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Immunogenic properties of a trimeric gp41-based immunogen containing an exposed membrane-proximal external region

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ABSTRACT

The membrane-proximal external region (MPER) of HIV-1 gp41 is an attractive target for vaccine development. Thus, better understanding of its immunogenic properties in various structural contexts is important. We previously described the crystal structure of a trimeric protein complex named gp41-HR1-54Q, which consists of the heptad repeat regions 1 and 2 and the MPER. The protein was efficiently recognized by broadly neutralizing antibodies. Here, we describe its immunogenic properties in rabbits. The protein was highly immunogenic, especially the C-terminal end of the MPER containing 4E10 and 10E8 epitopes (^{671}NWFDITNWLWYIK^{683}). Although antibodies exhibited strong competition activity against 4E10 and 10E8, neutralizing activity was not detected. Detailed mapping analyses indicated that amino acid residues critical for recognition resided on faces of the alpha helix that are either opposite of or perpendicular to the epitopes recognized by 4E10 and 10E8. These results provide critical information for designing the next generation of MPER-based immunogens.

Introduction

The envelope glycoprotein of human immunodeficiency virus type 1 (HIV-1) serves a critical role of mediating virus entry into host cells. This protein is also immunologically important because it is the sole target against which neutralizing antibodies are elicited in infected individuals. The two subunits of the envelope glycoprotein, gp120 and gp41, pose different sets of challenges for HIV-1 vaccine design. Besides being heavily glycosylated and highly variable, the gp120 subunit contains many immunodominant epitopes that act as decoys, which provide limited, if any, protection (Pantophlet and Burton, 2006; Sodroski et al., 1998; Wei et al., 2003). While several anti-gp120 broadly neutralizing antibodies (bnAbs) have been isolated from infected individuals (Blattner et al., 2014; Buchacher et al., 1994; Burton et al., 1991; Diskin et al., 2011; Falkowska et al., 2014; Klein et al., 2012; Scharf et al., 2014; Scheid et al., 2011; Walker et al., 2011, 2009; Wu et al., 2010), most of the epitopes targeted by these antibodies are non-linear and highly conformational. Hence, designing gp120 antigens that can present the neutralizing epitopes in the correct conformation, while limiting response to other non-protective immunodominant epitopes, has been a difficult task.

In comparison, gp41 is smaller, less variable and less glycosylated. It contains a highly conserved domain (~22 amino acid residues) called the membrane-proximal external region (MPER) that lies between heptad repeat region 2 (HR2) and the transmembrane (TM) domain. This MPER contains linear epitopes targeted by a number of bnAbs, including 2F5, Z13e1, 4E10 and 10E8 (Huang et al., 2012; Kwong et al., 2013; and reviewed in Montero et al., 2008; Purtscher et al., 1994; Stiegler et al., 2001; van Gils and Sanders, 2013; Zwick et al., 2001). Unfortunately, the structure of gp41 is thought to be highly dynamic, undergoing significant conformational changes upon receptor binding and during the fusion process (Mao et al., 2013; Melikyan, 2008). In the native, pre-fusion state, gp41 presumably exists in a metastable conformation that stores the free energy needed for membrane fusion. Following gp120 binding to CD4 and to a co-receptor, gp41 transforms into a fusion-active intermediate in which the N-terminal fusion peptide (FP) inserts into the host-cell membrane. Subsequently, the two heptad repeat regions, HR1 and HR2, are brought together to form a highly stable six-helix bundle, which concomitantly leads to the formation of a hairpin structure that completes the fusion of the viral and cellular membranes (Melikyan, 2008). This metastable and transient nature of the gp41 structure has made it difficult to design vaccine antigens that can present epitopes in their native form so as to generate potent bnAbs.

Significant efforts have been made for developing MPER-based vaccines (Montero et al., 2008). Some of the vaccine candidates

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evaluated so far include immunogens based on short MPER peptides, either alone or coupled to carrier proteins (Decroix et al., 2001; Joyce, 2002; Liao et al., 2000; Matoba et al., 2006; McGaughey et al., 2003; Ni et al., 2004); the use of artificial scaffolds containing stabilized MPER epitopes (Correia et al., 2010; Guenaga et al., 2011; Ofek et al., 2010); hybrid/fusion proteins (Coëffier et al., 2000; Hinz et al., 2009; Krebs et al., 2014; Law et al., 2007; Liang et al., 1999; Mantis et al., 2001; Strasz et al., 2014); chimeric viruses or virus-like particles displaying MPER epitopes (Arnold et al., 2009; Benen et al., 2014; Bomsel et al., 2011; Eckhart et al., 1996; Jain et al., 2010; Kamdem Toukam et al., 2012; Kim et al., 2007; Luo et al., 2006; Marusic et al., 2001; Muster et al., 1995; Ye et al., 2011; Yi et al., 2013; Zhang et al., 2004); and presentation of MPER peptides on liposomes (Dennison et al., 2011; Hanson et al., 2015; Hulsik et al., 2013; Lai et al., 2014; Matyas et al., 2009; Mohan et al., 2014; Serrano et al., 2014; Venditto et al., 2013, 2014). Despite these efforts, none of them succeeded in inducing bnAbs against the MPER, albeit a few recent studies reported induction of modest levels of cross-clade neutralizing activity (Hulsik et al., 2013; Krebs et al., 2014; Lai et al., 2014; Ye et al., 2011; Yi et al., 2013). These results highlight the difficulty in eliciting anti-MPER bnAbs through vaccination.

There likely are multiple reasons for these unsuccessful attempts. Short peptide-based immunogens might be lacking helper T cell epitopes that are needed to induce robust $CD4^+$ T cell immunity. In addition, peptides, in the absence of neighboring domains, might not fold into the conformation that may exist in the native trimeric envelope spikes on virus particles. However, merely mimicking bnAb-bound conformations might not be sufficient to elicit such antibodies since MPER epitopes constrained in artificial scaffolds also failed to elicit bnAbs (Correia et al., 2010; Guenaga et al., 2011; McGaughey et al., 2003; Ofek et al., 2010). Chimeric viruses with MPER grafts have shown to induce poor anti-MPER antibody titers (Eckhart et al., 1996; Kusov et al., 2007; Luo et al., 2006; Zhang et al., 2004). This could be due to the presence of other epitopes that might be more immunogenic than the MPER and distract immune responses away from it. Thus, the relative immunogenicity of the target epitope is important when in the context of a large protein. Finally, choosing appropriate adjuvants could also be an important factor, not only for enhancing immune responses *per se*, but also for making sure that the adjuvant being used is able to preserve the correct conformation of critical neutralizing epitopes.

