Supplementary Information

A Shared Structural Solution for Neutralizing Ebolaviruses

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Prefusion structure with modeled HR2

Cathepsins remove glycan cap and mucin-like domain

Arg 89 releases fusion loops, fusion loops begin to unwind

Fusion loops and HR1 unwind from GP1 base

Freed GP1 opens allowing neighboring HR1 to uncurl and adopt single rod-like helix. Fusion loops penetrate host membrane.

Rotation about the GP2 linker brings HR2 upward to form the post-fusion six-helix bundle. It is unclear if GP1 must be removed for membrane fusion. Structural modeling suggests GP2 could rearrange around a still-attached GP1.
**Supplementary Figure 1.** Proposed steps by which GP rearranges from its pre-fusion, viral surface conformation to its postfusion six-helix bundle conformation. (a) SUDV GP prefusion structure. GP is colored by domain: GP1 glycan cap (cyan), GP1 receptor-binding head (blue), GP1 base (green), GP2 fusion loop (orange), GP2 heptad repeat 1 (HR1; yellow), GP2 linker (tan); GP2 HR2 (brown, modeled). The mucin-like domain is attached to the glycan cap, but is not shown here. HR2 is disordered in the structure, but has been modeled according to secondary structure predictions. (b) Cathepsins in the host endosome cleave GP1 around residue 201 (ref. 1) in a disordered linker connecting the GP1 base to the GP1 head. This cleavage event removes the mucin-like domain and glycan cap from GP1. (c) As the next step in conformational change, the fusion loops must unwind from the outside of the trimer, after being released from Arg 89 of the neighboring monomer’s GP1 head. It is currently unknown if the low pH of the endosome has any effect on interactions of Arg 89 with the fusion loop. (d) The fusion loops and their attached HR1s unwrap from around their GP1 bases. (e) HR1 forms the single rod-like helix observed in post-fusion crystal structures, projecting the fusion loops upwards toward the target cell membrane. It is likely that unwinding of the fusion loops and HR1s is coupled to or concomitant with outward rotation of the GP1s in the trimer, because the GP1 of one monomer must open outwards in order to free the space required for the neighboring monomer’s HR1 in the rearrangement. Further, the disulfide-anchored linker region links GP1 to HR2 so that outward and downward movement of GP1 is coupled with upward and inward rotation of HR2. (f) Post-fusion six-helix bundle with modeled fusion loops. It is currently unknown whether GP1 is fully released from GP2 by a disulfide reductase or continued proteolytic digestion, or if GP1 remains associated with GP2 as the post-fusion structure forms. Our structural modeling suggests that GP1 release or digestion is not necessarily required structurally and that the conformational changes in GP2 could occur if GP1 remains attached and simply rotates outward.
Supplementary Figure 2. Possible anchors of the pre-fusion conformation of GP, and electron density for attached carbohydrate. (a) Arg 89 of the GP1 head (blue) anchors the fusion loop of the neighboring GP$_{1,2}$ monomer (orange) to the outside of the trimer via a network of hydrogen bonds and van der Waals contacts. This interaction is conserved between SUDV and EBOV GP$_{1,2}$ structures and must be released for the fusion loops and HR1 to unwind from the GP$_{1,2}$ trimer. (b) The cathepsin cleavage loop is disordered in both SUDV and EBOV structures but links Thr 192 (GP1 base, green) to Tyr 212 (GP1 head, blue), in a polypeptide (not to scale) that extends over the outside of the GP2 fusion loop (orange). It is possible that cleavage of this loop may free constraints on the fusion loop allowing it to fully unwind from the GP$_{1,2}$ trimer. Thr 192 and Tyr 212 are the limits of this loop observed in the SUDV structure. In the EBOV structure, Ala 189 and Tyr 214 are the visible limits of this loop (190-213 are disordered). (c) The antibody 16F6 binds a conformational epitope on the membrane-proximal side of GP. Here, one monomer of SUDV GP is shown as a molecular surface colored as in Supplementary Figure 2: GP1 glycan cap (cyan), GP1 head (dark blue), GP1 base (green), GP2 fusion loop (orange), GP2 heptad repeat 1 (yellow). 16F6 is shown as a ribbon model with the heavy chain in dark grey and the light chain in light grey. The molecular surface of the 16F6 paratope is indicated by a speckled pattern and key Fab residues that contact SUDV GP are labeled. The shape complementarity, $S_c^2$, is 0.61. (d) Electron density for the glycan NAG 700-701, which is covalently linked to Asn 563 (GP2), 2Fo-Fc sigma a weighted map contoured at 1.0$\sigma$. This glycan is one of two resistant to treatment with PNGase F.
Supplementary Figure 3. Surface representation of SUDV and EBOV GP$_{1,2}$ in two different orientations. (a,b) The domains of SUDV GP$_{1,2}$ monomer are outlined in black with subdomains indicated. (c,d) Sequence conservation between SUDV and EBOV GP$_{1,2}$, mapped onto the SUDV structure. Residues that are identical between SUDV and EBOV are colored red, while these that are different are colored yellow. (e,f) Electrostatic potential representation of the SUDV GP$_{1,2}$ surface with limits +/- 10 keT. (g,h) Electrostatic potential representation of the EBOV GP$_{1,2}$ surface with limits +/- 10 keT.
**Supplementary Figure 4.** Sequence alignment of Sudan virus (Sudan) and Ebola virus (Zaire) GP. Observed secondary structural elements are indicated. Residues that are identical are colored red, residues that are similar are colored green, and residues that are different are colored white. Secondary structural elements observed in the SUDV structure are illustrated below the sequence and colored according to subdomain (green=GP1 base, dark blue=GP1 head, cyan=GP1 glycan cap, orange=GP2 fusion loop, yellow=GP2 heptad repeat 1).
Anti-ebolavirus antibodies used

