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Mapping Immunological, Host Receptor Binding Determinants, and Cathepsin Cleavage Site of EBOV Glycoprotein Utilizing the Qubevirus Platform

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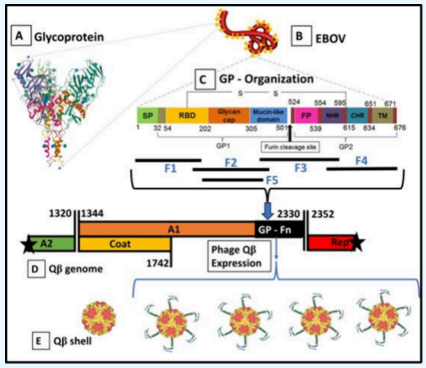
ABSTRACT: Ebola virus (EBOV) remains a highly infectious human pathogen that causes a severe and lethal disease known as Ebola virus disease (EVD), despite recent progress in vaccine development based on its only surface glycoprotein (GP). In this study, we modeled and inserted four overlapping fragments (F1−4) of the EBOV GP at the C-terminus of the A_1 protein of Qubevirus ($Q\beta$) and used the platform to investigate the tropism and immunological functions of the GP by displaying the peptides with 30 overlapping amino acids. The resulting recombinant phages were used to determine their reactivity with GP-specific antibodies and their binding to the recombinant Niemann-Pick C1 (rNPC1) receptor in an immunoassay. In addition, modified, truncated, and C-terminus-tagged fragment F1 named F5 was utilized to map the cathepsin cleavage sites in an enzymatic assay. We demonstrated that a large GP peptide of 200 AA could be fused to A_1 and exposed on the $Q\beta$ platform in an accessible manner without significantly affecting its viability and infectivity. Fragments F1 (GP1−200), F2 (GP170−370), and F3 (GP350−550) were shown to contain important immune epitopes through binding to anti-GP-specific antibodies. Further, F1 was found to bind rNPC1, thereby suggesting a receptor binding determinant of the GP that was further confirmed in a competitive assay where the recombinant phages bearing the F1 fragment reduced the infectivity of EBOV pseudovirus by 27%. In addition, the viral infectivity was shown to be reduced by 46.39% by a cyclic peptide selected from an RNA $Q\beta$ library. Finally, F5 showed the cleavage sites to be AA191−192 and AA194−195 for CatB and L, respectively, which were further validated using a recombinant EBOV glycoprotein. These results provide insights into the antigenicity and tropism characteristic of the glycoprotein, with implications for the development of subunit vaccines or other biologics against Ebola virus disease.



1. INTRODUCTION

The Ebola virus disease (EVD) outbreak between 2013 and 2016 in West Africa, caused by Ebolavirus (EBOV) is highly contagious with a high case-fatality rate and has triggered global health concerns.[1,2] Additionally, more cases outside Africa have occurred.[3,4] Currently, countries reporting both endemics and imported cases have witnessed more deaths than all previous outbreaks combined.[2,5,6] EVD is a severe hemorrhagic fever, with a mortality rate between 70 and 90%, and was first reported in 1976 as a zoonotic disease affecting human and nonhuman hosts.[2,7−9] EBOV is a member of the *Filoviridae* family with five species belonging to the Ebolavirus genus identified already.[10−12] The EBOV particle contains a nonsegmented, negative-sense RNA together with seven viral proteins consisting of the nucleoprotein (NP), RNA-dependent-RNA-polymerase (RdRp) cofactor (VP35), viral matrix protein 40 (VP40), glycoprotein (GP), transcriptional activator 30 (VP30), the minor matrix protein 24 (VP24), and the RNA polymerase (L) that is an RdRp.[12,13] Among the seven structural proteins encoded from the fragmented open reading frames (ORFs) of the EBOV genome, only the GP is the most exposed multifunctional protein involved in viral replication, tropism, and immunogenicity.[14,15]

The glycoprotein gene segment is cotranslationally processed and cleaved from a precursor (GP_0) to an N-terminus (G_N) of 140 kDa and a C-terminus (G_C) of 26 kDa.[16] The cleavage occurs in the Golgi apparatus at the amino acid position 501 by a cellular protease furin to yield GP1 and GP2 subunits, which are linked via a disulfide bond between Cys53 and Cys609.[16,17] The surface unit GP1 is the most exposed, and the transmembrane unit GP2 is anchored to the membrane through its domain. The GP1-GP2 complex exists as heterodimers assembled into trimers that are eventually transported to the host cell surface where they are finally

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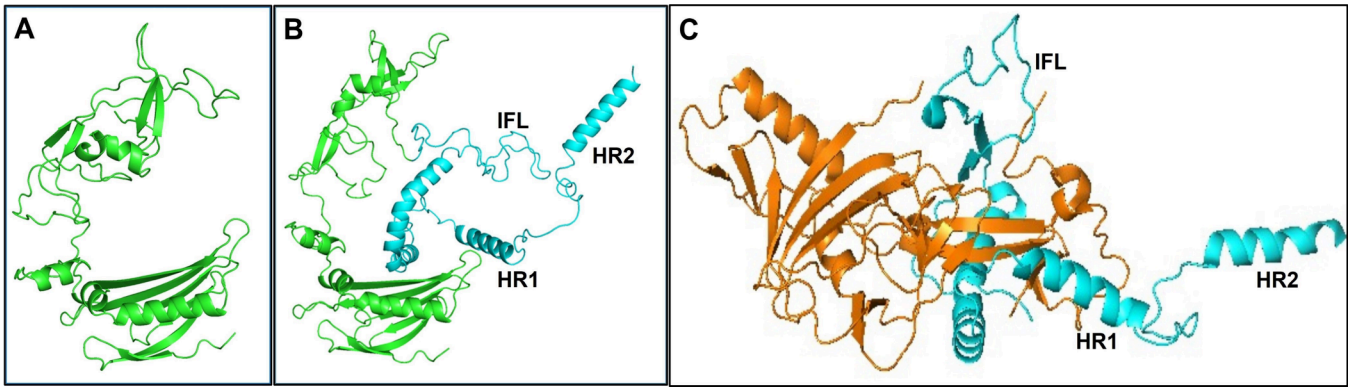

Figure 1. Modeling of the Qβ minor coat protein A1 with EBOV glycoprotein fragments. (A) wild type A1; (B) A1 fused with fragment F4 (cyan) corresponding to the entire GP2 of EBOV GP; (C) Crystal structure of the EBOV GP monomer from protein data bank (PDB ID: 7JPI); GP1 is shown in orange and GP2 in cyan. The four major α-helixes (including heptad repeat regions HR1 and HR2) and the internal fusion loop (IFL) are conserved in the model of F4 with A1(B).

incorporated into the virion envelope.[16,17] The domain organization of GP starts with a signal peptide (SP), followed by a receptor binding domain (RBD), a heavily N- and O-glycosylated mucin-like domain (MLD), an internal fusion loop, and two heptad repeat regions (HR1, HR2), and finishes with a transmembrane domain (TMD). During the infection following uptake, Ebola virions are trafficked to late endosomes where the GP is primed by the cysteine proteases, mainly cathepsin B and L (CatB and L) using their endopeptidase activity.[18,19] The GP primed by CatB/L triggers membrane fusion for the final viral tropism and entry by interacting with Niemann Pick C1 (NPC1), the natural host receptor of EBOV.[18,19] The multifaceted roles of GP in viral pathogenesis imply the existence of recognition sites (amino acid residues), binding domains or fragment motifs, and epitopes of this immunogenic protein and an active small receptor binding motif for the viral entry which has been mapped out in this study using our Qubevirus platform.

Qubevirus (Qβ) is a small, positive-sense, single-stranded RNA genome infecting *E. coli* that is found throughout the world in bacteria associated with sewage and animal feces.[20,21] Qβ encodes 4 genes within a 4220 nucleotide genome consisting of a subunit II (β) of replicase, a major coat protein (Cp), a maturation protein (A_2), and a read-through protein (A1).[22−24] Importantly, the minor coat protein A_1 is the key target in this study. The A_1 protein shares the same initial codon with Cp and is produced during the translation. The Cp stop codon UGA triplet is suppressed by a low level of ribosome mis-incorporation of tryptophan at the end of Cp stop to produce A_1.[25] We previously demonstrated that (i) the A_1 minor coat protein can be extended by up to 100 amino acids without affecting its function and (ii) the A_2 protein can initiate recombinant Qβ phage infection while binding to an immobilized target. We have thus optimized a unique novel in vitro evolutionary technology through biopanning based on elution by infection. In previous reports from our group, plasmids harboring the full cDNA of Qβ phage were used,[26,27] which allowed genetic construction of the vector for the expression of hybrid phages with surface-displayed overlapping GP library peptides.

