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Modulating immunogenic properties of HIV-1 gp41 membrane-proximal external region by destabilizing six-helix bundle structure

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Abstract

The C-terminal alpha-helix of gp41 membrane-proximal external region (MPER; ^{671}NWFDITNWLWYIK^{683}) encompassing 4E10/10E8 epitopes is an attractive target for HIV-1 vaccine development. We previously reported that gp41-HR1-54Q, a trimeric protein comprised of the MPER in the context of a stable six-helix bundle (6HB), induced strong immune responses against the helix, but antibodies were directed primarily against the non-neutralizing face of the helix. To better target 4E10/10E8 epitopes, we generated four putative fusion intermediates by introducing double point mutations or deletions in the heptad repeat region 1 (HR1) that destabilize 6HB in varying degrees. One variant, HR1- 10-54K, elicited antibodies in rabbits that targeted W672, I675 and L679, which are critical for 4E10/10E8 recognition. Overall, the results demonstrated that altering structural parameters of 6HB can influence immunogenic properties of the MPER and antibody targeting. Further exploration of this strategy could allow development of immunogens that could lead to induction of 4E10/10E8-like antibodies.

Keywords

HIV-1; gp41; MPER; fusion intermediate

Introduction

It is widely hypothesized that a successful AIDS vaccine should induce antibodies that can neutralize a large number of HIV-1 isolates from multiple clades. However, such broadly neutralizing antibodies (bnAbs) have been observed only in a small fraction of HIV-1 infected patients (Y. Li et al., 2007; Simek et al., 2009), suggesting that the generation of

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these bnAbs is a complex, difficult process. Nevertheless, efforts to develop immunogens and/or vaccine strategies that can elicit bnAbs must continue.

Recent isolation and characterization of potent bnAbs from patients has helped the field of vaccine research immensely by providing better understanding of both the epitopes targeted and the unique features of antibodies that contribute to their broad neutralizing ability. Most of the bnAbs that target gp120 recognize highly conformational, non-linear epitopes that might involve tertiary and/or quaternary structures (for a review, see (Mouquet, 2014)). These epitopes include the CD4 binding site, the glycan associated V1/V2 loops, the V3 loops, and a glycan only epitope targeted by 2G12. In addition, the glycan associated bridging region between gp120 and gp41 has also been identified as a target for multiple bnAbs recently (Blattner et al., 2014; Falkowska et al., 2014; Scharf et al., 2014). In contrast, those that target gp41 recognize linear epitopes that are structurally simpler and reside in a highly conserved, ~22 amino acid long domain called the membrane proximal external region (MPER) (for a review, see (Montero et al., 2008). These bnAbs are thought to inhibit conformational changes that are critical for fusion between viral and cellular membranes. A more recent discovery of the highly potent and broadly neutralizing 10E8 mAb (Huang et al., 2012), along with previously characterized 2F5, 4E10 and Z13e1 (Purtscher et al., 1994; Stiegler et al., 2001; Zwick et al., 2001), has renewed interests in designing MPER-based immunogens.

To date, eliciting anti-MPER bnAbs through vaccination has been elusive. Several approaches have been examined, including (1) immunization with 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), (2) neutralizing epitopes presented on scaffolds (Correia et al., 2010; Guenaga et al., 2011; Ofek et al., 2010), (3) MPER peptides delivered 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), (4) MPER containing hybrid/fusion proteins, and (5) chimeric viruses or virus like particles (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; E. Kim et al., 2014; Luo et al., 2006; Marusic et al., 2001; Muster et al., 1995; Ye et al., 2011; Yi et al., 2013; Zhang et al., 2004). Although a handful of studies have shown induction of modest levels of neutralizing activity with limited breadth against tier 1 HIV-1 isolates (Hulsik et al., 2013; Krebs et al., 2014; Lai et al., 2014; Ye et al., 2011; Yi et al., 2013), neither the identity of antibodies responsible for neutralization nor the mechanistic nature of inhibition have been further demonstrated.

Multiple crystal structures of short MPER peptides in complex with bnAbs have been solved (Cardoso et al., 2007; Huang et al., 2012; Julien et al., 2008; Ofek et al., 2004). Despite simpler epitope structures, the difficulty in designing immunogens that can induce similar antibodies lies partly on the fact that the MPER structure in the context of a native trimeric envelope spike on virus particles remains unknown. In this regard, it is possible that antibody-bound MPER structures do not accurately represent the MPER conformation on native trimers. Furthermore, gp41 undergoes large structural changes during the fusion process (Pancera et al., 2014), and it is likely that the MPER also assumes several different

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conformations. Thus, studies that characterize the structural and immunological properties of MPER in context of larger gp41-based proteins are much needed.

As an initial effort, we generated a soluble gp41 construct named gp41-HR1-54Q consisting of heptad repeat regions 1 and 2 (HR1and HR2, respectively) and the MPER (Shi et al., 2010). While the HR1 and HR2 domains formed a stable six-helix bundle (6HB), much of the MPER domain remained quite flexible and free from association with the 6HB (Shi et al., 2010). Surprisingly, this protein induced strong antibody responses against a peptide that encompass 4E10/10E8 epitopes (^{671}NWFDITNWLW^{680}; (Habte et al., 2015). Further analyses indicated that these antibodies targeted the non-neutralizing face of the C-terminal α-helix, but partly overlapping with 4E10/10E8 epitopes. One possible reason for the preferential targeting of the non-neutralizing face of the helix could be its orientation when the MPER is presented in the context of a stable 6HB structure, which represents a post-fusion conformation. It had been suggested that MPER might be more exposed during the fusion process as gp41 undergoes conformational changes (Chakrabarti et al., 2011; de Rosny et al., 2004; Dimitrov et al., 2007; Finnegan et al., 2002; Frey et al., 2008; M. Kim et al., 2011).

Recent crystal structures of BG505 SOSIP gp140 provided partial information on the pre-fusion state of gp41 (Julien et al., 2013; Pancera et al., 2014). However, fusion intermediate structures are completely unknown. In particular, there is no information on how the MPER is oriented relative to the rest of the protein, and when and whether it make any contact with the rest of gp41 or gp120, prior to the post-fusion state. The only certainty is that HR1 and HR2 are in the process of coming together to form 6HB. As such, we took an empirical approach of generating four variants of gp41-HR1-54Q that might represent different stages of fusion process by disrupting 6HB formation in varying degrees. Biochemical, antigenic, and immunogenic properties of these putative fusion intermediates (PFIs) were characterized. Although we did not succeed in inducing bnAbs against the MPER in rabbits, the results from the study should facilitate development of improved MPER immunogens.

