Host-Primed Ebola Virus GP Exposes a Hydrophobic NPC1 Receptor-Binding Pocket, Revealing a Target for Broadly Neutralizing Antibodies

Zachary A. Bornholdt,a Esther Ndungo,b Marnie L. Fusco,a Shridhar Bale,a Andrew I. Flyak,c James E. Crowe, Jr.,c,d,e Kartik Chandran,b Erica Ollmann Saphirea,b,†

Department of Immunology and Microbial Science, The Scripps Research Institute, La Jolla, California, USA; Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, New York, USA; Department of Pathology, Microbiology and Immunology; Department of Pediatrics; and Vanderbilt Vaccine Center, Vanderbilt University, Nashville, Tennessee, USA; The Skaggs Institute for Chemical Biology, The Scripps Research Institute, La Jolla, California, USA.

Z.A.B. and E.N. contributed equally to this article.

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ABSTRACT The filovirus surface glycoprotein (GP) mediates viral entry into host cells. Following viral internalization into endosomes, GP is cleaved by host cysteine proteases to expose a receptor-binding site (RBS) that is otherwise hidden from immune surveillance. Here, we present the crystal structure of proteolytically cleaved Ebola virus GP to a resolution of 3.3 Å. We use this structure in conjunction with functional analysis of a large panel of pseudotyped viruses bearing mutant GP proteins to map the Ebola virus GP endosomal RBS at molecular resolution. Our studies indicate that binding of GP to its endosomal receptor Niemann-Pick C1 occurs in two distinct stages: the initial electrostatic interactions are followed by specific interactions with a hydrophobic trough that is exposed on the endosomally cleaved GP1 subunit. Finally, we demonstrate that monoclonal antibodies targeting the filovirus RBS neutralize all known filovirus GPs, making this conserved pocket a promising target for the development of panfilovirus therapeutics.

IMPORTANCE Ebola virus uses its glycoprotein (GP) to enter new host cells. During entry, GP must be cleaved by human enzymes in order for receptor binding to occur. Here, we provide the crystal structure of the cleaved form of Ebola virus GP. We demonstrate that cleavage exposes a site at the top of GP and that this site binds the critical domain C of the receptor, termed Niemann-Pick C1 (NPC1). We perform mutagenesis to find parts of the site essential for binding NPC1 and map distinct roles for an upper, charged crest and lower, hydrophobic trough in cleaved GP. We find that this 3-dimensional site is conserved across the filovirus family and that antibody directed against this site is able to bind cleaved GP from every filovirus tested and neutralize viruses bearing those GPs.

E bola virus (EBOV) and Marburg virus (MARV) are both members of the Filoviridae family of enveloped negative-strand RNA viruses and are the causative agents of a highly lethal disease for which no approved vaccines or treatments are currently available (1, 2). Due to their virulence and biothreat potential, filoviruses are classified as category A pathogens. The ongoing EBOV epidemic in West Africa is the longest and most widespread filovirus outbreak on record (3).

Like all filoviruses, EBOV displays a single virus-encoded protein, the viral glycoprotein (GP), on the surface of the virion. EBOV GP is a 676-residue class I membrane fusion glycoprotein. However, EBOV GP differs from canonical class I fusion proteins, such as those of human immunodeficiency virus and influenza A virus, in that the architecture of its fusion loop more closely resembles those of class II and III glycoproteins (4, 5). EBOV GP is synthesized as a precursor polypeptide, GP0, which assembles into trimers in the endoplasmic reticulum. Each GP0 subunit is then posttranslationally cleaved by the Golgi endoprotease furin to yield disulfide-linked GP1 (≈55 kDa) and GP2 (≈20 kDa) subunits. The final GP assembly, which is an ≈450 kDa trimer of GP1,2 heterodimers, is then displayed on the surface of mature EBOV virions (4, 5). GP1 contains the receptor-binding site and regulates the triggering of the membrane fusion machinery in the GP3 subunit (6).

The GP1 structure can be divided into three subdomains: the mucin domain, glycan cap, and GP1 core. The outer mucin domain (GP1 residues 313 to 464), is predicted to be loosely structured and heavily glycosylated, incorporating five N-linked gly-
cans and 12 to 17 predicted O-linked glycans (5). Interior to the mucin-like domain is the glycan cap (GP1 residues 227 to 313), which sits atop the GP1 core. The glycan cap is more ordered than the mucin-like domain and contains four N-linked glycosylation sites. Neither the mucin nor the glycan cap domain is essential for viral entry. Indeed, removal of these domains enhances infection by viruses pseudotyped with EBOV GP (7–9). Therefore, it is currently hypothesized that a primary function of the mucin domain and glycan cap is to shield the GP1 core from immune surveillance (4, 5, 10, 11).