| Antibody Name | Species | Raised against | Epitope | Reference(s) |
|---------------|---------|----------------|---------|--------------|
| 16F6          | Murine  | Sudan          | base of GP | 1            |
| KZ52          | Human   | Ebola          | base of GP | 2-3          |
| 13F6          | Murine  | Ebola          | mucin-like domain, GP 405-413 | 4-5 |
| 13C6          | Murine  | Ebola          | unmapped conformational | 4       |
| 133/3.16      | Murine  | Ebola          | unmapped conformational (escape at 340) | 6       |
| 42/3.7        | Murine  | Ebola          | unmapped conformational | 6       |

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Supplementary Figure 5. *In vitro* and *in vivo* activity of mAb 16F6. (a) mAb 16F6 specifically stains Vero E6 cells infected with live SUDV (left, dotted red line), while a EBOV-specific mAb 13F6<sup>3</sup> specifically stains Vero E6 cells infected with EBOV (right, solid green line). (b) Neutralization of infectious SUDV by mAb 16F6 in the absence (red diamonds) or presence (orange squares) of complement. 90% neutralization is achieved at ~10 µg ml<sup>-1</sup> of 16F6. By contrast, mAb 13C6, which weakly cross-reacts with SUDV GP<sub>1,2</sub> (ref. 3) is non-neutralizing for SUDV. (c) SCID mice were infected IP with 500 pfu “SCID-adapted” SUDV homogenate. At 5, 10, 15 and 20 days post challenge, groups of SCID mice were treated IP with 100 µg either 16F6 (red diamonds) or 13F6 (EBOV-specific, green triangles), (n=15) or PBS (grey squares) (n=10) in volume of 200 µl. Mice were monitored for 25 days for morbidity and mortality. Data are presented on a Meier-Kaplan curve as % survival for each group. A t-test of p<0.0001 for each competition group indicates a significant difference in mean time-to-death between mice that received 16F6 vs. control antibody or PBS. (d) Neutralization of SUDV (Sudan) GP- and EBOV (Zaire) GP-pseudotyped VSIV by 16F6 and KZ52 IgGs, respectively. Virus particles were pre-incubated with the indicated concentrations of antibody for 1 h at room temp, and then exposed to Vero cells at 37°C. Viral infectivity was scored at 16 h post-infection by GFP-positive cells. (e) Table of the anti-ebolavirus antibodies used in this manuscript.
**Supplementary Figure 6.** mAbs 16F6 and KZ52 neutralize at a post-internalization, non-catL cleavage step. (a) Left of molecular mass markers: VSIV pseudotyped with EBOV or SUDV GP<sub>1,2</sub> attach to Vero E6 cells at 4°C in the presence of KZ52, 16F6 or a negative control anti-dengue murine IgG<sub>1</sub> 3H5. Lysates were probed for VSIV M (~28kDa). E(-)=EBOV+3H5. E•KZ52= EBOV+KZ52. Ø=no virions. S(-)=SUDV+3H5. S•16F6=SUDV +16F6. Right of molecular