Despite recent progress in drug (interferon, toremifene, clomiphene, and favipiravir having a weak effect on viral replication) and vaccine development, with up to eight licensed vaccines with the most promising rVSV-ZEBOV[28−30] approved by the US Food and Drugs Administration (FDA), GP remains druggable and a target for vaccine development. The initial focus is to identify biological drug targets, specific motifs, and residues of EBOV GP that are druggable and can initiate infection and stimulate desirable immune responses for incorporation into novel subunit vaccine development. The present study utilized this newly developed Qubevirus display platform through the engineering of its minor coat protein A_1 by mutation deletion and insertion. Four overlapping fragments of the EBOV glycoprotein were inserted separately into their corresponding recombinant phage expression system and subsequently used for target recognition studies. An optimized phage titer greater than 10^4 pfu/mL was obtained for each fragment and utilized for biopanning or ELISA against anti-GP-specific antibodies. Another ELISA was performed using a recombinant NPC1 based on the recently reported copy number of the phage minor coat protein of 12.[31,32] First, one fragment motif was found with an activity mimicking the binding to the human NPC1 receptor. Interestingly, the recombinant phages bearing this fragment were shown to reduce the viral infectivity in a competitive assay with the Ebola pseudovirus. Second, three fragments recognized and bound the cognate anti-GP antibodies and therefore contain antigenic epitopes of EBOV, but with different affinities. Finally, a fragment was also identified bearing the cathepsin B and L recognition and cleavage sites and confirmed using site-directed mutagenesis.

2. RESULTS

2.1. Simulation of A_1 Displaying the EBOV GP Fragments Showed No Significant Effect on Its 3D Structural Modeling for the Recombinant Phage Expression. We previously showed that up to 100 foreign amino acid residues (100 AA) can be inserted into the A_1 protein stably without affecting its functionality, and in this work, the number of inserted residues is increased substantially. The 3D structural modeling indicated that the EBOV GP fragment is displayed on the C-terminal end of the minor coat protein A_1. The potential recombinant A_1 model revealed slight changes in its conformation when compared to that of the wild type of the minor coat protein only. The critical N-terminal end for phage functionality did not significantly change with the conservation of its α-helixes and β-sheets numerically in all models (Figure 1A,B). The only impact

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observed was on the C-terminal with a much larger insertion peptide upon the rotation of its other domain (Figures 1 and S1). Additionally, we have further deleted the C-terminal of A_1 and inserted a linker peptide (GGSSGG) with the NotI restriction site that brings the additional GGR peptide extension appropriate for display purposes. The EBOV GP fragments of about 150, and 200 amino acid sequences separately, were predicted to be successfully inserted into all generated forms of the minor coat protein A_1. This model increases the number of amino acid residues that can be modeled and simulated substantially and reveals the rigidity of the N-terminal of the A_1 for this RNA phage display technology. The simulation of the A_1 and GP fragments was confirmed to be present at the C-terminal, in which the modeling of the recombinant A_1 protein revealed the exposition to the outer surface of the major capsid protein as shown in Figure 1. Additionally, the secondary structure of the recombinant RNA was analyzed with RNAfold and shown not to destroy the major features of the phage's functionality (the replicase gene ribosomal binding section).

2.2. Construction of the Recombinant Phage Vectors with EBOV GP Fragments. We have demonstrated in our earlier reports that the minor coat A_1 protein could be engineered to display foreign peptides with 50 and 100 amino acids corresponding to the gp4 of HIV-1 and spike protein of the SARS-CoV envelope, respectively.[27,32] Still, the maximum number of amino acids to be inserted in A_1 and displayed on the surface of the RNA Qβ phage remains unclear. Using the same procedure, different primers (Table 2) were designed and used to copy four distinct overlapping fragments of the EBOV *gp* gene corresponding to 200 amino acids each (with 30 amino acids overlapping sequence) and fused separately at the C-terminus of A_1, hereby increasing the total length of the peptide to be displayed at the end of A_1 so far in our research group. Similarly, another fragment of 145 AA (F5), a truncated modified F1 and its mutants were fused at the end of A_1 and cloned in the same vector template, separately. The electropherogram (Figure 2 Panel A) showed the expected gene size of each fragment of about ∼600 bp (F1−4) or 400 bp (F5, F5CatB, and F5CatL) while enzymatically restricted in our analyses. Each gene fragment was flanked with a unique restriction enzyme (NotI) inserted into the pBRQβ expression cassette and cloned into *E. coli* HB101, in our design, it was revealed. Positive recombinant plasmids were determined on the agarose gel electrophoresis following digestion into ∼7 kbp and ∼700 or ∼400 bp fragments, respectively, by NotI (Figure 2 Panel B). The resulting plasmids were further sequenced by the Sanger method, analyzed, and confirmed the appropriate insertion of all seven gene fragments of the EBOV GP, separately. Herein, the results confirmed the design and fusion in the frame of the GP gene fragments separately, with the C-terminus of the minor coat protein A_1. Interestingly, these results showed that we have successively generated all overlapping GP fragment genes, constructed recombinant and stable plasmids bearing the full length of the Qβ phage genome and containing them all, and validated them through sequencing.

2.3. Recombinant Phage Expression Displaying EBOV GP Fragments, Morphology, and Titer. The sequenced recombinant plasmids were successfully used to transform F^- bacteria for phage expression production. To avoid any possible reinfection after recombinant phage obtention, the host, F^- *E. coli* HB101 bacteria lacking the pilus appendage,

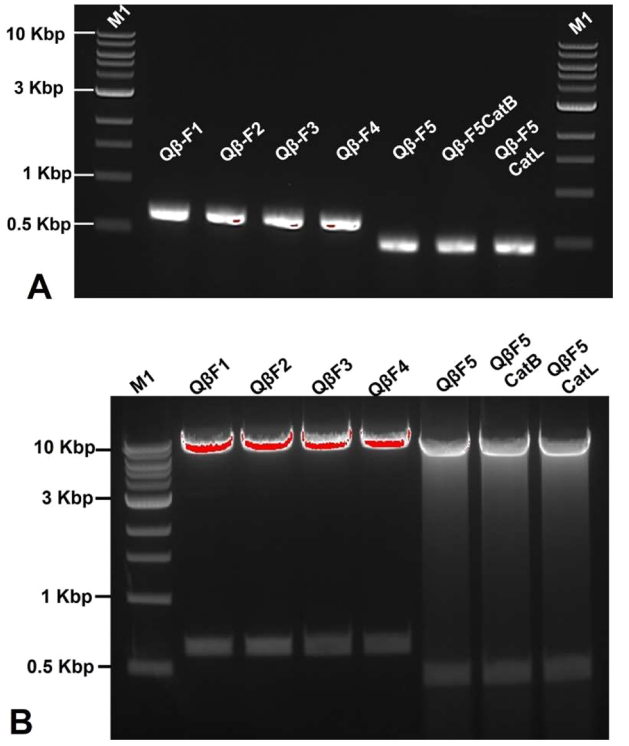

Figure 2. Electropherogram of the PCR product of different fragments of GP and the restriction digestion of the recombinant plasmids. (A) (1.2% agarose gel) \Fragments F1−4 showed the expected size of about 600 bp; fragment F5 and the resulting mutants were ∼400 bp and smaller than the parent fragment (F1) as expected. (B) (0.8% agarose gel) Clone selection of different plasmids containing GP fragments digested with NotI: small fragments of about 600 bp (QβF1 to F4) and about 400 bp (QβF5 and mutants) were removed by the restriction digestion, confirming the two restriction sites of the enzyme flanked to the insert.

was used for this production of the first generation of phages. The phage expression was under the control of the promotor T7 as previously reported, within the recombinant plasmids constructed.[26] After the transformation of *E. coli* HB101, clones were selected on 2YT media supplemented with ampicillin (100 mg/mL). The resulting recombinant phages from plasmids were purified separately from the bacteria cell debris after precipitation and showed a titer of 10^3−10^5 pfu/mL (Table 1) on a lawn of *E. coli* Q13 in a plaque assay, with the plaque size of 0.5 to 3 mm in diameter (Figure 3). Overall, each recombinant phage containing the fragment of EBOV GP

Table 1. Titer and Diameter of Phages[a]

phage	titer (pfu/mL)	diameter (mm)
Qβ	10^5	3.2 ± 1.3
QβF1	10^4	3.3 ± 1.3
QβF2	10^3	2.1 ± 0.7
QβF3	10^4	2.0 ± 0.6
QβF4	10^4	2.4 ± 0.6
QβF5	10^3	2.4 ± 0.9
QβF5CatB	10^3	3.0 ± 1.1
QβF5CatL	10^2	3.05 ± 1.7

[a]The titer was determined from the phage expressed in *E. coli* HB101. The diameter of each phage is the average of 10 random plaques. The plaque size varied from 0.5 to 5 mm.

