Ebolavirus Entry Requires a Compact Hydrophobic Fist at the Tip of the Fusion Loop

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ABSTRACT
Ebolavirus is an enveloped virus causing severe hemorrhagic fever. Its surface glycoproteins undergo proteolytic cleavage and rearrangements to permit membrane fusion and cell entry. Here we focus on the glycoprotein’s internal fusion loop (FL), critical for low pH-triggered fusion in the endosome. Alanine mutations at L529, I544, and particularly the double mutant L529/I544 compromised viral entry and fusion. The NMR structures of I544A and L529A/I544A in lipid environments showed significant disruption of a three-residue scaffold that is required for the formation of a consolidated fusogenic hydrophobic surface at the tip of the FL. Biophysical experiments and molecular simulation revealed the position of the WT FL in membranes and showed the inability of the inactive double mutant to reach this position. Consolidation of hydrophobic residues at the tip of FLs may be a common requirement for internal FLs of class I, II, and III fusion proteins.

IMPORTANCE
Many class I, II, and III viral fusion proteins bear fusion loops for target membrane insertion and fusion. We determined structures of the Ebolavirus fusion loop and found residues critical for forming a consolidated hydrophobic surface, membrane insertion, and viral entry.
Introduction

Ebola virus (Ebov) is a filovirus that causes severe hemorrhagic fever with mortality rates between 25-90% (1, 2). Outbreaks involving human fatalities have occurred in sub-Saharan Africa since 1976, the last two being in Uganda and the Democratic Republic of the Congo in 2012 (3). Ebola virus is also a much feared potential agent of bioterrorism. However, there are still no FDA-approved treatments or vaccines for these devastatingly morbid infectious agents.

One area of therapeutic interest is to target the viral entry machinery that governs virus-host membrane fusion. This would halt infection before initiation of viral replication and subsequent cell destruction. To guide therapeutic design, a detailed knowledge of Ebov entry and membrane fusion is needed, and currently little is known about Ebov virus-host membrane interactions.

Entry of Ebov is mediated by glycoprotein (GP) spikes that protrude from the virus particle (4-8). Like most other class I viral fusion proteins, GP is composed of a receptor binding (GP1) and a fusion (GP2) subunit. After binding to cell surface receptors, GP mediates virus uptake through a macropinocytotic-like process (9, 10). Viral fusion ultimately occurs in endosomes where GP1 is cleaved by cathepsins B and L to an ~19 kDa species (11, 12) which engages Niemann-Pick C1, a late endosomal protein essential for Ebov entry (13-15). A final unknown trigger causes conformational changes in GP (16, 17) that expose the fusion loop (FL) in GP2. The Ebov FL is clamped by a disulfide bond and has a hydrophobic region at its tip. It is thought to be functionally equivalent to the linear hydrophobic and glycine-rich fusion peptides found at the N-termini of most other class I fusion proteins (18-22).

It is clear that the unique internal fusion loop is critically involved in mediating fusion of Ebov with late endosomes, but we do not fully understand which specific protein-lipid interactions give rise to this activity. In pursuit of this question, we previously determined two solution NMR structures of the FL in a membrane mimetic (20). One was determined at low pH (similar to
endosomal conditions) and represents the active conformation. The other structure was
determined at neutral pH and represents a fusion-incompetent state. In the fusion-competent
state, the hydrophobic residues of the FL form a structure that resembles a hydrophobic ‘fist’
that we hypothesized to be the membrane-penetrating entity of the FL.

Here we report evidence that the Ebov FL ‘fist’ is, indeed, required for virus entry and is the
portion of the FL that embeds in the target membrane. Through analysis of our previous
structures, we identified FL residues likely critical for function and tested them using alanine
mutants and liposome fusion assays. We then verified the functional importance of these
residues to cell entry using virus-like particles (VLPs) in cell culture. We determined the NMR
structures of the most debilitated FL mutants I544A and L529A/I544A at pH 5.5 and compared
them to the pH 5.5 WT structure. The single mutation at 544 and double mutations both
severely disrupted the hydrophobic surface associated with the critical fist structure, which is
supported by a scaffold formed among residues L529, F535, and I544. These residues appear
to interact in an hierarchical order to form the required hydrophobic surface. Additional
biophysical and computational studies further revealed how the fusion loop inserts into lipid
bilayers to induce fusion, explaining why this activity is disrupted by the single and double
mutations.

Materials and Methods

Lipids and Detergents. 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-
2-oleoyl-sn-glycero-3-(1-phosphoglycerol) (POPG), 1,2-dioleoyl-sn-glycero-3-
phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-DOPE), 1,2-dioleoyl-sn-
glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (Rh-
DOPE), 1-palmitoyl-2-(6,7-dibromo)stearoyl-sn-glycero-3-phosphocholine (6,7-Br PC), 1-
palmitoyl-2-(9,10-dibromo)stearoyl-sn-glycero-3-phosphocholine (9,10-Br PC), 1-palmitoyl-2-(11,12-dibromo)stearoyl-sn-glycero-3-phosphocholine (11,12-Br PC) were purchased from Avanti Polar Lipids. Dodecylphosphocholine (DPC) was purchased from Anatrace.

**Site-directed Mutagenesis of Ebov FL.** Mutants were prepared using site-directed mutagenesis on the WT Ebov FL which was cloned into a pET-41 vector containing a T7 promoter and kanamycin resistance. Primers were designed to make the described Ala and single Trp and combination mutants. Stratagene QuikChange Site-Directed Mutagenesis Kit # 200518 was used to make point mutations to the wild type Ebov FL.

