Supplemental Information

Structure of the Ebola virus envelope protein MPER/TM domain and its interaction with the fusion loop explains their fusion activity

*Jinwoo Lee[1,2], David A. Nyenhuis[1,3], Elizabeth A. Nelson[1,4], David S. Cafiso[1,3], Judith M. White[1,4], and Lukas K. Tamm[1,2]**

Center for Membrane and Cell Physiology[1], Departments of Molecular Physiology and Biological Physics[2], Chemistry[3], and Cell Biology[4], University of Virginia, Charlottesville, Virginia 22908, U.S.A.

Supplemental Methods

Cloning of GP2 constructs

Forward and reverse primers were designed (shown in **Supplemental Figure 12**) to obtain the MPER/TM or GP2 target DNA sequences by PCR from full-length GP1/GP2 DNA (Zaire strain) that was available in Dr. J. White's lab. The primers were prepared at 125 ng/μL in sterile distilled water. The PCR reaction was run with 1 μL of each primer, 2 μL of 10 ng/μL template DNA, 5.0 μL of 10X pfu polymerase buffer, 10 μL of dNTPs (100 mM total), and 1 μL of pfu polymerase in a total volume of 50 μL as adjusted with sterile water. The thermal cycler was set to the following cycling conditions: 1 cycle at 95 °C for two minutes, 5 cycles at 95 °C for 15 seconds, 45 °C for 15 seconds, 72 °C for 1 minute, 1 cycle at 72 °C for 5 minutes, and 4 °C for an indefinite period. The PCR product was purified using the "PCR quick cleanup procedure" described in the Qiagen Gel Extraction kit. The purified PCR product was digested with the restriction enzymes BamH1 and EcoR1 to create sticky ends, which allowed the gene to be incorporated into the pET-24a vector. The vector was also digested with BamH1 and EcoR1 in a separate reaction. These digest reactions were carried out in a volume of 50 μL overnight at room temperature. The reactions contained 42.5 μL of PCR product or pET-24a vector, 5 μL of 10X EcoR1 buffer, 1 μL of BamH1, 1 μL of EcoR1, and 0.5 μL of 100X BSA. The DNA encoding the MPER/TM domain or GP2 and the pET-24a vector were purified by 1% agarose gel electrophoresis and DNA concentrations were measured on a Nanodrop. The ligation was performed at room temperature overnight using MPER/TM or GP2 and vector DNA at a ratio of 5:1. The product was transformed into XL-Blue cells for sequencing and BL21(DE3) cells for protein production. All mutant sequences were generated following procedures of the Stratagene QuikChange site-directed mutagenesis kit 200518 as described previously (1).

Expression and Purification of GP2 and its MPER/TM domain

In brief, cells were grown to an $O.D._{600}$ of 0.6-0.8 and induced with a final concentration of 1 mM IPTG. Cells from a 1 L culture were harvested 4 hours after induction and growth at 37 °C. The fusion protein expressed in inclusion bodies and was purified in three steps (**Supplemental Figure 1**). First, harvested cells were resuspended in 100 mL 20 mM Tris buffer pH 8.0 containing 100 mM NaCl and 10% sucrose, lysed by sonication until homogeneous, and centrifuged for 30 min at 40,000 g at 10 °C. Second, the washed pellets, which contained the inclusion bodies, were solubilized in 100 mL 20 mM Tris buffer pH 8.0 containing 100 mM NaCl, 8 M urea, and 1% Triton X-100 by following the same sonication and centrifugation procedure as in the sucrose wash step. The supernatant was incubated with 5 mL of Ni affinity beads for at least 1 hour at 4 °C. Third, a urea gradient (total 500 mL of 8 to 0 M urea in Tris buffer) wash was performed on the Ni column to remove urea and Triton X-100. The MPER/TM domain or GP2 were then cleaved from the Trp leader protein that was still bound to the resuspended Ni beads with 100 mL of a 5 mg/ml thrombin stock in 25 mL of 20 mM Tris buffer pH 8.0 containing 100 mM NaCl and 0.2 % DPC. The reaction was allowed to proceed for at least 2 hours under rotation at room temperature. The MPER/TM domain or GP2 were liberated from the Ni beads and washed with another 20 mL of 0.2% DPC buffer to collect all cleaved MPER/TM or GP2 protein. The collected fractions were pooled, concentrated, and run over a Superdex 200 size exclusion column in the final required buffer with 0.2% DPC. For example, for NMR studies, the final buffer was 20 mM Na phosphate pH 7 or pH 5.5 containing 100 mM NaCl and 0.2% DPC. SDS-PAGE showed that the target protein was pure and quantitatively cleaved under the employed reaction conditions and MALDI-TOF confirmed the identity of the purified MPER/TM domain or GP2 subunit. For NMR sample preparation, cells were grown in minimal media containing $^{15}NH_4SO_4$ and ^{13}C-glucose and induction was performed 25 °C overnight.