EBOV virions are internalized into cells via a macro-pinocytosis-like mechanism and undergo trafficking to late endosomes (12–15). There, host endosomal cysteine proteases, including cathepsins L (CatL) and B (CatB), cleave GP1 to remove the mucin and glycan cap domains. Cleavage generates a fusion-competent GP trimer (GPCL) comprising the 19-kDa GP1 core domain and GP2 (8, 9, 16). Cleavage of GP1,2 to GPCL is a prerequisite for viral recognition of the host endosomal receptor Niemann-Pick C1 (NPC1) (10, 17–20), strongly suggesting that the receptor-binding site in the GP1 core structure is unmasked by the cleavage of GP1, in late endosomes. Thus, GPCL represents the structure of EBOV GP in a conformation that is competent for receptor binding. In order to observe possible structural changes in GPCL and to illustrate definitively which surfaces and residues are unveiled upon endosomal proteolysis, we determined the crystal structure of the EBOV GPCL trimer at a resolution of 3.3 Å, in the presence of the neutralizing human antibody Fab KZ52 (21).

Our results thus suggest a novel approach for developing engineered Mabs with broad-spectrum activity against filoviruses.

RESULTS

The crystal structure of EBOV GPCL reveals the NPC1 receptor-binding site that is unmasked upon endosomal cleavage. Purified EBOV GPCL ectodomains (expressed without mucin-like domains; hereinafter referred to as GP) were treated with thermolysin, which mimics host endosomal protease processing of EBOV GP (8), in order to generate EBOV GPCL trimers for crystallization. EBOV GPCL crystallizes in the space group H3 (R3-H) with four GP monomers and four KZ52 Fabs in the asymmetric unit (ASU). The ASU contains one full GP trimer and one remaining GP monomer, which itself forms a biologically relevant trimer with two symmetry-related protomers about a crystallographic 3-fold axis. The overall changes to the tertiary structure upon cleavage of GP are minimal, reflected in a root mean square deviation (RMSD) of 0.419 Å compared to the structure of uncleaved EBOV GP (Fig. 1A) (4). This finding corroborates a previous model of EBOV GPCL, which suggested only limited changes in the GPCL structure upon thermolysin digestion (23). The structure of EBOV GPCL is more compact than that of EBOV GP and exhibits more stable crystal packing, less disorder, and improved resolution of X-ray diffraction over that of the previously determined uncleaved GP (4).

New regions of EBOV GP can now be visualized in the EBOV GPCL structure. These include C-terminal residues of GP2, the disulfide link between C53 of GP, and C609 of GP2, and an intra-GP2 disulfide bond between C601 and C610. As EBOV GP2 descends from the base of the GP trimer structure, it forms a tightly ordered loop structure that is stabilized by the intra-GP2 disulfide bond between C601 and C610. This disulfide link turns the peptide chain back toward the body of GP where it is anchored to GP1 by the C53-C609 inter-GP1,2 disulfide bond prior to turning downward toward the transmembrane domain and viral membrane (Fig. 1B).

The most striking structural feature of GPCL is the full exposure of a charged hydrophilic crest and a large hydrophobic trough structure in immediate proximity to the GP2 fusion loop. The trough becomes exposed upon proteolytic excision of the glycan cap from EBOV GP and is 13 Å wide, 23 Å long, and 10 Å deep (Fig. 1C). Residues I113 and L111 form an exposed hydrophilic face inside the trough, while residues V79, T83, W86, F88, L122, V141, and I170 line the bottom of the trough.

Mutation of GP residues exposed after removal of the glycan cap affects viral infectivity and binding to the filovirus receptor NPC1. Previous work utilizing scanning mutagenesis of EBOV GP identified multiple residues important for viral infectivity (24–26). These studies were carried out prior to the availability of a crystal structure of EBOV GP (4) or GPCL (this work) and prior to identification of the endosomal receptor, NPC1 (17, 18). Here, we map these residues onto the crystal structure of EBOV GPCL, and determine whether mutations in EBOV GP that reduce infectivity specifically correlate with defects in GPCL-NPC1 binding. Previous work identified three lysines at positions 114, 115, and 140 (16, 25) and hydrophobic residues F88, L111, and L122 (25–27) for which mutation to alanine diminishes infectivity (16, 25). These deficits in infectivity correlate with reductions in NPC1 binding, as determined by co-immunoprecipitation (28).

The crystal structure of EBOV GPCL illustrates that K114, K115, and K140 lie along the crest and F88, L111, and L122 lie along the trough of EBOV GPCL. These hydrophobic residues are buried in uncleaved EBOV GP (4) but become solvent exposed in the trough of EBOV GPCL. We systematically mutated residues that the GPCL crystal structure shows to be surface-exposed after cleavage, in order to determine their importance for NPC1 binding and viral infectivity and to define the GP1 receptor-binding site (RBS).