As a part of our efforts to develop gp41-based HIV-1 vaccine candidates, one of our goals has been to better understand the relationship between antigenic structures and their immunogenic properties. Towards this goal, we generated various gp41 constructs containing the MPER. One of these constructs, gp41-HR1-54Q, contains a portion of HR1 connected to HR2 by a short linker, followed by the MPER and a 6xHis tag. Not surprisingly, structural analyses of this construct indicated that it forms a stable six-helix bundle, which represents a post-fusion state (Shi et al., 2010). However, considering that the MPER was extended away from the six-helix bundle and that it was efficiently recognized by bnAbs 2F5, Z13e1 and 4E10 (Shi et al., 2010), we evaluated its immunogenic properties in rabbits. Although our antigen elicited strong antibody responses against the C-terminal end of the MPER that harbors 4E10 and 10E8 epitopes, no neutralizing activity was detected. Despite this failure, the results of our study demonstrate that the region targeted by 4E10 and 10E8 can be made highly immunogenic, even in the context of a large protein.

Results

Rationales for gp41-HR1-54Q design and its structure

For designing an immunogen based on gp41, we wanted to (1) incorporate as much of gp41 as possible in order to provide sufficient helper T cell epitopes; (2) make sure that critical neutralizing epitopes on the MPER are accessible (*viz.* 2F5, Z13e1 and 4E10; 10E8 was not discovered at the time this study began); (3) ensure that the antigen is expressed efficiently, rendered soluble and easy to purify; and (4) minimize the immunodominant epitopes that induce non-neutralizing antibodies. One of the constructs we generated, gp41-HR1-54Q, is shown in Fig. 1A. The immunodominant C-C loop between the HR1 and HR2 was replaced with a GGGGS linker. Concomitantly, the C- and N-terminal ends of HR1 and HR2 were also trimmed by six and two amino acids, respectively. While this flexible linker allowed the HR1 and HR2 domains to freely interact with each other, we hypothesized that replacement of the C-C Loop with the

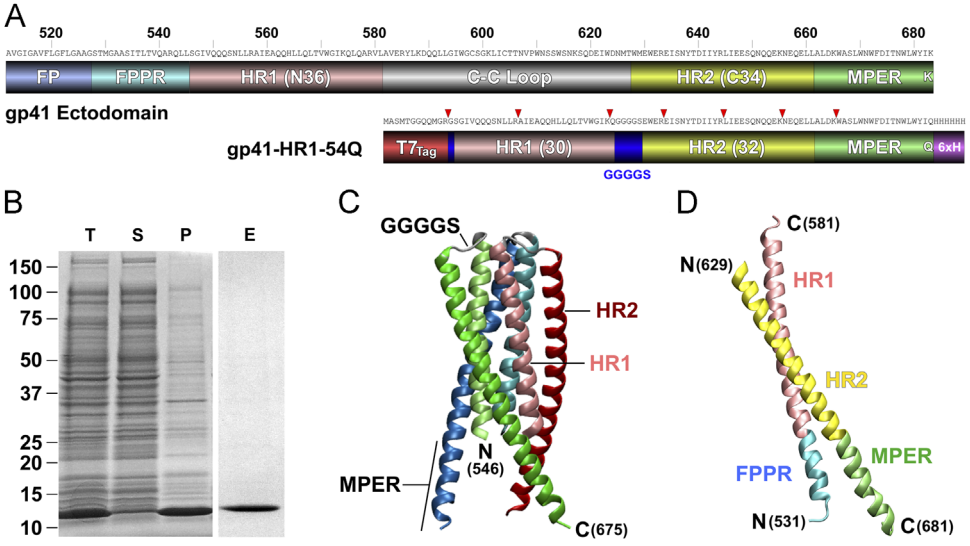

Fig. 1. Generation of gp41-HR1-54Q. (A) A domain structure of gp41 ectodomain is shown at the top consisting of FP (fusion peptide), FPPR (fusion peptide proximal region), HR1 (heptad repeat region 1), immunodominant C-C loop, HR2 (heptad repeat region 2) and MPER (membrane-proximal external region). In comparison, gp41-HR1-54Q consists of shortened HR1 and HR2 domains linked together by a GGGGS linker in place of the C-C loop. The construct also has an N-terminal T7 expression tag, K683Q substitution, and a C-terminal 6xHis tag. (B) SDS-PAGE of the expressed and purified protein stained with Coomassie blue showing total (T), supernatant (S), pellet (P) and elution (E) fractions. (C) A crystal structure of the gp41-HR1-54Q (pdb: 3K9A) (Shi et al., 2010) indicating individual domains. (D) A crystal structure of the post fusion complex (pdb: 2 × 7R) formed by two peptides containing the FPPR-HR1 and HR2-MPER domain (Buzon et al., 2010).

linker would avoid diverting immune responses away from the MPER domain. Secondly, the fusion peptide (FP) was removed to enhance solubility. Furthermore, the fusion peptide-proximal region (FPPR) between FP and HR1 was removed to eliminate any possible interactions between FPPR and MPER, which could interfere with recognition by bnAbs.

As shown in Fig. 1B, gp41-HR1-54Q was expressed at high levels (> 120 mg/l of purified protein). Although the protein fractionated in insoluble inclusion bodies, the protein could be readily solubilized with urea, refolded by step-wise removal of urea, and purified to homogeneity (Shi et al., 2010). Although our original intent was to remove the $T7_{Tag}$ by cleaving it with trypsin, as we previously observed that other potential digestion sites were resistant (data not shown), the tag also could not be cleaved, suggesting inaccessibility of the site. As shown by the crystal structure of the protein (Fig. 1C; (Shi et al., 2010)), HR1 and HR2 domains formed a highly stable six-helix bundle structure. The N-terminal eight amino acids of MPER were also highly ordered (^{662}ALDKWASL^{669}). The N-terminal 12 residues containing the $T7_{Tag}$, as well as the last eight residues (^{676}TNWLWYIQ^{683}) and the 6xHis tag were not ordered and their structures could not be defined. In addition, the side chains of six residues at the end (^{670}WNWFDI^{675}) could not be resolved, suggesting some flexibility. In contrast to the structure of our gp41-HR1-54Q, a crystal structure of two peptides encompassing FPPR-HR1 (a.a. 528–581) and HR2-MPER (a.a. 628–683) regions (Fig. 1D; (Buzon et al., 2010)), which was reported nearly at the same time of our structural study, indicated that FPPR interacts with MPER to enhance stability of the six-helix bundle. As a result, the MPER region became highly ordered and its structure could be resolved further downstream to Y^{681}. Thus, the structural state of our immunogen might represent a "near post-fusion", rather than the "post-fusion", in regards to the MPER.

