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The role of amino acid changes in the human immunodeficiency virus type 1 transmembrane domain in antibody binding and neutralization

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ABSTRACT

The detailed interactions between antibodies and the HIV-1 envelope protein that lead to neutralization are not well defined. Here, we show that several conservative substitutions in the envelope gp41 led to a ~100 fold increase in neutralization sensitivity to monoclonal antibodies (MAbs) that target gp41: 4E10 and 2F5. Substitution at position 675 alone did not impact neutralization susceptibility to MAbs that recognize more distal sites in gp120 (b12, VRC01, PG9). However, changes at position 675 in conjunction with Thr to Ala at position 569 increased the neutralization sensitivity to all gp41 and gp120 MAbs and plasma, in some cases by more than 1000-fold. Interestingly, the T569A change had a dramatic effect on b12 binding, but no effect on neutralization sensitivity. This finding suggests that antibody neutralization may occur through a multi-step pathway that includes distinct changes in envelope conformation that may affect binding but not neutralization susceptibility.

Introduction

Neutralizing antibodies (NAbs) play an important role in driving viral escape during HIV-1 infection (reviewed in (Burton et al., 2005)). HIV-1 specific NAb also have the potential to contribute to protection from infection, as evidenced by studies showing that passively administered HIV-1 specific monoclonal antibodies (MAbs) can prevent SHIV infection in non-human primates (reviewed in (Hu, 2005; Mascola, 2003)). However, antibodies capable of neutralizing a diverse spectrum of HIV-1 variants will be needed to achieve significant protection against circulating strains of HIV-1. While there are some HIV-1 specific NAbs that have broad specificity (Scheid et al., 2011; Walker et al., 2009, 2011; Wu et al., 2010, 2011), many virus isolates are not recognized by such MAbs, even those that target conserved regions of the virus (Blish et al., 2007, 2008, 2010; Scheid et al., 2011; Walker et al., 2009, 2011; Wu et al., 2010, 2011). The molecular basis for differences in neutralization sensitivity, especially in cases where the amino acid changes are outside of known epitope targets, remains poorly defined.

The envelope protein (Env) surface unit (gp120) and the transmembrane protein (gp41) are both targets of NAbs, including several MAbs that have been studied in some detail (reviewed in (Burton et al., 2004; Zolla-Pazner and Cardozo, 2010)). Two of the most intensively studied MAbs, 2F5 and 4E10, target adjacent conserved epitopes in the membrane proximal external region (MPER) of gp41: ELDKWA and NWF(D/N)IT, respectively; (Muster et al., 1993; Zwick et al., 2001). These MAbs bind to their peptide epitope target (Cardoso et al., 2005; Ofek et al., 2004), and they also bind weakly to membrane lipids but this binding alone does not induce neutralization (Julien et al., 2010; Xu et al., 2010).

There are also multiple antibody targets in the surface unit gp120. The IgG1 MAb b12 targets a discontinuous epitope overlapping the CD4 binding pocket (Burton et al., 1994; Roben et al., 1994). MAb b12 neutralizes a majority of subtype B variants (Binley et al., 2004; Burton et al., 1994), but fewer variants of other subtypes (Blish et al., 2007, 2009; Wu et al., 2006). More recently, VRC01, another MAb that targets the CD4 binding site, has been identified; VRC01 exhibits increased breadth and potency compared to b12 (Wu et al., 2010). A collection of related MAbs targeted to a different epitope in gp120 but with similar breadth as VRC01, have also been described recently (Walker et al., 2009). These MAbs, PG9 and PG16, recognize an epitope formed by conserved regions of V2 and V3 (Walker et al., 2009).

Early studies of antibody binding to HIV envelope focused on lab-adapted HIV-1 envelopes variants derived from virus grown in cell lines, which generally use the CXCR4 receptor. The study of these lab-adapted envelopes suggested that antibody neutralization correlated with binding to the envelope monomer (Parren et al., 1998a; Roben et al., 1994; Sattentau and Moore, 1995). Results of subsequent studies of envelope variants from viruses grown in primary cells, including CCR5-tropic variants that are more common in HIV-1 infection, suggested that antibody binding to monomeric envelope did not reliably predict neutralization potential (Fouts et al., 1997). Binding to the oligomeric form of envelope found on the virion has been correlated with neutralization sensitivity (Fouts et al., 1997; Sattentau and Moore, 1995; Stamatatos and Cheng-Mayer, 1995; Sullivan et al., 1995). However, there are numerous examples of MAbs that bind to virion-associated envelope protein,

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but do not neutralize the corresponding virus, suggesting that MAb binding alone is not sufficient to promote neutralization (Cavacini and Posner, 2004; Herrera et al., 2005; Leaman et al., 2010; Moore et al., 2006; Nyambi et al., 2000; Parren et al., 1998b). There is also evidence for binding between some trimeric envelope proteins and non-neutralizing antibodies in virion-capture assays (Poignard et al., 2003). Some of this binding can be explained by the potential of non-neutralizing antibodies to bind uncleaved envelope— an interaction that would not impact infectivity (Dey et al., 2009; Pancera and Wyatt, 2005). Thus, in examining differences in binding, the amounts of cleaved versus uncleaved envelope protein must be considered. Because many of the most detailed studies have focused on laboratory-adapted isolates or primary isolates obtained after culture, less is known regarding the NAb-binding properties of envelope variants obtained directly from infected individuals.

In a study of two closely related HIV-1 Env variants (Q461e2 and Q461d1) cloned directly from the blood of a subtype-A-infected individual, two amino acid differences were identified that altered neutralization sensitivity to a variety of NAb (Blish et al., 2008). Specifically, two changes in gp41, a T to A at position 569 (T569A) and an I to V change at position 675 (I675V) increased neutralization sensitivity to a variety of NAbs, including those that target gp41 as well as those that recognize distal sites in gp120 (Blish et al., 2008). HIV envelope mutations that impact neutralization susceptibility at distal sites had been reported prior to this study (Back et al., 1993; Klasse et al., 1993; Park et al., 1998, 2000; Reitz et al., 1988; Thali et al., 1994; Wilson et al., 1990; Zwick et al., 2005) and more recently (O'rourke et al., 2009; Shen et al., 2010) although these amino acid changes typically did not demonstrate the magnitude and/or breadth of the effects caused by the T569A and I675V changes. The T569A and I675V changes showed similar effects on neutralization sensitivity when introduced into other envelope variants, including variants from different subtypes (Blish et al., 2008; Wu et al., 2009). These findings suggest that the two amino acid positions in gp41 have a global impact on envelope structure that results in better exposure of epitopes throughout the protein. In this study, we took advantage of the differences in neutralization sensitivities conferred by these amino acid changes to examine how specific changes at distal sites impact antibody binding and neutralization. Our results suggest that for some antibodies the specific amino acid change have a concordant effect on both envelope protein binding and virus neutralization. However, in other cases, the effects of each change on binding and neutralization could be separated, suggesting a two-component model for the interaction of these amino acids, one to promote binding and the other to increase neutralization susceptibility.