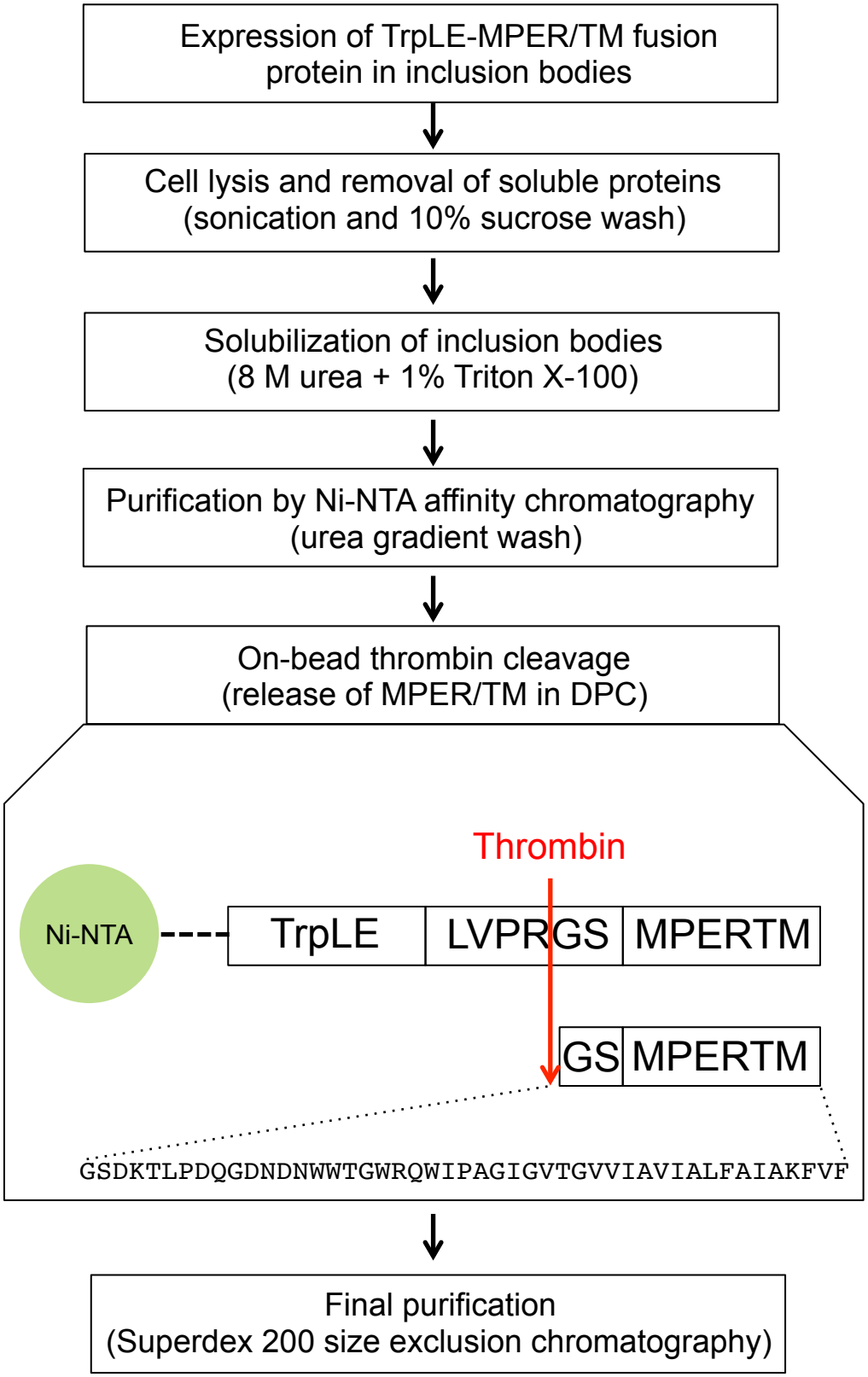
NMR distance and dihedral angle restraints

Distance restraints	
Total NOE	696
Intra-residue	336
Inter-residue	360
Sequential (i-j=1)	237
Medium-range (i-j≤4)	114
Long-range (i-j≥5)	9
Total dihedral angle restraints	
Φ	22
Ψ	22

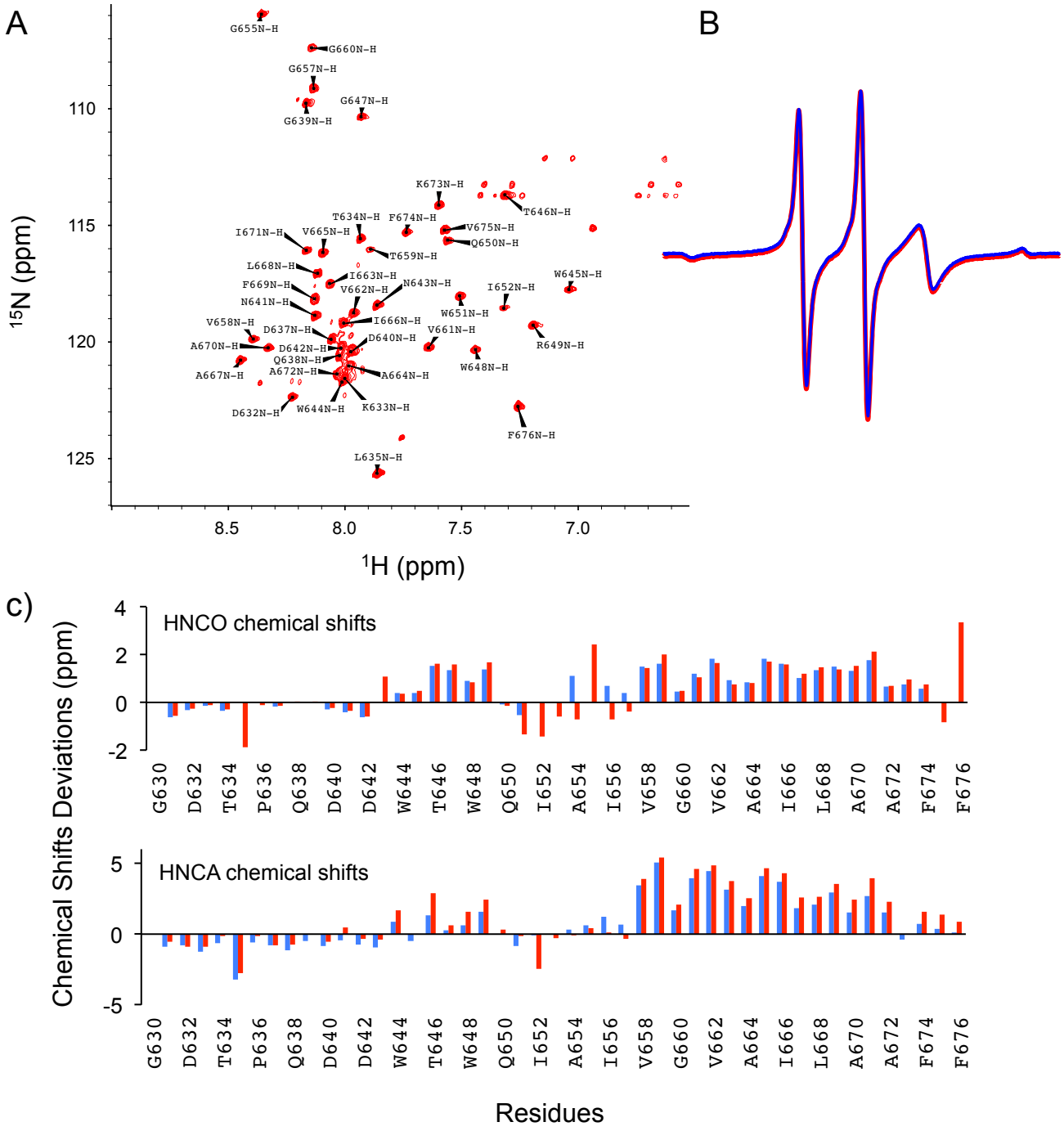
Structure statistics

Violations (mean and s.d.)	
Distance restraints (Å)	0.029 ± 0.001
Dihedral angle restraints (°)	0.33 ± 0.03
Max. dihedral angle violation (°)	0
Max. distance restraint violation (Å)	0
Deviations from idealized geometry	
Bond lengths (Å)	0.00247 ± 0.00005
Bond angles (°)	0.36 ± 0.01
Impropers (°)	0.20 ± 0.01
Average pairwise r.m.s. deviation* (Å)	
Heavy	1.64 ± 0.55
Backbone	2.06 ± 0.56
Ramachandran map analysis	
Most favored regions (%)	63.3
Additionally allowed regions (%)	20.7
Generously allowed regions (%)	16
Disallowed regions (%)	0