Antigenicity and immunogenicity of gp41-HR1-54Q

We have previously shown that gp41-HR1-54Q could be efficiently recognized by three bnAbs against MPER (2F5, Z13e1 and 4E10; (Shi et al., 2010)). 10E8, which was more recently isolated, also binds the protein, albeit with lower affinity (data not shown; Fig. 5). This is likely due to the fact that our immunogen contains K683Q substitution and that K or R683 is one of the amino acid residues recognized by 10E8 (Huang et al., 2012). Since these results indicated that the epitopes targeted by the bnAbs were accessible and could fold into correct conformations, we proceeded to evaluate the immunogenicity of gp41-HR1-54Q.

Six rabbits were immunized with gp41-HR1-54Q. Zn-chitosan was used as an adjuvant/delivery platform, which we have recently shown to induce strong antibody responses against gp120-based antigens (Qin et al., 2014a). Zn-chitosan was particularly well suited for our immunogen compared to many adjuvants that are oil/lipid-based considering that the MPER regions is highly hydrophobic. Rabbits were immunized four times subcutaneously on weeks 0, 4, 9 and 15. Pre- and post-immune sera (2 weeks post-immunization) were collected and antibody titers were determined by ELISA against the immunogen (Fig. 2). Strong antibody responses were observed in all of the animals. In particular, we were quite surprised to see end-point antibody titers approaching nearly 1×10^7 even after only a single immunization. Antibody titers increased substantially after the second immunization in most of the animals resulting in end-point titers between 1×10^7 and 1×10^8; however, titers did not increase further after the third or the fourth immunizations, indicating that antibody responses reached the maximum level after two immunizations. Despite having induced high levels of antibodies against gp41-HR1-54Q, none of the sera exhibited neutralizing activity against HIV-1 pseudoviruses in a standard TZM-bl cell based neutralization assay (data not shown).

Detailed characterization of antibody responses

Despite failing to exhibit neutralizing activity, understanding the properties of antibodies elicited is important as they may provide hints as to why they failed to neutralize, and facilitate designing better immunogens. Towards this goal, immunogenic epitope mapping analyses were conducted by ELISA using various protein fragments and peptides spanning different segments of gp41-HR1-54Q (Fig. 3).

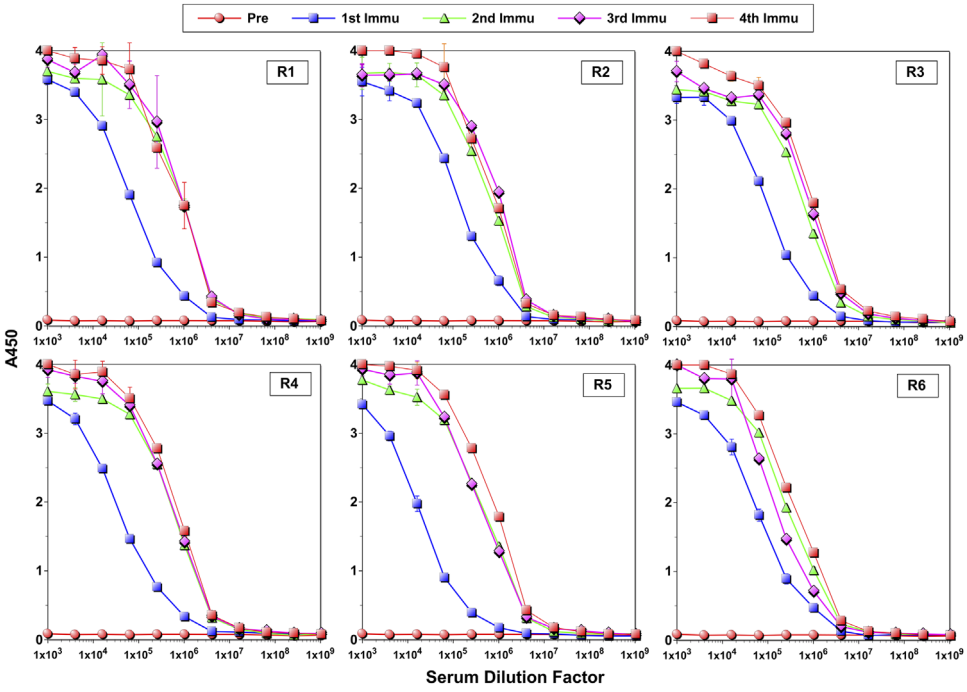

Fig. 2. Antibody Titers. Sera from six immunized rabbits (R1-R6) were tested for binding to gp41-HR1-54Q after each of the four immunizations. Pre-immune serum was used as a negative control.