Results

Characterization of the neutralization sensitivities of Q461 variant Envs

The neutralization sensitivity of the original Q461 variants was compared to variants engineered to contain other changes at position 675, which resides within the 4E10 epitope [Fig. 1; (Blish et al., 2008)]. Similar to previously published results, the combination of a change from Thr to Ala at position 569 and Ile to Val at position 675 introduced into the neutralization-resistant Q461e2 (Q461e2TAIV) resulted in a dramatic increase in neutralization sensitivity to various NAbs, including both MAbs and polyclonal NAbs in plasma pooled from HIV-positive individuals (Table 1). The T569A change alone had little impact on neutralization sensitivity: neither the pooled plasma nor MAb b12 achieved 50% neutralization of Q461e2TA while the MAbs 4E10 and 2F5 resulted in detectable but modest neutralization (IC_{50} values of 18.8 and 12.6 μg/ml, respectively). Virus with only the Ile to Val change at position 675 (Q461e2IV) showed >250-fold increase in neutralization sensitivity to the gp41-directed MAbs, 4E10 and 2 F5 (IC_{50} values of 0.1 μg/ml) when compared with Q461e2. However, this virus was resistant to both MAbs directed to gp120 as well as to the NAbs in pooled plasma as observed previously (Blish et al., 2008).

To examine whether the increase in neutralization sensitivity of the I675V variant to gp41-specific MAbs was due to the loss of an Ile or the presence of a Val at position 675, we investigated the neutralization sensitivity of Q461e2-derived viruses with other uncharged amino acids at this position, either alone or in combination with the T569A change. Viruses with a change from Ile to either an Ala or Thr at position 675 (Q461e2IA and Q461e2IT, respectively) showed similar neutralization profiles as Q461e2IV with IC_{50} values ranging from 0.04 to 0.3 μg/ml with 4E10 and 2F5. As observed for Q461e2IV, there was no detectable neutralization of Q461e2IA or Q461e2IT by either b12 or the pooled plasma at the highest concentrations tested (25 μg/ml for b12 and 1:50 dilution for the plasma pool).

Viruses with a change from Ile to either an Ala or Thr at position 675 paired with the T569A change (Q461e2TAIA and Q461e2TAIT, respectively), also showed similar neutralization profiles to a variant encoding the Val at 675 (Q461e2TAIV). The Q461e2TAIV, Q461e2TAIA, and Q461e2TAIT variants were ~6-fold more sensitive to neutralization by the gp41-directed MAbs 2 F5 and 4E10 than the corresponding variants that did not have the secondary mutation at position 569, with IC_{50} values ranging from less than 0.02–0.03 μg/ml. There was also an increase of ~10-fold or more in neutralization sensitivity to b12 of viruses with both mutations (Q461e2TAIA and Q461e2TAIT) compared to viruses with only the mutation at position 675 (Q461e2IA and Q461e2IT); in this case, the IC_{50} values ranged from 1.2 to 3.3 μg/ml. The effect of the combination of mutations at positions 569 and 675 was much more dramatic when tested against the neutralization activity of the plasma pool: no detectable neutralization was observed with the pooled plasma against the viruses with either of the single mutations at positions 569 or 675, whereas the IC_{50} values ranged from a dilution of 1547–5587 for the viruses with both changes.

In all cases, the IC_{50} values were similar (within 4-fold) when comparing viruses with these three different amino acid changes at 675, either alone or in combination with the Thr to Ala change at position 569. Thus, the presence of an Ile at position 675 is specifically associated with a neutralization-resistant phenotype; a variety of other amino acids at that position can confer neutralization susceptibility.

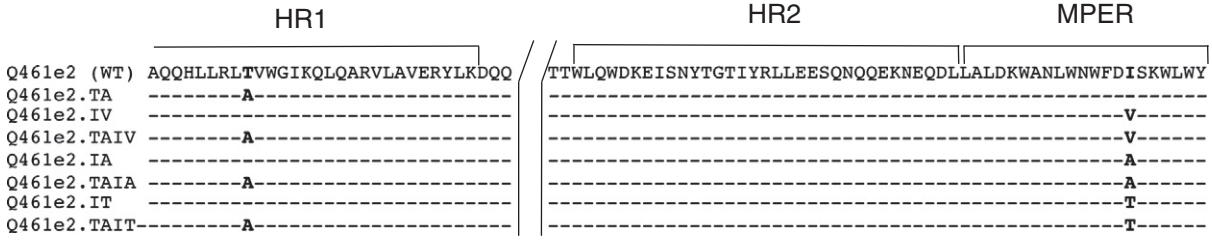

Fig. 1. Sequence changes of the Q461e2 mutants. The amino acid sequence of the parental, wild type Q461e2 in the relevant regions of the transmembrane domain, gp41, is shown. Differences in the mutant envelope variants are indicated. The clone names are shown to the left. HR1 and HR2 refer to heptad repeats 1 and 2, respectively and MPER refers to the membrane proximal external region of gp41.

Table 1
IC_{50} values for the MAb and plasma against pseudoviruses with the Q461 Env mutants.

Q461e2 mutant	MAb			Plasma pool[a]
	4E10[a]	2F5[a]	b12[a]	
e2	>25	>25	>25	<1:50
TA	18.8	12.6	>25	<1:50
IV	0.1	0.1	>25	<1:50
IA	0.1	0.04	>25	<1:50
IT	0.3	0.1	>25	<1:50
TAIV	0.03	<0.02	3.3	1547
TAIA	<0.02	<0.02	2.7	2080
TAIT	<0.02	<0.02	1.2	5587

[a] All values are concentraions of the MAb (in μg/ml) or reciprocal dilutions of the plasma required to achieve 50% neutralization. IC_{50} values are the arithemetic mean averages of three independent experiments performed in triplicate.