**Site-directed Mutations of Membrane-Anchored Trimeric GP1/GP2:** Primers were designed to mutate residues L529 and I544 to alanine. L529A Forward- G GAT GAA GGT GCT GCA ATC GGA GCG GCC TGG AT C CC. I544A Forward- GCA GCC GAG GGA ATT TAC GCA GAG GGG CTA ATG C. pfu Ultra HF DNA polymerase (Agilent #600380-51) was used to make point mutations in a trimeric GP (WT) mammalian expression vector. Maxi-preps of the sequence-confirmed DNA were then prepared using an Invitrogen HiPure Plasmid Maxiprep kit (#K210007).

**Expression and Purification of Ebov FL.** Ebov FL WT and its mutants were expressed and purified as previously described with several modifications(20). Cell cultures grown at 37°C were induced with IPTG at A_{600} 0.7-0.9 OD and expression was conducted at 30°C for 2-4 hours. β-mercaptoethanol was removed from the digestion buffer to increase the efficiency of His-tag cleavage by Factor Xa.

**Production and Purification of Ebola Virus Like Particles (VLPs).** VLPs were produced essentially as previously described(23). In brief, HEK293T cells were plated and allowed to grow until the cells were 70-80% confluent. A mixture of β-lactamase-VP40, Cherry-VP40, VP-
40 and GP delta WT, L529A, I544A, or L529A/I544A DNA was incubated with Polyethylenimine (PEI; Polysciences Inc) and added to the cells. After incubation at 37°C for 48 hours, the media were harvested and cleared of cell debris via centrifugation. Cleared media were overlayed onto a 20% sucrose cushion and ultracentrifuged. Pellets were resuspended and stored at -80°C. Samples were analyzed on western blots probed with anti-Ebov GP and anti-Ebov VP40 antibodies, followed by appropriate secondary antibodies. Blots were then imaged on an Odyssey Infrared Imaging System (LI-COR) and analyzed for ratios of GP to VP40 using Odyssey software v3.0.

**Fluorescence Lipid-Mixing Assay.** A 96-well plate reader format of the lipid mixing assay was developed from previously described methods(20). Large unilamellar vesicles (LUVs) composed of POPC:POPG (85:15) were labeled with 1.5 mol % of both Rh-DPPE and NBD-PPPE. Experiments were performed with 100 μM lipid and 5 μM Ebov FL at pH 7.4 and 5.0. Fluorescence measurements were recorded using a Molecular Devices SpectraMax M5 plate reader fluorometer at room temperature with a 1 second mixing agitation between each scan. Excitation and emission wavelengths were set at 460 nm and 538 nm, respectively. All mutants were normalized to WT lipid mixing values. Experiments were repeated in triplicate for all FLs with at least two different sets of liposome preparations.

**Ebola VLP internalization.** VLP internalization assays were performed essentially as described(23). In brief, 10 μL of WT, L529A, I544A, and L529A/I544A Ebola GP VLPs were bound to confluent CHOK1 cells by low speed centrifugation and washed on ice. Internalization took place for 1 hr at 37°C. 150 μL of 0.5% phenol red-free trypsin was added to cells for 30 minutes and cells were washed to remove uninternalized VLPs. Cells were then lifted, washed, and analyzed on an LSRFortessa cytometer (Becton Dickinson flow cytometer) for mCherry fluorescence (representing internalized VLPs). Cells that did not receive any VLPs and were warmed for 1 hr and were used for gating. All data were analyzed using FlowJo software.
**VLP entry assay.** VLP entry assays were performed essentially as described(23). Post centrifugation (described in VLP internalization section), VLPs were allowed to enter into cells for three hours at 37°C. A β-lactamase substrate, CCF2-AM (Invitrogen) was added and cells were incubated in the dark at room temp for 1 hr. The CCF2 solution was then removed and the cells were washed once and incubated overnight in the dark at room temperature. Cells were lifted, fixed, and analyzed using a FACS Calibur flow cytometer. The shift in fluorescence due to cleavage of the CCF2 substrate in the cell cytoplasm by beta-lactamase tagged VP40 was used to measure the extent of entry(24). Cells treated with CCF2-AM alone were used for gating. All data were analyzed using FlowJo software.

**NMR Experimental and Structure Determination.** Experiments were performed at 30°C on a Bruker Avance III 600 spectrometer equipped with a cold probe. $^{15}$N-$^1$H HSQC(25) and aliphatic and aromatic $^{13}$C-$^1$H HSQC(25, 26) were collected to show N-H and C-H correlations. CBCANH and HNCO(27) experiments were performed to obtain backbone assignments. Side chain assignments were obtained from CCONNH(28), HCCONNH(29), and HCCH-TOCSY experiments. $^{15}$N-edited NOESY(30) and aliphatic and aromatic $^{13}$C-edited NOESY(25, 31) with mixing times of 100 ms were used to collect NOEs. Samples contained 600 µM protein at pH 5.5 in a solution buffered with 30 mM sodium phosphate, 50 mM NaCl and contained 150 mM d$_{38}$-DPC. Data were processed with NMRPipe(32) and analyzed using Sparky(33). TALOS plus(34) was used to calculate the dihedral angle restraints. Cyana(35) was used to calculate the initial structures, followed by calculation of 200 structures with CNS(36). 20 conformers with the lowest target function were chosen for representation of the overall structure. Regular secondary structure was determined in MOLMOL by an algorithm utilizing hydrogen-bonding patterns(37). All structures were rendered using PyMOL as the molecular graphics system(38).

