAPSGSGSGSGSPVIHIRSRGDIPRAC