Effects of changes at position 569 and 675 on the Env content of virions

To determine if the differences in neutralization sensitivities were associated with differences in the levels Env on the viral particles, we performed western blots on the purified pseudoviral particles and quantified the Env and Gag p24 protein levels using a rabbit polyclonal antisera, which recognizes both uncleaved (gp160) and cleaved (gp120) forms of Env (Doria-Rose et al., 2005) and an anti-p24-specific MAb (Fig. 2). The majority of Env detected was uncleaved gp160 protein (Fig. 2a). In general, viruses encoding the Thr to Ala change at position 569 had the least amount of cleaved Env, whether T569A was alone or in combination with the second change at 675. In some cases, such as TAIT, the percent cleavage may be slightly overestimated because the signal for the uncleaved envelope is near saturation for that Env. Thus, the percentage of cleaved Env ranged from ≤21–26% among the variants encoding Ala at 569, compared to 39–50% among viruses with a Thr at position 569.

The levels of total Env protein on pseudoviruses derived from the mutant envelopes varied by less than 4-fold from that of the parental Q461e2 (Fig. 2b). The neutralization sensitive virus Q461e2TAIT had the highest amount of Env (≥3.5-fold more than Q461e2), mostly in the uncleaved form. Another neutralization-sensitive variant, Q461e2TAIV, along with the neutralization-resistant parental virus, Q461e2, had the lowest levels of total Env protein. Similar results were observed for uncleaved Env (gp160; Figs. 2a and b). In the case of cleaved Env, which is the presumed functional target of the neutralizing antibodies, the levels were within ~2-fold to those of the parental Q461e2 virus for the other viruses. Q461e2TAIV had the lowest amounts of cleaved Env and Q461e2TAIT had the greatest amounts of cleaved Env .

Similar patterns of expression were observed when the Env levels were normalized to Gag (p24) protein (Fig. 2c). The amounts of total Env/p24 varied ~2-fold compared to Q461e2; Q461e2TAIT had the greatest amount of total Env/p24 (~2-fold more than Q461e2) and Q461e2TAIA had the least amount of total Env/p24 (~half the amount of Q461e2). The amounts of cleaved Env/p24 was highest for Q461e2IT (~2-fold more than Q461e2) and lowest for Q461e2TAIA (~one-third that of Q461e2). Overall, there was no apparent correlation between the patterns of Env expression and the neutralization properties of the virus.

Effect of amino acid changes at position 569 and 675 on sensitivity to broadly neutralizing MAbs, PG9 and VRC01

Since the first description of the effect of the T569A and I675V amino acid changes on neutralization sensitivity (Blish et al., 2007), several potent MAbs have been isolated, notably VRC01 and PG9 (Walker et al., 2009; Wu et al., 2010). To determine if the amino acid changes at 569 and 675, which are outside of these MAb recognition sites, impact neutralization sensitivity, we examined the ability of the MAbs to neutralize virus bearing the original neutralization-resistant Q461e2 Env and associated mutants (Table 2). The pattern of neutralization susceptibility with these MAbs was generally similar to that with other MAbs: Q461e2 was more difficult to neutralize than Q461e2TAIV. However, the difference in neutralization sensitivity was relatively modest. In the case of PG9, 50% inhibition was not achieved for Q461e2 at the maximum concentration tested (10 μg/ml). The MAb PG9 did neutralize Q461e2TAIV with modest potency (IC_{50} 2.6 μg/ml); and there was also some detectable neutralization of the variant bearing the T569A mutation alone (IC_{50} 7.2 μg/ml), but neutralization was not achieved at the highest concentration tested (10 μg/ml) for the variant bearing Env with a change at position 675. Similar results were observed with VRC01: the combination of T569A and I675V increased neutralization susceptibility compared to the parental Q461e2 (IC_{50} 0.3 μg/ml and 1.6 μg/ml, respectively); the individual mutants had an intermediate effect (Table 2).

Effect of mutations that alter neutralization sensitivity on binding to b12, a MAb targeted to gp120

A flow cytometric assay using 293T cells expressing the various Env variants was performed to determine the binding properties of NAbs to the Env mutants, and to examine whether the mutations that confer enhanced neutralization sensitivity also increase binding to the same NAbs. IgG b12, which binds to an epitope overlapping the CD4 binding site in gp120 (Burton et al., 1994; Roben et al., 1994), was used to probe the effects of the two gp41-associated changes on NAb binding at distal sites in gp120. SF162, a highly neutralization-sensitive Env (Binley et al., 2004; Saunders et al., 2005), was used as a positive control for the flow cytometry experiments. As shown in Fig. 3, b12 binding was evident by an increase in fluorescence of cells expressing SF162 Env compared to mock transfected cells. There was no evidence of binding to cells transfected with SIV Env, suggesting that the b12 binding was specific for HIV-1 Env. In the case of cells expressing Env variants encoding only the change at position 675 (Q461e2IV, Q461e2IA, Q461e2IT), there was no clear shift in fluorescence intensity in the presence of b12. However, cells expressing the Env variant with the change from Thr to Ala at position 569 (Q461e2TA) showed a clear and reproducible shift in fluorescence intensity, indicating that this variant is capable of binding b12 MAb even though it is not sensitive to neutralization by this NAb. The most pronounced binding was observed with the neutralization-sensitive variants encoding both changes in the gp41 (Q461e2TAIV, Q461e2TAIA and Q461e2TAIT), resulting in clear shifts in fluorescence intensities similar to that of cells expressing SF162 Env.