**NMR Dynamics.** The $^{15}$N $T_1$ and $T_2$ relaxation and $^1$H-$^{15}$N heteronuclear NOE data were
recorded at 30°C on a Bruker Avance III 600 spectrometer equipped with a cold probe using three-dimensional TROSY-HNCO based pulse sequences(39). (\(^1\)H)-\(^{15}\)N heteronuclear NOE experiments were acquired as two interleaved 3D spectra (NOE and reference spectra, respectively), with a 5-s saturation delay. Relaxation delay times of 50, 120, 300, 600, 1,000, 1,500, and 3,000 ms and 0, 17, 34, 68, 119, 170, 255, 340, and 511 ms were used in the R1 and R2 experiments, respectively.

**Isothermal Titration Calorimetry.** Measurements were made using a MicroCal VP-Isothermal Titration Calorimeter (MicroCal, Northampton, MA). Small unilamellar vesicles (SUVs) were prepared as previously described(20). 6 \(\mu\)L of lipid injections of SUVs composed of POPC:POPG 85:15 were titrated to a fixed amount of protein. Stock lipid and protein concentrations were optimized for each mutant and wild-type according to their lipid affinities. The concentrations were as follows: 2.5 mM lipid into 20 \(\mu\)M WT pH 5.0 and WT 7.4, 10 mM lipid into 50 \(\mu\)M L529A pH 5.0, 20 mM lipid into 50 \(\mu\)M I544A pH 5.0, and L529A/I544A pH 5.0. Heats were integrated and fitted with a Wiseman isotherm(40).

**Tryptophan Quenching Experiments.** Fluorescence measurements were made in a 384 well plate using a Molecular Devices SpectraMax M5 plate reader. Tryptophan excitation was set to 295 nm and emission was detected at 330 nm. SUVs were composed of POPC:POPG 85:15 for control samples or POPC:POPG:Br-PC 55:15:30 where the lipids were brominated at 6,7-, 9,10-, or 11,12-positions. 500 \(\mu\)M lipid was incubated with 5 \(\mu\)M protein at pH 5.0. Relative intensities at 330 nm were calculated for each bromine position and fit using distribution analysis(41). A list of sequences for single Trp mutants is shown in Figure 6c below.

**Molecular Dynamics Simulations.** All atomistic simulations were performed using Gromacs 4.5(42) and the Charmm36(43) force-field for protein and lipid interactions. Temperature was...
controlled at 300 K using the velocity-rescaling thermostat (44) and pressure was maintained at 1 bar using the Parrinello-Rahman barostat (45). All covalent bonds were constrained using P-LINCS (46), and long-range electrostatics were computed every step using PME (47). The lipid bilayer was composed of a 512-lipid patch (POPC:POPG 85:15). The system included approximately 34,000 TIP3p waters. The program g_membed (48) was used for inserting the FL into the bilayer. To model a pH of 5.5, residues H516, H549, and E545 were protonated. Docking of the WT pH 5.5 structure was performed using the best-fit Trp insertion depths (see Table 3 below). Each mutant structure was initially placed in the bilayer via global rigid-body alignment to the WT structure. Following insertion, each system was energy minimized for 500 steps. Systems were then equilibrated for 40 ns using the Gromacs pull code to maintain residues W518, W531, Y534 and Y543 at their initial distances to the phospholipids headgroups, and also using local bond restraints (restraining distances for each pair of backbone atoms within 7Å of each other to their original distance) to maintain the initial structure. Both sets of restraints were then released, and production runs were carried out for >500 ns in each case. Equilibration and production runs were performed with the same settings for electrostatics and van der Waals (vdW) interactions, using a short-range electrostatic cutoff of 1.2 nm, while vdW interactions were switched off between 0.8 and 1.2 nm. The time-step in the atomistic simulations was 2 fs.

Coarse-grained simulations were performed using Gromacs 4.5 and the MARTINI force-field (49, 50). An initial patch with the same constituents as for the atomistic simulations was produced using the g_fg2cg (51) tool. A coarse-grained representation of the FL was then placed out of the membrane, at a center of mass-distance to the phospholipid headgroups of approximately 3 nm. Local structural restraints were put in place to maintain the NMR structure when in solution conditions. Eleven runs of each system (WT, L529A/I544A) were completed. Each system was
Results

Identification of Residues Required for Liposome Fusion and VLP Cell Entry. Analysis of FLs containing mutations at 13 hydrophobic residues revealed several partially inactive single mutants and one completely inactive double mutant (Fig. 1a and b). The WT NMR structure showed long-range hydrophobic interactions between residues L529 and I544(20) that we hypothesized might be defining its structure and hence critical for membrane fusion and cell entry. To test this hypothesis, we focused here on two partially inactive mutants, L529A and I544A, and the completely inactive double mutant, L529A/I544A. Activity of FL mutants was first determined using a liposome-based FRET fusion assay (Fig. 1b). The results showed that the double mutant L529A/I544A was most compromised for FL activity: only 2% of WT lipid mixing activity was observed with this mutant. Single alanine mutations at L529 and I544 showed 68% and 22% lipid mixing activity, respectively. These mutations were next introduced into a construct encoding transmembrane-anchored GP1/GP2 trimeric spikes, and co-expressed with Ebov VP40 in HEK293 cells to form viral like particles (VLPs)(23). The GPs containing mutations at 529 and/or 544 were successfully incorporated into VLPs and supported VLP internalization into cells as efficiently as WT GP (Fig. 1d and 1e). Nonetheless, as seen in Fig. 1c, the trimeric membrane-anchored GP mutants showed similar defects in supporting VLP entry into the cytosol of target cells (a measure of VLP fusion with late endosomes) as their respective FLs showed for liposome fusion, although I544A was somewhat more impaired for VLP entry. The W518A FL mutant, which showed significantly impaired lipid mixing (Fig. 1b), was not further characterized because this mutation perturbed incorporation of GP into VLPs.
Secondary Structure Analysis of Ebov FL Mutants by NMR. To probe for long-range effects of alanine mutations on secondary structure in the FL constructs, we used NMR spectroscopy to gather structural information on each residue in the L529A, I544A, and L529A/I544A FLs, and compared the data to those for the WT FL. Heteronuclear single-quantum correlation (HSQC) spectra at pH 5.5 for WT, L529A, I544A and L529A/I544A all showed well dispersed spectra indicative of the presence of well defined secondary structure (Fig. 2 and Fig. 3).