Results

Designing gp41-HR1-54Q variants with destabilized 6HB

The trimeric structure of gp41-HR1-54Q is stabilized by both inter- and intramolecular interactions between HR1 and HR2 (Shi et al., 2010). The exact order of molecular interactions between HR1 and HR2 that leads to 6HB formation is not known, although a leading working model suggests a zipping process along HR1-HR2 that begins at the C- and N-termini of respective domains in an anti-parallel fashion, with a central trimeric HR1 core (Markosyan et al., 2009). As such, we hypothesized that it might be possible to generate partially opened hairpin loop structures that might simulate fusion intermediates if HR1-HR1 or HR1-HR2 interactions were destabilized.

Four variants were generated by either introducing point mutations or deletions (Fig. 1A). All mutations were restricted to the HR1 only, so as to avoid altering the native conformation of the HR2 or MPER domains. The C-terminal half of HR1 was mutated by introducing two point mutations (HR1-AA-54Q and HR1-EE-54Q), whereas the N-terminal

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half of HR1 was mutated more drastically by deletions (HR1 10-54K and HR1 17-54K). The HR1-AA-54Q variant contained L565A and L568A mutations with an intent of weakening hydrophobic interactions with I635 and Y638 residues on HR2 (Fig. 1B). In contrast, HR1-EE-54Q contained L568E and K574E mutations designed destabilize 6HB formation by introducing intra- and inter-molecular charge-charge repulsions with E634 and E632 residues on HR2, respectively (Fig. 1C). Deleting the N-terminal 10 or 17 amino acids of HR1 is designed to allow initiation of 6HB formation, but halt the zipping process in the middle to generate structures that might resemble fusion intermediates (Figs. 1D and 1E, respectively). For these constructs with deletions, the terminal 683Q residue was reverted back to the wild type lysine as it was later reported to be critical for 10E8 binding (Huang et al., 2012). Although we do not know whether any of these constructs would mimic true fusion intermediates, they will be referred herein as putative fusion intermediates (PFIs) for simplicity.

Biochemical and antigenic properties of PFIs

Similar to gp41-HR1-54Q, all of the PFIs were insoluble when expressed in *E. coli*. However, they could be solubilized through a urea denaturation/renaturation process as we previously described for gp41-HR1-54Q (Habte et al., 2015). Unlike gp41-HR1-54Q and PFIS containing point mutations, however, HR1 10-54K and HR1 17-54K were prone to aggregation, especially upon freeze-thawing, and appeared as polydispersed oligomers.

As we previously reported, gp41-HR1-54Q is highly resistant to trypsin digestion (Habte et al., 2015), presumably due to its rigid structure. To determine how mutations might affect the protein, trypsin sensitivity of the PFIs was assessed. As shown in Fig. 2A, gp41-HR1-54Q was completely resistant to trypsin digestion even after one hour. In contrast, PFIs exhibited varying degrees of trypsin sensitivity. Not surprisingly, HR1-AA-54Q was least sensitive. Unexpectedly, however, HR1-EE-54Q was most sensitive and that HR1- 10-54K was more sensitive than HR1- 17-54K. The differences in trypsin sensitivity among the PFIs suggested that they likely have folded into structures different from each other, and certainly different from gp41-HR1-54Q.

Next, the PFIs were probed with NC-1, a mouse monoclonal antibody (mAb) that recognizes post-fusion 6HB structure (Jiang et al., 1998). NC-1 recognizes amino acid residues from 643 to 655 within HR2 (Yuan et al., 2009), which is present in all four PFIs. Thus, any changes in NC-1 binding should be the result of conformational changes induced by the mutations. As shown in Fig. 2B (left panel), NC-1 binding was completely abolished for HR1-EE-54Q, HR1- 10-54K and HR1- 17-54K. Although HR1-AA-54Q could be recognized, the binding was substantially weaker than gp41-HR1-54Q. These results indicate that introduced mutations were able to disrupt formation of the post-fusion 6HB conformation.

Next, the PFIs were probed with 126-7, a human mAb (an IgG2 version of 126-6) that only recognizes a trimeric conformation of gp41 shared between both pre- and post-fusion state (Gorny et al., 1989; Robinson et al., 1991; Tyler et al., 1990; Xu et al., 1991; Yuan et al., 2009). It recognizes residues from 641 to 648 in the cluster II of gp41. As shown in Fig. 2B (right panel), gp41-HR1-54Q, HR1-AA-54Q and HR1-EE-54Q bound nearly equally to

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126-7 suggesting that the trimeric conformations of these proteins were similar (at least at the 126-7 epitope). Not surprisingly, 126-7 failed to recognize both HR1- 10-54K and HR1- 17-54K. The elimination of three and five helical turns in HR1, respectively, likely prevented formation of stable trimeric HR1 core.

To examine whether epitopes recognized by MPER bnAbs remained conformationally intact and accessible on PFIs, they were probed with 2F5, Z13e1, 4E10 and 10E8 by ELISA (Fig. 2C). For 2F5, there were only minor differences between HR1-54Q and PFIs. The results were similar for Z13e1, although HR1-EE-54Q showed slightly weaker binding. The reduced binding to HR1-EE-54Q was more pronounced with 4E10 and 10E8. To a lesser extent, binding was also reduced for HR1-AA-54Q. In general, antibody binding to HR1- 10-54K and HR1- 17-54K was quite similar to gp41-HR1-54Q, except for 10E8, for which there was significantly better binding. However, this enhanced binding is most likely due to reverting back to lysine at position 683, rather than deletions themselves, since K683 is one of the critical residues that 10E8 recognizes. Taken together, these results suggest that destabilization of 6HB, depending on the approach taken, could potentially affect MPER structure, which could in turn alter conformation or accessibility of epitopes targeted by bnAbs.

Immunogenic properties of PFIs

Immunogenic properties of PFIs were evaluated in rabbits as previously described for gp41-HR1-54Q (Habte et al., 2015). For this initial study, two animals were used for each of the four PFIs. Serum samples were collected two weeks after each immunization (on weeks 0, 4, 9 and 15). Antibody titers against autologous antigens were assessed by ELISA (Fig. 3). Overall, antibody responses against the PFIs were weaker than gp41-HR1-54Q, which induced end point antibody titers greater than 5×10^6 even after a single immunization (Habte et al., 2015). PFIs with point mutations were more immunogenic than the ones with deletions, which was more noticeable after the first immunization. The reduced antibody responses could be due in part to elimination of helper T cell epitopes, especially for the deletion mutants, in addition to altered conformations or loss of epitopes.