Env levels in cells used in these flow cytometry experiments were evaluated by western blot using a rabbit polyclonal antisera (Fig. 3b). As seen previously, the envelope proteins of the subtype B envelope variant SF162 have increased mobility compared to Q461e2, which is common among HIV envelope variants because of differences in envelope length and glycosylation (Blish et al., 2007). The overall levels of total Env expressed in 293T cells were similar across all Q461e2 variants (within 2-fold), but there was variation up to ~5-fold in the fraction of Env that was cleaved. Q461e2 had the highest percentage (~75%) of cleaved Env. Cells expressing Env encoding the T569A change, either alone or in combination with a change at 675, had the smallest percentage of cleaved Env. The percentage of cleaved Env ranged from ~15–17% among cells expressing Envs with an Ala at 569, compared to ~66–75% among the cells expressing Env proteins with a Thr at position 569. The cells expressing the envelope variants with the highest level of binding to b12 had the highest levels of unprocessed Env and the lowest levels of Env gp120. However, it is important to note that even for the 461e2 and I675 envelope variants that did not bind b12 in this assay, there was a substantial amount of unprocessed envelope, in some cases, similar (within 2-fold) to the levels found in envelope variants that did bind b12 (compare Q461e2IV and Q461e2TAIV). This suggests that differences in b12 binding cannot simply be due to binding to unprocessed envelope protein.

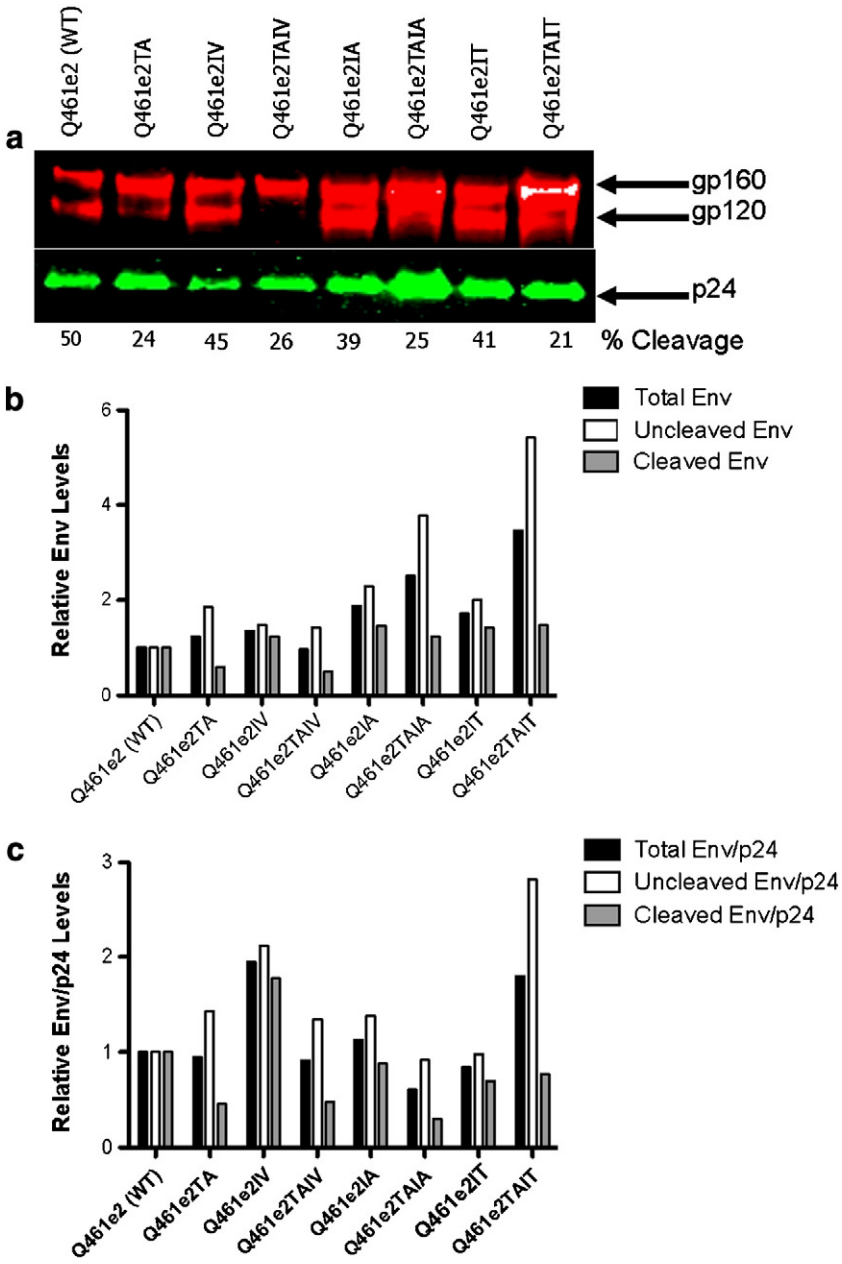

Fig. 2. Western blot analyses of Env (i.e., gp160 and gp120) and Gag (i.e., p24) of Q461e2-associated pseudoviral supernatants. (A) Western blot of Q461e2-associated pseudoviral supernatants probed with a combination of anti-Env polyclonal rabbit primary Ab, which recognizes uncleaved Env (i.e., gp160) and cleaved Env (i.e., gp120), and anti-p24 mouse Ab for the detection of Gag (i.e., p24). The Env tested is shown above and the % Env cleavage is shown below. Percent cleavage was calculated as the amount of gp120/(gp120 + gp160). (B) Comparison of Env levels of mutants relative to Q461e2. For each variant (designated below bar graph) the total, cleaved, and uncleaved Env levels were calculated relative to Q461e2, which thus has a level of 1. (C) Comparison of Env/p24 levels of mutants relative to Q461e2. For each variant (designated below the bar graph) the total, cleaved and uncleaved Env levels divided by p24 levels are shown relative to Q461e2.

Table 2
IC_{50} values for the MAbs PG9 and VRC01 against pseudoviruses with the Q461 Env mutants.

Q461e2 mutant	PG9[a]	VRC01[a]
e2	>10	1.6
TA	7.2	0.7
IV	13.5	0.8
TAIV	2.6	0.3

[a] All values are concentraions of the MAb (in μg/ml) required to achieve 50% neutralization. IC_{50} values are the arithemetic mean averages of three independent experiments performed in triplicate.