mass markers: After attachment, cells were warmed to 37°C to permit internalization, then treated with proteinase K to remove bound, non-internalized virus. Cell lysates were probed for the presence of VSIV M. As a control, virus-cell complexes that were chilled to prevent internalization were also treated with proteinase K (E•prot.K or S•prot.K). (-) IgG is 3H5 alone. (b) Top, ΔVP30 EBOV in the presence of one of several antibodies was allowed to attach to Vero E6 cells at 4°C. Cells were washed of unbound material, warmed to 37°C and plaqued. mAbs 133/3.16<sup>4</sup> and KZ52 neutralize, whereas 42/3.7<sup>4</sup> and anti-HA do not. Bottom: ΔVP30 EBOV was attached to Vero E6 cells at 4°C, washed, incubated with mAb, washed again, warmed to 37°C to permit internalization, and plaqued. KZ52 still neutralized whereas 133/3.16 did not, indicating that KZ52 neutralizes at a post-attachment step whereas 133/3.16 neutralizes at the attachment step. Note that there is currently no VP30-deleted SUDV model, so only VP30-deleted EBOV was analyzed in these experiments. Antibodies used are described in Supplementary Fig. 5e.
**Supplementary Figure 7.** KZ52 and 16F6 do not prevent cleavage of virion-surface GP_{1,2}. (a-c) A 10-fold molar excess of KZ52 and 16F6 does not prevent cleavage of recombinant GP used for crystallization. EBOV or SUDV GP is incubated with CatL in the presence (+) or absence (-) of KZ52, 16F6 or a control murine anti-dengue virus antibody 1B7 in a 1:10 molar ratio (GP: mAb). (a) EBOV GP +/- human IgG\_1 KZ52. (b) SUDV GP +/- murine IgG\_1 16F6. (c) SUDV GP +/- anti-dengue virus murine IgG\_1 1B7. Although any antibody, even the control 1B7, slightly slows CatL cleavage, no antibody prevents it. Time points are for EBOV GP: 10, 20, 60, and 90’ and SUDV GP: 8, 15, 30, and 60’. Ø= no CatL control. CatL cleavage products are detected by blotting with HRP-conjugated anti-HA, and are ~22 kDa because of the HA tag and linker region added to the N terminus of GP1 in this construct. Note that SUDV GP is more susceptible to CatL cleavage than EBOV GP. (d) EBOV GP_{1,2}-pseudotyped VSIV was incubated with increasing concentrations of cathepsin L in the presence of 100 µg ml^{-1} KZ52 or isotype-matched control human IgG1κ. Intact GP1 (~150 kDa) is initially cleaved to a ~50 kDa intermediate, and ultimately to an ~18 kDa product. (e) SUDV GP_{1,2}-pseudotyped VSIV was incubated with increasing concentrations of cathepsin L in the presence of 100 µg ml^{-1} 16F6 or isotype-matched control mouse IgG1. The ~18kDa GP_{1,2} cleavage product is indicated at bottom. Blots were incubated with rabbit anti-GP_{1,2} polyclonal sera and probed with an HRP-conjugated anti-rabbit antibody.