To better understand how mutations on PFIs altered immune responses, immunogenic linear epitopes were mapped by ELISA using overlapping biotinylated peptides as we previously described (Habte et al., 2015). Since mutations and deletions were in the HR1 domain, we focused on antibody responses directed against the HR2 and the MPER. Notwithstanding some animal-to-animal variations, the immunogenic epitope profile of HR1-AA-54Q was somewhat similar to that of gp41-HR1-54Q, with 671 peptide (^{671}NWFDITNWLW^{680}) being highly immunogenic in both animals. Interestingly, antibody responses against HR1-EE-54Q were directed towards N-terminus of HR2 and the C-terminus of MPER with little to no antibodies against peptides spanning the cluster II region (^{644}RLIEESQNQQEKNEQELLAL^{663}) that typically elicits non-neutralizing antibodies (Alam et al., 2008; Frey et al., 2010; Hioe et al., 1997). Compared to gp41-HR1-54Q, one major difference in immune responses against HR1- 10-54K is strong antibody responses against the N-terminal end of HR2. Although the 671 peptide remained immunogenic, peptides 629 (^{629}MEWEREISNY^{638}), 632 (^{632}EREISNYTDI^{641}) and 635

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(^{635}ISNYTDIIYR^{634}) were clearly immunodominant. By far, the most striking change in immunogenic epitope profile was with HR1- 17-54K. Virtually all of the peptides, except for peptides 665, 668 and 674, were highly immunogenic in one or both of the rabbits.

Despite strong antibody responses against the 671 peptide (^{671}NWFDITNWLW^{680}) that contained all or most of 4E10 and 10E8 epitopes, none of the rabbit sera exhibited neutralizing activity when tested against HIV-1 pseudoviruses SF162 (tier 1A, clade B), MW965.26 (tier 1A, clade C), and MN.3 (tier 1A, clade B) in a standard TZM-bl assay.

Detailed analyses of antibodies targeting near 4E10/10E8 epitopes

Although we did not succeed in inducing bnAbs against gp41 MPER, better characterization of antibody responses near 4E10/10E8 epitopes could facilitate designing of better immunogens. In particular, we were curious to see whether and how epitope targeting was altered for PFIs compared to gp41-HR1-54Q. Towards this goal, we conducted fine epitope mapping analyses using alanine-scanning mutants of a 13-mer peptide (^{671}NWFDITNWLWYIK^{683}), which we previously used to characterize antibody responses against gp41-HR1-54Q (Habte et al., 2015). Initially, antibody reactivity against the wild-type 13-mer 671 peptide was measured (Fig. 5). Interestingly, some of the antisera reacted poorly to the 13-mer peptide when compared to their reactivity against the 10-mer 671 peptide (Fig. 4). This was particularly severe with rabbit #2 immunized with HR1- 17-54K, and, to a lesser degree, rabbit #2 immunized with HR1-AA-54Q. Since ELISA using 10-mer peptides (Fig. 4) was done with a mixture of both N- and C-terminally biotinylated peptides, and that the 13-mer is biotinylated at the C-terminal K683 residue, it was possible that the orientation of the peptide attachment to an ELISA plate, could have affected antibody binding. However, this might not be the case since antibodies reacted strongly to the 10-mer peptide that was biotinylated at the C-terminus (data not shown). Thus, the reason for the discrepancy is unknown at the present time.

With the caveat that we would be evaluating only a subset of antibodies targeting near 4E10/10E8 epitopes, we proceeded to characterize antibodies using the panel of 13-mer mutant peptides. All sera, except from rabbit #2 immunized with HR1- 17-54K, were analyzed. In doing so, serum samples were first normalized to yield comparable signals when bound to the wild-type peptide (Fig. 6A). For all rabbits tested, D674 residue was critical for antibody binding, which is likely due to a critical role it plays in maintaining a helical conformation of the peptide (Brunel et al., 2006). For HR1-AA-54Q, the two animals exhibited different antibody epitope profiles (Fig. 6B); such animal-to-animal variations have been observed with gp41-HR1-54Q also (Habte et al., 2015). Interestingly, similar patterns were also observed in animals immunized with HR1-EE-54Q (Fig. 6C). This might suggest structural similarity of the C-terminal MPER for HR1-AA-54Q and HR1-EE-54Q. For the both groups, the profile shown on the top panels resembled one of the patterns observed from animals immunized with gp41-HR1-54Q. The profile shown on the bottom panels (critical residues being D674, W678, L679, and I675 for HR1-AA-54Q) was not observed in any of the six animals immunized with gp41-HR1-54Q. Thus, the latter profile could be specific to antibody responses against PFIs. Coincidently, a similar profile was observed for a rabbit immunized with HR1- 17-54K (Fig. 6E). The same four residues were

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also critical for antibodies induced by HR1- 10-54K (Fig. 6D). In addition, antibodies induced by HR1- 10-54K also targeted W672, which may be highly significant since this residue was never targeted by antibodies induced with gp41-HR1-54Q or with any of the other PFIs. More importantly, this is one of the critical residues recognized by both 4E10 and 10E8.

To better compare antibodies induced by gp41-HR1-54Q and HR1- 10-54K, amino acid residues critical for binding were visualized on a structure of a peptide co-crystallized with 4E10 (Fig. 7; Cardoso et al., 2007). This analysis revealed that antibodies induced by HR1- 10-54K targeted nearly the opposite face of the helix compared to those induced by HR1-54Q with W678 being targeted by both. More importantly, HR1- 10-54K-induced antibodies targeted three of the five most critical residues for 4E10 binding (**W672**, F673, **I675**, T676 and **L679**). So, although we were not able to induce nAbs, the results of our study demonstrate that it is possible to alter immunogenicity of epitopes simply by changing structural context of an immunogen.

Discussion

In previous studies, we described structural and immunological properties of gp41-HR1-54Q, which likely represents a near-post-fusion conformation of gp41 (Habte et al., 2015; Shi et al., 2010). Although antibodies elicited in rabbits by this antigen bound epitopes that partially overlap with those targeted by 4E10 and 10E8, they were largely directed against the non-neutralizing face of the helix and failed to exhibit neutralizing activity. It had been speculated that anti-MPER bnAbs primarily target fusion intermediate forms of gp41 (Chen et al., 2014; Dimitrov et al., 2007; Frey et al., 2008). However, their structures are not yet known, except for those of short peptides bound to the antibodies. As such, we decided to take an empirical approach of generating fusion intermediates with a simple assumption that they would have minimal or partial HR1-HR2 pairing and 6HB formation. We further assumed that the MPER would likely exist in a conformation that is different from the one observed on gp41-HR1-54Q. With these assumptions, four putative fusion intermediates (PFIs) were generated by introducing double point mutations or deletions into the HR1 of gp41-HR1-54Q to destabilize HR1-HR1 and HR1-HR2 interactions, and their biochemical and immunological properties were evaluated in this study.

Although we do not have structural evidence, the increased sensitivity of the PFIs to trypsin digestion and their altered reactivity to NC-1 and/or 126-7 mAbs demonstrated that the structure of 6HB on PFIs has been disrupted in varying degrees. Not surprisingly, the four PFIs revealed different immunological profiles with respect to the overall immunogenicity (*i.e.* total antibody titers induced; Fig. 3), immunodominance of epitopes across the HR2 and MPER (Fig. 4), and specific amino acid residues targeted by antibodies directed against the C-terminal region containing 4E10/10E8 epitopes (^{671}NWFDITNWLWYIK^{683}; Fig. 6). Together, these results indicate that immunogenicity of HR2 and MPER domains are highly dependent on the structural context in which they are presented.