Characterization of the binding of Q461 Envs to MAbs targeting the gp41 protein, 2F5 and 4E10

We also examined the binding properties of the gp41-directed MAbs, 2F5 and 4E10, using cells expressing the same panel of Env variants. In the case of 2 F5, there was a subtle but reproducible shift in fluorescence for cells expressing the positive control, SF162 Env (Fig. 4a). For the parental, neutralization-resistant Env Q461e2 and the Q461e2TA variant, which is also relatively resistant to 2F5 neutralization, the fluorescence intensity of cells expressing these Envs was similar to or just slightly greater than the fluorescence intensity of mock-transfected cells and cells expressing the SIV Env. There was more significant and consistent shift in fluorescence intensity for each of the viruses encoding a change from Ile at position 675,

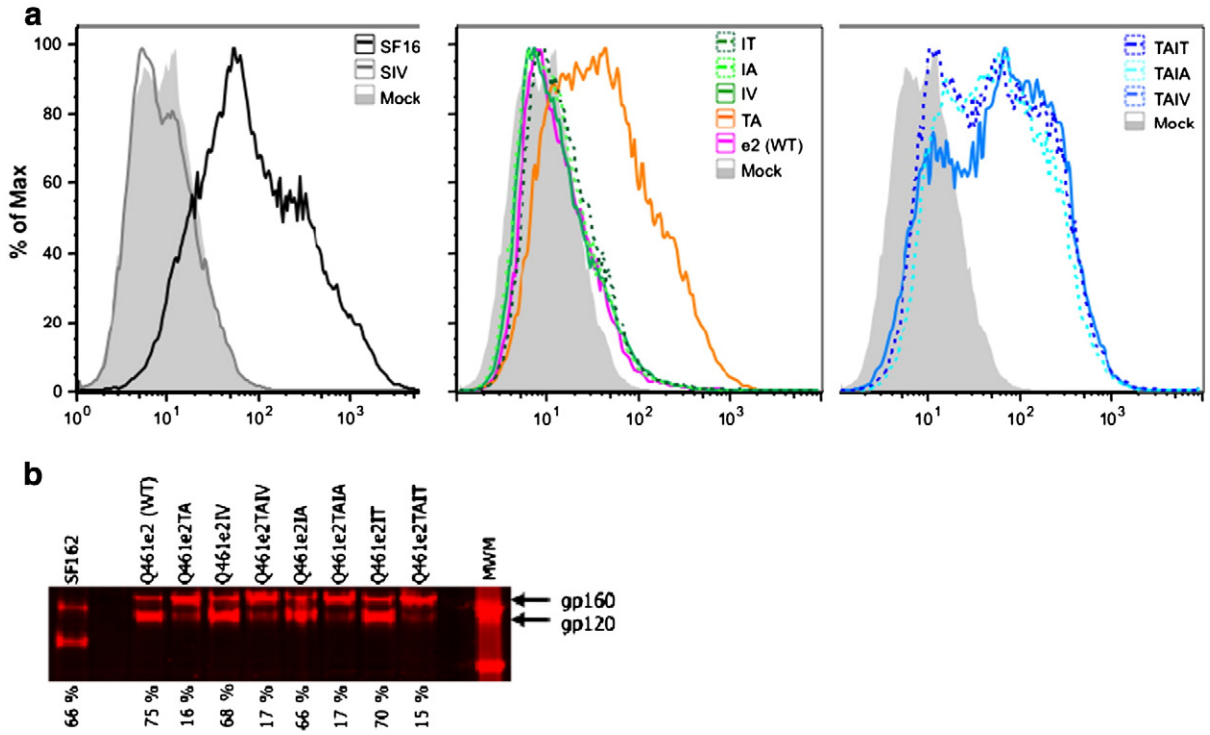

Fig. 3. (A) Flow cytometry analysis of b12 binding to cells expressing Q461e2 Env variants. The gray shaded profile denotes fluorescence for cells transfected in the absence of plasmid (Mock transfected cells). The color designation for the fluorescence profile for the various Envs is shown within each panel. (B) Western blot of cells expressing the Q461e2-associated Envs probed with an anti-Env polyclonal rabbit primary Ab, which recognizes uncleaved Env (i.e., gp160) and cleaved Env (i.e., gp120). The bands corresponding to gp160 and gp120 are designated to the right. For each lane, the Env tested is shown above the blot, with the wild type (WT), parental Q461e2 indicated. The percent of Env cleavage is shown below. Percent cleavage was calculated as the ratio of gp120/(gp120 + gp160). MWM indicates molecular weight marker lane, with markers shown 181 kDa and 115 kDa.

either alone (Q461e2IV, Q461e2IA, Q461e2IT) or in combination with the Thr to Ala change at position 569 (Q461e2TAIV, Q461e2TAIA, Q461e2TAIT), suggesting increased binding compared to the parental Env. There was little to no detectable binding to 4E10 for cells expressing any of the envelope variants (data not shown), perhaps reflecting the poor binding of this MAb to its MPER peptide target when the Env is in its native, untriggered, membrane-bound conformation or steric occlusion of the epitope at the cell surface.

Similar patterns of Env expression were observed in the cells used for the gp41-MAb flow cytometry experiment (Fig. 4b) to those used to examine b12 binding (Fig. 3b). Because the target for binding in this experiment was the gp41, we performed Western blot analysis of the cells using a cocktail of MAbs that detects various epitopes in Env including gp41 (Fig. 4c) (Moore et al., 2006). The g41 protein appeared as a doublet, which has been attributed to different glycolsylated forms of gp41 in prior studies (Phogat et al., 2008). Cells expressing any envelope with the T569A change had the lowest amount of cleaved Env, ranging from 22 to 23% of the total Env (Fig. 4c). While the relative amounts of total Env were generally similar across the virus panel between the MAb cocktail and the polyclonal rabbit sera primary antibodies, the estimated amount of cleaved Env differed by as much as ~2-fold depending on the antibody used. In some case, a portion of the difference may be due to saturation of the signal from the uncleaved Env in the Western analysis using the MAb cocktail, which could lead to overestimates of the ratio of cleaved to uncleaved Env. Importantly, the overall findings are the same with the different antibody probes: the levels of cleaved Env are consistently lower for variants with the T569A mutation. However, the magnitude of the difference between the T569A and other variants may be as little as ~2-fold or as much as ~5-fold. These data also highlight the potential differences in detection of specific protein variants depending on the antibody used for analyses (Herrera et al., 2005; Moore et al., 2006).