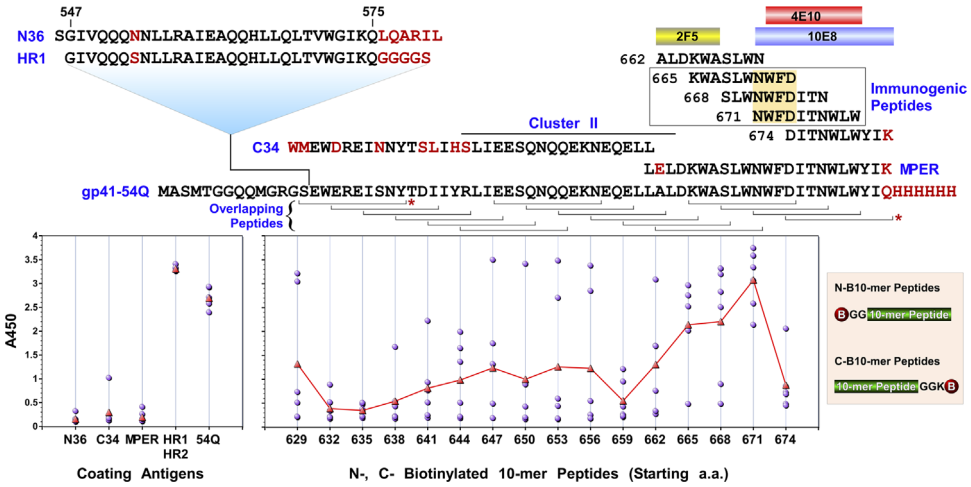

Fig. 3. Mapping of Immunogenic Epitopes. Sera after fourth immunization were tested for binding against N36 peptide (HR1), C34 peptide (HR2), MPER peptide, gp41-HR1-HR2 (comprised of HR1 and HR2 domains) and gp41-54Q (comprised of HR2 and MPER domains). Purple spheres indicate ELISA A450 values for individual rabbits while the average values are plotted with red triangles. The amino acid sequences of N36, C34 and MPER, are compared with our antigen above the graph (conserved residues in black; differences in red). ELISA was also performed against biotinylated 10-mer peptides spanning both HR2 and MPER domains. For each 10-mer, a mixture of N-terminus biotinylated (N-B10-mer) and C-terminus biotinylated (C-B10-mer) peptides were used. The amino acid sequence of each 10-mer peptide is indicated by horizontal brackets. The first peptide (MEWEREISNY) and terminal peptides (DITNWLWYIK) are marked with an asterisk to indicate slight sequence differences from the original antigen. The three most immunogenic peptides, along with two adjacent peptides, are indicated separately and the important binding residues are highlighted. The core binding epitopes for 2F5, 4E10 and 10E8 bnAbs are also indicated.

First, ELISAs were done with three long peptides available from the NIH repository, that cover the entire length of the immunogen: HR1 (N36), HR2 (C34) and MPER (^{661}LELDKWASLWNWFDITNWLWYIK^{683}). Despite some sequence differences in the N-terminal half of the C34 peptide, it was used since the cluster II region was quite conserved. Although not unexpected, antibodies against N36 were not detected. Considering that HR1 forms the inner core of the six-helix bundle, it is possible that they are simply not exposed enough to elicit antibody responses. In this regard, it was surprising to see little to no reactivity against C34 or MPER peptides since they are well exposed. This lack of reactivity could be due to a possibility that the vast majority of the antibodies are against non-linear epitopes and that these peptides do not contain the full structural elements necessary to form the epitopes. Alternatively, these peptides simply might not be able to fold into conformations that mimic the structure of the whole protein. Yet, another possibility is that the way in which they are coated onto the surface of ELISA plates hides the epitopes or sterically hinder efficient antibody binding. Some differences in the amino acid sequences in the N-terminal half of the C34 peptide with our immunogen could also contribute.

To further characterize antibodies, two larger protein fragments were used: gp41-HR1-HR2 and gp41-54Q, which are similar to gp41-HR1-54Q but lack either MPER or HR1, respectively (Fig. 3). Not surprisingly, HR1-HR2, which would form a stable six-helix bundle, was efficiently recognized, indicating that a large proportion of antibodies recognize non-linear, or highly conformational epitopes on the six-helix bundle. But, what was interesting was that gp41-54Q, which is unable to form a six-helix bundle, was also well recognized. This suggested that gp41-54Q folded into a structure that is different from C34 or MPER peptides individually. Alternatively, although not exclusively, the two segments joined together may have allowed the protein to expose epitopes when coated onto the ELISA plate.

To identify epitopes recognized by antibodies that bind gp41-54Q, we conducted ELISA with overlapping "10-mer" peptides (Fig. 3). However, rather than coating plates with peptides directly using the traditional method, peptides were biotinylated and layered onto streptavidin-coated plates. Considering that the peptides are very short, we suspected that direct coating of the peptides onto plates could potentially mask epitopes. Since antibodies could bind at either N- or C-terminal ends of the peptides, peptides were biotinylated at either ends of the peptides, thereby generating two sets of biotinylated peptides. We rationalized that using two different sets of the peptides would enhance our ability to detect antibody binding. Furthermore, two glycine residues were inserted as a spacer to avoid steric clashes between antibodies and the plate. To minimize the amount of work, wells were coated with both types of peptides simultaneously. Surprisingly, high levels of antibodies were detected against a number of peptides (Fig. 3). Although there were some animal-to-animal variations, overall, the MPER was more immunogenic than HR2. The three most immunogenic peptides were ^{671}**NWFD**ITNWLW^{680}, followed by ^{668}SLW**NWFD**ITN^{677} and ^{665}KWASLW**NWFD**^{674}. The common amino acid residues on these peptides are ^{671}NWFD^{674}, suggesting they might play a critical role. Consistent with this interpretation, the reactivity of adjacent peptides that lack NWFD (^{662}ALDKWASLWN^{671} and ^{674}DITNWLWYIK^{683}) decreased precipitously.

Quantification of antibodies against 671 peptide

The 671 peptide (^{671}**NWFD**ITNWLW^{680}) encompasses the entire 4E10 epitope and most of the 10E8 epitope, which extends further out to K/R^{683} (Cardoso et al., 2005; Huang et al., 2012). Since it was the most immunogenic peptide in the region that encompasses HR2 and MPER, we were curious about the amount of antibodies directed at this peptide. Antibody levels were compared with those directed against HR1-HR2 six-helix bundle. As shown in Fig. 4 (left panel), all six animals mounted strong antibody responses against the six-helix bundle with end-point titers reaching 2×10^5. While this is high, it was at least 100-fold less than the titer against the whole immunogen (Fig. 2), indicating that there are significant levels of antibodies directed against other epitopes. In contrast, antibody levels against the 671 peptide varied from animal-to-animal, with end-point titers ranging from about 1×10^4 to greater than 2×10^5 (Fig. 4, right panel). Considering that the 671 peptide is significantly smaller than HR1-HR2 six-helix bundle (~7-fold), this result indicates that the peptide is highly immunogenic in the context our gp41-HR1-54Q.

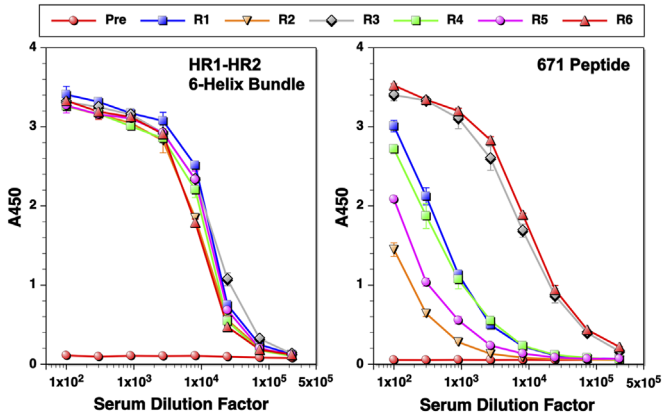

Fig. 4. Antibody titers against six helix bundle and MPER peptide. Sera after fourth immunization showed strong binding antibody titers against gp41-HR1-HR2. Binding antibody titers were also high against the biotinylated, 10-mer 671 peptide that harbors the complete 4E10 epitope and the partial 10E8 eptiope suggesting strong response against MPER.