While we did not succeed in inducing bnAbs, detailed epitope mapping analyses revealed a few important findings about antibodies induced by PFIs, HR1- 10-54K in particular. First,

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antibodies induced in both rabbits immunized with HR1- 10-54K targeted W672. Targeting of this residue was never observed in any of the six rabbits immunized with gp41-HR1-54Q (Habte et al., 2015; Shi et al., 2010) or in any of the animals immunized with other PFIs. Replacement of this residue with an alanine has been reported to reduce 4E10 binding by over 1000-fold, highlighting its overarching importance (Brunel et al., 2006). W672 is also critical for 10E8 binding (Huang et al., 2012). Second, animals immunized with HR1- 10-54K and HR1- 17-54K induced antibodies that bound strongly to I675 and L679, both of which line up with W672 along the same side of the helix and contribute significantly to 4E10 and 10E8 binding (Brunel et al., 2006; Cardoso et al., 2005; Huang et al., 2012). Targeting these three residues highlights a remarkable shift from the binding pattern of antibodies elicited by gp41-HR1-54Q. Third, while gaining recognition of these three residues, antibodies induced by HR1- 10-54K seemed to have lost recognition of F673 and T676, which were quite well recognized by antibodies induced with gp41-HR1-54Q and targeted by both 4E10 and 10E8.

One cautionary note for interpreting the results of our study is that the epitope mapping analyses shown in Fig. 6 were conducted with polyclonal antibodies. Thus, the phenotypic changes we observed are average of all antibodies that bind the peptide. Accordingly, when there are many antibodies that bind to different epitopes, we might not see significant reduction in antibody binding for mutations at any given position; this might be the case for rabbit #1 of HR1-AA-54Q and HR1-EE-54Q. Significant reduction in binding would be seen only when the antibody response is homogeneous or when all or most antibodies target same residues. In this regard, characterizing antibodies at the monoclonal level would provide a more accurate assessment.

In the absence of a crystal structure of HR1- 10-54K, it is hard to speculate how deleting ten residues from the N-terminal end of HR1 (with a potential contribution from K683) influenced the overall MPER conformation to promote such a major shift in antibody response. Nevertheless, our results clearly demonstrated that changes in HR1, which in turn affect stability of 6HB, significantly influence how antibodies target the MPER. Considering the difficulties in crystallizing proteins that contain the hydrophobic C-terminal ectodomain of gp41, MPER-based vaccine development may have to rely on reiterative, empirical approaches. Based on the results from this study, HR1- 10-54K would be an excellent starting point.

To improve the immunogen, one factor that could be adjusted is the length of HR1. In this study, we deleted 10 or 17 residues, which account for roughly 3 and 5 helical turns, respectively. As shown in Fig. 4, the antibodies induced by HR1- 17-54K were drastically different from all others, which we believe is due to complete disruption of 6HB, thereby rendering the protein highly flexible and inducing greater diversity of antibodies. By adding two helical turns (HR1- 10-54K), which likely increased the stability of 6HB, antibody responses against HR2 was largely restricted to the N-terminal end and W672 could be targeted. This raises a question as to what would happen to the antibody response if less than 10 residues were deleted. Would any of them allow targeting of F673 and T676 (as did gp41-HR1-54Q), while also targeting W672, I675 and L679?

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Another factor that could be considered for improving antibody responses is to minimize immunogenicity of W678. As shown in Fig. 6, W678 was targeted on all of the PFIs as well as on gp41-HR1-54Q (Habte et al., 2015), suggesting its dominant role in determining antibody responses. Most likely, W678 is not exposed during the natural course of the fusion process, rendering these antibodies useless. Thus, preventing antibody responses against W678 could improve the chance of inducing bnAbs. Perhaps substituting W678 with a less immunogenic residue (*e.g.* glycine or alanine) might redirect the immune system to shift the focus away from W678 towards F673 and T676.

In all of our immunization studies, we used Zn-chitosan not only as an adjuvant, but also as an antigen delivery platform. Although all of our PFIs, as well as gp41-HR1-54Q, are soluble proteins, they all have 6×His tag at the C-terminus, which was used to affix the proteins to Zn-chitosan. Thus, the flexibility of the C-terminal end of the MPER was most likely limited. However, neither the spatial distribution nor the orientation of the MPER when it is bound to Zn-chitosan is known. More importantly, how it would affect MPER immunogenicity is completely unknown. In this regard, it would be worthwhile to evaluate immunogenicity of some of the PFIs in the context of lipid membranes (*e.g.* delivered on liposomes or expressed directly on the cell surface), which would better resemble the MPER structure and the microenvironment of virus particles. Although the discovery of 10E8 (Huang et al., 2012) has shown that MPER-directed antibodies do not have to interact with membranes to exhibit neutralizing activity, it is possible that anchoring the MPER onto the membrane (*via* a transmembrane domain with or without a cytoplasmic tail) could (1) stabilize the correct conformation of the neutralizing epitope, (2) provide rigidity and limit the mobility of the region, and/or (3) allow better orientation/exposure of the neutralizing face of the helix, thereby facilitate induction of 4E10/10E8-like antibodies.

Conclusion

Previously characterized gp41-HR1-54Q, which likely exists in a near post-fusion state, induced antibodies primarily against the non-neutralizing face of the C-terminal α-helix that contains 4E10/10E8 epitopes. To generate immunogens that might better resemble fusion intermediates, four constructs were generated by introducing double point mutations or deletions in the HR1 of gp41-HR1-54Q. Different mutations disrupted the six-helix bundle (6HB) structure in varying degrees and differentially affected immunogenicity of the MPER. Antibodies induced by one of the variants, HR1- 10-54K, targeted three residues critical for recognition by 4E10 and 10E8. Further exploration of this strategy could lead to development of immunogens that could elicit 4E10/10E8-like antibodies.

Materials and Methods

Cloning, Expression and Purification of PFIs

To generate PFI constructs with point mutations, the QuikChange® XL Site directed mutagenesis kit was used as per the manufacturer's instructions using the original gp41-HR1-54Q plasmid as the template (Shi et al., 2010). For HR1-AA-54Q, the mutations L565A and L568A were introduced using the sense primer 5'-GAGGCCCAGCAGCAC**GCC**CTGCAG**GCC**ACCGTGTGGGGCATC-3' and the

antisense primer 5'-GATGCCCCACACGGT**GGC**CTGCAG**GGC**GTGCTGCTGGGCCTC-3'. For HR1-EE-54Q, the mutations L568E and K574E were introduced using the sense primer 5'-GCACCTGCTGCAG**GA**GACCGTGTGGGGCATC**G**AGCAGGGAGGAGG-3' and the antisense primer 5'-CCTCCTCCCTGCT**C**GATGCCCCACACGGTC**TC**CTGCAGCAGGTGC-3'.