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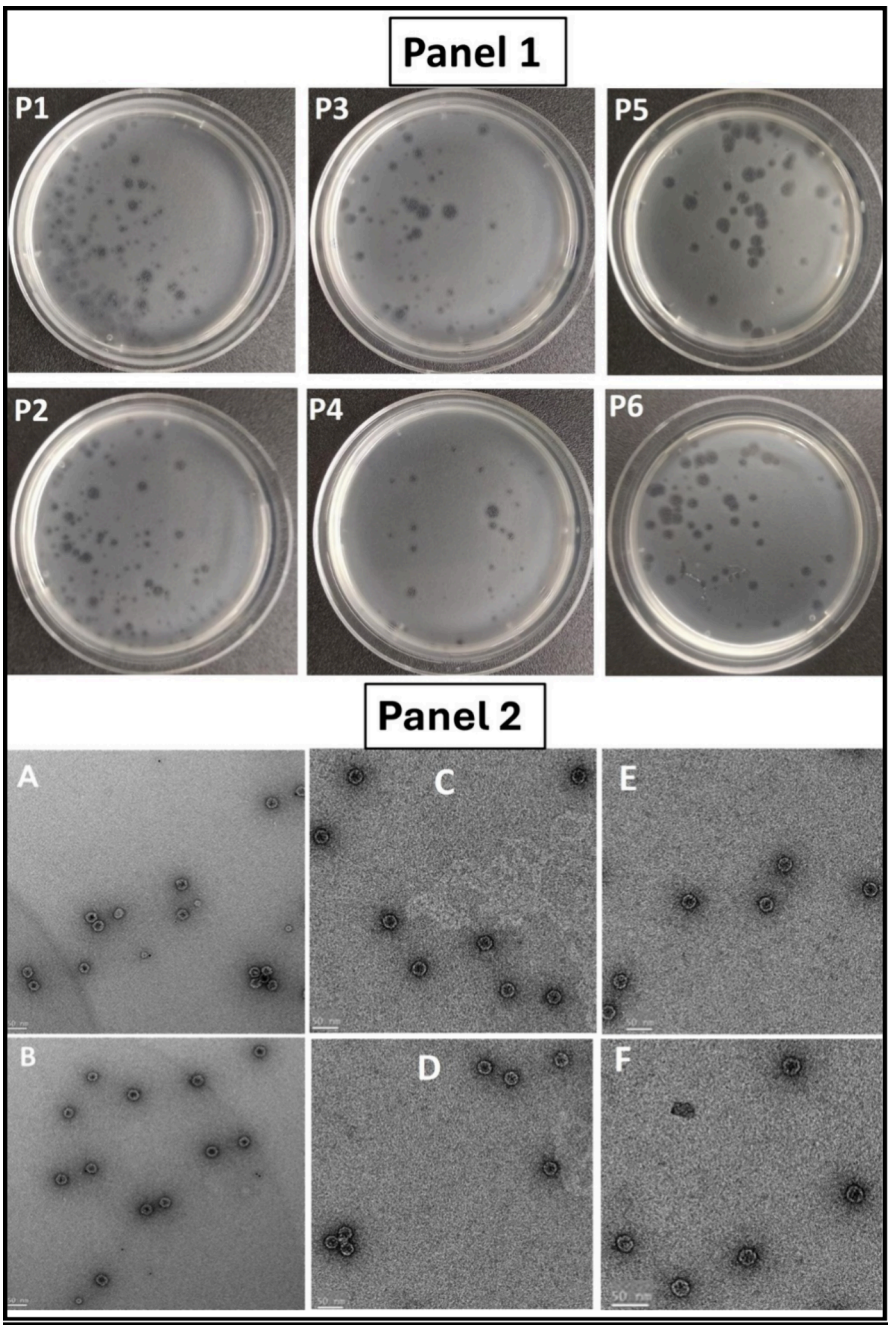

Figure 3. Morphology of Qβ and recombinant phage derivatives. Panel 1: Phage plaques on the lawn of *E. coli* Q13. (P1) Wild type Qβ; (P2) QβF1; (P3) QβF2; (P4) QβF3; (P5) QβF4; and (P6) QβF5. All of the recombinant phages conserved their infectivity by inducing *E. coli* Q13 lysis (formation of clear zones or plaques). A mixture of plaques of different sizes was also found in the plates containing the recombinant phages compared to the wild type. Panel 2: Negative stain electron micrograph of phages. (A) Wild type Qβ; (B) QβF1; (C) QβF2; (D) QβF3; (E) QβF4; and (F) QβF5. Nickeled gold of 10 nm (black dots) was used for detection. The scale bars on the micrographs are 50 nm. The size of the recombinant phages was almost identical to the wild type.

(F1−4) exhibited predominantly large plaques as compared to the wild type Qβ (Figure 3 Panel 1) and their morphology did not significantly change when observed under an electron microscope (Figure 3 Panel 2). This first generation of the recombinant phages bearing separately the overlapping fragments of EBOV GP was subjected to two more rounds of 3 h amplification using *E. coli* Q13 as the host cell. This resulted in an increased titer of up to $10^{10}-10^{12}$ pfu/mL after precipitation/purification, which is our working conventional phage titer. Herein, the results show that the scaled working phage titer was obtained with a large, inserted entity (200 amino acids). In our previous study, we noticed that the recombinant phages bearing the transmembrane portion of the spike protein of SARS-CoV were poorly expressed in *E. coli* HB101.[27] In contrast with the present study, a phage titer of up to 10^4 pfu/mL was obtained with fragment F4 containing the transmembrane domain of the Ebola glycoprotein. To confirm the appropriate insertion of each EBOV fragment fused at the C-terminus of the minor coat protein A_1 of Qβ, a sequenced cDNA was prepared from the recombinant phages

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and amplified using RT-PCR. The results of sequencing reactions confirmed the gel analysis of their size checked on the agarose gel electropherogram to be approximately 1.5 kbp and above the cDNA of the wild type. The obtained cDNA for each round of amplification was further sequenced and analyzed, and the result showed the appropriate insertion of each GP fragment gene fused in frame with the C-terminus of A_1 of Qβ phage and defining the genotype of the recombinant phages. These results demonstrated the possibility of obtaining viable and infectious recombinant Qβ phages cDNA containing a large gene sequence fused at the end of the minor coat protein gene.

2.4. Avidity of Recombinant Phages to Anti-EBOV GP Antibody. The Ebola glycoprotein is a membrane-anchored protein, exposed on the surface of the virus, with antigenic sites that can be recognized and bound by the antibodies.[10] Mostly, the GP epitopes have been shown to be masked by the heavily glycosylated mucin-like domain, hence, hiding them from the immune system of the host.[13,14] Linearizing the overlapping fragments of the EBOV GP on the Qβ platform would lead to the identification of its immunogenic portions. In our previous reports, we developed and optimized a protocol used to probe recombinant phages bearing immunogenic fragments of pathogens with their cognate antibodies.[27,33] The same strategy was used by immobilizing recombinant phages bearing EBOV GP fragments on the nitrocellulose membrane and probing with anti-EBOV GP antibody in a dot blot assay. By exposing the membrane to chemiluminescence after incubating with a secondary antibody conjugated with horseradish peroxidase, three displayed overlapping fragments including F1 (Met1-His200), F2 (Tyr170-Val370), and F3 (Val350-His550) were shown to bind to the anti-GP antibody (Figure 4A), with F2 and F3 showing higher affinity. The wild type Qβ phages were used as a control and showed no affinity to the anti-GP specific antibody. Based on the results, the GP fragments located between Met1-Arg500 are determined to contain major epitopes (Figure abstract Panel C) corresponding to the GP1 subunit of Ebola virus glycoprotein. The positive reaction of phages bearing F1−3 with anti-GP antibody confirmed the presence and affinity of the EBOV GP fragments displayed on the surface of the Qβ phage to the target. The recombinant phages were subjected to quantitative ELISA subsequently. The results showed an increased absorbance (OD) with the increasing amount of anti-GP antibody, with fragment F3 having the highest affinity compared to F1 and F2 (Figure 4B). A plateau was observed with fragment F2 after 0.5 μg/mL of antibody. The reaction of the recombinant phage surface to the anti-GP antibody is functional and reveals the phenotype of the phage display correlating with the expected genotype. Our RNA Qβ phage display system was successfully used to map and identify the immunogenic portion of the Ebola glycoprotein within each fragment (F1−3).