The backbone resonances of each mutant, assigned using standard triple resonance techniques on $^{15}$N- and $^{13}$C-labeled samples, showed marked differences in peak position when compared to WT. Chemical shift differences are plotted in Fig. 2c and Fig. 3c and d to highlight the observed $^1$H and $^{15}$N chemical shift changes.

As expected, significant changes in chemical shift were observed directly around each mutation site. However, changes above 0.2 ppm were also apparent for L529A extending out to Y534, and, in the case of I544A, increased chemical shifts were observed down to residue G536. In the double mutant, significant changes were observed for the majority of the residues that compose the primary hydrophobic region, i.e., A525 through A539, as well as the downstream I542 to I544 region, which was previously shown to contribute hydrophobicity to the fist structure(20).

Solution NMR Structure of I544A and L529A/I544A. To further scrutinize the molecular reasons for the moderate and severe fusion and entry defects of the I544A and L529A/I544A mutants, we determined their solution NMR structures in the presence of DPC micelles at pH 5.5. To do so, 306 and 447 NOEs, respectively, were collected from $^{15}$N-edited and $^{13}$C-edited NOESY experiments. Structures were calculated using these and additional dihedral angle restraints (Table 1). Fig. 4b and c show the 20 lowest-energy conformers of I544A and L529A/I544A and compare them to the 20 lowest-energy conformers of WT, which was
previously determined in DPC micelles at pH 5.5 (Fig. 4a)(20). Complete $^{15}$N relaxation data show that all structures are fairly well ordered throughout the molecules (see online Supplemental Relaxation Data). For example, except for the terminal residues, all ($^1$H)-$^{15}$N NOE values are in the range 0.4 to 0.85, reaching their theoretical rigid limit, and most of them are between 0.6-0.8, which is typical for lipid micelle-associated proteins and polypeptides(52, 53). Compared to WT, the double mutant structure shows a significantly flattened tip and lacks the characteristic 90° bend. The single mutant also lacks the 90° bend, but still shows an interaction between Ala544 and Leu529 (Fig. 4e) not seen in the double mutant (Fig. 4f). As predicted from the chemical shift data, there is a significant structural change in the hydrophobic region located at the tip of the FL. A direct comparison of the WT, single, and double mutant fusion loop tips is shown face-on in Fig. 4d-f. The double mutant clearly shows a flat elongated tip where the highlighted residues 529, 535, and 544 are spread out and no longer form the hydrophobic scaffold of residues L529, F535 and A544 that we propose is required for the formation of the fist seen in WT. As seen in Fig. 4g-i, the hydrophobic residues, red, are therefore broadly distributed at the face of the double mutant, while they are close-packed at the face of the compact WT structure and intermediate in the single mutant.

**Binding and Penetration of the Ebov FL into Membranes.** We next asked whether the fusion and entry defects of the mutants were primarily due to a different strength of membrane binding or a different mode of penetration into the membrane or both. To address the first question, we measured binding of the WT and mutant FLs to liposomes by isothermal titration calorimetry (ITC). ITC binding isotherms are shown in Fig. 5. The data were analyzed with a partition model as indicated in Methods and the resulting apparent partition coefficients are shown in Table 2. Experiments were performed for WT, the L529A and I544A single mutants, and the double mutant at pH 5.0 and for WT at pH 7.4. Binding was not detected for either WT at pH 7.4 or L529A/I544A at pH 5.0. The apparent partition coefficients were 112,000 M$^{-1}$ for WT, but only
30,000 and 5,400 M$^{-1}$, respectively, for the two single mutants at pH 5.0. Thus, the partitioning of these FLs into bilayers was weaker than for WT, following the same trends as seen in the lipid mixing and VLP entry assays.

To measure penetration of the FL into lipid bilayers, we measured the quenching of tryptophan (Trp) residues by membrane-bound brominated lipids(54). The fusion loop contains two natural Trp residues (518 and 531) and two natural tyrosines (534 and 543). To separately measure the penetration depth at each of these positions we made four single Trp mutants. To produce the single Trp518 and Trp531 mutants, residues 531 or 518 were individually converted to phenylalanines. To produce the Trp534 and Trp543 mutants, the two tyrosines were individually converted to Trp residues in a background in which Trp 518 and 531 were both mutated to phenylalanines. To ensure that Trp substitutions did not affect function, lipid mixing control experiments were performed for the single Trp mutants in the WT backbone (Fig. 6d). Analogous single Trp mutants were also made for the L529A, I544A, and L529A/I544A mutants.

For clarification the sequences of all 16 FL constructs are shown in Fig. 6c.