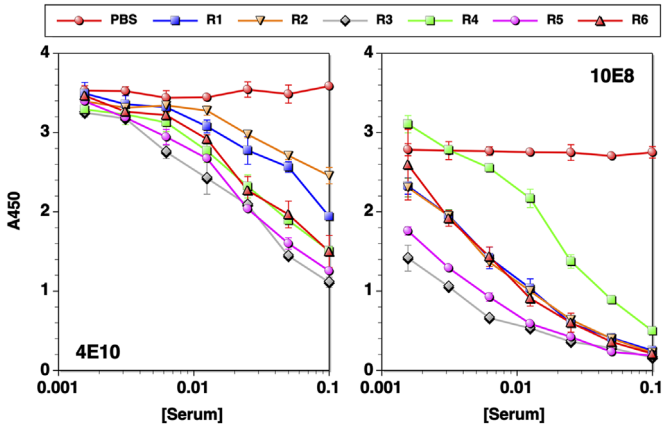

Fig. 5. Competition assay against bnAbs. Sera after fourth immunization could compete against both 4E10 and 10E8 for gp41-HR1-54Q binding.

Competition analyses with bnAbs 4E10 and 10E8

Although antibodies bound biotinylated 671 peptides, they did not bind the full length, unbiotinylated MPER peptide. To determine whether antibodies that target the 671 peptide could indeed bind the epitopes recognized by 4E10 or 10E8 in the context of gp41-HR1-54Q, we conducted antibody competition analyses with the two mAbs. As shown in Fig. 5, both 4E10 and 10E8 could be competed away with antisera in a dose-dependent manner. 10E8 was more easily competed, which is likely due to the fact that gp41-HR1-54Q has Q at position 683, instead of K or R, which is one of the residues important for 10E8 binding. Although the assay might not prove that antibodies bind exactly at the same epitope, it does confirm that antibodies do indeed bind at or near the 4E10 and 10E8 binding site close enough to compete.

The antibody titers against the 671 peptide and their ability to compete with 4E10 or 10E8 did not seem to have clear correlation. For example, rabbit #3, which showed the highest antibody titer, was best able to compete with 4E10 or 10E8. However, there were a few notable exceptions. For example, although rabbit #5 had lower antibody titer against the 671 peptide than rabbits #1, #4 and #6, antibodies from the animal were better able to compete with 4E10 and 10E8. Another example is rabbit #2, which showed the lowest antibody titer against the peptide. While it competed poorly against 4E10, it competed better than rabbit #4 and competed equally with rabbits #1 and #6 against 10E8. These results reveal complexity in evaluating antibody responses and that multiple parameters must be considered, including quantity, affinity, epitope targets and antigens being used for analyses.

Fine mapping analyses of antibodies targeting near 4E10/10E8 epitopes

To further define amino acid residues critical for antibody recognition, ELISA was conducted using a panel of 13-mer (^{671}NWFDITNWLWYIK^{683}) alanine scanning mutant peptides. First, ELISA was done with the wild type peptide (Fig. 6A). Due to significantly higher levels of antibodies against the peptide for rabbits #3, #5 and #6, higher dilution of antisera was used for the three rabbits (1:2700 compared to 1:100 for the other rabbits) to avoid oversaturation. As a positive control, effects of mutations on 4E10 binding were evaluated. As shown in Fig. 6B, mutations at N671, W672, D674, and T676 severely affected 4E10 binding. Mutations at F673, I675 and L679 also affected binding to a lesser extent. It has been shown that mutations at N671 and D674, both of which lie on the non-neutralizing face, affect 4E10 binding because these residues are critical for maintaining the alpha helical conformation of C-terminal MPER peptides (Brunel et al., 2006). Although W680 is important for neutralization, it is not critical for binding (Brunel et al., 2006; Zwick et al., 2005). Thus, these results are consistent with previously published report (Brunel et al., 2006) and validate our assay.

ELISA results from the six rabbits varied significantly from animal-to-animal. In general, three patterns were observed: (1) rabbits #1 and #2, (2) rabbits #3 and #4, and (3) rabbits #5 and #6. For rabbits #1 and #2, mutations at D674 and N671 affected binding the most, followed by mutations at F673, N677, W678 and L679 (Fig. 6C). Mutations at I675 and T676 also affected, albeit weakly. For rabbits #3 and #4, mutations at D674 and N671 also affected binding (Fig. 6D). However, none of the mutations at other sites (with the exception of F673) significantly affected binding. It should be noted that these assays were conducted with polyclonal sera. Thus, one possible explanation is that a large diversity of antibodies was induced in these animals such that a mutation at a single site would not result in significantly reduced binding. In contrast, the affects of mutations on antibody binding were quite severe for rabbits #5 and #6 (Fig. 6E); virtually all mutations, except for W672 and I682, had affected binding. As with all other rabbits, the mutation at D674 affected binding most severely, possibly due to the importance of this residue for folding into a stable alpha helix. Other critical residues were N671, F673, T676, N677 and W678. Mutations at L679 and W680 also affected, albeit weakly. Y681 was also critical, but only for rabbit #5. The fact that mutations strongly affected antibody binding for rabbits #5 and #6, in contrast to rabbits #3 and #4, suggested that a limited number of highly dominant antibodies might have been generated in rabbits #5 and #6.