For the deletion variants, 10 and 17 residues were deleted from the N terminus end of the HR1 domain as shown in Fig 1A. Both constructs were synthesized from IDT (Integrated DNA Technology) in the pUC57 backbone with flanking restriction sites for BamHI and EcoRI at the 5' and 3' ends of the constructs, respectively. The sequence was also altered to encode terminal 683K residue instead of the 683Q as in gp41-HR1-54Q. These constructs were cloned into the pET-21a vector (Novagen; cat#69740-3) using BamHI and EcoRI. All constructs were expressed and purified similar to gp41-HR1-54Q (Shi et al., 2010). The final proteins were dialyzed into 1× PBS (pH 8.0) and stored at −80 degrees.

Trypsin sensitivity assay

All PFIs were incubated with trypsin at 1:100 (enzyme:protein) mass ratio for one hour at 37 °C. 3 μg of untreated and trypsin treated samples were then run on a Novex® 10-20% tricine gel (Thermo Fisher Scientific; cat# EC6625BOX).

Rabbit immunization

Eight New Zealand white female rabbits (2.5 to 3 kg) were purchased from Charles River (USA), housed under specific pathogen free environments and used in compliance with the animal protocol approved by IACUC of Iowa State University. Two animals were immunized with each antigen using Zn-chitosan as an adjuvant. The immunization protocol including the adjuvant preparation, antigen/adjuvant dosage, the immunization and bleeding schedule were all exactly the same as that previously described for gp41-HR1-54Q (Habte et al., 2015).

Enzyme-linked immunosorbent assay (ELISA)

All ELISAs were performed using the standard protocol described for gp41-HR1-54Q (Habte et al., 2015) except for the use of an alternate blocking buffer consisting of PBS (pH 7.5) with 2.5% skim milk and 5% calf sera. For ELISAs testing the binding of antibodies NC-1, 126-7, 2F5, 4E10, Z13e1 and 10E8, coating antigen amounts for all other antigens equimolar to 30 ng/well of gp41-HR1-54Q using the same coating conditions as described for gp41-HR1-54Q. In order to determine end point titers, all antigens were coated at 30 ng/well. The end-point ELISA titers were defined as serum dilution factor that gave readings of average + 2×SD (standard deviation) of the background as described previously (Qin et al., 2014). Coating for linear epitope mapping using 10-mer biotinylated peptides and 13-mer alanine scanning was also performed as previously described (Habte et al., 2015).

Neutralization assays

Neutralization assays were performed in TZM-bl cells as previously described (M. Li et al., 2005; Qin et al., 2014; Wei et al., 2002). Viruses tested included SF162 (tier 1A, clade B),

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MW965.26 (tier 1A, clade C), and MN.3 (tier 1A, clade B). Murine leukemia virus Envpseudotyped virus was used as a negative control.

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Highlights

- Four gp41 MPER-based immunogens that resemble fusion intermediates were generated.
- C-terminal region of MPER that contains 4E10/10E8 epitopes was highly immunogenic.
- Altering 6HB structure can influence immunogenic properties of the MPER.
- Induced antibodies targeted multiple residues critical for 4E10/10E8 binding.
- Development of immunogens based on fusion intermediates is a promising strategy.

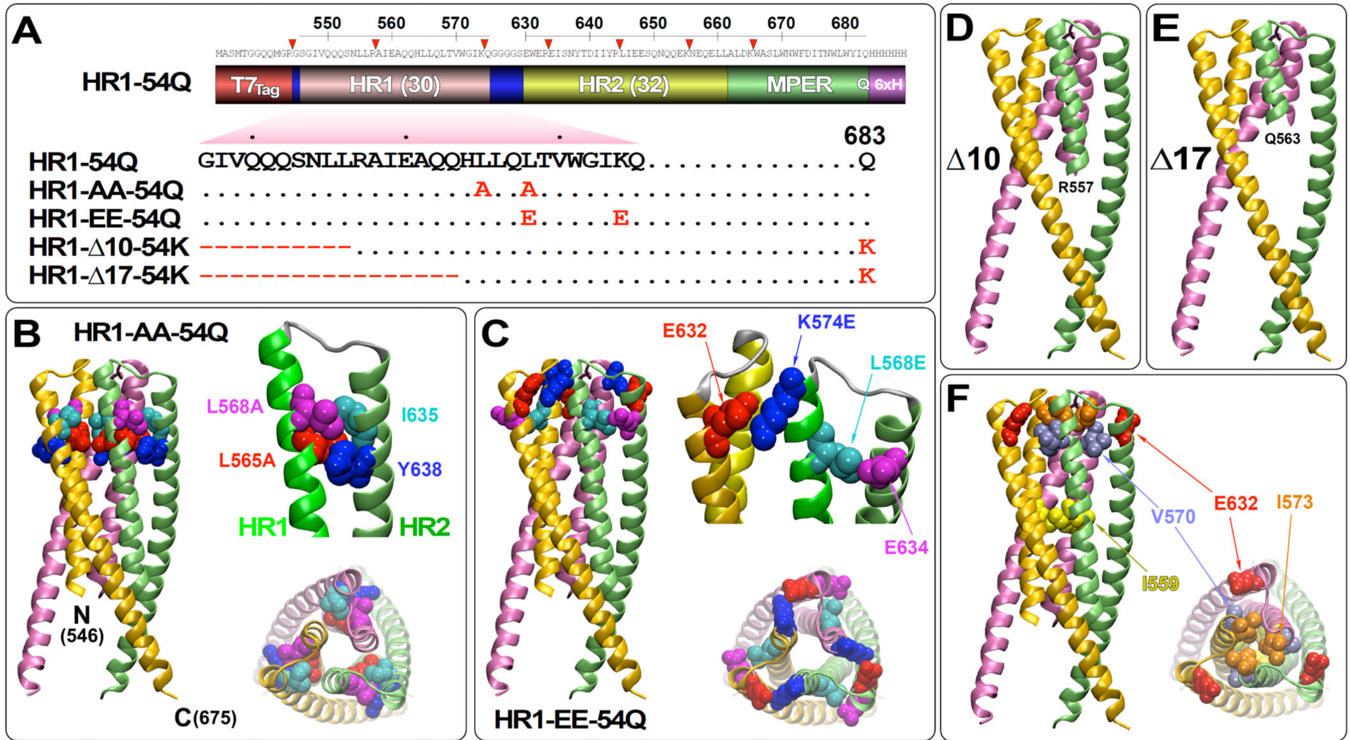

Fig 1.