Insertion depths of the 16 resulting single Trp mutants were measured via collisional fluorescence quenching with bromine, using liposomes containing bromo-PC substituted with bromines at acyl chain (6,7), (9,10), or (11,12) positions and fitting the data by distribution analysis as described in Methods. The results for WT show that residues Trp518 (green symbols) and Trp531 (blue symbols) do not penetrate the bilayer; no significant interaction with the lipid acyl chains was observed (Fig. 6a). However, Trp534 (cyan line) and Trp543 (red line) showed quenching profiles indicative of acyl chain penetration (Fig. 6a). Quenching profiles for L529A showed similar results to WT, where residues 534 and 543 are well associated with the membrane and 518 and 531 are not (Fig. 6b). We therefore concluded that L529A has comparable FL orientation and membrane insertion to WT. As expected from the ITC results, no considerable insertion was detected for I544A or for L529A/I544A. The maximum-likelihood
depths of penetration for each single Trp construct for WT and L529A, expressed as distance from the bilayer center or surface, are shown in Table 3. The most notable differences occur at Trp534 and Trp543: Trp534 and Trp543 were located approximately 7.9 Å and 8.8 Å from the center of the bilayer for WT, and 9.3 Å and 7.9 Å, respectively, for L529A. Assuming that the thickness of our bilayers is 42 Å (phosphate-to-phosphate distance), Trp534 and Trp543 penetrate into the first leaflet of the lipid bilayer approximately 13.1 Å and 12.2 Å for WT and 11.7 Å and 13.1 Å for L529A, respectively.

Molecular Dynamics Simulations to Dock Positions of Ebov FL in Lipid Bilayers.
Molecular dynamics simulations were used to test the physical compatibility of the FL structures determined in micelles with bilayer insertion data as well as the degree to which the hydrophobic fist structure influences membrane interaction. The WT NMR structure at pH 5.5 was docked into a POPC:POPG 85:15 lipid bilayer based on the experimental tryptophan depth approximations (Fig. 6a), and the I544A and L529A/I544A structures were aligned to the docked WT. Each docked structure was then simulated via atomistic molecular dynamics for 500 ns (See online Supplemental Video 1, Video 2 and Video 3 for WT, I544A and L529A/I544A simulations, respectively). As shown in the simulation snapshots taken after 400 ns, each mutant stabilized to a slightly different insertion mode (Fig. 7a-c). The number of membrane-inserted residues was not statistically different between the wild-type peptide and the I544A mutant over the 500-ns simulation trajectories, but the wild-type peptide retained its "fist" structure, whereas I544A never assumed such a structure in the unrestrained simulations. The L529A/I544A mutant, by contrast, maintained insertion of only a short part of the loop, and the number of inserted residues was significantly fewer than either the wild-type or the I544A mutant (p < 10⁻⁵ via Wilcoxon Rank Sum Test). The experimental NMR structures were not constrained in these simulations. Nonetheless, all three molecules maintained their structures throughout the course of the simulation with calculated Cα (C₅₁₁-C₅₅₆) r.m.s.d. values of 2.2 Å,
1.7 Å, and 2.6 Å for the WT, I544A, and L529A/I544A, respectively. We did not observe gross disruption or curvature induction of the bilayers in these simulations, but we cannot rule out that such effects could happen with much more extensive sampling and additional copies of FLs in the membrane.

Coarse-grained simulations were also conducted where the structures were placed in solution and allowed to interact with a bilayer. In these coarse-grained simulations, each sequence was constrained to hold its respective structure determined via NMR. The hydrophobic portion of the WT fist structure (A525-I544) (Fig. 7d and e) bound and penetrated bilayers more often and more deeply than the corresponding region of the open L529A/I544A structure, which only associated minimally with the phospholipid headgroups.

**Discussion**

Virus-host membrane fusion is an essential step in the lifecycle of every enveloped virus. One of the first events in initiating membrane fusion is the interaction of viral fusion peptides or fusion loops, found in all viral fusion proteins, with the target membrane. The fusogenic peptide from Ebov is somewhat unique among Class I fusion proteins in being an internal loop maintained by a disulfide bond, as opposed to being a more typical N-terminal fusion peptide. To investigate structural properties that are critical for the interaction of the internal Ebov FL with target membranes and their functional consequences we employed solution NMR, fluorescence spectroscopy, virus-like particle cell entry, calorimetry, and molecular simulation methods. Although membrane interactions of N-terminal short fusion peptides of many viruses have been studied in the past in much detail, the current work represents the first comprehensive investigation of the interaction of a large disulfide-clamped fusion loop with lipid membranes. Using liposome fusion and Ebov-GP VLP entry assays we first identified L529 and I544 as key...
residues for virus fusion and entry. L529 is located in the previously recognized major hydrophobic region of the FL, while I544 is located in an additional short hydrophobic segment located C-terminal to the primary hydrophobic region. These results confirm our hypothesis that L529 and I544 engage in a hydrophobic interaction (hydrophobic bridge) that is critical for the development of the fusion-active hydrophobic surface of the FL. The current study further demonstrates that F535 needs to interact with the hydrophobic bridge to form a hydrophobic triad that supports the bent fist structure of the fully functional fusion loop. The I544A mutant forms a smaller hydrophobic bridge (between A544 and L529), to which F535 no longer binds. Therefore, this mutant no longer forms either the triad or the bent fist. In the double mutant, there is no interaction between residues 529, 535 and 544 and, hence, this mutant is severely altered in structure and does not interact with membranes to any significant extent.