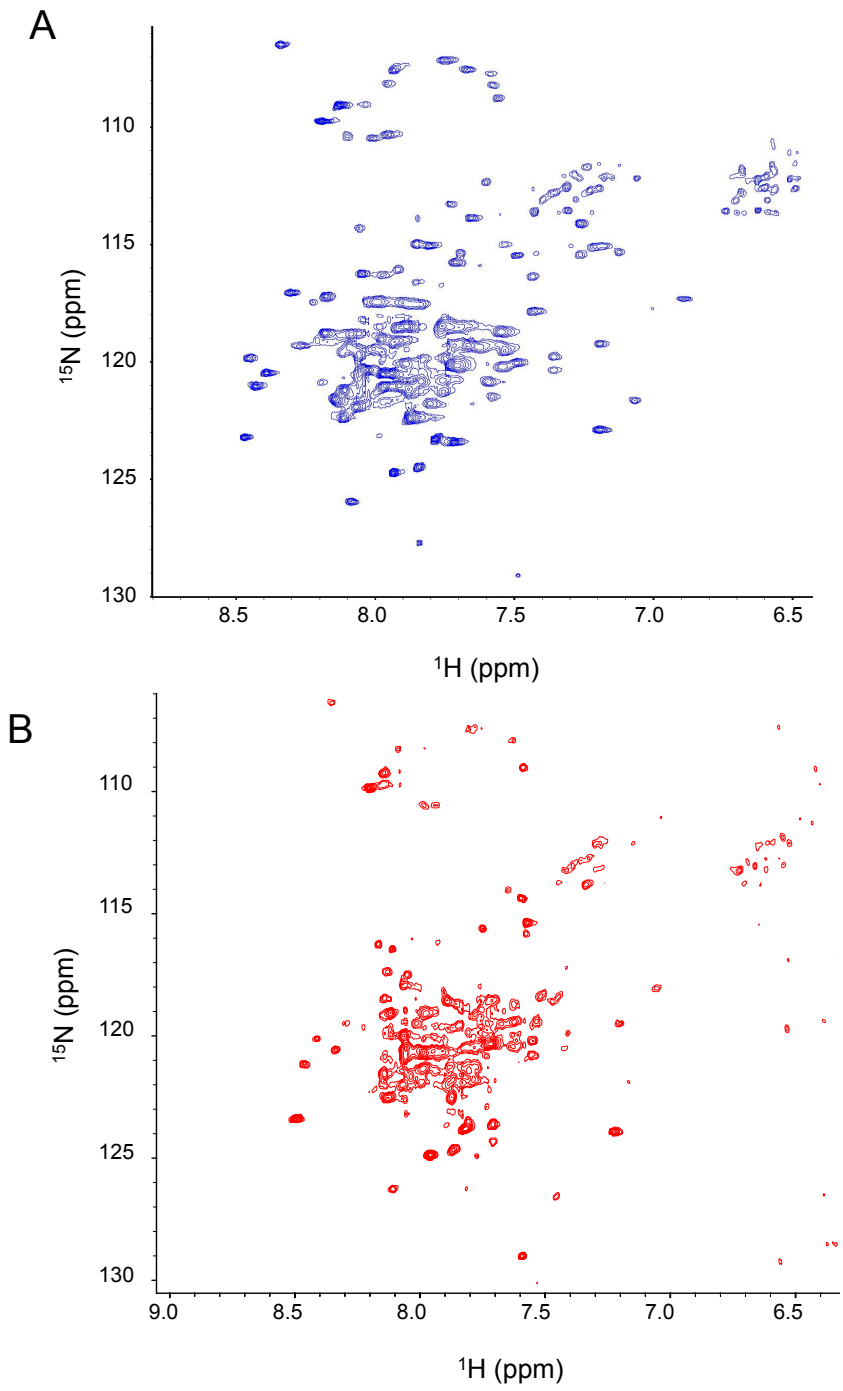
Supplemental Table 1. NMR refinement statistics for the EBOV MPER/TM domain in DPC micelles. Asterisk denotes calculated from the 20 lowest-energy CNS conformers out of 200.



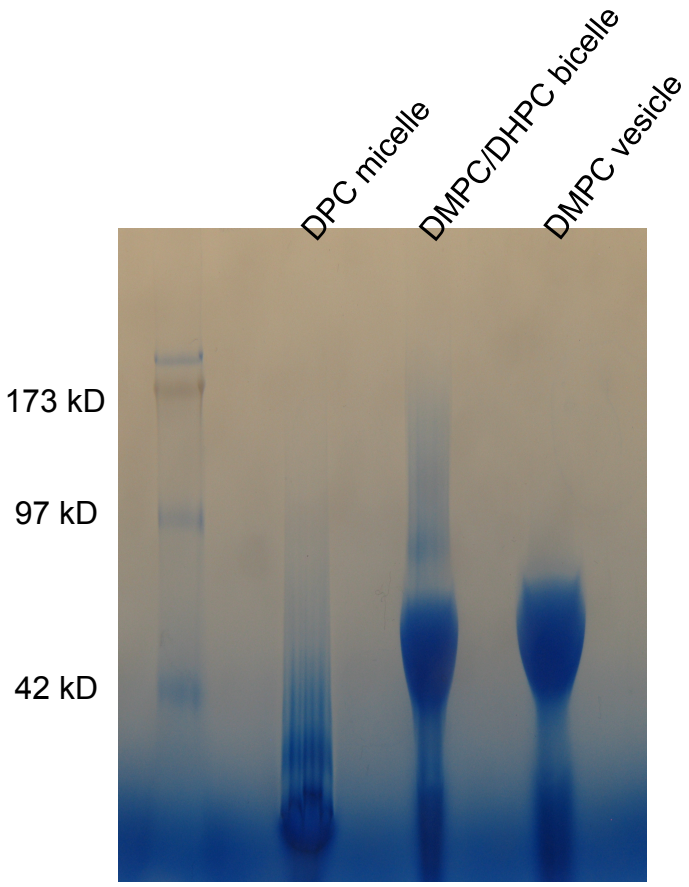
Supplemental Figure 1. Flow chart of the production and purification of the EBOV MPER/TM domain. This method was adopted to also produce and purify other constructs such as full-length GP2.