Design of putative fusion intermediates of gp41-HR1-54Q. (A) A domain structure of gp41-HR1-54Q (indicated as HR1-54Q for simplicity) consisting of the $T7_{Tag}$, heptad repeat region 1 (HR1), GGGGS linker, heptad repeat region 2 (HR2), membrane-proximal external region (MPER) and the 6× His tag is shown. The HR1 domain sequence, along with the terminal 683Q residue, is indicated for gp41-HR1-54Q. Point mutations and deletions introduced into the HR1 domain to generate variants HR1-AA-54Q, HR1-EE-54Q, HR1- 10-54K and HR1- 17-54K are indicated. The terminal 683Q residue was reverted back to 683K in HR1- 10-54K and HR1- 17-54K. (B) The mutations introduced in HR1-AA-54Q (L565A and L568A) are plotted on the gp41-HR1-54Q crystal structure (pdb: 3K9A) (Shi et al., 2010) to highlight the proximity of these residues to the neighboring I635 and Y638 residues located on the HR2 domain. Structures of the unmutated amino acids are shown. (C) The mutations introduced in HR1-EE-54Q (L568E and K574E) are plotted on the gp41-HR1-54Q crystal structure to display the proximity of these residues to E632 and E634 residues. The truncations introduced at the N-terminal end of the HR1 domain are plotted onto the gp41-HR1-54Q structure simply to show the point of deletion for (D) HR1- 10-54K and (E) HR1- 17-54K. (E) As a point of reference, other residues that have been previously shown to destabilize 6HB when mutated (I559P, V570D and I573D; (Kesavardhana and Varadarajan, 2014; Sanders et al., 2002)) are indicated.

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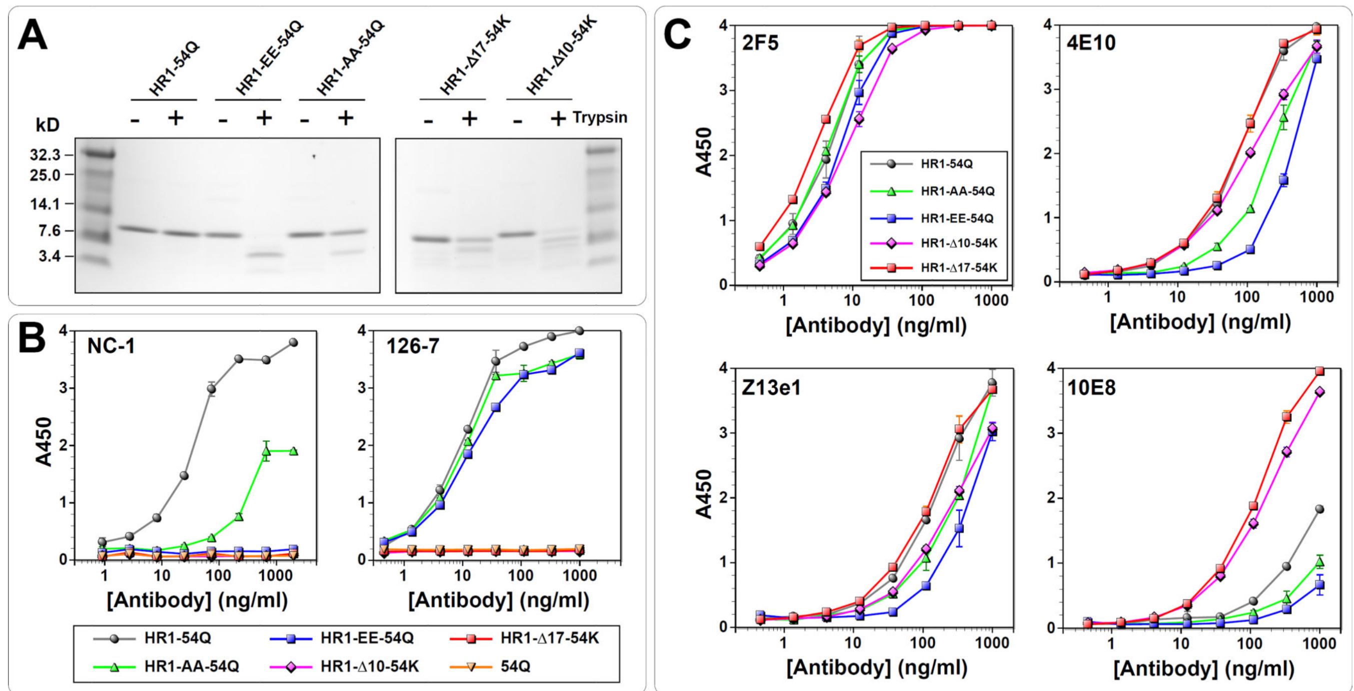

Fig 2.

Biochemical and antigenic properties of putative fusion intermediates. (A) Evaluation of trypsin sensitivity of PFIs in comparison to gp41-HR1-54Q. (B) ELISA with mAbs NC-1 and 126-7 to monitor effects of the mutation on six-helix bundle formation. gp41-HR1-54Q was used as a positive control, while another protein (gp41-54Q) that lacks the HR1 domain was used as a negative control. (C) The antigenic integrity of the variants was tested by performing ELISA with bnAbs 2F5, 4E10, Z13e1 and 10E8.

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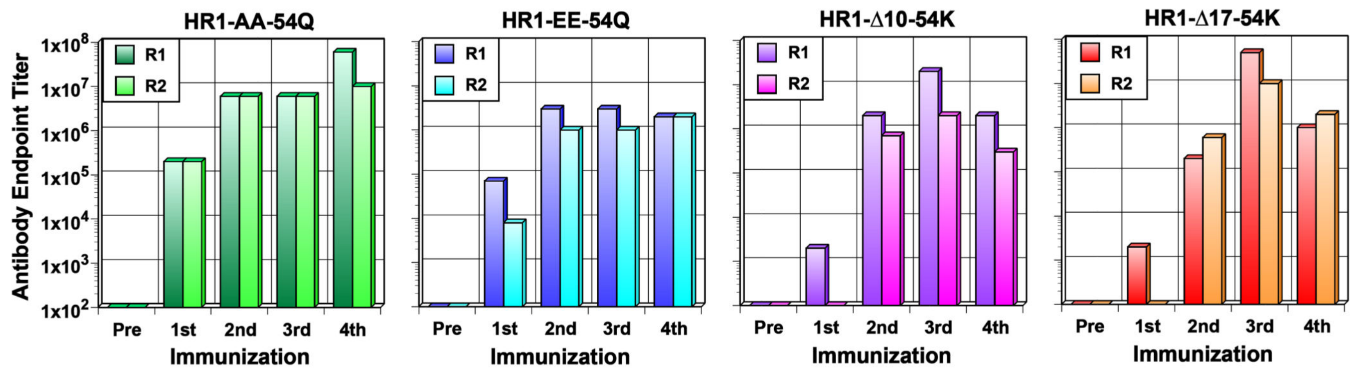

Fig 3.

Antibody end-point titers induced by putative fusion intermediates. Serum samples collected two weeks after each immunization were evaluated by ELISA to determine the end-point antibody titers against autologous antigens. Pre-immune serum was used as a negative control.