2.5. Interaction of EBOV GP with the Host Receptor NPC1. The human Niemann-Pick C1 (NPC1) has been identified as the natural host receptor of the Ebola virus. During infection prior to the fusion with the host cellular membrane, the virus binds to the C domain of the receptor through the GP1 subunit of its surface glycoprotein (GP).[34,35] In this study, we used our optimized expression cassette containing the full-length cDNA of Qβ phage to clone, express, and display different EBOV GP-derived fragments that were further screened for their ability to bind to the recombinant

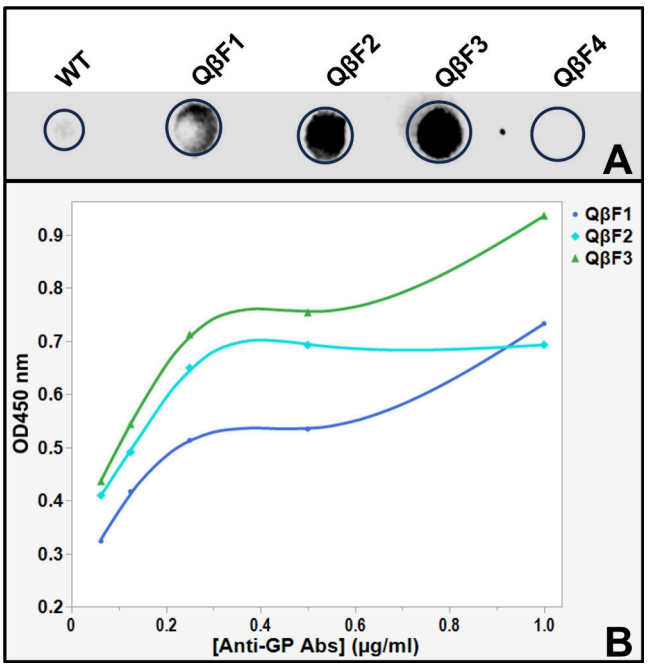

Figure 4. Binding activity of Ebola glycoprotein identified on the RNA Qβ phage platform to anti-EBOV GP antibody. (A) Recombinant phages QβF1, QβF2, QβF3, and QβF4 were subjected to immunoblotting assay (dot blot) with anti-EBOV GP antibody in comparison with the wild type Qβ (WT). (B) Quantitative ELISA of the anti-EBOV GP antibody with the immunogenic fractions on Qβ phage.

receptor NPC1. In earlier reports, we developed and optimized an ELISA protocol to test the binding affinity of a recombinant Qβ phage bearing the receptor binding motif of SARS-CoV spike protein with the recombinant host receptor angiotensin-converting enzyme 2 (rhACE2).[27] A similar approach was used in this study to test the NPC1 receptor binding affinity of the EBOV GP by immobilizing the recombinant phages bearing different fragments of the glycoprotein in separate wells of a 96-well plate and exposing them to the recombinant host receptor (rNPC1) fused with a His-tag. The detection after subjecting the complex to anti-His antibody conjugated with horseradish peroxidase (HRP) showed that only F1 (Met1-His200) bound to NPC1 whereas other fragments (F2−4) did not show any reactivity with the receptor (Figure 5A). Similarly, the wild type Qβ phage served as a control and showed no binding reactivity to NPC1. To confirm the binding reactivity of the EBOV GP fragment exposed on the surface of the phages, a quantitative ELISA was performed with a 2-fold serial dilution of the receptor. The results show an increase in the absorbance (OD) of HRP conjugated to anti-His antibody with increasing concentration of the recombinant receptor NPC1 for recombinant phages harboring F1 and none for other phages (F2−F4). A plateau was observed at a concentration of NPC1 greater than 2 μg (Figure 5B), showing a saturation of the receptor. In this study, by expressing and exposing large fragments of the EBOV GP on the Qβ platform, we identified and situated the receptor binding domain or motif of the glycoprotein on the N-terminus of the GP1 subunit, within the AA1−170. Additionally, F2 containing 30 amino acids overlapped with F1 showed no reactivity to NPC1 thereby excluding this portion from the EBOV GP receptor binding domain.

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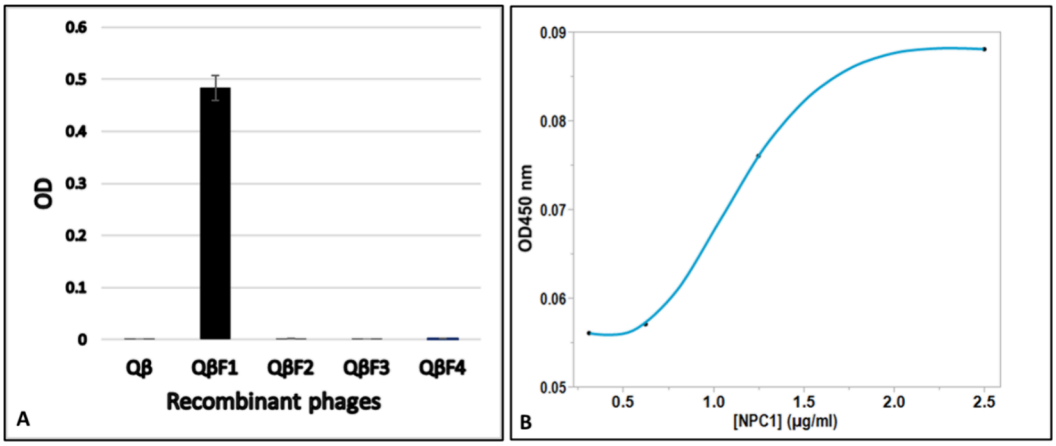

Figure 5. Reactivity of Ebola glycoprotein fragments with the host receptor NPC1. (A) ELISA of the interaction of the recombinant phages (QβF1, QβF2, QβF3, QβF4) bearing the EBOV GP fragment with the recombinant host receptor, the receptor was detected with anti-6xHis-tag antibody conjugated with HRP; Qβ is the wild type phage used as control. (B) quantitative ELISA of NPC1 bound QβF1 containing the receptor binding domain.

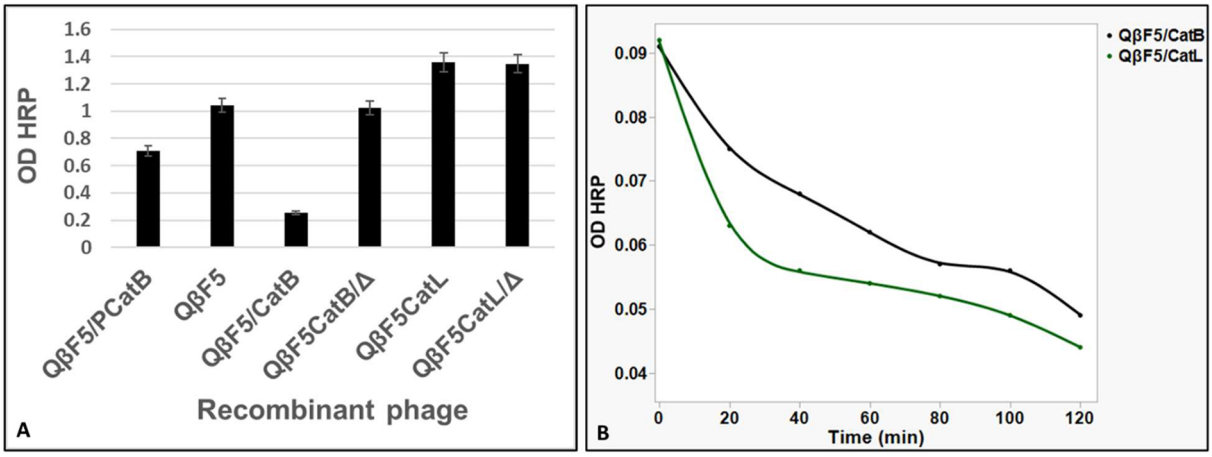

Figure 6. Cathepsin cleavage activity on EBOV GP fragment displayed on the Qβ platform. In A, the fragment predigested (QβF5/pCatB) or postdigested (QβF5/CatB) with CatB was compared to the control (QβF5); the recombinant phages with the mutations at the cleavage site were also digested with CatB (QβF5CatB/Δ) and compared with the control (QβF5); Phages with the mutated cleavage site of CatL were digested with the enzyme (QβF5CatL/Δ) and compared with the nondigested mutants (QβF5CatL). In B, a kinetic reaction was set to determine the specificity of the enzymes; the GP fragment was cleaved separately with CatB (QβF5/CatB) or CatL (QβF5/CatL).