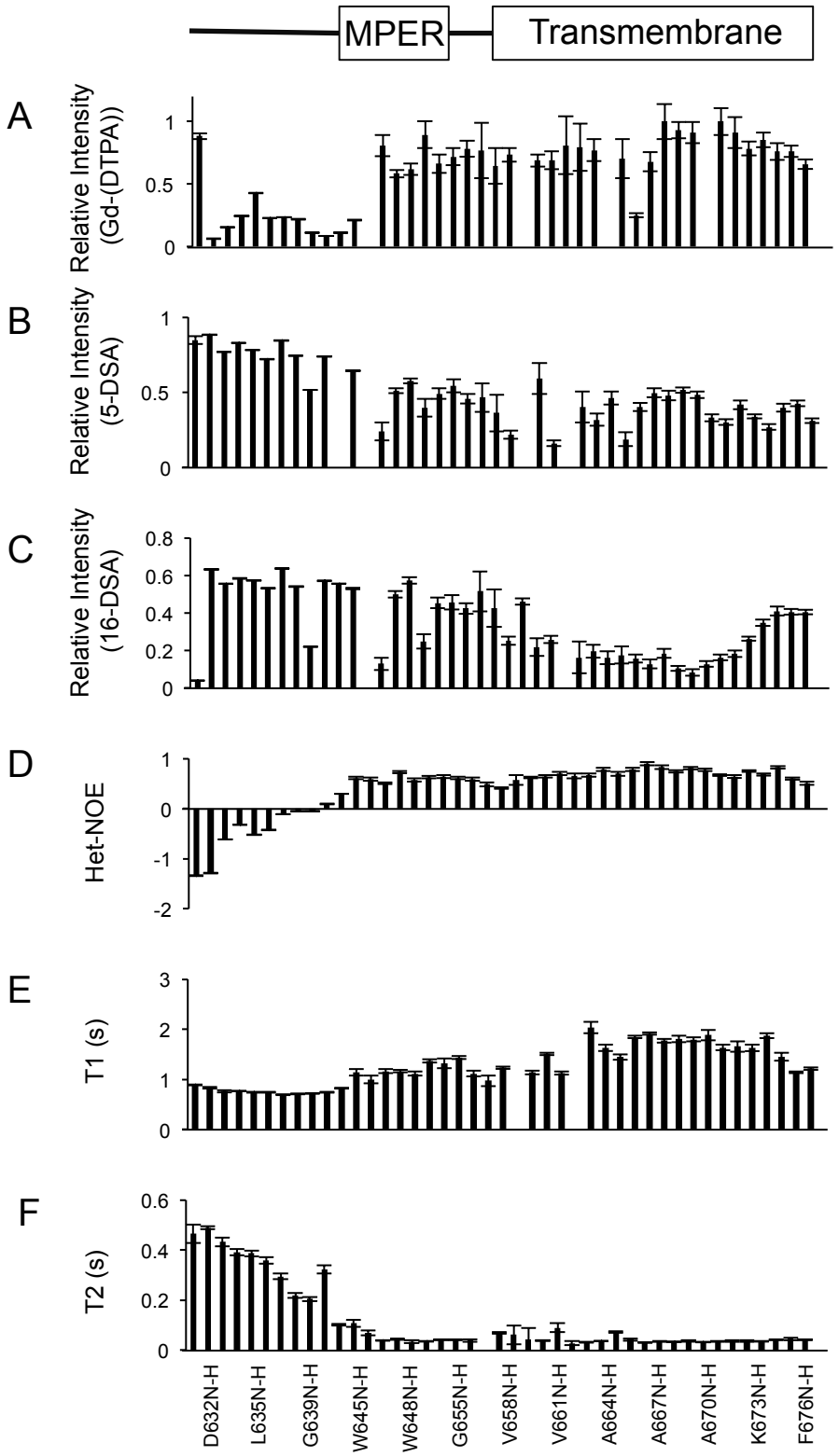
Supplemental Figure 2. Comparison of EBOV MPER/TM domain in lipid bicelles and micelles. (A) Fully assigned HSQC spectrum of MPER/TM in q=0.5 DMPC/DHPC bicelles at pH 5.5, 45 °C. (B) CW-EPR spectra (150 G full-width) of MPER/TM domain labeled with MTSL at Cys 677 in DPC micelles (red) and q=0.5 DMPC/DHPC bicelles (blue). The two spectra are indistinguishable. The small features in the wings of the spectra may have arisen from capillary sealant and are not part of the nitroxide spectra. (C) Comparison of EBOV MPER/TM chemical shifts in DPC micelles (red) and q=0.5 DMPC/DHPC bicelles (blue).



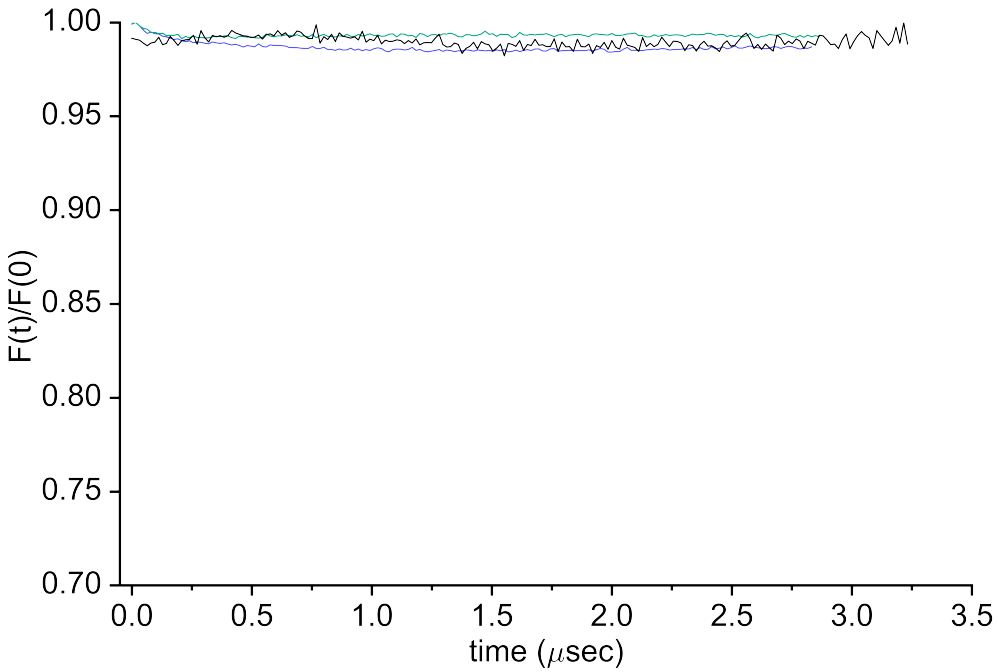
Supplemental Figure 3. HSQC spectra of EBOV GP2 in lipid micelles and bicelles. (A) HSQC spectrum in DPC at pH 5.5, 45 °C. (B) HSQC spectrum of GP2 in 15 %, q=0.5 DMPC/DHPC bicelles at pH 5.5, 45 °C.