Although work by others, using denatured antibodies as controls, suggested that EBOV GP_{1,2} cleavage is prevented by KZ52 (ref. 5), we found that no antibody prevents cleavage of GP_{1,2}. Instead, all fully native (non-denatured) antibodies, even those directed against irrelevant antigens, slightly delay cleavage in a non-specific, concentration-dependent manner.
### Supplementary Table 1. Data collection and refinement statistics

| Data collection | Value |
|-----------------|-------|
| **Space group** | I23 |
| **Cell dimensions** | 193.59, 193.59, 193.59 |
| **Resolution (Å)** | 46-3.35 (3.47-3.35)* |
| **R_{sym} (%)** | 8.8 (82.5) |
| **I/σI** | 10.1 (1.7) |
| **Completeness (%)** | 97.4 (99.1) |
| **Redundancy** | 5.3 (5.1) |

| Refinement | Value |
|------------|-------|
| **Resolution (Å)** | 50.00-3.35 |
| **No. reflections** | 15735 |
| **R_{work}/ R_{free}** | 22.55/27.68 |
| **No. atoms** | |
| **Protein** | 6043 |
| **Glycan** | 56 |
| **B-factors (Å²)** | |
| **Protein** | 121.8 |
| **Glycan** | 176.5 |
| **R.m.s. deviations** | |
| **bond lengths (Å)** | 0.009 |
| **bond angles (°)** | 1.23 |

*Values in parentheses are for highest-resolution shell.*
METHODS

Animals. Female BALB/c mice and severe combined immunodeficiency (SCID) mice (5 to 8 weeks old) were obtained from the National Cancer Institute (Frederick, MD) and housed under specific-pathogen-free conditions.

Vaccinations for antibody generation. BALB/c mice were vaccinated subcutaneously in the dorsal neck region with Venezuelan equine encephalitis replicons (VRP) (2 x 10^6 focus-forming units (ffu)/mouse) expressing the glycoprotein of Sudan virus (strain Boniface). The mice were boosted three times over consecutive months with this VRP (1 x 10^7 ffu/mouse). One month after the last subcutaneous boost, mice were intravenously injected with inactivated, irradiated SUDV-Boniface.

Hybridoma cell lines. Spleens were removed from mice 7 days after the final vaccination and splenocytes were prepared using standard techniques. Antibody specificity of the supernatants of the cell lines was determined by ELISA against irradiated-whole SUDV-Boniface and irradiated-whole EBOV.

Plaque assays. Four tenfold serial dilutions of mAbs (starting at 200 µg/ml) were mixed with 100 plaque-forming units (pfu) of Sudan virus (strain Boniface) at 37°C for 1 h in the presence of absence of 5% guinea pig complement (Cedarlane) and used to infect Vero cell monolayers. Cells were overlaid with agarose and a second overlay containing 5% neutral red was added 8 days later. Plaques were counted the next day. Neutralization titers were determined to be the last dilution of mAb that reduced the number of plaques by 80% compared with control wells.

In vivo activity. Wild-type SUDV-Boniface is non-lethal for wild-type mice and only lethal for SCID mice at day 60-65 postinfection. To generate a mouse-adapted lethal Sudan virus with a similar time to death as that observed in humans, we successively passaged wild-type SUDV-Boniface from liver/spleen homogenates of SCID mice. This passaged virus was now lethal for SCID mice in 16-18 days. SCID mice were challenged by the intraperitoneal route with passaged SUDV-Boniface. After challenge, SCID mice were treated on days 5, 10, 15 and 20 with 100 µg 16F6 diluted in 200 µl of PBS. Mice were monitored daily for morbidity and mortality. Time to death was determined for the antibody-treated mice and compared to untreated and mock-treated controls to determine statistical significance.

GP_{1,2} expression and purification
SUDV glycoprotein (GP_{1,2}) (strain Gulu) was cloned into the pDISPLAY vector (Invitrogen) for expression in mammalian cells. The crystallized construct comprises residues 33-313 of GP1 and 473-637 of GP2. The mucin-like domain and the transmembrane domain (residues 314-472 and 640-676, respectively) were excised from the construct to improve solubility and homogeneity of the glycoprotein. The recombinant SUDV-Gulu glycoprotein (residues 33-313, 473-637) was transiently expressed in HEK293T cells, captured by anti-HA affinity, and natively deglycosylated using Peptide:N-glycosidase F (PNGaseF) at room temperature.
Crystallization and data collection
SUDV GP\textsubscript{1,2} trimers were incubated with Fab fragments of mAb 16F6 for 1 h. Complexes were purified by gel filtration and concentrated up to 10 mg ml\textsuperscript{-1} for crystallization in 15% PEG 3350 and 0.2M lithium citrate. Benzamidine hydrochloride was added directly to the drop to a final concentration of 1% to promote nucleation. The final pH of the crystallization solution was 8.4. The crystals were cryoprotected with 17.5% (v/v) glycerol plus mother liquor before flash cooling in liquid nitrogen. These crystals diffracted beyond 3.2Å resolution, but due to radiation damage, a complete dataset was collected with good statistics up to 3.35Å, at a wavelength of 0.9793Å using APS beamline 19-ID. All data were indexed, integrated, and scaled using HKL2000 (ref. 6) to 3.35Å resolution (see Supplementary Table 1).