We pseudotyped vesicular stomatitis virus (VSV) particles with 73 mutant GP proteins and tested them for viral incorporation of GP relative to the incorporation of the wild-type (WT) protein, and for binding to the conformational antibody KZ52 (4, 21), which only recognizes properly folded GP (see Fig. S4 in the supplemental material). The 68 VSV-GP mutants that met these quality benchmarks were then evaluated for their capacity to recognize a purified, soluble form of human NPC1 domain C in an
enzyme-linked immunosorbent assay (ELISA), as described previously (27, 29). We report that WT EBOV GPCL binds to NPC1 domain C with a 50% effective concentration (EC50) of \(0.5\) nM, consistent with a high-avidity binding interaction between these proteins. In comparison, we find that mutants that demonstrate reduced infectivity are also defective for binding to NPC1 domain C (see Fig. S1 and S2 in the supplemental material). Furthermore, a few single point mutations that cause drastic reductions (>10-fold) in the GPCL-NPC1 domain C-binding EC50 are located in or around the hydrophobic trough and hydrophilic crest. These mutants allow us to map those residues of EBOV GP1 that are critical to NPC1 domain C onto the EBOV GPCL structure and to better

FIGURE 1  Crystal structure of ebolavirus GPCL. (A) The trimeric EBOV GPCL structure is shown, with GP1 colored teal, GP2 colored light blue, the fusion loop colored orange, and disulfide bonds displayed as sticks and colored gold. The former position of the glycan cap, now absent in the GPCL structure, is illustrated in semitransparent red and is derived from an alignment with the uncleaved EBOV GP structure (PDB code 3CSY). (B) Additional residues at the C terminus of GP2 are now visible in this higher-resolution structure. These residues include C601-C608, contained within GP2, as well as the C53-C609 disulfide bond that cross-links GP1 and GP2 together. (C) The structure of EBOV GPCL is displayed to the right, with the same coloring as described for panel A. An enlarged illustration of the putative EBOV GP1 RBS is shown to the left, in two orientations. Residues forming the hydrophilic crest and hydrophobic trough are labeled and colored green and purple, respectively. The disulfide bonds present around the crest and trough, C108-C135 and C121-C145, are colored gold.
define the RBS (see Fig. S1 and S2). Interestingly, mutation to alanine of two trough residues, F88 and L111, reduces viral infectivity dramatically (by >3 log_{10} units) but has more modest effects on GP_{CL}-NPC1 binding (see Fig. S2A). The disparity between strong reduction in infectivity but modest effect on NPC1 binding suggests that these residues may be important for steps in viral entry post-NPC1 binding and prior to membrane fusion, such as conformational changes or release of GP2.

The hydrophobic trough exposed on GP1 upon endosomal cleavage is the primary binding site of NPC1 domain C. We performed further mutagenesis of the hydrophobic trough to better define its precise role. Since most of the point mutations to alanine within the hydrophobic trough had only modest effects, we postulated that replacing them with bulkier methionine residues would more completely occlude the trough and prevent GP-NPC1 binding. We selected two trough residues, T83 and I113, which did not inhibit NPC1 binding when mutated to alanine, for additional mutagenesis to methionine (Fig. 2A and B). To prevent misfolding or disruption of the GP structure, we engineered compensatory mutations with interacting residues of the glycan cap to fit the larger methionine residues and prevent steric clashes. We engineered the following mutants: I113M (trough)/F225A (cap), T83M (trough)/F225V+Y232F (cap), and T83M+I113M (trough)/F225A+Y232F (cap) (Fig. 2C to E). For simplicity, since the compensatory mutations are removed along with the glycan cap upon proteolysis, we will only refer to these mutants by the mutations remaining on EBOV GP_{CL}: T83M, I113M, and T83M+I113M. All engineered VSV-GP mutants maintain high levels of incorporation compared to the incorporation of WT GP (see Fig. S2 in the supplemental material). As posited, the single T83M and I113M mutations, as well as the T83M+I113M double mutation, lead to defects in NPC1 domain C binding and pseudo-virus infectivity by GP_{CL} bearing them (Fig. 2A to C). We further find that a single point mutation, L122A, located in the bottom of the trough, abrogates both NPC1 domain C binding and pseudo-virus infectivity (Fig. 2E). The position of L122 suggests that it has a structural role; the L122A mutation may destabilize the local trough structure, preventing NPC1 binding and subsequent infectivity. Together, these findings provide evidence that supports a direct correlation between NPC1 binding and infectivity and effectively maps the GP_{CL} trough as a critical component of the NPC1-binding site.

An overall basic charge on the GP1 crest is required for GP binding to NPC1 domain C and viral infectivity. Experiments performed prior to the identification of the filovirus endosomal receptor NPC1 demonstrated that K114A, K115A, and K140A mutations (now mapped to the GP_{CL} crest) significantly reduce viral infectivity (16, 25). Here, we investigated whether the observed reductions in viral infectivity from these mutations correlate with defects in binding to NPC1 domain C. We show that while the individual mutations K114A and K115A have only modest effects (see Fig. S1 and S2 in the supplemental material), the double mutation (K114A+K115A) dramatically inhibits GP_{CL}-NPC1 domain C binding and viral entry (Fig. 3A and B). In contrast, the K140A mutant showed no significant defect in viral infectivity or NPC1 domain C binding (see Fig. S2). To test the hypothesis that these crest residues participate in electrostatic interactions with NPC1 during virus-receptor engagement, we engineered and analyzed VSV-GPs in which these lysines were replaced with either basic or acidic residues. The K114R+K115R double mutant, which maintains the basic charge, remains fully functional. In contrast, the K114E+K115E double mutant, which reverses charge, displays an even greater deficit in receptor-binding function and entry activity than the neutral K114A+K115A mutant (Fig. 3A and B). To determine whether it is the overall charge of the site or specific basic residues within the site that are important, we mutated two glutamic acid residues in proximity to positions 114 and 115 to alanine. The resulting quadruple mutant (K114A+K115A+E112A+E120A), which is predicted to have WT-like electrostatics, exhibits receptor-binding activity and infectivity at nearly WT levels (Fig. 3A and B). The importance of a set of basic residues but lack of a specific requirement for any one of them individually suggest a need to maintain an overall basic charge on the GP_{CL} crest (Fig. 3C).