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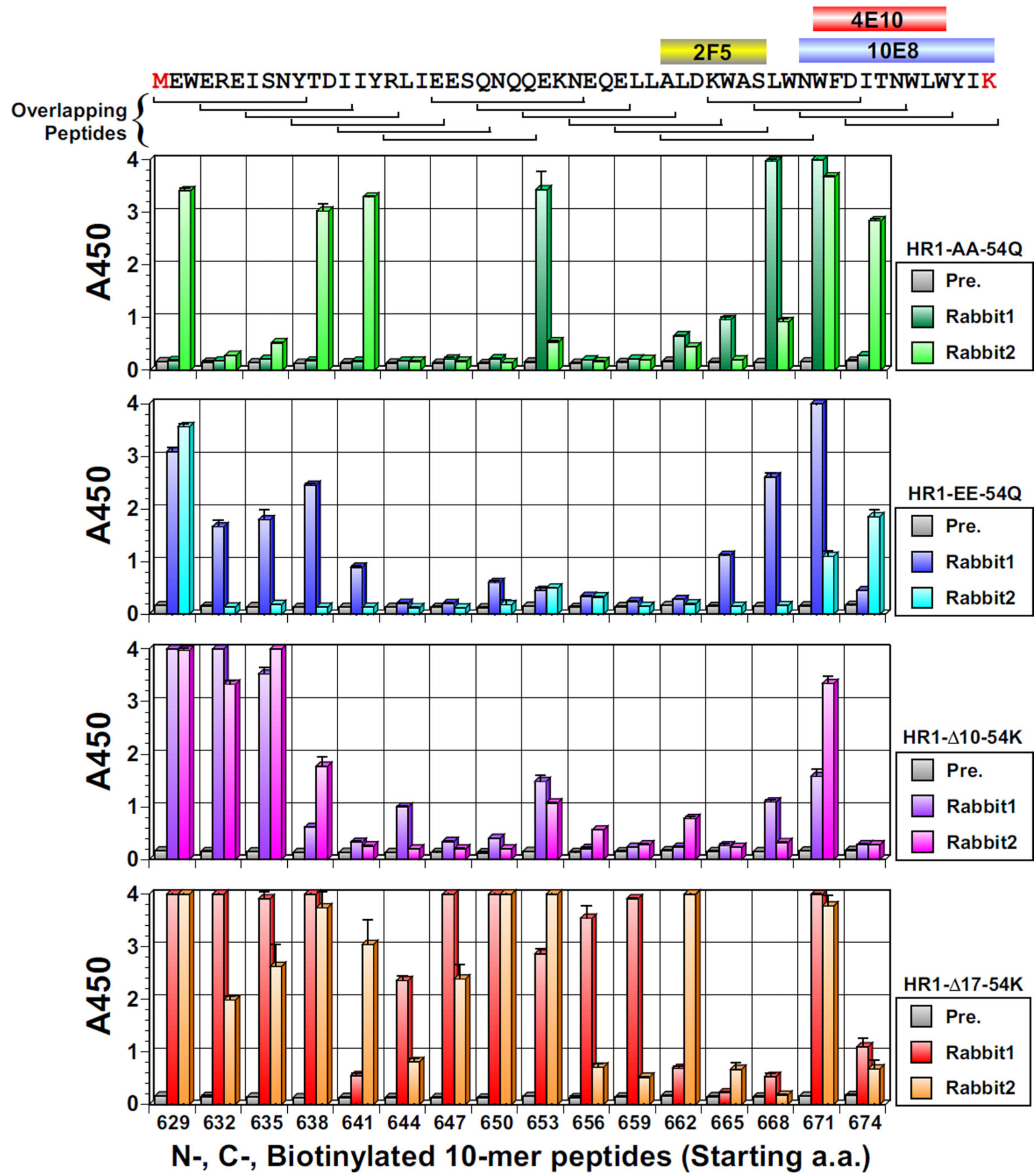

Fig 4.

PepScan analyses using linear, overlapping peptides. Serum samples collected after the fourth immunization were evaluated for reactivity against biotinylated 10-mer peptides (a mixture of peptides biotinylated at the N-terminal and C-terminal ends) spanning both HR2 and MPER domains. The amino acid sequence of each peptide is indicated by horizontal brackets. The first and last residue in the peptide panel is indicated in red as they can differ from the immunogens. The core binding epitopes for 2F5, 4E10 and 10E8 bnAbs are also indicated. Pre-immune serum was used as a negative control.

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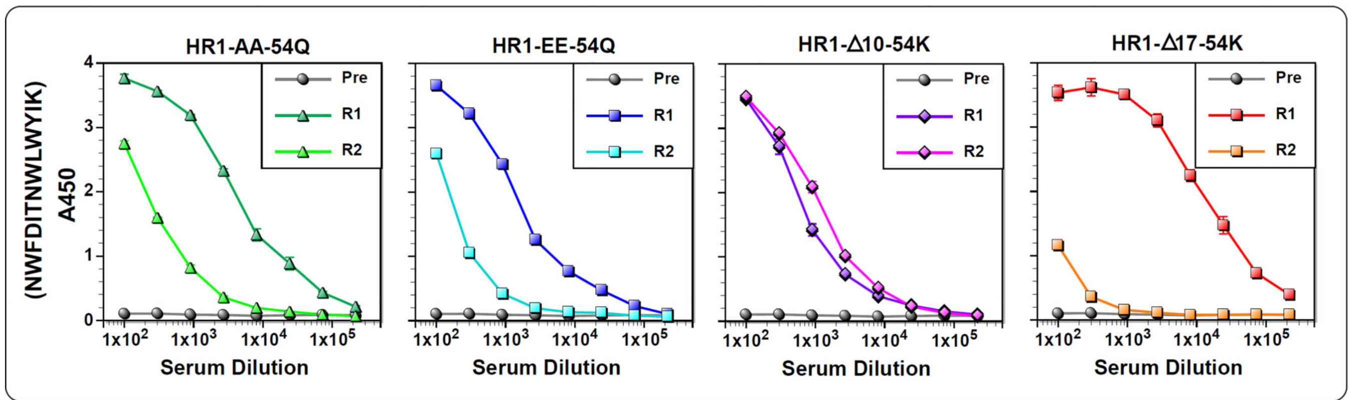

Fig 5.

Antibody titers against a wild type peptide containing C-terminal 13 amino acids. Serum samples collected after the fourth immunization was evaluated for binding biotinylated 13-mer peptide (^{671}NWFDITNWLWYIK^{683}) that contains 4E10/10E8 epitopes. Pre-immune serum was used as negative control.