The epitope recognized by 4E10 is ^{672}**WF**D**IT**NW**LW**^{680} (Cardoso et al., 2005). 10E8 has a slightly larger footprint, ^{671}**NWF**DI**T**NWLWYI**R**^{683} (Huang et al., 2012). The results from the ELISA with alanine scanning mutant peptides clearly showed that residues important for recognition by the rabbit sera overlap with those critical for 4E10 and 10E8 binding (bold/underlined). Despite this, we did not detect any neutralizing activity in our rabbit sera. To better understand possible reason(s) for the lack of neutralizing activity, the amino acid residues critical for binding were plotted onto a peptide that was co-crystallized with 10E8 (Fig. 7). The analyses were done based on an assumption that the C-terminal 13-mer peptide used for the ELISA, and the corresponding residues on gp41-HR1-54Q, also existed in an alpha helix. The analyses revealed that the critical residues for 4E10/10E8 and rabbit antibodies were on different faces of the alpha helix. For rabbits #5

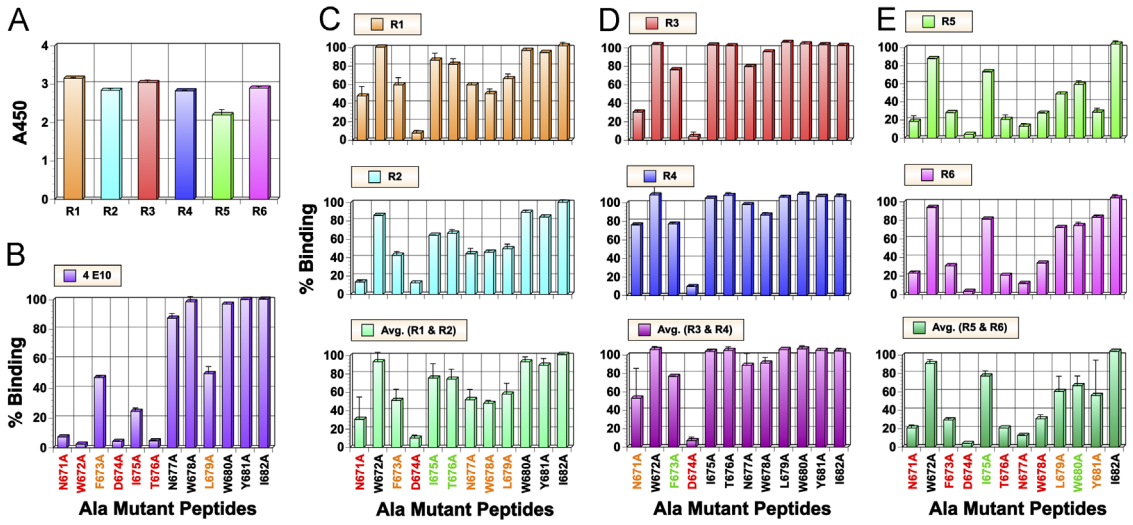

Fig. 6. PepScan analysis of the C-terminus end of MPER. (A) Sera after fourth immunization were tested for binding to a biotinylated wild type 13-mer peptide (^{671}NWFDITNWLWYIK^{683}). The sera dilutions were normalized to give comparable binding signal (R1, R2 and R4 were tested at 100-fold dilution while R3, R5, and R6 were tested at 2700-fold dilution). (B) Binding of 4E10 (1 ug/ml) to mutant peptides was evaluated as a positive control. (C–E) The same dilutions of rabbit serum samples were tested for binding mutant peptides. Results are shown as the percentage of binding to the wild type peptide shown in panel (A). Three different patterns of antibody responses are shown on different columns with the average calculated at the bottom. The labeling of the mutant peptides are color coded based on the extent of reduction in binding as follows: red: $<31\%$; orange: 31–61%; green 61–80%.

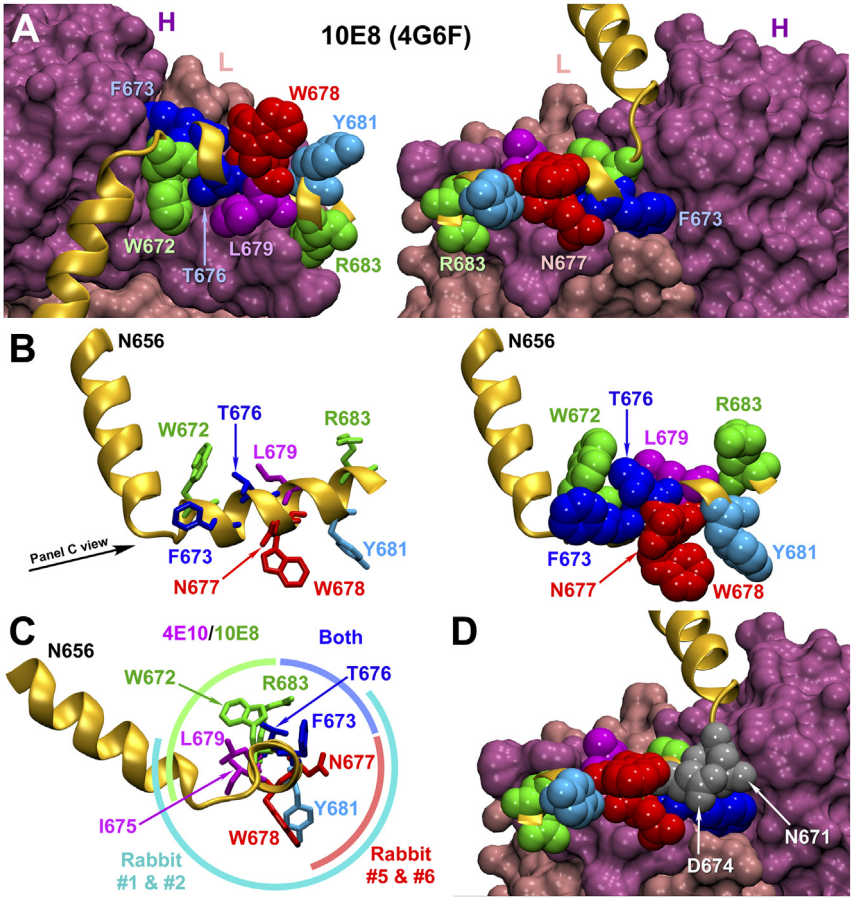

Fig. 7. Structural comparison of critical residues targeted by antibodies induced in rabbits with those of 10E8 and 4E10. (A) A co-crystal structure of a peptide bound to 10E8 is shown (pdb: 4G6F). Amino acid residues critical for binding by 10E8 (W672 and R683) and antibodies from rabbits #5 and #6 (N677 and W678) are shown in green and red, respectively. The residues important for binding by both antibodies (F673 and T676) are shown in blue. Only the most critical residues are shown. N671 and D674 are not shown, as their affect on binding may be indirect. L679, which moderately affects binding of 4E10, as well as rabbits #1, #2, #5, and #6, is shown in magenta. Y681 (cyan), which affected rabbit #5 quite significantly is also shown. Heavy (H) and Light (L) chains are indicated. (B, C) Views of the peptide bound to 10E8 from different angles. Panel C shows a view through the axis of the alpha helix at the C-terminus (from N671 to R683), which reveals that residues recognized by 4E10/10E8 and rabbit antibodies are situated on different faces of the helix. (D) A crystal structure of a peptide bound to 10E8 illustrating the locations of N671 and D674, which shows that these two residues lie on the binding face of the helix for rabbit antibodies. Thus, they could be directly involved in binding antibodies in addition to being important for maintaining alpha helix conformation of the peptide.

and #6, they were separated by about 90° with overlap at F673 and T676 (Fig. 7C). For rabbits #1 and #2, they were completely on the opposite side, with overlap at I675 and L679 on one side and F673 on the other. Thus, the likely reason why rabbit antibodies failed to neutralize HIV-1 is because the faces of the alpha helix recognized by them might not be fully accessible on the trimeric envelope structure on the virion surface.