Neutralizing antibodies raised from a Marburg virus survivor demonstrate potential panfilovirus neutralization activity. The high degree of sequence and structural conservation in the NPC1-binding site of filovirus glycoproteins makes it an attractive target for the development of broadly neutralizing MAbs with therapeutic potential (see Fig. S3 in the supplemental material). Unfortunately, no such MAbs against ebolaviruses have been isolated. Instead, most known neutralizing anti-ebolavirus MAbs target a conformational epitope at the base of the GP_{CL} trimer (4, 5, 30, 31). Recently, however, several MAbs isolated from a human survivor of MARV infection were found to recognize the hydrophobic GP_{CL} trough and inhibit GP-NPC1 domain C binding (11, 22). Of significance, one anti-MARV MAb from that study, MR72, cross-reacts with purified GP and GP_{CL} of EBOV, while three other MAbs, MR78, MR111, and MR191, cross-react only with EBOV GP_{CL} (22). MR72, MR78, MR111, and MR191 bind to similar locations on MARV GP but approach from significantly different angles (22). The third complementarity-determining region of the heavy chain variable region (CDRH3) of MR78 binds into the expected MARV GP_{CL} RBS (see Fig. S3) (11).

As the RBS is conserved in sequence and structure across known filoviruses, we evaluated the capacity of MR72 and two additional GP_{CL}-reactive antibodies, MR78 and MR191 (22), to recognize and neutralize VSV bearing GP_{CL} from four ebolaviruses (Sudan virus [SUDV], Bundibugyo virus [BDBV], Tai Forest virus [TAFV], and Reston virus [RESTV]) and the cuevavirus Lloviu virus (LLOV) (2, 32). Remarkably, we find that MR72 effectively neutralizes VSVs pseudotyped with GP_{CL} derived from all known filoviruses (Fig. 4A). In contrast, MR191 neutralizes VSV bearing other filovirus GP2s only weakly, and MR78 fails to neutralize VSVs bearing GP_{CL} derived from any species other than MARV. We speculate that the steeper angle of approach of MR191 to MARV GP_{CL} compared to that of MR78 may enhance the breadth of neutralization by improving access to the shared RBS (Fig. 4A and S3). Of significance, we found that MR72 failed to bind VSVs bearing uncleaved EBOV GP on the surface (see Fig. S3 in the supplemental material). This finding is in contrast to a previous observation of MR72 binding to uncleaved soluble EBOV GP ectodomain (see Fig. S3) (22). It is likely that there are differences in the presentation of EBOV GP on the surface of actual virions that prevent MR72 from binding and effectively neutralizing either wild-type EBOV or VSV bearing uncleaved EBOV GP.

The contrasting neutralization breadth properties of MR72 and MR78, despite their similar binding angles and shared epitope, led us to explore our panel of GP_{CL} mutations to identify
FIGURE 2 Mutagenic occlusion of the EBOV GP₁ receptor-binding site. (A) Alanine or methionine mutations were made to key residues in the RBS. The affinities of wild-type and mutant GP₁ for NPC1 domain C were analyzed via ELISA. Note that the L122A and T83M+I113M mutations significantly reduce binding to NPC1 domain C. Means ± SD (n = 4) from a representative experiment are shown. (B) Graph displaying titers of VSV pseudoviruses harboring GP₁ RBS mutations. Means ± SD (n = 2–4) from a representative experiment are shown. (C) A semitransparent surface has been placed over the cartoon model of the WT RBS on EBOV GP₁ to display the RBS pocket (within the dashed oval outline). Residues T83 and I113 are illustrated as sticks (black). (D) Model of EBOV RBS bearing the mutations T83M and I113M (red). The longer side chains of the introduced methionine residues fill the RBS pocket and likely prevent NPC1 domain C binding by occluding the NPC1 binding site. (E) The buried location of L122 (black) is displayed in the EBOV GP₁, RBS. See also Fig. S1, S2, and S4 in the supplemental material.
specific residues in the GP RBS that can affect MR78’s neutralization of EBOV GP-C (see Fig. S2 in the supplemental material). We find that a single point mutation, V79A, allows MR78 to neutralize EBOV GP-C: although MR78 cannot neutralize VSV bearing wild-type EBOV GP-C, it can neutralize V79A-bearing VSV-EBOV GP-C (Fig. 4B). Position 79 in EBOV GP is equivalent to position 63 in MARV. Structural alignment of EBOV GP-C with MARV in the MARV GP-MR78 crystal structure (11) suggests that the wild-type V79 may sterically clash with the light chain of MR78. Replacement of valine with the smaller alanine residue (V79A) may improve neutralization by relieving the steric clash (Fig. 4B).