Characterization of the binding of 4E10 to Q461 peptides using SPR

The I165V change lies within epitope for 4E10, and thus could impact 4E10 binding. To examine the direct effect of amino acid changes at position 675 on the binding of 4E10, we performed SPR equilibrium experiments on immobilized peptides spanning the linear 4E10 epitope of Q461e2 and the IV, IA and IT variants plus four C-terminal lysine residues added to facilitate coupling (NWFD**I**SKWLWY*IKKKK*; Fig. S1; (Blish et al., 2007)). Binding analyses with Fv versus Fab forms of 4E10, representing independent determinations of the interactions, agreed within ~two-fold, showing good reproducibility and agreement, consistent with previous analyses ((Xu et al., 2010); Table 3 and Figs. S1a and b). 4E10 Fv and Fab analytes bound to peptides representing the conserved Q461e2 sequence, containing an Ile at position 675, no more than ~seven-fold more tightly than peptides representing any of the neutralization-sensitive sequences with a Val, Ala, or Thr at this position, with the largest differences between the Q461e2 and Q461e2IT peptides. Such small differences in affinity could easily be accounted for by slight entropic effects from changes in conformational flexibility (substituting an alanine for β-branched residues) or hydrophobicity (substituting more polar alanine and threonine residues for isoleucine or valine).

Discussion

An understanding of the mechanisms of HIV-1 neutralization susceptibility, and how natural genetic variation in HIV-1 affects this process, are important for rationale design of HIV-1 envelope immunogens. The studies presented here suggest that two positions (569 and 675) in the Env gp41 that alter neutralization susceptibility to a range of broadly neutralizing antibodies do so by inducing changes in Env conformation. Changes at positions 569 and 675, which were first identified among envelope variants cloned directly from an infected individual (Blish et al.,

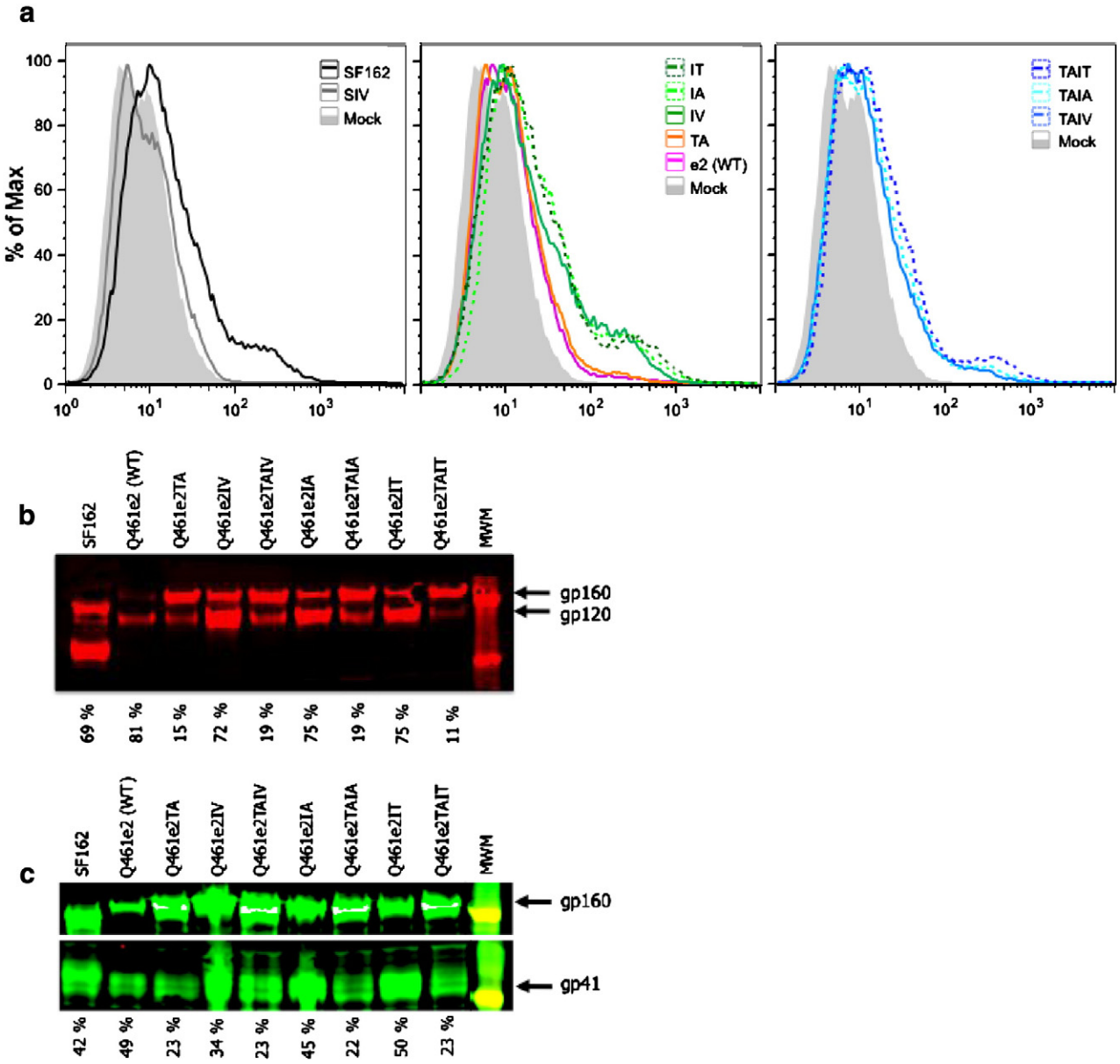

Fig. 4. (A) Flow cytometry analysis of 2F5 binding to cells expressing Q461e2 Env variants. The gray shaded profile denotes fluorescence for cells transfected in the absence of plasmid (Mock transfected cells). The color designation for the fluorescence profile for the various Envs is shown within each panel. (B) Western blot of cells expressing the Q461e2-associated Envs probed with an anti-Env polyclonal rabbit primary Ab. The Env tested in shown above the blot with the parental wild type (WT) Q461e2 indicated; the percent Env cleavage is shown below. The bands corresponding to gp160 and gp120 are designated. Percent cleavage was calculated as the ratio of gp120/(gp120 + gp160). MWM indicates molecular weight marker lane, with markers shown 181 kDa and 115 kDa. (C) Western blot analysis of the same cell lysates as in panel B using a MAb cocktail. The MAb cocktail included 1 μg/ml of 2 G12, b12, 2 F5, and 4E10, which recognizes uncleaved Env (i.e., gp160) and cleaved Env (i.e., gp41(Moore et al., 2006)), Designations are as in panel B. MWM indicates molecular weight marker lane, with markers shown 181 kDa in the upper panel and 48.8 and 37.1 KDa in the lower panel.