Structure Determination and Refinement
The structure was determined by molecular replacement using using PHASER\textsuperscript{7} with an edited polyalanine model derived from the previously determined EBOV GP\textsubscript{1,2} (pdb: 3CSY\textsuperscript{8}). This partial solution was fixed and the Fab position was determined using a polyalanine model of a different Fab structure (1JLP, ref. 9), with complementarity-determining regions (CDRs) excised, and split into separate variable and constant domains. After an initial step of rigid-body refinement in Phaser, the structure was then refined with PHENIX\textsuperscript{10} using torsion-angle simulated-annealing refinement with a maximum-likelihood amplitude target. The initial model was improved in an iterative process of visual inspection and model building using COOT\textsuperscript{11}, and cycles of refinement using PHENIX\textsuperscript{10} using riding hydrogens, grouped atomic displacement and TLS parameters. In the final rounds of refinement, the structure was refined with BUSTER followed by REFMAC\textsuperscript{12,13}. The final R\textsubscript{work} and R\textsubscript{free} are 22.17% and 27.76%, respectively (Supplementary Table 1), and the final model contains SUDV GP\textsubscript{1,2} residues 32–192, 212–287, 300–311 and 510–615, and Fab 16F6 residues 1–220 (heavy chain) and 1–212 (light chain). Electron density was missing for GP\textsubscript{1,2} residues 193–211, 288-299, 312–313, 473–509 and 616–637. Weak or discontinuous electron density was observed in the outer regions of the glycan cap (residues 266–287 and 300–311) and these regions were tentatively assigned mainly as polyalanine fragments. Despite the treatment of the GP\textsubscript{1,2} with PNGaseF, two glycan chains resistant to deglycosylation at N257 on GP1 and N563 on GP2 are visible. These glycans show higher B values than the overall average of the molecule (glycan average B= 176 Å\textsuperscript{2}; overall average B=122 Å\textsuperscript{2}), but there is unambiguous electron density for the first two NAG residues of these glycans (Supplementary Fig. 2d). 80.4% of residues are present in most favored regions, 15.6% in less favored regions, 2.4% in generously allowed regions, and 1.6% in disallowed regions of the Ramachandran plot. Final refinement statistics and analysis are presented in Supplementary Table 1. Atomic coordinates and structure factors are deposited in the RCSB Protein Data Bank under accession number 3S88. All structure figures have been created using PyMOL\textsuperscript{14} or VMD\textsuperscript{15}.

Attachment Assays, Pseudovirions: Vesicular stomatitis virus (VSV) pseudotypes bearing EBOV or SUDV GP\textsubscript{1,2} were generated and concentrated by pelleting as described\textsuperscript{16,17}. Vero cells were plated 24 h in advance at 2x10\textsuperscript{5} Vero cells/well of a 12-well plate and were chilled to 4°C before addition of VSV pseudovirus-antibody complexes. 0.7 mg antibody (IgG 16F6 for SUDV and IgG KZ52 for EBOV) was complexed with 800 µl each pseudovirus at 2 x10\textsuperscript{5} transduction units/ml for 1 h at 37°C.
Complexes were chilled and added to the cold cells for 1 h at 4°C. For attachment assays, cells were washed five times with cold PBS to remove any unbound virus and subsequently lysed off the plate with 40 µl of CytoBuster (Novagen).