2.6. Identification and Validation of the Cleavage Site of Cathepsins. Ebola virus glycoprotein (GP) is a trimeric surface protein with each monomer formed by two subunits of GP1 and GP2. The GP is the most abundant surface antigen that plays a critical role in viral infection by binding to the host receptor. GP1 has been identified to be processed by endosomal enzymes cathepsins (CatB and L) prior to binding to the host receptor NPC1.[35] The potential cleavage sites of these enzymes have been identified to be located between amino acid residues 190 and 194.[19,36] To investigate the cleavage site of the cathepsins, we constructed, expressed, and exposed Qβ-tagged truncated F1 fragments of EBOV GP (F5) with mutations introduced at the predicted cleavage sites. In our design strategy, we constructed an LPETG-tagged fragment (F5) and obtained purified recombinant Qβ phages. After subjecting the recombinant phages to CatB cleavage for 1 h (QβF5/CatB) before labeling the LPETG with a peptide-HRP conjugate by sortase A, the OD was shown to be reduced by 25% compared to the control (nondigested QβF5). When the HRP was attached to the peptide before digesting with CatB (QβF5/CatB), the OD was significantly reduced by up to 75% compared to the control (Figure 6A). In contrast, phages bearing GP peptide with mutations at position AA191−192 did not show any cleavage (no reduction in OD) when treated with CatB (QβF5CatB/Δ) compared to the control (QβF5). Similarly, the recombinant phages bearing the mutations at the CatL cleavage site did not show any difference when treated with the enzyme (QβF5CatL/Δ) compared to the nondigested mutants (QβF5CatL) (Figure 6A). This result showed that cathepsins, especially CatB, were able to recognize and cleave the EBOV GP peptide exposed on the surface of recombinant Qβ phages at the predicted site toward the C-terminus. To confirm the effectiveness of the cleavage activities of both CatB and CatL, a kinetic experiment was further performed with the recombinant phages without mutation (QβF5). The results showed a decrease in OD with time when the wells were washed every 20 min to remove the cleavage product containing the HRP at the C-terminus of the displayed peptide (Figure 6B). This decrease in absorbance confirmed that CatB and CatL effectively cleaved the EBOV GP peptide exposed on the surface of recombinant Qβ phages at the

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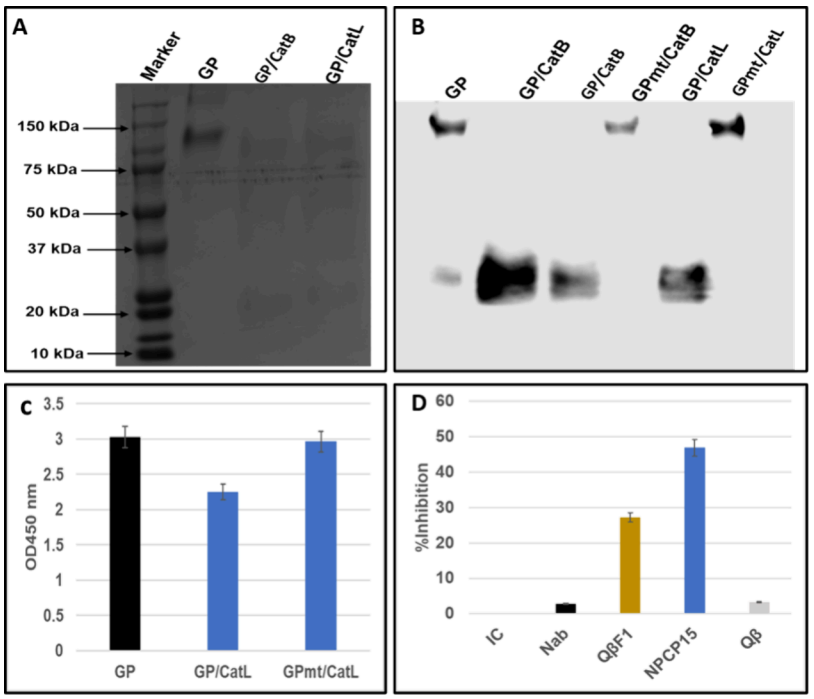

Figure 7. Cathepsin cleavage and EBOV Pseudovirus inhibitory effects. (A) SDS-PAGE of the recombinant EBOV GP digested with CatB (GP/CatB) and CatL (GP/CatL); GP is the nondigested glycoprotein used as control. (B) Western blot of EBOV GP; GP/CatB is 5 and 2.5 μg of GP digested with CatB, respectively; GP/CatL is 2.5 μg of GP digested with CatL; GPmt/CatB and GPmt/CatL are mutated CatB and CatL cleavage sites digested with CatB and CatL, respectively. (C) ELISA of the recombinant EBOV GP and its mutant (GPmt) digested with CatL (GP/CatL and GPmt/CatL, respectively); GP is the nondigested EBOV glycoprotein used as control. (D) Competitive and inhibitory assays of the recombinant phages and peptide with Ebola pseudovirus; (IC) infected cell alone, (Nab) anti-GP antibody was added to the cell culture, (QβF1) recombinant phages were added to cell culture, (NPCP15) cyclic peptide added to the cell culture, and (Qβ) wild type phages were added to the cell culture. All of the tests were performed in triplicate.

expected sites, notably, Lys191-Lys192 and Phe193-Phe194, respectively. Therefore, F5 contains the appropriate binding motif for the CatB/L activities. To further validate the cathepsin cleavage activity, a full-length recombinant EBOV GP and its derived mutants (in which CatB and L cleavage sites were mutated) were subjected to CatB and L digestion separately. SDS-electropherogram (Figure 7A) shows the appearance of a cleavage product of approximately 25 kDa compared to the negative control well in which only one band of about 145 kDa was observed, corresponding to the parent recombinant GP. Similar results were obtained in Western blot (Figure 7B) with the recombinant GP digested with CatB or CatL. The mutants did not show any cleavage when subjected to CatB or CatL, with a band detected at the same position as the nondigested GP. The ELISA further demonstrated the cleavage activity of the cysteine proteases, with a decrease in OD when the recombinant GP was digested with CatL (Figure 7C). Collectively, these results support the CatB/L cleavage activity at the predicted sites.

2.7. . Inhibition of the EBOV Pseudovirus by the Recombinant Phage and Cyclic Peptide. EBOV infection is initiated by the attachment of the virus to the cell surface receptor(s) followed by the binding of the viral glycoprotein to the host endosomal/lysosomal receptor NPC1. The recombinant phage (QβF1), bearing the EBOV GP receptor binding domain and a cyclic peptide (NPCP15) was evaluated for its ability to inhibit ebola pseudovirus entry into Vero E6 cells. The recombinant phages were shown to reduce the infectivity of the pseudovirus by 27% when 10 μL of the phages were added to the culture medium, compared to the culture containing wild type phage (Qβ). Similarly, the cyclic peptide was shown to inhibit the pseudovirus infectivity by 46.93% at 0.5 μM when added to the culture media compared to the control (Figure 7D). Therefore, these results indicate that the recombinant phages displaying F1 mimic the GP RBD structure and exhibit an affinity for the EBOV host receptor.