2008), had variable impacts on binding to different antibodies. Increased antibody binding did not always result in increased neutralization sensitivity and increased neutralization sensitivity was not always reflected by increased binding to the epitope target. These findings support a model in which gp41 changes alter Env conformation to expose a variety of epitopes on both gp41 and gp120, but in a manner that is not directly correlated to changes in MAb binding.

A highly conserved Ile at position 675 contributes to a neutralization-resistant phenotype. A change from Ile to either Ala, Thr or Val increased susceptibility to the gp41-specific antibodies 4E10 and 2F5, but did not affect susceptibility to gp120-directed MAbs, b12, VRC01 or PG9, unless paired with a second change at position 569. The Ile 675 lies within the core epitope for 4E10 (NWFN(D)IT(S); (Zwick et al., 2001)), and it is part of the hydrophobic face that contacts the 4E10 Mab (Cardoso et al., 2005). However, the dramatic increase (~400-fold) in neutralization sensitivity that results from a change away from Ile is not due to increased binding affinity for the core epitope, because the amino acid changes at 675 have a very small effect on 4E10 Fv binding to peptide. These results using quantitative SPR binding analyses suggest that differences in

Table 3
K_D values for 4E10 Fv and Fab analytes interacting with epitope peptides.

Peptide	Fv 4E10 (nM)	Fab 4E10 (nM)
e2	20 ± 2	10 ± 1
IV	53 ± 3	25 ± 3
IA	39 ± 2	40 ± 2
IT	71 ± 3	64 ± 3

neutralization due to a change away from Ile 675 cannot be explained by differences in binding to the specific peptide target. Previous studies suggested that neutralization differences also cannot be explained by a prolonged exposure of the peptide during fusion (Blish et al., 2008). Therefore, these findings support a model in which an amino acid other than Ile at position 675 causes a conformational change that in some manner allows better access of a variety of antibodies to their epitopes.

We detected weak binding of 2F5 to Env on the cell-surface, and there is some evidence that 2F5 binds both the unliganded trimer as well as a fusion intermediate (de Rosny et al., 2004), although other studies have not detected 2F5 binding, even to viral Envs that were sensitive to neutralization by this Mab (Cavacini and Posner, 2004). There was a subtle signal suggesting binding of 2F5 to neutralization resistant variants, including the parental envelope, Q461e2, but the shift in fluorescent signal was less than for variants encoding the Ile 675 changes. Overall, the data suggest that the change from Ile at position 675 results in a modest increase in binding to 2F5 that corresponds to a dramatic increase in neutralization sensitivity. However, due to the poor signal to noise ratio in the 2F5 flow cytometry experiments, we interpret this finding with caution.

The Thr to Ala at position 569 is sufficient to permit antibody binding to gp120, as evidenced by binding to the MAb b12. b12 targets a discontinuous epitope overlapping the CD4 binding pocket in gp120 (Burton et al., 1994; Roben et al., 1994)). There are no differences in the CD4 binding loop sequence among the variants studied here; all the viruses differs from the canonical CD4 binding loop sequence at positions 369 and 372, both of which are variable among natural isolates and not directly linked to neutralization sensitivity(Wu et al., 2009). Thus, differences in binding cannot be ascribed to differences in the target sequences that bind to the b12 MAb. Differences in binding are also not due to differences in the amount of total or cleaved Env on the cell surface as the T569A variant had similar total Env and lower levels of cleaved Env compared to Q461e2.

The percentage of uncleaved, gp160 protein was high for the Q461e2TA variant, 2–5-fold higher than the levels found on cells expressing variants that did not bind b12 (e.g. Q461e2 and the I675A/T/V mutants). Because non-neutralizing antibodies can bind uncleaved envelope (Dey et al., 2009; Pancera and Wyatt, 2005), we considered the more trivial explanation that the increased binding to the T569A mutant at least partially reflects binding to uncleaved, non-functional trimers (Herrera et al., 2005; Moore et al., 2006). If this were the case here, one would expect to see at least some relationship between the amounts of uncleaved envelope and the extent of the shift in fluorescence intensity due to specific antibody binding, perhaps in addition to the desired read-out of binding due to any specific interactions with the cleaved trimer. However, there was no detectable b12 binding to the parental Q461e2 envelope and variants with changes at 675, even though as much as 51% of the envelope protein was uncleaved, depending on the antibody used for Western analysis. Thus, if the increased binding due to the T569A mutation was simply due to increased levels of uncleaved envelope, we would expect that there would be detectable b12 binding to all the Env proteins. This was not the case, and the magnitude of the increase in fluorescent signal for the Q461e2TA versus the wild-type and 675 mutant envelope proteins do not support the model that increased binding of b12 to Q461e2TA is simply due to increased amounts of uncleaved Env. Rather, these data suggest that there is a conformational effect that results from the T569A amino acid change in the gp41 that leads to b12 binding to Env expressed on the cell surface.

Studies of Wu et al., 2009 (Wu et al., 2009), demonstrated that the T569A change, when introduced into a b12-resistant subtype B variant (CAAN5342.A2), enhanced neutralization sensitivity to b12 and other MAbs. Thus, the T569A change may impact b12 sensitivity in the absence of other changes in certain viral contexts, but not in others (e.g. Q461e2). In the study of Wu et al., it was not possible to discern the effects of this mutation on Env-b12 binding because only binding to monomeric gp120 was examined, which would not reflect conformational changes contributed by the T569A change in gp41. However, the findings that T569A can increase neutralization sensitivity support the model that this amino acid change may induce a conformational change that allows antibody access to key b12 binding residues, resulting in b12 binding to the membrane-associated form of the envelope protein. In the context of some Env variants, such as the subtype B virus CAAN5342.A2, this interaction may be sufficient to promote neutralization. However, in other cases, such as the subtype A, Q461e2 variant studied here, the T569A may increase b12 binding, but the second mutation at I675 is needed to render the envelope sensitive to b12 neutralization. A second, as yet unidentified amino acid position in other virus variants, such as the subtype B virus mentioned above, may be playing a similar role. Interestingly, the I675 changes alone do not impact either b12 binding or neutralization. These data therefore suggest that these gp41 changes participate in a two step process to promote neutralization by b12 antibody — one, induced by the T569A change, is a conformational change that permits b12 access to key binding residues; another, which results from a I675A/T/V change only occurs in the context of the conformational change induced by the T569A change. For the I675 changes, in the presence of T569A, but not in its absence, there is both increased binding and enhanced susceptibility to b12 neutralization.