To permit internalization of attached virions, cells were incubated at 37°C for 1 h. Cells were washed twice with cold PBS and bound virions were stripped from the cell surface by incubating with 0.1 mg ml⁻¹ of Proteinase K in PBS for 30 min at room temperature. Cells were washed five times with PBS to remove stripped virions. Cells were lysed in 40 ml CytoBuster. Cell lysates were run onto SDS-PAGE gels, blotted, and probed with IgG 23H12, which was directed against the matrix protein M of the VSV pseudovirus framework (gift of Michael Whitt, Univ. of Tennessee Health Science Center and Douglas Lyles, Wake Forest University School of Medicine).

**Attachment assays, ΔVP30 Ebola virus:** A biologically contained Ebola virus containing green fluorescent protein (GFP) instead of the essential viral protein VP30, was rescued and propagated in VeroVP30 cells as described previously. For the neutralization assay, EbolaΔVP30-GFP virus was diluted in medium (2% fetal calf serum in minimum essential medium) to approximately 90-100 pfu. Diluted virus was incubated with the indicated purified monoclonal antibodies (10 µg ml⁻¹) for 1 h at 37°C. The virus-antibody mixture was added to VeroVP30 cells in triplicate, allowed to absorb for 1 h at 37°C, then cells were washed three times, and overlaid with 1.5% methylcellulose. Five days after infection, plaques were counted. For the post-attachment neutralization assay, the same steps were followed as described above with the following modifications. Medium and VeroVP30 cells were prechilled to 4°C. The same amount of EbolaΔVP30-GFP virus was added to cells in triplicate and virus adsorption was allowed for 1 h at 4°C. Cells were then washed three times with chilled medium and the indicated monoclonal antibodies (10 µg ml⁻¹) were added to cells. Virus-antibody complexes were allowed to form for 1 h at 4°C after which, cells were washed three times with chilled medium. Cells were warmed up to 37°C for 1 h, then washed three times with room temperature medium, and overlaid with 1.5% methylcellulose. Five days after infection, plaques were counted.

**Cathepsin L cleavage, recombinant ectodomain:** 1µg of recombinant GP₁₂ (EBOV-Mayinga or SUDV-Bon) was incubated with a tenfold molar excess IgG KZ52 (for EBOV) or 16F6 (for SUDV) at 37°C for 1 h before digestion. IgG-GP₁₂ complexes were digested for 30 min at 37°C with 1µl of Cathepsin L (50 µg/ml) in 50 mM HEPES, 50 mM MES pH 5.5. Mock reactions were incubated in identical conditions but in the absence of enzyme. Reactions were terminated by the addition of 0.4M Tris HCl pH 9.0 and SDS loading buffer. Reactions were reduced, run on an SDS–PAGE gel, blotted and probed with an anti-HA antibody to detect the N-terminally tagged GP1.

**Cathepsin L cleavage, VSV- GP₁₂ -antibody mixtures:** EBOV/SUDV GP₁₂ -specific (KZ52 or 16F6) or isotype-matched control antibodies (human IgG1, Southern Biotech or mouse IgG1 anti-human IL-2, Pharmingen) were incubated at a final concentration of 100 µg/ml with VSV-GP₁₂ (1-2 µl of concentrated virus preparations) in NT buffer (10 mM Tris [pH 7.5], 135 mM NaCl) in a total volume of 20 µl for 2 h at 4°C. Recombinant CatL (R&D biosystems) was diluted into 100 mM MES [pH 5.5] buffer containing 2 mM EDTA and 5 mM DTT to yield the enzyme concentration range (0, 0.3125, 0.625, 1.25,
2.5, 5, and 10 µg/mL). Virus-antibody mixtures (2 µl) were added to 18 µl of the diluted CatL and the reactions were incubated at 37°C for 30 min. Reactions were then quenched on ice with 50 µM E64 and 250 mM HEPES [pH 7.5] buffer. To detect GP1, proteins were transferred to nitrocellulose membranes and incubated with a rabbit polyclonal antibody produced against GP1 residues 83-97 (1:10,000 dilution; a gift from Dr. James M Cunningham, Brigham and Women’s Hospital, Boston, MA).
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