Discussion

Despite many failures to induce potent bnAbs against gp41 MPER during the past decades, it remains an important goal towards developing a protective AIDS vaccine. Towards this goal, we have been designing various MPER-based immunogens, one of them being gp41-HR1-54Q. We had previously reported its crystal structure (Shi et al., 2010) and its immunogenicity was examined in this study. Although we failed to induce bnAbs using this construct, we believed it was important to characterize its immunogenic properties in detail to learn why it may have failed. Indeed, we have made a number of important observations, which we believe would facilitate future vaccine development efforts.

First, strong immune responses were induced against gp41-HR1-54Q in rabbits. The antibody titers elicited seemed to be much stronger than previously characterized gp41-based immunogens, reaching nearly 1×10^7 end-point titers even after a single immunization. This could be attributed to a potent adjuvant effect of Zn-chitosan (Qin et al., 2014a; Seferian and Martinez, 2000). It could also be attributed to a stable structure of the six-helix bundle formed by HR1 and HR2. Strong antibody responses against the six-helix bundle, especially against the cluster II region within HR2, have also been observed in HIV-1 infected patients (Alam et al., 2008; Frey et al., 2010). It should be noted, however, that the end-point antibody titers against HR1/HR2 six-helix bundle were only 2×10^5 (Fig. 4), about 100-fold less than the titers against the whole antigen. This suggests that other regions/conformations of the antigen were also immunogenic. Indeed, significant levels of antibodies were also detected against gp41-54Q (Fig. 3), a construct that contains just HR2 and MPER and would not be able to form the six-helix bundle. Substantial antibody levels were also detected against MPER using biotinylated 10-mer peptides, although not when the 23 amino acid MPER peptide was used. In this regard, it should be pointed out that antibody detection by ELISA depends significantly on what protein or peptide fragments are used and how they are attached to plates (*i.e.* direct coating vs. using biotinylated peptides).

Although we were able to induce high titers of antibodies against MPER using gp41-HR1-54Q, they failed to exhibit neutralizing activity. Detailed mapping analyses indicated that the antibodies targeted epitopes that overlap with those of 4E10 and 10E8. However, the critical residues of the epitopes seemed to lie on the face of the MPER alpha helix perpendicular to, or opposite side of, the residues recognized by 4E10 and 10E8 (Figs. 6 and 7). The crystal structure revealed that the MPER region of our gp41-HR1-54Q is highly flexible and disordered; the very C-terminal eight residues of MPER as well as the 6xHis tag could not be observed and the last eight residues that could be seen could be resolved only at the level of the backbone atoms (Shi et al., 2010). Being in a "near post-fusion" state without FPPR, it appears that MPER on our immunogen is flexible enough to be recognized by 4E10 and 10E8 as well as the antibodies induced in rabbits. However, on the native trimeric envelope structure on virus particles, flexibility of MPER is likely more limited being not only bound to the membrane, but also connected to a large cytoplasmic domain. In such a rigid state, it is possible that the epitopes being recognized by the rabbit antibodies are not fully exposed on either the pre-fusion structure or on fusion intermediates that may exist during the fusion process. Alternatively, these epitopes might be exposed, but the angle of approach required for binding might not be possible in the context of the protein situated on the viral membrane.

Given that 4E10 and 10E8 epitopes are accessible on our immunogen, we are unsure as to why antibody responses were not induced against these epitopes. One possibility is that the epitopes that induced antibodies is inherently and overwhelmingly more immunogenic such that faster antibody responses against these epitopes prevented any immune responses being mounted against the 4E10 or 10E8 epitopes (due to steric competition). If this was the case, perhaps reducing immunogenicity of the epitopes by amino acid substitutions or by masking (*e.g.* by glycosylation, PEGylation or immune complexing) could render 4E10/10E8 epitopes more immunogenic. Another possibility is that the epitopes on our HR1-54Q that induced antibodies are more favorably targeted than 4E10/10E8 epitopes when presented in the context of a stable six-helix bundle. In this case, immunogens with less completely formed (or less stable) six-helix bundle structures that might mimic fusion intermediates could be better immunogens. Alternatively, although not exclusively, immunogens that include the transmembrane domain (with or without the cytoplasmic tail) might be necessary to provide proper rigidity of, or spacing between, the three MPER on a trimeric structure that would hide the non-neutralizing face of the 4E10/10E8 peptide.

Recently, there have been a couple of reports describing immunogenic properties of antigen constructs very similar to ours, which also contained HR1, HR2 and MPER domains. In a report by Vassell *et al.* (Vassell et al., 2015), authors evaluated immunogenicity of several constructs comprised of MPER with different lengths of HR1 and HR2 in rabbits. Constructs were made with or without two different trimerization domains (GCN4 or foldon). The immunogens were based on HIV-1_{HXB2} strain, in contrast to ours, which was based on M group consensus sequence (MCON6). Compared to our study, antibody responses were significantly weaker with end-pointers reaching only $4–8 \times 10^4$. More importantly, antibody titers directed against MPER ranged only between about 100 and 5,000, which are 100- to 1000-fold less than what we observed. Two notable differences between the two studies are (1) we used 200 μg of antigen per immunization while they used 50 μg, and (2) we used Zn-chitosan as an adjuvant in contrast to their study, which used complete/incomplete Freund's adjuvant. Thus, our study demonstrates that it is possible to overcome poor immunogenicity of MPER by using a suitable antigen with appropriate dosage and an adjuvant. In this regard, one interesting observation from their study is that of all the immunogens they evaluated, the construct that induced best antibody responses against MPER was FDA26, which lacked a C-terminal trimerization domain, suggesting that rigidity of the region makes it less immunogenic.