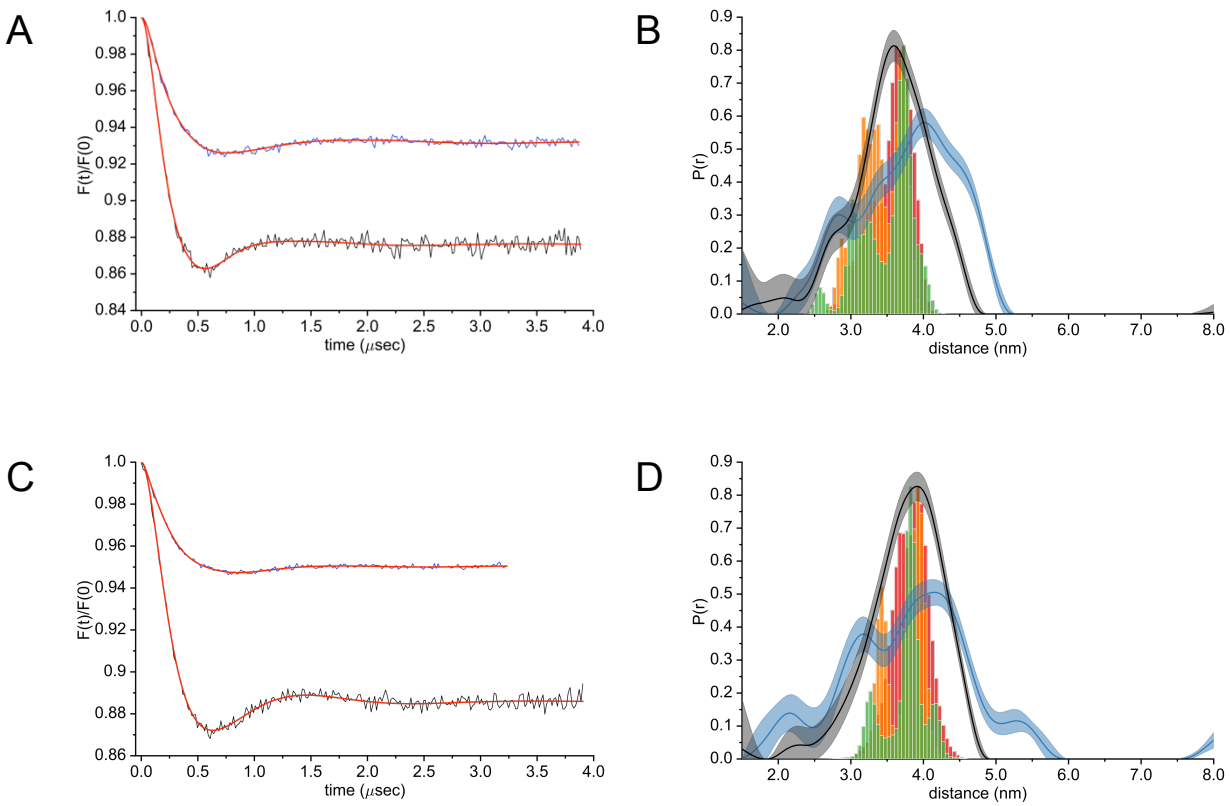
Supplemental Figure 4. Native gel showing oligomerization state of GP2 in different membrane mimetics. GP2 is monomeric in DPC micelles and likely trimeric in q=0.5 DMPC/DHPC bicelles and in DMPC lipid bilayers.