Furthermore, previous studies have shown that, unlike MR72, MR78 fails to block NPC1 domain C binding to EBOV GP-C (11). Therefore, we performed NPC1 domain C competitive-binding assays to determine whether MR78 neutralizes EBOV GP-C-V79A by inhibiting GP-NPC1 binding. Curiously, even though MR78 is now able to neutralize VSV bearing EBOV GP-C-V79A, it remains unable to prevent binding of NPC1 domain C to EBOV GP-C, or EBOV GP-C-V79A (Fig. 4B). MR72, however, does block NPC1 binding to EBOV GP-C. Therefore, our data suggest that MAbs MR72 and MR78 may neutralize by distinct mechanisms. MR72 effectively blocks GP-C-NPC1 binding for all filoviruses, whereas MR78 does not block EBOV GP-C-NPC1 binding. We speculate that MR78 neutralizes EBOV entry by inhibiting viral membrane fusion downstream from virus receptor recognition.

In order to gauge the neutralization potentials of MR72 and MR78 relative to those of other MAbs with demonstrated protective efficacy in vivo, we performed a comparative analysis with the combined MAbs of the EBOV-specific ZMapp cocktail: 2G4, 4G7, and 13C6 (31, 33), as well as with KZ52, a known neutralizing MAb from a human survivor (21). Our analysis demonstrates that MR72 can neutralize pseudoviruses at 10-fold lower concentrations of antibody than are required for KZ52 and the ZMapp cocktail (Fig. 4C). Thus, MAbs such as MR72, which target the highly conserved GP1 RBS, represent a novel avenue for both broad and potent neutralization of filoviruses, if they can be delivered to the endosomal compartments where GP-C is generated during entry.

**DISCUSSION**

In this study, we present the 3.3-Å crystal structure of thermolysin-cleaved EBOV GP (GP-C), which is primed for interaction with the filovirus receptor, NPC1. Thermolysin has previously been demonstrated to mimic host CatB and CatL proteolytic processing of
EBOV GP, which occurs in the endosome and is required for receptor binding and membrane fusion (8–10, 16). This high-resolution structure of EBOV GP_{CL} has now defined the intermolecular disulfide bridge between C53 in GP\(_1\) and C609 in GP\(_2\), a region previously unresolved for EBOV GP. The disulfide bridge likely contributes to the inherent stability of ebolavirus GP despite proteolytic processing. This stability is reflected in a high degree of structural conservation between uncleaved EBOV GP (4) and

**FIGURE 4** Monoclonal antibodies targeting the conserved GP\(_1\) RBS demonstrate panfilovirus neutralization activity. (A) VSV pseudotyped with GPs from different species of filovirus (as indicated in the key to the right) were preprimed with thermolysin to expose the GP\(_1\) RBS and then analyzed for reduction in relative infectivity following treatment with MR72 or MR78. (B) The graph to the left shows a comparative analysis of the neutralization of VSV-EBOV GP\(_{CL}\) and VSV-EBOV GP\(_{CL}\)-V79A by MR72 and MR78. The graph to the right displays the results of competitive binding assays detecting NPC1 domain C binding in the presence of increasing concentrations of MR72 or MR78 for EBOV GP\(_{CL}\) and EBOV GP\(_{CL}\)-V79A. The key for both graphs is on the far right. (C) Graph showing the results of comparative infectivity assays of nonprimed VSV pseudotyped with EBOV GP treated with MAbs from the ZMapp cocktail (2G4, 4G7, and 13C6) (33) or the neutralizing EBOV antibody KZ52 (21). MR72 neutralizes primed EBOV GP\(_{CL}\) pseudovirions at 10-fold lower concentrations than are required for ZMapp or KZ52 to neutralize EBOV GP pseudovirions. See also Fig. S3 in the supplemental material. Means ± SD (n = 2–4) from a representative experiment are shown in each panel.
GP<sub>CL</sub>; the aligned structures have an RMSD of 0.419 Å. The crystal structure of EBOV GP<sub>CL</sub> presented here also illustrates how proteolytic priming removes the glycans of EBOV GP<sub>P</sub> to expose the binding site for the filovirus receptor NPC1. The GP<sub>CL</sub> crystal structure suggests that the glycans can act as a final layer of defense, shielding the critical and conserved NPC1 domain C binding site from host immune surveillance prior to cellular entry. We show that this RBS has a crest-and-trough morphology and exists at the apex of the GP<sub>CL</sub> trimer.