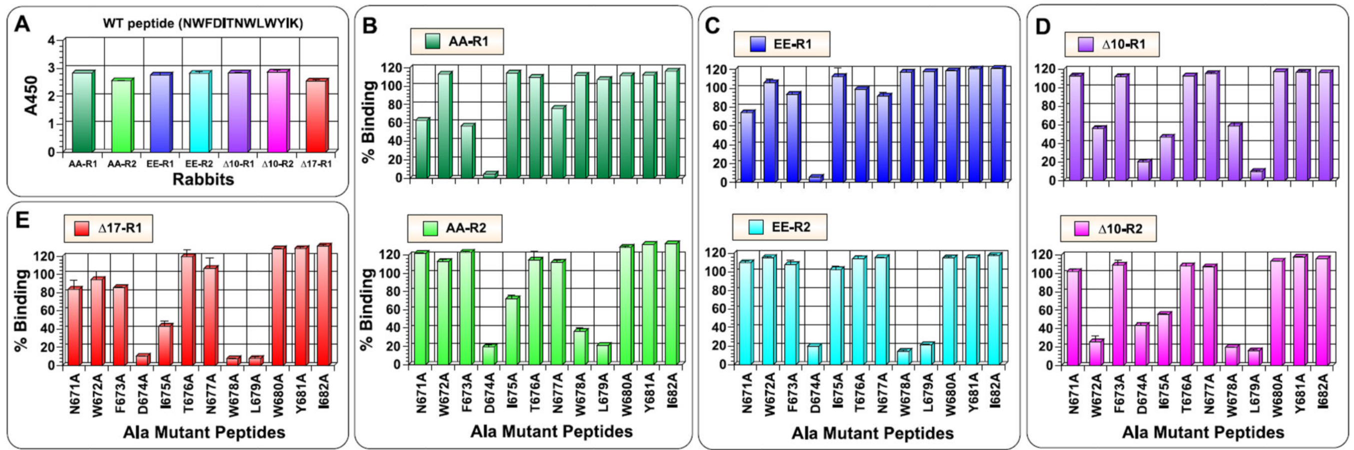

Fig 6.

Detailed epitope mapping analysis of antibodies against the C-terminal 13 amino acid residues using alanine-scanning mutant peptides. (A) Serum samples after the fourth immunization were examined for binding biotinylated 13-mer peptide (^{671}NWFDITNWLWYIK^{683}). The analyses were done using normalized serum samples to yield comparable binding signal (AA-R1 at 1:2000 dilution; AA-R2 and EE-R2 at 1:100 dilution; EE-R1 at 1:600 dilution; 10-R1 at 1:300 dilution; 10-R2 at 1:400 dilution; and 17-R1 at 1:5000 dilution). (B–E) The same dilutions were tested for binding to mutant peptides. Results are shown as the percentage of binding to the wild type peptide observed in panel (A).

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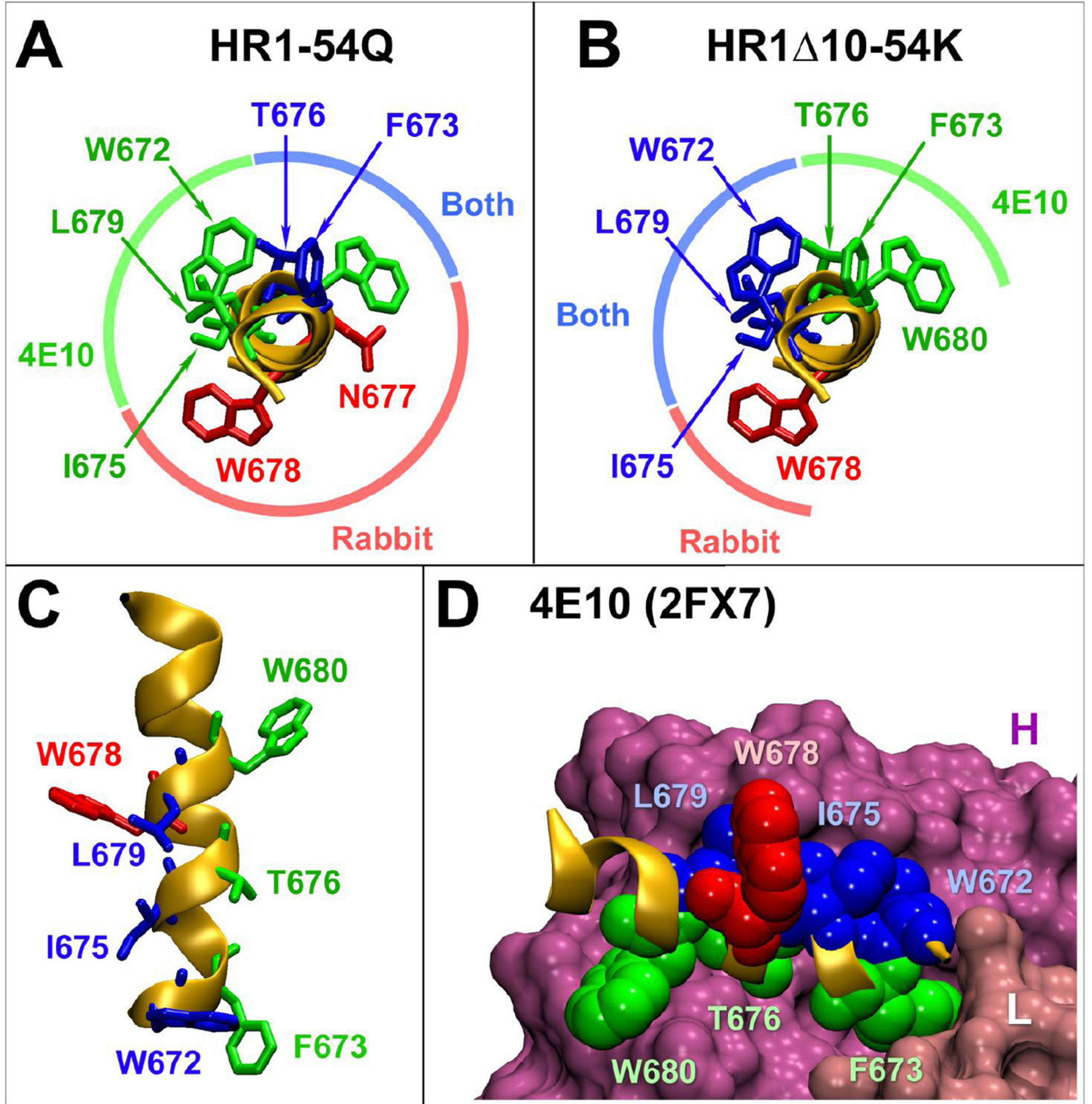

Fig 7.

Comparison of critical binding residues for antibodies induced by HR1-Δ10-54K and gp41-HR1-54Q relative to 4E10. (A) Critical binding residues for 4E10 and antibodies induced by gp41-HR1-54Q are plotted onto the peptide co-crystalized with 4E10 (pdb: 2FX7) (Cardoso et al., 2007). Residues critical for 4E10 or rabbit antibody only are shown in green and red, respectively. Residues important for both are shown in blue. (B) Critical binding residues for antibodies induced by HR1-Δ10-54K were also plotted onto the same peptide revealing significant difference from the pattern observed with gp41-HR1-54Q. (C) A lateral view of

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the peptide displaying critical binding residues for 4E10 and HR1- 10-54K-induced antibodies. (D) Position of all the HR1- 10-54K critical residues in context of the 4E10 bound peptide. The heavy and light chains for the antibody are indicated as H and L.