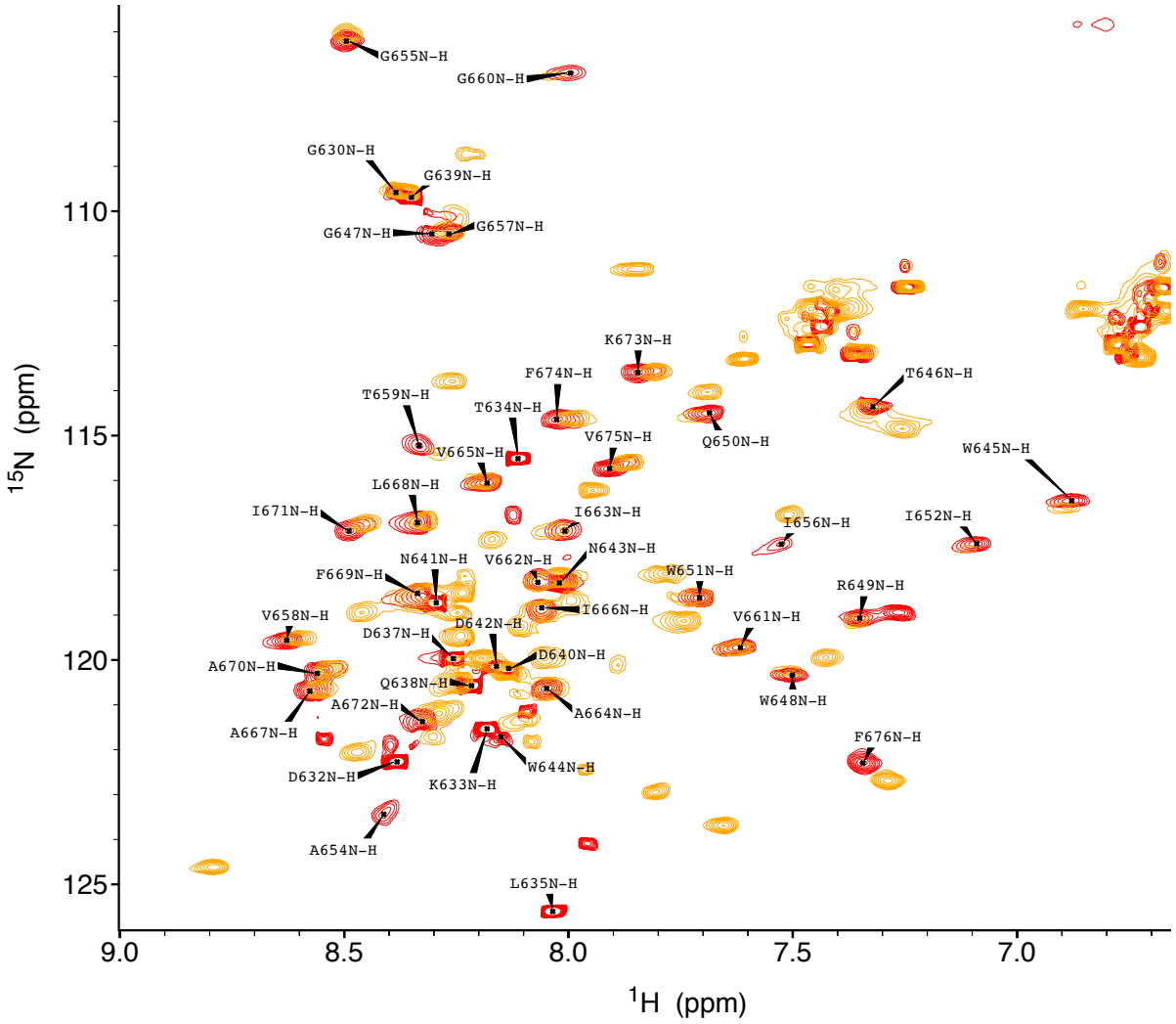
Supplemental Figure 5. Paramagnetic relaxation enhancement (PRE) and dynamics measurements of the MPER/TM domain in DPC micelles at pH 5.5, 30 °C. The PRE experiments were performed in the presence of (A) soluble Gd-(DTPA), (B) 5-doxyl stearic acid, and (C) 16-doxyl stearic acid. (D) Heteronuclear $\{^1H\}^{15}N$-NOEs. (E) ^{15}N T_1 spin-lattice and (F) T_2 spin-spin relaxation times.



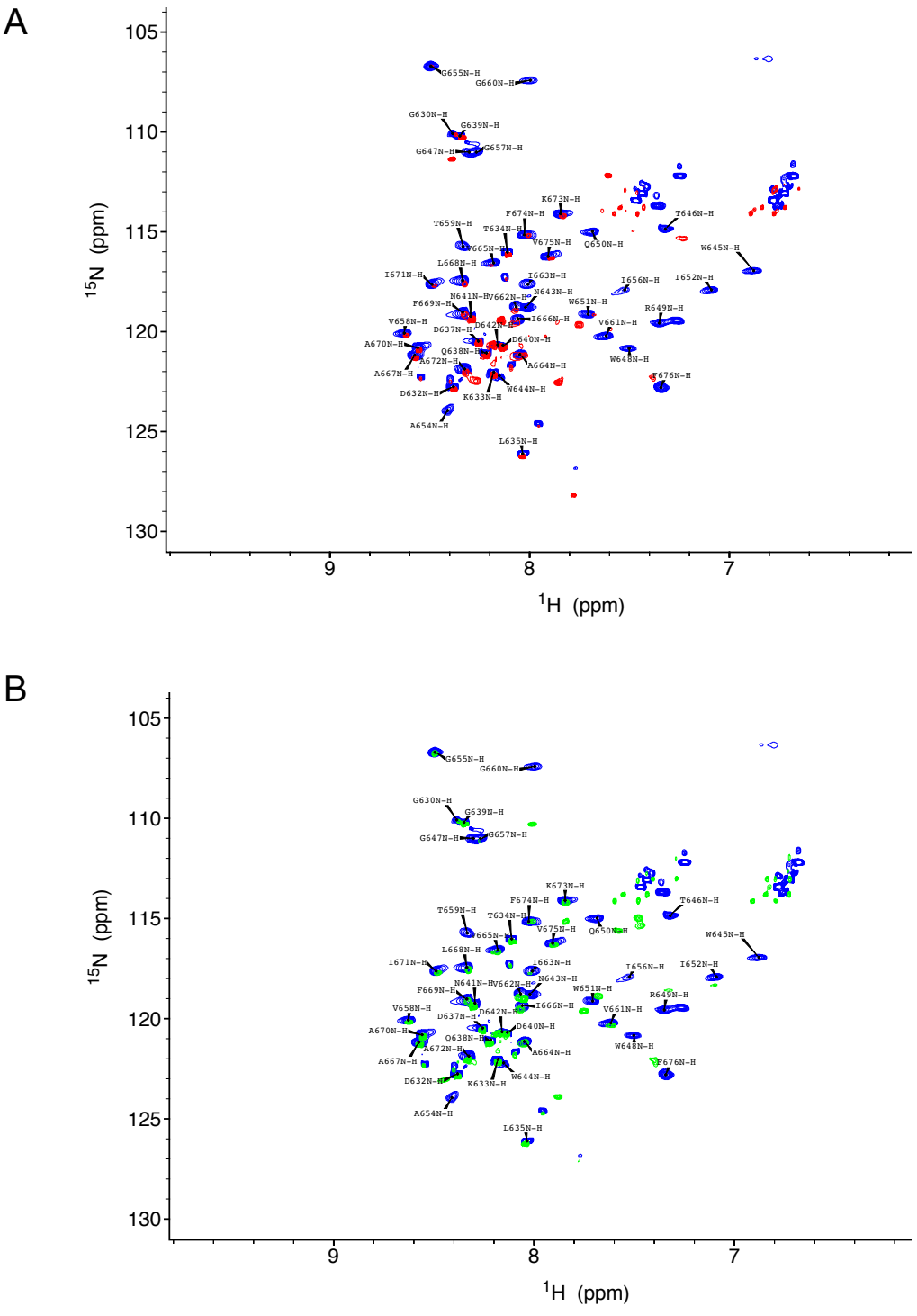
Supplemental Figure 6. Background-corrected DEER data obtained for a single MTSL label attached to Cys677 of the EBOV MPER/TM construct. No spin interactions were detected in these DEER experiments in DPC (black trace), DHPC:DMPC bicelles (green trace), or DMPC/MSP1D1 nanodiscs (blue trace) indicating that the MPER/TM domains exist as monomers in lipid micelles, bicelles, and in lipid bilayer nanodiscs.