The crest is lined with hydrophilic basic residues, while the trough is recessed and entirely hydrophobic. Mutagenic analysis of EBOV GP<sub>CL</sub> demonstrates that the crest is involved in nonspecific electrostatic interactions with NPC1, requiring an overall basic charge to facilitate binding of NPC1 domain C. Mutations in EBOV GP (such as K114E+K115E) that reverse the electrostatic charge on the GP<sub>CL</sub> crest consistently abrogate receptor binding and reduce infectivity. In contrast, the GP<sub>CL</sub> trough is involved in more specific hydrophobic interactions with NPC1 domain C. Structure-based mutants with mutations designed to obstruct the structure of the trough (such as T83M+I113M) diminish the affinity of GP for NPC1 domain C and severely restrict the infectivity of VSV pseudotypes bearing these mutations. Based on the crystal structure and results of mutagenesis reported here, we propose that the NPC1 receptor binds GP in a two-stage process. First, GP<sub>CL</sub> recruits the NPC1 domain C receptor through nonspecific electrostatic interactions between NPC1 and the basic crest region on GP<sub>CL</sub>. Without this interaction, there is no detectable GP-receptor binding. Next, specific hydrophobic interactions are initiated between the GP, RBS trough and NPC1 domain C. The specificity of these interactions likely explains the differential effects of individual mutations in the trough (Fig. 3 and 4), whereas the effects of mutations in the crest were determined by charge, not specific amino acid position.

We also further analyzed two mutants with a mutation in the hydrophobic trough, F88A or L111A, which have been described previously as unable to support infection (25, 26). These two mutants are outliers in our analysis. Their infectivities are reduced by more than three log<sub>10</sub> infectious units relative to that of WT EBOV GP, despite only modest defects in binding of NPC1 domain C. We postulate that these mutants are defective at a step downstream from NPC1 binding. They will provide useful tools to further decipher precisely how GP<sub>CL</sub>-NPC1 binding facilitates fusion triggering and membrane fusion.

Recent work has identified multiple neutralizing MAbs from a patient who survived MARV infection. The MAbs from those studies were found to bind to the apex of MARV GP (11, 22)—the site we have confirmed here to be the filovirus GP<sub>CL</sub>-receptor-binding site. Since ebolavirus, marburgvirus, and cuevavirus GP proteins all use the NPC1 protein as a receptor, it is not unexpected that the structure of the GP<sub>P</sub> RBS would be highly conserved across all filoviruses (10, 17–19, 32). Thus, we hypothesized that the MAbs identified by Flynn et al. (22), shown to target the RBS trough on MARV GP<sub>P</sub> (11), should be broadly neutralizing. However, unlike the GP proteins of marburgviruses, those of the ebolaviruses and cuevavirus maintain a glycan cap structure that effectively shields the GP<sub>P</sub> RBS from immune surveillance. Therefore, by proteolytically priming filovirus GP<sub>P</sub> on the surface of VSV particles (such as VSV-EBOV GP<sub>CL</sub>-), we were able to analyze the neutralization potential of MAbs targeting the otherwise-occluded filovirus RBS. Analysis of GP<sub>CL</sub>-bearing VSVs would tell us if it was worthwhile to target such antibodies to the endosome as future therapeutics.

Of the panel of neutralizing MAbs from an MARV survivor described by Flyak et al. (22), only one, MR72, demonstrates significant cross-reactivity to uncleaved EBOV GP. Three other MAbs, MR78, MR111, and MR191, only react with EBOV GP<sub>CL</sub>. We focused our analysis on the EBOV GP-reactive MR72 and the EBOV GP<sub>CL</sub>-reactive MR78 and MR191, which approach GP from different angles. Here, we show that MR72 effectively neutralizes VSV pseudovirions bearing GP<sub>CL</sub> from EBOV, SUDV, BDBV, TAFV, RESTV, or LLOV. MR191 neutralizes the EBOV, BDBV, TAFV, and LLOV VSV-GP<sub>CL</sub> virions weakly (and RESTV and SUDV GP<sub>CL</sub> not at all), with infectivity never reduced below 50% (see Fig. S3 in the supplemental material). In contrast, MR78 can only neutralize MARV GP<sub>CL</sub>. It cannot neutralize EBOV GP<sub>CL</sub>-bearing VSVs, even though it is able to bind them.

The crystal structures of MARV GP<sub>CL</sub> and EBOV GP<sub>CL</sub> bound to MR78 suggest that MR78 binds the same site in both viruses. However, the resolution of the EBOV GP<sub>CL</sub>-MR78 complex was too low to identify subtle differences imposed by sequence deviations from MARV GP that might explain why MR78 fails to neutralize EBOV GP<sub>CL</sub> (11). Hence, we used a panel of VSV-EBOV GP RBS mutants to understand which sequence variations could prevent MR78-mediated neutralization of EBOV GP<sub>CL</sub>. Surprisingly, the introduction of a single point mutation (V79A) within the EBOV RBS allows MR78 to neutralize VSV-EBOV GP<sub>CL</sub>-V79A. Of significance, BDBV, TAFV, and RESTV GP<sub>P</sub> also encode valine at this position, while SUDV and LLOV GP<sub>P</sub> encode isoleucine and leucine, respectively. The larger Val, Ile, and Leu aliphatic residues encoded by the ebolaviruses and cuevavirus may prevent MR78 from neutralizing their GP<sub>CL</sub>-bearing particles (Fig. 4A; see also Fig. S3 in the supplemental material). We note that, unlike MR78, MR191 exhibits no improvement in neutralization of VSV-EBOV GP<sub>CL</sub>-V79A (see Fig. S3 in the supplemental material). How MR72 but not MR78 is able to overcome divergent amino acids at position 79 to broadly neutralize filovirus GP<sub>CL</sub> is the subject of continued structural and biochemical study.