In contrast, Wang *et al.* (Wang et al., 2011) reported the elicitation of neutralizing antibodies, albeit with limited breadth and potency, in rabbits upon immunization with a similar gp41 antigen named NCM(TAIV). This construct, based on HXB2 strain, contains N36 HR1 connected to C34 HR2 *via* a GGGKLGGG liner followed by MPER. It also carries two point mutations: T569A and I675V, which have been reported to increase the exposure of the neutralizing epitopes in the MPER region (Blish et al., 2008). Interestingly, the same construct without the mutations or with a single mutation individually, induced much weaker antibody responses, especially against MPER region. The exact mechanism of enhanced immune responses rendered by these mutations, or the nature of neutralizing activity, currently remains unknown. Furthermore, the absence of detailed epitope mapping data in the report and the lack of further follow up studies limit our ability to fully compare immunogenic properties of NCM(TAIV) and gp41-HR1-54Q.

In recent years, significant advances have been made in discovering potent bnAbs against HIV-1 (Bonsignori et al., 2011; Gaebler et al., 2013; Gray et al., 2011; Scheid et al., 2009; Walker et al., 2009; Wardemann et al., 2003) and determining high-resolution structures of the bnAbs (Huang et al., 2012; Julien et al., 2013b; Pejchal et al., 2011; Scharf et al., 2014; Zhou et al., 2010) as well as novel envelope antigens (*e.g.* germline targeting eOD-GT6 and stable trimeric SOSIP gp140; (Bartesaghi et al., 2013; Jardine et al., 2013; Julien et al., 2013a; Lyumkis et al., 2013)). While structure-based, rational immunogen design can facilitate vaccine development efforts, much of vaccine research still remains an empirical process because immunology still is a "black box" and we are unable to predict immunological responses to a given immunogen with any precision. As such, vaccine development efforts remain a reiterative process for which understanding why a vaccine candidate failed to induce desired immune response is important. Our study reveals detailed information on immunogenic properties of gp41-HR1-54Q. The availability of its crystal structure allows us to have better understanding of the relationship between antigenic structures and their immunogenic properties. We hope to use this information to design next generation of MPER-based immunogens.

Conclusion

A novel, M group consensus sequence-based HIV-1 gp41 MPER immunogen (gp41-HR1-54Q) was generated and its immunogenicity was evaluated in rabbits. Our vaccine regimen using Zn-chitosan as an adjuvant induced potent antibody responses against the immunogen, especially against the C-terminal 13 amino acid peptide that contains epitopes recognized by bnAbs 4E10 ad 10E8. Neutralizing activity was not detected. However, detailed epitope mapping analyses revealed amino acid residues recognized by induced antibodies. These results provide critical information for designing the next generation of MPER-based immunogens.

Materials and methods

Rabbit immunization

Female New Zealand white rabbits (2.5–3 kg) were purchased from Charles River or Myrtle's Rabbitry and housed under specific pathogen free environments. Rabbits were cared for and used following animal protocols approved by IACUC at Case Western Reserve University or Iowa State University. To evaluate immunogenic properties of gp41-HR1-54Q, which was expressed and purified as previously described (Shi et al., 2010), rabbits were immunized subcutaneously with the protein four times (weeks 0, 4, 9 and 15) using Zn-chitosan as an adjuvant. Zn-chitosan was prepared and used as previously reported (Qin et al., 2014a). The protein was loaded onto Zn-chitosan at a ratio of 200 μg to 200 mg, respectively, in phosphate-buffered saline (PBS, pH 8.0) by continuous agitation for three hours at room temperature. Rabbits were immunized with 200 μg of gp41-HR1-54Q per each immunization.

Enzyme-linked immunosorbent assay (ELISA)

To determine the end point titers, gp41-HR1-54Q was coated onto 96-well Nunc-Immuno Plates (Nunc; # 439454) at 30 ng/well using antigen coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 3 mM NaN_3, pH 9.6) overnight at 4 °C. Uncoated surface was blocked using 200 μl of PBS (pH 7.5) containing 2.5% skim milk and 25% Fetal Bovine Serum (FBS) for 1 h at 37 °C. The plates were subsequently washed 10 × with 0.1% Tween 20 in PBS. Rabbit sera were serially diluted (three folds) in the blocking buffer, and 100 μl was added to each well and incubated for 2 h at 37 °C. The plates were washed 10 ×, and horseradish peroxidase (HRP)-conjugated secondary antibody (goat anti-rabbit, 1:3000 dilution; Thermo Scientific; Cat #31430) was added to each well and incubated (100 μl, for 1 h at 37 °C). Wells were washed 10 × and developed by adding 100 μl TMB HRP-substrate (Bio-Rad) for 10 min. Reactions were stopped with 50 μl of 2 N H_2SO_4. Plates were read on a microplate reader (Versamax by Molecular Devices) at 450 nm. All experiments were performed in duplicates.

For ELISA with other proteins (gp41-HR1-HR2, gp41-54Q) and peptides (N36, C34), coating antigen amounts used were molar equivalents to that used for gp41-HR1-54Q (30 ng/well). The details of the expression and purification of gp41-HR1-HR2 and gp41-54Q will be described elsewhere. As described in the results section and Fig. 3, constructs for these proteins were the same as gp41-HR1-54Q, except for the lack of MPER or HR1 domain respectively. The gp41-HR1-HR2 protein ended at L661 with RSELVPR thrombin cleavage site at the C-terminus. For ELISA with overlapping peptides, 10-mer peptides were biotinylated with EZ-Link Sulfo-NHS-LC Biotin as per the manufacturer's instructions (Thermo Scientific, Cat #21327). A mixture of both N- and C-terminally biotinylated peptides (100 ng each) were used for coating onto streptavidin-coated plates (Thermo Scientific, Cat #15500). For alanine scan analysis, 13-mer ^{671}NWFDITNWLWYIK^{683} peptides were also biotinylated at the C-terminal K683. All other steps for ELISA were the same as described above.

Competition assays

For competition assays, plates were coated overnight with 30 ng/well of gp41-HR1-54Q. Antibodies used for competition included 4E10 (Stiegler et al., 2001) and 10E8 (Huang et al., 2012) at a final concentration of 1 μg/ml. The rest of the assay was performed as previously described (Qin et al., 2014b).

Neutralization assays

TZM-bl cell-based HIV-1 pseudovirus neutralization assays were done as previously described (Li et al., 2005; Qin et al., 2014a; Wei et al., 2002). Viruses tested were SF162 (tier 1A, clade B), MW965.26 (tier 1A, clade C), and MN.3 (tier 1A, clade B). Murine leukemia virus Env-pseudotyped virus was used as a negative control.

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