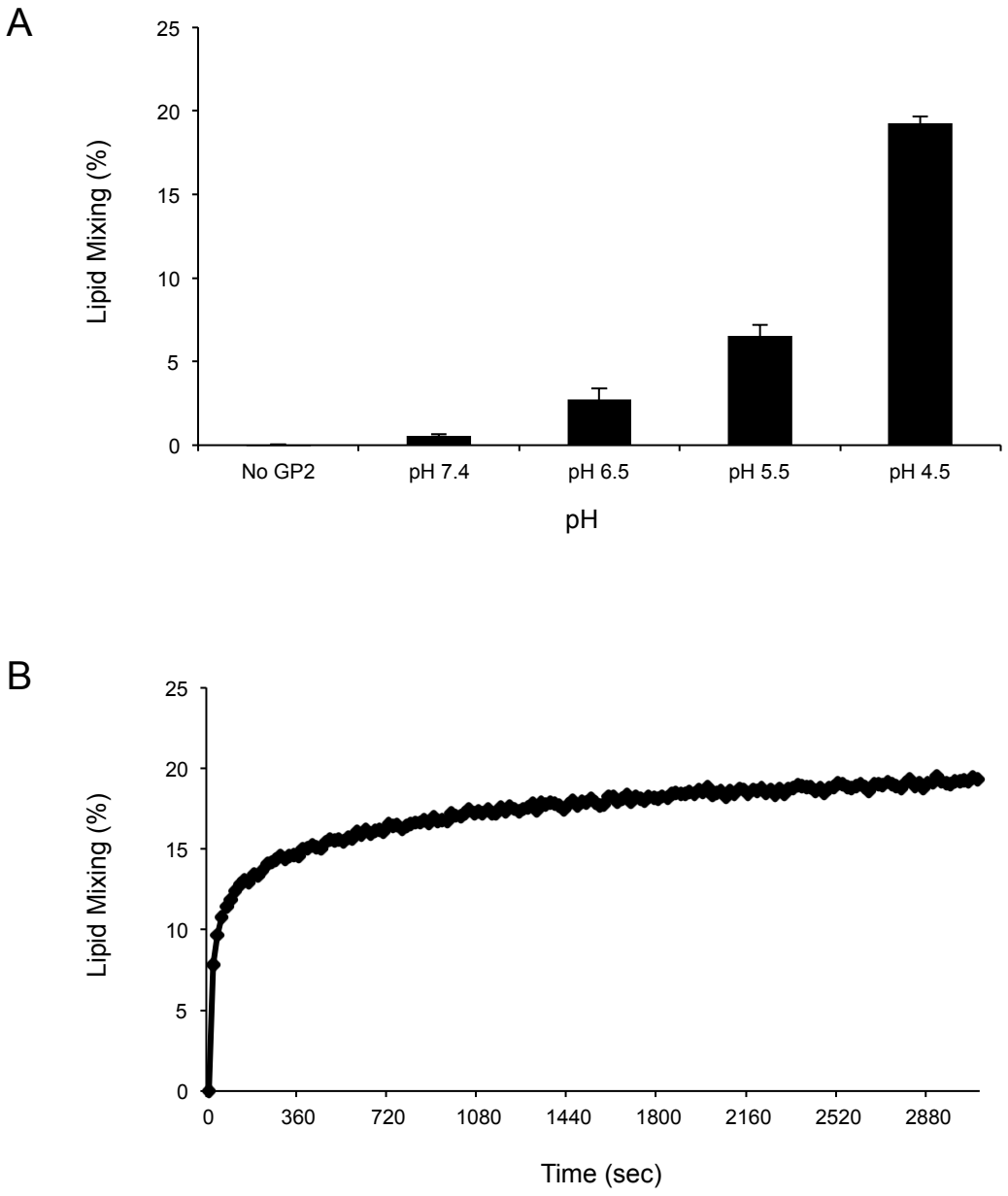
Supplemental Figure 7. Distance information obtained using double-MTSL-labeled EBOV MPER/TM. (A) Background-corrected DEER data of EBOV MPER/TM_C643/C666 in DPC (black) and DMPC/MSP1D1 nanodiscs (blue). (B) Distance distribution obtained from DEER data of EBOV MPER/TM_C643/C666 in DPC (black) and nanodiscs (blue). Fitting of calculated MTSL distances from 3 lowest-energy EPR-aided structures (red, orange, and green) show agreement with the experimental DEER distances. (C) Background-corrected DEER data of EBOV MPER/TM_C643/C670 in DPC (black) and DMPC/MSP1D1 nanodiscs (blue). (D) Distance distribution obtained from DEER data of EBOV MPER/TM_C643/C670 in DPC (black) and nanodiscs (blue). Fitting of calculated MTSL distances from 3 lowest-energy EPR-aided structures (red, orange, and green) show agreement with the experimental DEER distances.



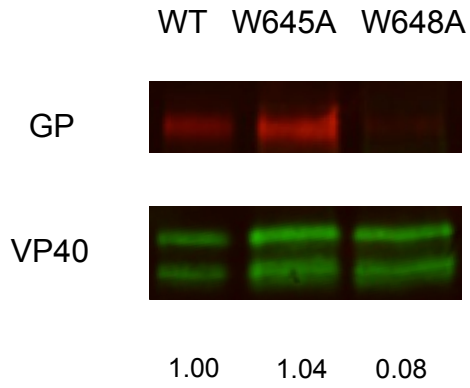
Supplemental Figure 8. Comparison of HSQC spectra of the MPER/TM domain (red) with the GP2 fragment comprising the CHR/MPER/TM domains (orange). Most cross-peaks of the MPER/TM domain overlay with peaks having the same chemical shifts in the CHR/MPER/TM fragment, which also shows numerous additional peaks that most likely originate from the CHR domain. This indicates that MPER/TM construct captures the essence of this structure without the CHR domain.



Supplemental Figure 9. HSQC spectra of EBOV MPER/TM mutants show no major changes compared to spectra of wild-type. (A) Overlay of HSQC spectra of EBOV MPER/TM W645A (red) and wild-type (blue). (B) Overlay of HSQC spectra of EBOV MPER/TM W648A (green) and wild-type (blue).