3. DISCUSSION

Licensed vaccines available to date are rVSV, a live replicating virus expressing the EBOV glycoprotein susceptible to mutations, which dampen or eliminate the vaccine efficacy and efficiency. Earlier reports implicated the glycoprotein in vaccine efficacy but using crystallography a few immune epitopes have been identified mainly situated at the base and top of its structure.[10] Hence, very little is known about the tropism and antigenicity of the EBOV glycoprotein. Recently, the Qβ platform was used to map the SARS-CoV spike protein epitopes[27] and showed that they were buried within its full length. In some of our earlier reports, we have demonstrated that Qβ phage can be engineered to display a total of 12 copies of a desired peptide per recombinant phage equally distributed upon its surface. In addition, this peptide would also mimic the natural conformation and functionality of the foreign peptide. Also, epitopes of *Plasmodium falciparum* displayed on the Qβ phage platform were recently used in a study to quantify the levels of antibodies in pregnant women and their neonates.[37] From our initial publications, we have sequentially increased the size of the peptide to be displayed upon the phage to over 100 AA without affecting its viability and infectivity. In this study, the peptide size was further increased up to 200 amino

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acids, being the first time an RNA phage Qβ has been used to construct a library of large overlapping fragments from the entire EBOV glycoprotein. Simultaneously, we have also identified GP-located immunogenic epitopes by probing each segment with the anti-EBOV GP antibody. During this study, we first identified three immunogenic fragments, namely, F1, F2, and F3, which were located toward the N-terminus of the glycoprotein between Met1-Arg500 corresponding to the GP1 subunit. The 30 amino acids overlapped in each fragment present no activity toward the anti-GP antibody. By mapping the EBOV glycoprotein, three main regions of GP1 were found to be immunogenic including the portion containing the receptor binding domain (F1), glycan cap (F2), and mucin-like domain (F3). Very little is known about the amino acids of the mucin-like domain involved in the immunogenicity of the GP. By linearizing the glycoprotein on the RNA Qβ phage platform, we identified for the first time the peptide sequence that contains the epitope(s) of this EBOV GP domain. Fragment F1 largely contains the receptor binding domain (Val79-Ile170), suggesting a possible immunological function of the GP RBD in addition to its interaction with the host receptor. Fragment F1 was less reactive to the antibodies than F2 and F3. In fact, the two fragments (F2 and F3) are located on the top of the glycoprotein (Glycan cape and mucin-like domain, respectively) and thus well exposed and accessible to the antibodies as compared to F1 located inside the chalice. Earlier reports have targeted the base, the chalice, and the top (glycan cap) of the GP1 as the binding sites of the neutralizing antibodies.[10,38,39] Shortening subsequentially these GP immunogenic fragments would allow to determine the smallest portion of the viral glycoprotein or the key amino acids (epitope) that are involved in its immunogenicity which could also be a further demonstration that epitopes might be buried in the full GP. No antigenicity was found in the F4 (Arg501-Leu676), fragment suggesting that this region corresponding to the GP2 subunit of EBOV GP is nonimmunogenic or might have no affinity with the anti-EBOV GP antibody, although some amino acid residues located in the region at the base of the internal fusion loop of GP2 have been suggested to be involved in the interaction with antibodies like KZ52, mAb100, C2G4, and C4G7 in a crystallography study.[10,40]

EBOV GP is known as the key viral surface protein involved in the infectivity and pathogenicity of the virus.[18,19] Recently, the RNA Qβ display platform was used to map the receptor binding small motif (RBSM) of SARS-CoV and study its reactivity with the host receptor ACE2.[27] In this study using a similar strategy and protocol, different GP-derived overlapping fragments were evaluated for their binding affinity with the recombinant host receptor NPC1. Out of the four overlapping fragments (F1−4) only one showed the ability to bind to the receptor, suggesting that this portion (F1) displayed on the Qβ surface contains the receptor binding domain (RBD). In fact, F1 contains the predicted receptor binding site (Val79-Ile179), and its large size (200 AA) may have led to the proper folding of the peptide displayed on the surface of Qβ, exposing the amino acids involved in the interaction with the receptor. This hypothesis is supported by the modeling of Qβ minor coat protein A_1 with fragment F4 of the GP that showed a mimicry of its folding by exposing α-helices comparable to heptad repeat regions 1 and 2 (HR1 and HR2). In addition, a portion like the internal fusion loop (IFL) was observed in the model of the fragment with A1. These results and observations in modeling strongly support our hypothesis that a peptide displayed on the Qβ platform might mimic the natural configuration and function of the parent protein. Interestingly, the recombinant phages (QβF1) were shown to reduce the Ebola pseudotyped virus, suggesting a competition inhibition of the phage against the virus at the host receptor binding site of the viral glycoprotein. The F2 contains 30 amino acids of F1 and did not show any affinity to the receptor NPC1. The portion of GP recognizing the receptor is thus between AA79−170. Previous studies have identified the amino acids that are involved in the interaction of the EBOV GP with the receptor NPC1[41] and it has been reported that a mutation of A82 V increases the entry of the Ebola virus into human cells.[41,42] Fragmentation and site-specific mutations of this GP portion (F1) would lead to the determination of the small motif and/or amino acids that constitute the key residues in binding of the EBOV GP to the host receptor NPC1 and that are druggable.

During EBOV infection, the glycoprotein is cleaved in the late endosome by cysteine proteases (CatB and CatL) prior to interaction with the host receptor NPC1. This cleavage that removes the mucin-like domain and glycan cap of the GP1 subunit is critical for virus infectivity. Hence, the cathepsin cleavage site is predicted to be within the GP1. CathB and CatL have been shown to cleave peptides at a Lys-Lys and Phe-Phe site, respectively.[18,19] In our design strategy, we targeted a portion of the EBOV GP1 (F5) with two Lys-Lys sites containing consecutive lysine residues (Lys115-Lys116 and Lys191-Lys192) with more focus on the cleavage site located toward the glycan cap. Similarly, we targeted Phe194-Phe195 located on the same fragment as the cleavage site of CatL. The resulting recombinant phages were exposed to the cathepsin activity separately after incorporating HRP into the LPETG tag at the C-terminus of F5 using sortase A. Washing the plate after incubating with the cysteine proteases showed a decrease in absorbance in treated samples compared to the control, suggesting the cleavage of the displayed peptide by the enzymes. To further confirm the cleavage sites of the enzymes, the targeted cleavage sites were mutated (K191R-K192R; F194Y−F195Y) in two different constructs. The resulting recombinant phages were further subjected to CatB and CatL activity, respectively. From the results, the absorbance remained constant compared to the controls, confirming the cleavage site of both enzymes and suggesting that the mutated amino acids are among the key residues involved in the cathepsin activity. The cleavage activity of CatB/L was further validated in vitro using the recombinant EBOV GP and its derived mutants. The active site of an enzyme consists of the binding and catalytic sites. Further site mutations/deletions of F5 displayed on the RNA Qβ system would lead to the identification of the amino acids or GP1 smallest motif involved in the binding and catalytic activity of CatB and CatL, respectively.

4. CONCLUSIONS

The glycoprotein of the Ebola virus is the key surface protein involved in the pathogenicity of the virus. We successfully used the RNA Qβ phage platform to display large overlapping fragments covering the full length of the EBOV GP and identified three major portions situated on the GP1 subunit that are implicated in viral immunogenicity. When displayed on the surface of Qβ, fragment F1 containing the amino acid sequence Met1-His200 mimicked the receptor binding function of the cathepsin-primed Ebola virus glycoprotein

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and bound to the recombinant host receptor NPC1. In addition, this fragment showed the immunological properties of the receptor-binding domain of EBOV GP. Furthermore, GP1 amino acids K191−K192 and F194−F195 were identified as the cleavage sites of cathepsins B and L, respectively, using the Qβ platform. Finally, fragmentation and mutation deletions of the immunogenic fragments and receptor binding domain of the displayed Ebola virus glycoprotein could result in the identification of the major amino acids and the small motif that is involved in the immunogenicity, host receptor binding function, and cathepsin binding motif respectively. The identified epitopes and small receptor binding motifs constitute potent targets that could lead to the development of subunit vaccines and drugs against Ebola virus diseases and for monitoring pathogen-specific antibodies in Ebola-exposed people or patients. In addition, we demonstrated for the first time in this study the inhibitory ability of the recombinant Qβ phages and a cyclic peptide from an RNA Qβ phage library that constitute potential leads in the development of phage- and peptide-based anti-Ebola drugs.

5. MATERIAL AND METHODS

5.1. Material. Restriction enzymes and other enzymes like alkaline phosphatase, Taq polymerase, and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA, USA). The kits for viral RNA extraction, bacterial plasmid isolation, and DNA gel extraction and purification were purchased from Qiagen (Valencia, CA, USA). Media for bacterial culture and enzymes for reverse transcription were purchased from Fisher Scientific (Pittsburgh, PA, USA) and Promega (Madison, WI, USA), respectively. Anti-EBOV GP antibody and recombinant human NPC1 were purchased from the R&D System (Minneapolis, MN, USA) and Sino Biological (Wayne, PA, US), respectively. Sortase A and cathepsin kits were purchased from Active Motif (Carlsbad, CA, USA) and ANASPEC (Fremont, CA, USA), respectively. The synthetic gene and oligonucleotides were obtained from Eurofins (Louisville, KY, USA). Wild type Ebola pseudovirus neutralization kit was purchased from Virongy Biosciences (Manassas, VA, USA). The wild type Qβ phage was obtained from ATCC (American Type Culture Collection) number: 23631-B1. Plasmids pQβ7[43] and pBRQβT7Qβ, and their derivative were used for the insertion of different fragments and portions of EBV GP.