Remarkably, enhanced neutralization of EBOV GP<sub>CL</sub>-V79A by MR78 was not accompanied by a commensurate increase in its capacity to block NPC1 binding of this GP (Fig. 4). This apparent uncoupling of neutralization and receptor blockage raises the possibility that MR78 may act as an allosteric inhibitor, preventing membrane fusion by binding to GP, subunits in the trimer that are not occupied by NPC1 domain C in the endosome. There, it may inhibit events that occur after receptor binding in order to trigger GP-mediated membrane fusion. Mutations like L111A, which eliminate infectivity without affecting receptor binding, may target these same post-receptor-binding steps.

Recent events, including the unprecedented EBOV epidemic in West Africa (3, 34), coinciding with human cases of MARV emerging in central Africa (35) and the emergence of BDBV (36) and re-emergence of SUDV (37) in this decade, highlight the urgent need for broad-spectrum antifilovirus therapeutics (38). Here, we demonstrate that the highly conserved binding site for the essential intracellular receptor NPC1 provides an attractive and underexplored target for broadly protective antibodies or small-molecule therapeutics. However, one crucial challenge to the development of such antibodies as therapeutics is an evolved feature of the filovirus entry mechanism—the unavailability of its NPC1-binding site to extracellular antibodies. The success of this
antiviral strategy therefore requires novel protein engineering approaches to deliver GP$_{\text{C}}$-specific MAbs to late endosomes and/or lysosomes, where the NPC1-binding site is unmasked by host proteases.

**MATERIALS AND METHODS**

**Expression and purification of GP$_{\text{C}}$-KZ52 complex for crystallization.** Ebola virus GP (lacking the mucin domain [residues 312 to 462]) was produced by stable expression in *Drosophila melanogaster* S2 cells. Briefly, Effectene (Qiagen) was used to transfect S2 cells with a modified pMT-puro vector plasmid containing the GP gene of interest, followed by stable selection of transfected cells with 6 μg/ml puromycin. Cells were cultured at 27°C in complete Schneider’s medium for selection and then adapted to Insect Xpress medium (Lonza) for large-scale expression in 2-liter Erlenmeyer flasks. Secreted GP ectodomain expression was induced with 0.5 mM CuSO$_4$, and supernatant harvested after 4 days. Ebola virus GP was engineered with a double Streptag at the C terminus to facilitate purification using Strept-Tactin resin (2-1201-010) (Qiagen) and then further purified by Superdex 200 size exclusion chromatography (SEC) in 10 mM Tris-buffered saline (Tris-HCl, pH 7.5, 150 mM NaCl [TBS]). EBOV GP$_{\text{C}}$ was produced by incubation of 1 mg GP with 0.02 mg thermolysin overnight at room temperature in TBS containing 1 mM CaCl$_2$ and purified by using Superdex 200 SEC. Trimeric EBOV GP$_{\text{C}}$ was then complexed with a KZ52 Fab fragment prior to crystallization as previously described (4).

**Crystallization, data collection, and structure determinations.** The purified EBOV GP$_{\text{C}}$-KZ52 Fab complex was concentrated to 3.5 mg/ml in TBS. The crystal drops consisted of a 1:1 ratio of protein/well solution. The well solution consisted of 25% polyethylene glycol monomethyl ether 550 (PEG MME 550), 10% 2-propanol, 5% ethylene glycol, 100 mM sodium acetate, pH 4.7, and 100 mM calcium chloride. Crystals grew over the course of a month and were flash frozen directly out of the crystal drop into liquid nitrogen for data collection. Data were collected remotely at the Argonne National Laboratory, Advanced Photon Source (APS), from the GM/CA beamline 23-ID-D. The structure was determined using molecular replacement with Phaser (39), within the CCP4 suite (40), using a modified EBOV GP-KZ2 complex model (PDB code 3CSY) with all the residues corresponding to the glycan cap removed (4). Refinement of the EBOV GP$_{\text{C}}$ crystal structure was done through iterative cycles of model building using COOT, followed by refinement with Refmac5 and PHENIX (41–43). The structure was determined using molecular replacement with Phaser (39), within the CCP4 suite (40), using a modified EBOV GP-KZ2 complex model (PDB code 3CSY) with all the residues corresponding to the glycan cap removed (4). Refinement of the EBOV GP$_{\text{C}}$ crystal structure was done through iterative cycles of model building using COOT, followed by refinement with Refmac5 and PHENIX (41–43).

**Cells and viruses.** African green monkey kidney (Vero) cells were maintained at 37°C and 5% CO$_2$ in high-glucose Dulbecco’s modified Eagle medium (DMEM; Invitrogen Corp., Carlsbad, CA) supplemented with 10% fetal calf serum. Replication-incompetent vesicular stomatitis virus (VSV) (serotype Indiana) pseudotyped viruses were generated as described (22). Expression and purification of GPCL-KZ52 complex for crystallization. EBOV GPCL was produced by incubation of 1 mg GP with 0.02 mg thermolysin overnight at room temperature in TBS containing 1 mM CaCl$_2$ and purified by using Superdex 200 SEC. Trimeric EBOV GP$_{\text{C}}$ was then complexed with a KZ52 Fab fragment prior to crystallization as previously described (4).