Supplemental Figure 10. Fusion of proteoliposomes with reconstituted full-length GP2 and protein-free liposomes is pH-dependent. The proteoliposomes and the protein-free liposomes were composed of POPC/POPG (85:15) and the protein-free liposomes additionally contained 1.5% each of NBD-DPPE and Rh-DPPE. (A) Effect of pH on GP2-mediated liposome fusion. (B) Representative experiment showing the kinetics GP2-mediated lipid mixing at pH 4.5.



Supplemental Figure 11. Representative Western blot of EBOV WT, W645A, and W648A VLPs. Incorporation of mutant GPs into VLPs were confirmed in the gel by assessing incorporation of mutant GPs into VP40-based VLPs. The normalized GP:VP40 incorporation ratios determined from densitometry of the respective bands were 1.0 for WT GP, 1.04 for W645A GP, and 0.08 for W648A GP.

A

ATGGGCGTTACAGGAATATTGCAGTTACCTCGTGATCGATTCAAGAGGACATCATTCTTTCTTTGGGTAATTATC
CTTTTCCAAAGAACATTTTCCATCCCACTTGGAGTCATCCACAATAGCACATTACAGGTTAGTGATGTCGACAAA
CTAGTTTGTCGTGACAAACTGTCATCCACAAATCAATTGAGATCAGTTGGACTGAATCTCGAAGGGAATGGAGT
GGCAACTGACGTGCCATCTGCAACTAAAAGATGGGGCTTCAGGTCCGGTGTCCCACCAAAGGTGGTCAATTATG
AAGCTGGTGAATGGGCTGAAAACTGCTACAATCTTGAAATCAAAAAACCTGACGGGAGTGAGTGTCTACCAGC
AGCGCCAGACGGGATTCGGGGCTTCCCCCGGTGCCGGTATGTGCACAAAGTATCAGGAACGGGACCGTGTGCC
GGAGACTTTGCCTTCCATAAAGAGGGTGCTTTCTTCCTGTATGATCGACTTGCTTCCACAGTTATCTACCGAGGA
ACGACTTTCGCTGAAGGTGTCGTTGCATTTCTGATACTGCCCCAAGCTAAGAAGGACTTCTTCAGCTCACACCCC
TTGAGAGAGCCGGTCAATGCAACGGAGGACCCGTCTAGTGGCTACTATTCTACCACAATTAGATATCAGGCTAC
CGGTTTTGGAACCAATGAGACAGAGTACTTGTTCGAGGTTGACAATTTGACCTACGTCCAACTTGAATCAAGATT
CACACCACAGTTTCTGCTCCAGCTGAATGAGACAATATATACAAGTGGGAAAAGGAGCAATACCACGGGAAAA
CTAATTTGGAAGGTCAACCCCGAAATTGATACAACAATCGGGGAGTGGGCCTTCTGGGAAACTAAAAAAAACC
TCACTAGAAAAATTCGCAGTGAAGAGTTCACAGTTGTATCAAACGGAGCCAAAAACATCAGTGGTCAGAGTCC
GGCGCGAACTTCTTCCGACCCAGGGACCAACACAACAACTGAAGACCACAAAATCATGGCTTCAGAAAATTCCT
CTGCAATGGTTCAAGTGCACAGTCAAGGAAGGGAAGCTGCAGTGTCGCATCTAACAACCCTTGCCACAATCTCC
ACGAGTCCCCAATCCCTCACAACCAAACCAGGTCCGGACAACAGCACCCATAATACACCCGTGTATAAACTTGAC
ATCTCTGAGGCAACTCAAGTTGAACAACATCACCGCAGAACAGACAACGACAGCACAGCCTCCGACACTCCCTC
TGCCACGACCGCAGCCGGACCCCCAAAAGCAGAGAACACCAACACGAGCAAGAGCACTGACTTCCTGGACCCC
GCCACCACAACAAGTCCCCAAAACCACAGCGAGACCGCTGGCAACAACAACACTCATCACCAAGATACCGGAG
AAGAGAGTGCCAGCAGCGGGAAGCTAGGCTTAATTACCAATACTATTGCTGGAGTCGCAGGACTGATCACAGG
CGGGAGAAGAACTCGAAGAGAAGCAATTGTCAATGCTCAACCCAAATGCAACCCTAATTTACATTACTGGACTA
CTCAGGATGAAGGTGCTGCAATCGGACTGGCCTGGATACCATATTTCGGGCCAGCAGCCGAGGGAATTTACAT
AGAGGGGCTAATGCACAATCAAGATGGTTTAATCTGTGGGTTGAGACAGCTGGCCAACGAGACGACTCAAGCT
CTTCAACTGTTCCTGAGAGCCACAACTGAGCTACGCACCTTTTCAATCCTCAACCGTAAGGCAATTGATTTCTTGC
TGCAGCGATGGGGCGGCACATGCCACATTCTGGGACCGGACTGCTGTATCGAACCACATGATTGGACCAAGAA
CATAACAGACAAAATTGATCAGATTATTCATGATTTTGTTGATAAGACCCTTCCGGACCAGGGGGACAATGACA
ATTGGTGGACAGGATGGAGACAATGGATACCGGCAGGTATTGGAGTTACAGGCGTTGTAATTGCAGTTATCGC
TTTATTCTGTATATGCAAATTTGTCTTTCCGCGGTTCGAAGGTAAGCCTATCCCTAACCCTCTCCTCGGTCTCGATT
CTACGCGTACCGGTCATCATCACCATCACCATTGA

B

```
Forward primer: 5’-CGCGGATCCGATAAGACCCTTCCGGACCAG-3’

Reverse primer: 5’-CCGGAATTCTTAAAAGACAAATTTGCATATAC-3’
```

C

```
Forward primer: 5’-CGCGGATCCGAAGCAATTGTCAATGCTCA-3’

Reverse primer: 5’-CCGGAATTCTTAAAAGACAAATTTGCATATAC-3’
```

Supplemental Figure 12. DNA sequence of EBOV GP. (A) Sequence of full-length EBOV GP comprising GP1 and GP2. The GP2 sequence is highlighted in yellow and the MPER/TM domain is highlighted in red. (B) Forward and reverse primers for generating EBOV MPER/TM construct. (C) Forward and reverse primers for generating EBOV GP2 construct.

References

1. Gregory SM*, et al.* (2014) Ebolavirus entry requires a compact hydrophobic fist at the tip of the fusion loop. *Journal of virology* 88(12):6636-6649.