5.2. Methods. *5.2.1. Prediction of the Fusion of Qβ Minor Coat Protein with EBOV GP Fragments.* The tridimensional (3D) computational structures of the minor coat protein A_1 of Qβ, the various fragments of the EBOV GP (F1−F5), and fusion A_1F*n*, where F is an EBOV GP fragment with "*n*" being the rank, were predicted from their various protein sequences using the Alphafold2 algorithm.[44] All of the structures were aligned to analyze the differences among the various models and how the fusion might affect the folding of the A_1 protein and the inserted GP fragment.

5.2.2. Plasmid Construction and Cloning. 5.2.2.1. Construction of Recombinant pQβ Plasmids Fused with Overlapping GP Fragments. Fusion PCR was used to generate each of the four overlapping fragments of the EBOV GP: F1 (Met1 to His200), F2 (Ile170 to Val370), F3 (Val350 to His550), and F4 (Asn501 to Leu676), using the natural *gp* gene (full length, 676AA) synthesized by Eurofins as a DNA template. Both reverse and forward primers (Table 2) for F1, F2, F3, and F4, respectively, were designed and flanked

Table 2. Primers Used in the Design and Construction of Recombinant Plasmids Containing*EBOV gp* gene Fragments

GP fragment	primer name	oligo sequence
F1	EFW1	5′ CCACGCGGCCGCATGGTGACCTCTGGCATCCTCCAACTTCCAAGGGAGAGGTTCAGGAA
	ERV1	5′ GGTGGCGGCCGCttatcaATGCAGTGGAGGGCTCTGGAAGAAGTCCTTCTTGGTCTCAGG
F2	EFW2	5′ CCACGCGGCCGCTACAGGAGCACCACCTTCTCTGAGGGAGTGGTGGCTTTCCTGATTCTGCC
	ERV2	5′ GGTGGCGGCCGCttatcaCACTGTGTCAGGAGGAGGTGTTGGCACTGTGTTCTCCCTTTGGAGG
F3	EFW3	5′ CCACGCGGCCGCGTCCAGGTGAGGGACCTCCAAAGGGAGAACACAGTGCCAACACCTCC
	ERV3	5′ GTGGCGGCCGCttatcaGTTGTGCATTATGCCCTCTGTGTAGATGCCCTCAGCAGCAGGTCCAAA
F4	EFW4	5′ CCACGCGGCCGC AGGGAGATTACCCTGAGGACCCAGGCTAAGTGTAACCCAAACC
	ERV4	5′AATGCGGCCGCttatcaTTACAGCAGGAACTTACAGATACACAGCAGGGCAATCACAGCATAATCACTCCTGTGATGCCAATGCC
F5	VgCatFw	5′ TATTGCGGCCGCGAGGGCAATGGAGTGGCTACAGATGTGC
	VgCatRev	5′CTTACTATGCGGCCGCTTATCAGCCGGTTTCCGGCAGGCTGCTGCCGCTGCCGCCATTGGCAGGTTCATGCAGTGGAGGGCTCTGGAAGAAGTCCTT
F5CatB	VgCatFw	5′ TATTGCGGCCGCGAGGGCAATGGAGTGGCTACAGATGTGC
	VgCatK	5′TTACTATGCGGCCGCTTATCAGCCGGTTTCCGGCAGGCTGCTGCCGCTGCCGCCATTGGCAG GTTCATGCAGTGGAGGGCTCTGGAAGAAGTCTCTTCTGGTCTCAGGCAGAATCAGGAAAGCC
F5CatL	VgCatFw	5′ TATTGCGGCCGCGAGGGCAATGGAGTGGCTACAGATGTGC
	VgCatF	5′TTACTATGCGGCCGCTTATCAGCCGGTTTCCGGCAGGCTGCTGCCGCTGCCGCCATTGGCAG GTTCATGCAGTGGAGGGCTCTGGTAGTAGTCCTTCTTGGTCTCAGGCAGAATCAGGAAAGCC

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at the 3′ and 5′ ends by the NotI restriction site. For each of the GP fragments, the PCR product (∼600 bp) was extracted from agarose gel after electrophoresis, digested with NotI, and purified. The pQβAd2 plasmid containing the full cDNA of the Qβ phage with a 150 AA deletion at the C-terminus of the *QβA1* gene, previously designed with a NotI restriction site inserted before the two natural stop codons TAGTAA within the $Q\beta A_1$ frame and containing an ampicillin-resistant gene, was used to obtain a Qβ-vector by a single NotI restriction digestion and dephosphorylation with the Quick CIP enzyme. The purified pQβAd2 vector was separately ligated with each of the GP inserts in a ratio of 1:3 overnight at 16 °C. The ligation products were transformed into competent *E. coli* HB101 cells. The transformed cells were plated on 2YT agar supplemented with 100 mg/g of ampicillin. For each transformation, 10 colonies were randomly picked, and the plasmid DNA was extracted and analyzed with NotI restriction digestion. The extracted plasmids were further subjected to Sanger sequencing to confirm the presence of each of the GP fragments within the frame of $Q\beta A_1$. The confirmed plasmids, subsequently named pQβF1, pQβF2, pQβF3, and pQβF4, were amplified and used for phage expression.

5.2.2.2. Construction and Analysis of Recombinant Plasmids Bearing the GPCatL/B Fragment and the LPTEG Tag for Determination of CatL/B Cleavage Sites. Many reports have identified the GP1 subunit of EBOV glycoprotein to contain the Cat B/L cleavage sites.[19,34,36] To construct this portion of EBOV GP (F5), fusion PCR was used with the natural *gp* gene as a template. Both forward and reverse primers (Table 2) were designed to copy the predicted portion of the fragment, flanked with NotI restriction sites at both the 3′ and 5′ ends, and ligated into the pQβAd2 vector as described above. To confirm the presence of this CatL/B cleavage site within this fragment, the sortase recognition motif LPETG was fused to the F5 fragment through a GSSGSS linker at the N-terminal. The sortase A easily recognizes this LPETG motif and labels it with a measurable polyglycine-horseradish peroxidase (HRP) molecule. The recombinant phage QβF5/LPETG/HRP label complex allows for the confirmation of the CatL/B cleavage sites. The reactivity of CatB/L allows peptide cleavage and washing off of the cleaved F5/LPTEG peptide motif on the surface of the Qβ phage, thereby preventing any subsequent absorbance of HRP. To identify the key amino acids involved in the reactivity of CatB and CatL, single amino acid mutations were made on the QβF5 plasmid, at predicted sites. For QβF5CatB, K191R and K192R mutations were generated on the QβF5 plasmid, and for QβF5CatL, F194Y and F195Y mutations were generated as well using site-directed mutagenesis. Recombinant Qβ phages for the plasmids constructed above were expressed as described below and tested for their cleavage by CatL and CatB.

5.2.3. Phage Expression and Purification. Phage expression and purification were performed following the same protocol as described in our earlier reports.[26,27] Briefly, each recombinant plasmid was used to transform *E. coli* HB101 and selected on 2YT agar supplemented with ampicillin. Two clones were aseptically inoculated into 1 L of 2YT broth and incubated at 37 °C for 24 h at 220 rpm. The cells were lysed, and phages were precipitated using PEG/NaCl at 4 °C overnight. Thereafter, the lysate was centrifuged, and the pellet was resuspended in phage buffer and spun at 4 °C at 12,000 rpm. The phages were recovered in the supernatant and scaled using *E. coli* Q13, as described elsewhere.[26] Purified phages were dialyzed against the phage buffer and stored at 4 °C for further use.

5.2.4. Morphological Characterization of Recombinant Phages. Pure phages from the recombinant plasmid expression were identified macroscopically using plaque assay on the lawn of *E. coli* Q13 as described elsewhere.[26] Before the experiment, the phages were subjected to a thousand-fold dilution, and the wild type Qβ served as a control. The microscopic morphology of the phages was identified using electron microscopy as described in our earlier reports.[26,27]

5.2.5. Probing of Recombinant Phages with Antibody. 5.2.5.1. Dot Blot Assay. Five microliters (5 μL) of pure phages at a titer of 10^{12} to 10^{13} pfu/mL were spotted on the nitrocellulose membrane and allowed to air-dry for 45 min. The membrane was subsequently blocked with 5% dry milk for 30 min followed by the incubation with anti-EBOV GP antibody on a rotary orbital at room temperature for 1 h. The solution containing the antibody was removed, the membrane was washed trice with TBST buffer, incubated with antimouse antibody conjugated with horseradish peroxidase at room temperature for 1 h, and washed again thrice with TBST. Finally, the membrane was exposed to luminol/p-coumaric acid and the chemiluminescence was recorded.