Beyond providing proof for the importance of the three-point hydrophobic scaffold in developing the functional fist structure of the Ebov FL, the calorimetric binding and fluorescence-based membrane insertion data show how this structure inserts into lipid bilayers, and that the [L529-I544]-F535 scaffold is required for the FL to properly bind and penetrate into model membranes. Moreover, the combined insertion and molecular dynamics studies demonstrate that the surface of the WT FL that interacts most extensively with the lipid bilayer is the front face, or tip, of the fist-shaped FL. I527, W531, I532, Y534, F535, I542 and Y543 are consolidated at this tip to form a contiguous hydrophobic surface for membrane insertion.

The hydrophobic scaffold residues L529, F535, and I544 all point inward in the fusion-active WT structure(20). When I544 or both I544 and L529 were mutated to alanines, the fusion activity of the FL, as well as VLP entry mediated by Ebov-GP, were both inhibited. While we saw only a minimal effect of mutating F535 to an alanine we saw a significant effect on function when F535 was mutated to a tryptophan, which is bulkier than phenylalanine. This example shows that the ability to fuse membranes is not simply an effect of the hydrophobicity of individual residues, but
that some mutations can have long-range effects in these larger FLs with tertiary structure. Mutation of F535 to an arginine was previously shown to significantly reduce GP-mediated virus entry as well as binding of the GP ectodomain to liposomes(16, 21). Collectively our findings indicate that the scaffold formed by L529, F535, and I544 is strictly required to form the fist with the consolidated hydrophobic surface at the tip of the FL and that this specific structure is crucial for a sufficiently deep membrane insertion of the FL, as well as its ability to promote membrane fusion and virus entry.

All fusion peptides and fusion loops are thought to function by inserting hydrophobic residues into the lipid bilayer of target membranes. However, the level of hydrophobicity and the distribution of hydrophobic residues needed to create a functional membrane-interacting surface are not known. In Fig. 8 we compare the structures of several FLs, most derived from the post-fusion structures of the corresponding viral fusion proteins. A number of different patterns emerge in terms of distribution of hydrophobic residues on the surfaces of these FLs. In some cases the hydrophobic residues form consolidated hydrophobic surfaces as we revealed for the Ebov FL at pH 5.5 in DPC micelles, but in other cases they are more sparsely distributed. Examples of consolidated hydrophobic surfaces can be found in class I (Ebov) (Fig. 8a), class II (Flaviviruses) (Fig. 8c) and class III (Vesicular stomatitis virus) (Fig. 8b) fusion proteins. On the other hand, some class II (Rubella virus) (Fig. 8e) and class III (Baculovirus and Herpes viruses) (Fig. 8e-h) fusion proteins display quite dispersed hydrophobic residues on their FL surfaces, while some class II alphaviruses (Fig. 8d) feature intermediate situations. Since most of these structures were obtained in the absence of a membrane mimetic, it is possible that further rearrangement of hydrophobic side chains on the surface may take place when these FLs bind to and penetrate membranes. An indication that a consolidation of hydrophobic residues may happen upon lipid binding is given by the comparison of two structures of the Chikungunya virus FL: the residues are more dispersed on the FL in the crystal structure of the full (pre-fusion)
fusion protein without lipids (55), but more consolidated, with clusters of two Phe and two Tyr residues, in an 18-residue peptide analogue inserted into DPC micelles for structure determination by NMR (Fig. 8d) (56). A similar consolidated hydrophobic grouping formed by Trp, Phe, and Leu residues was observed in the FLs at the tips of class II flavivirus fusion proteins (Fig. 8c) (57-59).

A consolidated hydrophobic surface may be required for the proper functioning of all FLs. Hence, as we found for the Ebov FL upon exposure to low pH (in a membrane mimetic) (20), rearrangements of hydrophobic residues have also been proposed (60) and experimentally supported (61, 62) for the fusion loops of the Herpes simplex virus gB fusion protein. Similarly, the bipartite FLs from Rubella virus E1 are proposed to form, along with an amphipathic α-helix, a large membrane-interacting surface, and this consolidation of hydrophobic regions is thought to be crucial for the infectivity of the virus (63). Alternatively, a greater number of FLs may be required to come together at the site of fusion if viral fusion peptides/loops have more sparsely distributed hydrophobic residues. This may be the case for Baculovirus, for which up to ten trimers of gp64 appear to be required (64).

Our structures of the Ebov GP2 FL are the first molecular depictions of a large disulfide-clamped FL in a lipid environment. Together with analyses of the structure-disrupting mutations, our findings clearly show that a continuous consolidated hydrophobic surface is necessary to promote fusion of this virus with its target membrane, both in vitro and in vivo. Our findings also indicate that an internal scaffold of three hydrophobic residues is needed to present the consolidated hydrophobic surface to the target membrane. It will be interesting to see whether other viruses have similar strict requirements on surface presentation of hydrophobic residues on their FLs to enable fusion. At least for the case of Ebov GP2, low pH combined with a lipid interface cooperate to trigger formation of the fusion-active conformation with a sufficiently large exposed hydrophobic surface area. More structural information in appropriate environments is
required for other viral fusion proteins before we can determine with certainty which features of FLs are common and required to render them fusogenic. The current work on Ebov GP2 not only represents a major step forward towards a thorough understanding of the structural features of the Ebov FL under fusion-competent conditions, but hopefully will also stimulate similar future work on the FLs of other viruses. Such information combined with structural knowledge of the fusion proteins in their pre-fusion states and pathways to post-fusion states, as is emerging for Ebov GP(4, 7, 11, 12, 16), may facilitate the development of therapeutics aimed against the fusion machinery.