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**Cells and viruses.** African green monkey kidney (Vero) cells were maintained at 37°C and 5% CO$_2$ in high-glucose Dulbecco’s modified Eagle medium (DMEM; Invitrogen Corp., Carlsbad, CA) supplemented with 10% fetal calf serum. Replication-incompetent vesicular stomatitis virus (VSV) (serotype Indiana) pseudotyped viruses were generated as previously described (49). The wild type (VSV-WT GP) encodes enhanced green fluorescence protein (eGFP) in place of the VSV-G gene to allow scoring of infection and bears the EBOV GP$_{\text{C}}$-muc gene (Mayinga isolate, GenBank accession number AF086833) but lacks the mucin-like domain (residues 309 to 489 [Δmuc]) (6). Point mutants and multiple mutants were generated by subcloning GP fragments containing the mutation(s) to replace EBOV GP$_{\text{C}}$muc. Cleaved VSV-GP$_{\text{C}}$ particles were generated by incubating VSV-GP$_{\text{C}}$ pseudotypes with thermolysin (250 μg/ml) for 1 h at 37°C. The reaction was stopped by adding phosphoramidon (1 mM) and incubating on ice for 5 min.

**Normalization of GP for ELISA.** Normalization of GP$_{\text{C}}$ amounts to be used in the binding experiments was done by ELISA, as illustrated in Fig. S4 in the supplemental material. Briefly, high-binding 96-well ELISA plates (Corning) were coated with serial dilutions of GP$_{\text{C}}$ in phosphate-buffered saline (PBS), and allowed to bind at 37°C for 1 h. The plates were blocked with PBS containing 3% bovine serum albumin (PBSA), followed by incubation with the anti-GP monoclonal antibody KZ52 (2 μg/ml in PBS) (21) and a horseradish-conjugated anti-human secondary antibody (Santa Cruz Biotechnology), which was detected by ultra-TMB (3,3’,5,5’-tetramethylbenzidine) substrate (Thermo Scientific). Absorbance readings were subjected to a nonlinear regression analysis (GraphPad Prism software) to generate binding curves and calculate an EC$_{50}$ value. Additionally, the virions were normalized for GP incorporation by comparing the amount of GP to the amount of the VSV matrix protein (M). Equal amounts of purified virions were resolved on SDS-PAGE and blotted for the VSV matrix protein using a mouse anti-VSV M antibody (23H12). Quantification was done using a LI-COR IR dye-conjugated anti-mouse Alexa Fluor 680 secondary antibody (Invitrogen) on the Odyssey Imaging Station and Image Studio 2.1 software (LI-COR Biosciences), and the results were normalized to the WT control. Virus particles that had less than 25% incorporation of mutant GP compared to the incorporation of WT GP or that were highly sensitive to proteolysis were excluded from our analysis.

**GP-NPC1 domain C capture ELISA.** Binding of GP to NPC1 domain C was performed as previously described (10, 32). Briefly, high-binding 96-well ELISA plates (Corning) were coated with the anti-GP monoclonal antibody KZ52 (2 μg/ml in PBS) (21). Following a blocking step, either uncleaved or in vitro-cleaved GP$_{\text{C}}$ pseudotypes were captured on the plate. Unbound GP was washed off, and serial dilutions of Tagged purified soluble human NPC1 domain C (0 to 40 μg/ml) were added. Bound NPC1 domain C was detected by a horseradish-conjugated anti-Flag antibody (Sigma-Aldrich), using ultra-TMB substrate (Thermo Scientific). EC$_{50}$s were calculated from binding curves generated by nonlinear regression analysis using GraphPad Prism software. Binding ELISAs were done in duplicate in at least two independent experiments. All incubation steps were done at 37°C for 1 h or at 4°C overnight.

**Pseudovirus neutralization assays.** Serial dilutions of MAbs and of a no-antibody control were mixed with either cleaved or uncleaved VSV-GP particles and allowed to bind for 1 h at room temperature. Monolayers of Vero cells were inoculated with the antibody-virus mixture in duplicate and incubated at 37°C in 5% CO$_2$. Infection was scored 12 to 16 h postinfection by enumeration of eGFP-positive cells under a fluorescence microscope. The ZMapp cocktail MAbs 2G4, 4G7, and 13C6, as well as MAb KZ52, prepared as previously described (33), were generously provided by Mapp Biopharmaceutical. MAbs MR78 and MR72 were prepared as previously described (22).

**Protein structure accession number.** Coordinates and structure factors have been deposited into the Protein Data Bank under accession number 5HJ3.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.02154-15/-/DCSupplemental.

Figure S1, PDF file, 2.7 MB.

Figure S2, PDF file, 0.3 MB.

Figure S3, PDF file, 1.2 MB.

Figure S4, PDF file, 0.2 MB.

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