5.2.5.2. ELISA. Quantitative ELISA was used to determine the binding affinity of the recombinant phages to the antibody using the same protocol as described in our earlier reports.[27] Briefly, phages displaying different fragments of GP at a titer of 10^2 pfu/mL were used to coat a 96-well plate separately at 4 °C overnight. The coating buffer was removed, and the plate was washed by using PBST (phosphate buffered saline, 0.1% Tween-20). Thereafter, the wells were blocked with 300 μL of 0.5% BSA dissolved in coating buffer at room temperature (RT) for 1 h. After washing the wells 5× with PBST, 100 μL of anti-EBOV GP antibody subjected to a 2-fold dilution ranging from 0.0625 to 1.0 μg/mL in PBS was added and incubated at RT for 1 h on an orbital shaker. Thereafter, the wells were washed 5× with PBST, and 100 μL of antibody conjugated with horseradish peroxidase (HRP) at 1:1000 dilution was added. The plate was incubated again at RT for 1 h, the content was removed, and the wells were washed 5× with PBST. Subsequently, 100 μL of 3,3′,5,5′-tetramethylbenzidine (TMB) was added to the plate, incubated at RT for 30 min, and the optical density (OD) of the plates was recorded at 450 nm using an ELISA plate reader. The test was performed in triplicate.

5.2.6. Screening of Recombinant Phages against the Host Receptor NPC1. Recombinant phages displaying different fragments of GP at a titer of 10^3 pfu/mL were used to coat a 96-well plate separately at 4 °C overnight. The coating buffer was removed and the plate washed using PBST (phosphate buffered saline, 0.1% Tween-20) followed by blocking with 0.5% BSA dissolved in coating buffer at room temperature (RT) for 1 h. After washing the wells, 2.5 μg of the recombinant NPC1 in PBS was added and incubated at RT for 1 h on an orbital shaker. The solution was removed, and the plate was washed 5× with PBST. Thereafter, anti-6xHis antibody-HRP conjugate was added and incubated at RT for another hour, the solution was removed, and the wells were washed 5× with PBST. Finally, the plate was treated with the horseradish peroxidase substrate (TMB) for 30 min, the stop reagent was added, and the OD was recorded at 450 nm using an ELISA plate reader. The wild type Qβ phages were used as a control. To determine the specific binding of NPC1 to the

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GP fragments displayed on the surface of the phages, different concentrations of the receptor ranging from 0.3125 to 2.5 μg/mL were prepared and used in a quantitative ELISA as described in our earlier report.[27] The test was performed in triplicate.

5.2.7. Identification of Cathepsin Cleavage Site(s). A construct from fragment F1 of EBOV GP subsequently named F5 containing the predicted cleavage sites of the proteases (CatB and L) was flanked with the LPETG tag (F5) at the C-terminus and inserted into the pBRQ7 vector. The recombinant plasmid was used for cloning and phage expression in *E. coli* HB101. Pure recombinant phages at a titer of 10^7 pfu/mL were used to coat and saturate a 96-well plate at 4 °C overnight. The coating buffer was discarded, the plate was washed 5× with the PBS buffer, blocked with 0.5% BSA in the coating buffer, and the exposed peptide was labeled with horseradish peroxidase (HRP) using the sortase A kit (Active Motif) in a total volume of 100 μL. After incubation at 30 °C for 1 h at 100 rpm, the plate was washed 6× with PBS and the wells were treated with 100 μL of TMB by incubating the plate at RT for 45 min. Thereafter, 50 μL of the stop solution was added, and the optical density (OD) was measured at 450 nm using a plate reader and used as a control. To determine the cathepsin cleavage activity, two reactions were set. In the first reaction, the recombinant phages bound to the well were subjected to cathepsin B (CatB) for 1 h using the kit (ANASPEC) and following the manufacturer's instructions. After washing the plate 6× with PBS buffer, the peptide was labeled, and the OD was recorded as described above. In the second reaction, the recombinant phages were first labeled with HRP using sortase A. After washing the plate with PBS, the phages were subjected to CatB cleavage at RT for 1 h. Thereafter, the wells were washed 6× with PBS and treated with TMB, and the OD was measured after adding the stop solution. To determine the specificity of the enzymes, a kinetic experiment was performed in which six different reactions were set with phages diluted to 10^3 pfu/mL. The displayed peptide was HRP-labeled as described above and subjected to CatB and CatL separately using the kits (ANASPEC). The wells were washed successively after every 20 min incubation at RT and the OD was recorded after 45 min incubation with TMB at RT. Each experiment was performed in triplicate.

5.2.8. Confirmation of Cathepsin Cleavage Site(s). New constructs of EBOV GP fragment flanked with LPETG were made with mutations K191R, K192R for CatB (F5CatB) and F194Y, F195Y for CatL (F5CatL) at the predicted cleavage sites. The recombinant plasmids were used for the cloning, phage expression, and purification using PEG/NaCl precipitation as described in our earlier reports.[26,27] The resulting recombinant phages (QβF5CatB and QβF5CatL) at titers of 10^4 and 10^6 pfu/mL, respectively, were subjected to sortase A for HRP labeling and cathepsin activities as described above. All the experiments were carried out in triplicate in a 96-well microtiter plate.

5.2.9. Validation of Cathepsin Cleavage Site(s) and the Receptor Binding Domain. To further validate the cathepsin cleavage activity, 2.5 μg of a full-length recombinant EBOV GP and corresponding mutants at the CatB/L cleavage sites were synthesized and subjected to enzymatic digestion separately at 30 °C for 2 h at 60 rpm. Subsequently, the samples were subjected to SDS-PAGE followed by western blotting onto a nitrocellulose membrane. The membrane was blocked with 5% dry milk and probed with anti-6XHis antibody conjugated with HRP. The chemiluminescence was recorded after adding the HRP substrate to the membrane. Nondigested GP was used as a negative control. Further validation was conducted via ELISA by coating a 96-well plate with 2.5 μg of GP and its mutant in separate wells at 4 °C overnight. After blocking with 0.5% BSA, the wells were washed with PBST, digested with CatL at 30 °C for 1 h, washed again 5 times with PBST, and incubated with anti-6XHis antibody-HRP conjugate at RT for 1 h. Thereafter, the plate was washed 5× with PBST, and 100 μL of TMB was added and incubated at RT for 30 min. The stop solution was added into the wells and OD was recorded at 450 nm using an ELISA plate reader.

To validate the receptor binding activity, a competitive assay was designed against the wild type Ebola pseudovirus using the recombinant QβF1 phages displaying the mapped receptor binding domain at a titer of 1×10^5 pfu/mL. Wild type phages at the same titer were used as a negative control. In addition, a cyclic peptide (NPCP15) selected from a Qβ phage library against EBOV host receptor NPC1 was assessed for its inhibitory effects against the pseudovirus entry. The tests were performed using a 96-well Rapid Pseudoviral Neutralization Kit from Virongy Biosciences following the manufacturer's instructions. The peptide was tested at a single concentration of 0.5 μM. All the tests were performed in triplicate.

■ ASSOCIATED CONTENT

Data Availability Statement

Data supporting the reported results can be found at Addgene (https://www.addgene.org, accessed on 19 September 2024) where the plasmids have been deposited under Deposit Number is **84896**.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.5c00408.

Modeling of EBOV GP fragments with the minor coat protein A1 of Qβ phage and the design of cathepsin cleavage sites (PDF)

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Author Contributions

A.B.W., A.N., and A.D. generated the library of glycoprotein fragments and variants of the ORF used for phage display

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selection assays; A.B.W. and A.N. conducted the structural and EM analyses; G.N., A.N., and A.D. conducted initial phage purification and analyses. A.N., A.D., and G.N. conducted dot blotting and ELISA analyses; A.B.W., A.N., and A.D. conducted the phage vector plasmid construction and expression. A.N. and A.B.W. conducted viral inhibition assay; A.B.W., A.N., and G.N. conducted phage display simulation and restriction assays; A.B.W., A.D. and A.N. wrote the original manuscript draft; all authors participated in finalization of the manuscript and approval of its final full text and figures.

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Notes

The authors declare no competing financial interest.

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