**Accession codes.** The atomic coordinates for the NMR structure of the Ebov FL structures for I544A and L529A/I544A have been deposited in the Protein Data Bank with accession codes **2MB1** and **2M5F**, respectively. The associated NMR chemical shifts have been deposited in the Biological Magnetic Resonance Bank with accession codes **19383** and **19052**, respectively.

**Acknowledgements:** We thank Van Ahn Do for experimental help preparing and assessing WT and mutant VLPs. This work was supported by NIH grants R01 AI030557 (to LKT), R21 AI103601 (to JMW), and R01 GM098304 (to PMK).
Table 1: NMR and refinement statistics for Ebola FL in DPC micelles

<table>
<thead>
<tr>
<th>NMR distance and dihedral restraints</th>
<th>I544A pH 5.5</th>
<th>L529A/I544A pH 5.5</th>
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<tr>
<td>Distance restraints</td>
<td></td>
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<tr>
<td>Total NOE</td>
<td>306</td>
<td>447</td>
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<tr>
<td>Intra-residue</td>
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<td>124</td>
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<tr>
<td>Inter-residue</td>
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<tr>
<td>Sequential (i-j = 1)</td>
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<tr>
<td>Medium-range (i-j ≤ 4)</td>
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<tr>
<td>Total dihedral angle restraints</td>
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<td>ψ</td>
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<tr>
<td>Structure statistics</td>
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<td>Violations (mean and s.d.)</td>
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<tr>
<td>Distance restraints (Å)</td>
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<td>0.031 ± 0.001</td>
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<tr>
<td>Dihedral angle restraints (°)</td>
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<tr>
<td>Max. distance restraint violation (&gt;0.2 Å)</td>
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<tr>
<td>Deviations from idealized geometry</td>
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<tr>
<td>Bond lengths (Å)</td>
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<td>Bond angles (°)</td>
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<td>Impropers (°)</td>
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<td>Average pairwise r.m.s. deviation* (Å)</td>
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<tr>
<td>Heavy</td>
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<td>1.30 ± 0.38</td>
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<tr>
<td>Backbone</td>
<td>1.98 ± 0.41</td>
<td>1.93 ± 0.38</td>
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Table 2: Partition Coefficients for Ebov FL Partitioning into POPC:POPG (85:15) Bilayers*

<table>
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<th>Fusion Loop</th>
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<tr>
<td>WT</td>
<td>5.0</td>
<td>1.12 ± 0.05</td>
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<tr>
<td>WT</td>
<td>7.4</td>
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<tr>
<td>L529A</td>
<td>5.0</td>
<td>0.30 ± 0.02</td>
</tr>
<tr>
<td>I544A</td>
<td>5.0</td>
<td>0.054 ± 0.002</td>
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<tr>
<td>L529A/I544A</td>
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<td>nd**</td>
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* not detected

Table 3: Distances of Ebov FL residues from Bilayer Center and Phospholipid Headgroup determined by fluorescence quenching

<table>
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<tr>
<th>Trp</th>
<th>Bilayer Center</th>
<th>Phospholipid Headgroup</th>
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<tr>
<td></td>
<td>WT  L529A</td>
<td>WT  L529A</td>
</tr>
<tr>
<td>Trp518</td>
<td>&gt;21 Å</td>
<td>&gt;21 Å</td>
</tr>
<tr>
<td>Trp531</td>
<td>&gt;21 Å</td>
<td>&gt;21 Å</td>
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<tr>
<td>Trp534</td>
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<td>Trp543</td>
<td>8.8 Å  7.9 Å</td>
<td>12.2 Å  13.1 Å</td>
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**Figure Legends**

**Fig. 1** Activity of WT and mutant Ebov fusion proteins. (a) Schematic of the Ebola FL sequence: hydrophobic regions in pink, disulfide-bonded cysteine residues in orange. Twelve residues were converted to alanine, and an alanine was converted to a leucine. Circles indicate: open ≥ 70% activity; crossed ≥ 30% activity; closed < 30% activity. (b) All mutants were analyzed for FL lipid mixing activity. (c) Lipid mixing activity of WT and key (*) in b) mutant Ebov FLs are compared to entry into cytoplasm of VLPs with trimeric Ebov GPs with WT or mutant (* ) fusion loops into CHOK1 cells. Results are shown normalized to WT. (d) Equivalent incorporation of mutant GPs into VLPs and equivalent internalization of all GP mutant VLPs compared to WT was confirmed in the gel assessing incorporation of mutant GPs into VP40-based VLPs. Normalized GP:VP40 incorporation values (numbers under gel lanes) are averages of three measurements. (e) Ability of VLPs bearing mutant GPs to be internalized from cell surface into CHOK1 cells. Error bars indicate standard deviations of experiments performed in triplicate. Any perceived differences between mutants and wild-type in panel e are not statistically significant.

**Fig. 2** Observed changes in chemical environment induced by alanine mutations. Assigned HSQC spectra of Ebov FLs in DPC micelles at pH 5.5. (a) WT and (b) L529A/I544A. (c) $^1$H and $^{15}$N chemical shift differences between L529A/I544A and WT Ebov FLs. Ala mutation sites are marked with red circles. Proline residues and other residues that could not be assigned are marked with an (x). Chemical shift differences were combined according to $\Delta\delta_{\text{comp}} = [\Delta\delta_{\text{HN}}^2 + (\Delta\delta_{\text{NN}}/6.25)^2]^{1/2}$. (65)

**Fig. 3** Observed changes in chemical environment induced by alanine mutations. Assigned HSQC spectra of Ebov FLs (a) L529A and (b) I544A in DPC micelles at pH 5.5. $^1$H and $^{15}$N chemical shift differences between (c) L529A or (d) I544A and WT Ebov FLs. Proline residues and other residues that could not be assigned are marked with an (x). Mutation sites are labeled with a red circle. All measurements were carried out in DPC micelles at pH 5.5.

**Fig. 4** NMR structures of WT (left), I544A (center), and L529A/I544A (right) Ebov FLs in DPC micelles at pH 5.5. The 20 lowest-energy conformers are rendered for (a) Ebov FL WT, (b) Ebov FL I544A, and (c) Ebov FL L529A/I544A. Irregular structured loops and turn regions are shown in grey, $\alpha$-helix in green, $\beta$-
sheet in blue, and the disulfide-linked Cys$_{511}$ and Cys$_{556}$ in orange. Lowest-energy conformers are shown in a forward-facing view of the fusion loop tip (residues I527-L547) for (d) WT, (e) I544A, and (f) L529A/I544A. Residues 529, 535, and 544 are shown in stick representation and colored blue, bright-orange, and purple, respectively. Hydrophobic surface representation at 20% transparency to reveal hydrophobic scaffold in stick representation, (g) WT, (h) I544A, and (i) L529A/I544A. Residues mutated to Ala are shown in yellow. Heteronuclear NOEs and R1 and R2 relaxation rates are presented for WT pH 7.0 and 5.5, I544A, and L529A/I544A in the online Supplemental Relaxation Data.

Fig. 5 Binding of WT and mutant FLs to lipid bilayers measured by ITC. (a) Ebov FL WT pH 5.0, (b) Ebov FL WT pH 7.4, (c) L529A FL pH 5.0, (d) I544A FL pH 5.0, (e) L529A/I544A FL pH 5.0. One exemplary titration is shown for each condition. At least two titrations were performed for each condition.

Fig. 6 Depth of membrane insertion by tryptophan fluorescence quenching. (a, b) Fluorescence quenching profiles of Ebov FLs containing a single Trp at positions 518 (green), 531 (blue), 534 (cyan), and 543 (red) for WT (a) and L529A (b). Profiles were generated by plotting relative fluorescence F(dQ)/F$_0$ as a function of the Br-quencher distance from the bilayer center. Experimental data were fit using the distribution analysis method (lines). 5 μM peptide was incubated with 500 μM SUVs composed of POPC:POPG 85:15 (control) or 55:15:30, where 30 mol % lipid was (6,7), (9,10), or (11,12) bromo-PC. (c) Sequences of single tryptophan mutants used in the Trp fluorescence quenching experiments. Alanine mutations are highlighted in red, single Trp mutations are green, and phenylalanines are yellow. (d) Lipid mixing of single Trp and the Phe double mutations in the WT backbone confirm that FL constructs with Trps at positions 518, 531, 534, and 543 behave like WT, and therefore could be used for fluorescence quenching analyses. F535W was not used for fluorescence quenching, but is included here to demonstrate the importance of this residue, which contributes to the triad of residues forming the structural scaffold of the FL fist structure. FLs (5 μM) were tested for fusion at pH 5.0 with POPC:POPG 85:15 liposomes (100 μM) and normalized to the activity of WT. All results were repeated in triplicate with at least two preparations of liposomes. Error bars indicate standard deviations.
Fig. 7 Molecular dynamics simulations showing insertion of Ebov FLs in POPC:POPG bilayers. (a to c) Atomistic simulations of (a) Ebov FL WT pH 5.0, (b) Ebov FL I544A at pH 5.0, and (c) Ebov FL L529A/I544A pH 5.0, all rendered after 400 ns of simulation. The I544A mutant displayed a “flatter” structure but was still membrane-associated, while the L529A/I544A mutant was both less structured and significantly less inserted (p < 10^{-5}). Coarse-grain simulations showing the binding of WT FL to a lipid bilayer starting from (d) a solution conformation of WT Ebov FL at t = 0 to form (e) a membrane-bound structure at t = 3 μs. The hydrophobic region, A525-I544, is colored green, while the other residues are shown in red for WT, magenta for I544A, and blue for L529A/I544A. Phospholipid headgroups are orange and in atomistic simulations the first leaflet of the lipid bilayer is shown in line representation. Movies of atomistic simulations of WT (Video 1), I544A (Video 2), and L529A/I544A mutant (Video 3) insertions are shown in the online Supplemental Video Data.

Fig. 8 Backbone and surface representations of viral fusion loops with hydrophobic residues highlighted. (a) Ebola virus GP2, Filoviridae, Class I, NMR in DPC at pH 5.5 (2LCY). (b) Vesicular stomatitis virus G, Rhabdoviridae, Class III (2CMZ). (c) Tick-borne encephalitis virus E1, (1URZ)*, West Nile virus E1, (2HG0), and Dengue virus E1, (1OK8). Flaviviridae, Class II. (d) Semliki Forest virus E1 (1RER)*, Sindbis virus E1, (3MUU), Chikungunya virus E1 (3N41) and Chikungunya virus E1, NMR in DPC at pH 5.0. (2RSW), Togaviridae, Class II. (e) Rubella virus E1, Togaviridae, Class II (4ADJ). (f) Baculovirus gp64, Baculoviridae, Class III (3DUZ). (g) Herpes Simplex virus gB, Herpesviridae, Class III (2GUM). (h) Herpes Simplex virus gB, Herpesviridae, Class III (3NWD). Trp, Tyr, Phe, Leu, and Ile are classified as hydrophobic, and their side-chains and surfaces are shown in red. (*) indicates crystal structures determined in the presence of detergent.
Figure 1
Figure 4
Figure 5
Figure 7
Figure 8