While the combination of the 569 and 675 changes had a dramatic effect on neutralization susceptibility to some MAbs and to polyclonal plasma antibodies from infected individuals, they had less effect on the more potent MAbs, PG9 and VRC01. Thus, while these two changes in gp41 may induce conformational changes that have a broad impact on neutralization susceptibility to a range of antibodies, their effect on antibody binding and neutralization varies depending on the epitope target. It is noteworthy that changes in neutralization sensitivity were not always reflected by changes in antibody binding, for example, in the case of b12, where a change at 569 that increased antibody binding was needed for the second 675 substitution to affect neutralization sensitivity. It may be that for some virus-antibody combinations, the Env conformation permits some aspects of this two-step process to occur in the absence of these amino acid changes. Thus, each change individually has a more modest effect on neutralization sensitivity. Overall, these findings suggest that antibody neutralization likely involves higher-order conformational changes that result from multiple amino acid changes in envelope acting in concert, only some of which directly impact antibody binding.

Materials and methods

Cloning and generation of HIV-1 viral envelope proteins

The full-length *env* clone Q461e2 was amplified directly from peripheral blood mononuclear cells from a Kenyan woman at 28 days post-infection (Long et al., 2002). The Quik-Change site-directed mutagenesis kit (Stratagene) was used to create Q461e2 *env* mutants using primers containing the desired mutations that encode amino acid changes at positions 569 and 675. Clones were sequenced to identify those that had solely the desired change and these were used in subsequent experiments.

Plasma samples and antibodies

The plasma pool was generated by pooling plasma collected between 1998 and 2000 from 30 HIV-1-positive individuals in Mombasa, Kenya (Blish et al., 2007). The MAbs used in the neutralization and flow cytometry experiments were as follows: 2F5 and 4E10 were provided by Hermann Katinger (Polymun Scientific, Vienna, Austria), and b12 was provided by Quality Biological Inc. (Gaithersburg, MA). PG9 was kindly provided by Dennis Burton and the IAVI team, VRC01 by John Mascola, and the rabbit polyclonal antisera generated to HIV-1 Env (Doria-Rose

et al., 2005) was kindly provided by Nancy Haigwood (Oregon Health and Science University).

Generation of pseudoviruses and infectivity assays

HIV-1 *env* clones, as well as SIVMne Clone 8 *env* (Pineda et al., 2007), were used to generate pseudoviral particles by cotransfection of 293T cells with the viral plasmid containing the *env* of interest and Q23Δ*env* (Long et al., 2002; Poss and Overbaugh, 1999) DNA, a subtype A HIV-1 proviral clone with a partial deletion in *env*. Transfections were performed using polyethylenimine (PEI; Polysciences, PA), and Q23Δ*env* DNA preparations were used at a 20:1 w/w backbone/*env* ratio to complement the *env* expression plasmids and create pseudovirus, as previously described (Blay et al., 2007). After 48 hours, viral supernatants were harvested through a 0.22 μm sterile filter and infectious titers were determined by infecting TZM-bl cells (NIH AIDS Research and Reference Reagent Program) in the presence of 10 μg/ml DEAE-dextran (Sigma) (Vodicka et al., 1997). All pseudoviral supernatants were concentrated prior to aliquoting using an Amicon Ultra centrifugal 100 kDa MWCO cellulose membrane filter device (Millipore), typically resulting in a four-fold increase in viral titer.

All neutralization assays were performed in triplicate with at least two independent preparations of pseudoviral stock, as previously described (Blish et al., 2007; Wu et al., 2006)). Median inhibitory concentrations (IC_{50}s) were defined as the concentration of MAb or the reciprocal dilution of plasma pool stock that resulted in 50% inhibition, calculated as described (Blish et al., 2007; Wu et al., 2006). When the virus was not neutralized to 50% at the highest neutralizing antibody concentration tested, the highest concentration tested (25 μg/ml) was used for calculating the mean IC_{50}.

Flow cytometry analysis

Cells expressing the relevant Env variant were generated by transfecting 293T cells with the viral Env DNA. Briefly, 4×10^6 cells were seeded the day before transfection, transfected with PEI and 12 μg of HIV-1 *env* DNA, replaced by medium within 16 h, and cells were harvested approximately 72 hours post-transfection. To stain cells for flow cytometry, cells were resuspended in wash buffer (WB; 2% fetal bovine serum in PBS; Gibco) then incubated at 37 °C for 60 min in primary antibody (e.g., MAb or plasma pool) at a final concentration of 20 μg/ml. Following primary antibody incubation, cells were washed and incubated at 37 °C for 40 min with secondary antibody, goat-anti-human IgG-PE (Santa Cruz Biotech, US), at a 1:100 dilution. Cells were rewashed, resuspended in 1% paraformaldahyde to fix, and stored at 4°°C in the dark until flow cytometry processing. All flow cytometry was performed on Becton-Dickinson FACS Calibur and data was analyzed using FlowJo software.

Quantified western blotting of pseudoviral envelope and envelope-expressed 293Ts

Purified pseudoviral preparations were prepared for Western blotting using a modification of a previously described protocol (Blish et al., 2007). The amount of virus used was normalized based on titer defined by infectivity in TZM-bl cells. Briefly, pseudoviral supernatants were pelleted by microcentrifugation at 16,000 *g* for 60 min at 8 °C and the viral pellet was resuspended in lysis buffer (1% Triton X-100 [Sigma]/PBS) to ablate viral infectivity. Samples were then boiled at 100 °C for 10 min, immediately put on ice, and then stored at −20 °C. Prior to loading on the gel, samples were thawed, thoroughly mixed with SDS loading buffer and reducing agent (Invitrogen), followed by an additional 10 min boil to ensure complete protein denaturation. Samples were resolved on a 4–12% gradient Bis-Tris polyacrylamide gel (Invitrogen), followed by electrotransfer to a nitrocellulose membrane. A dilution of 1:8000 of rabbit polyclonal antisera generated to HIV-1 Env (Doria-Rose et al., 2005) was used as a primary antibody and IRDye700DX-conjugated goat-anti-rabbit IgG at a dilution of 1:15,000 was used as a secondary antibody (Rockland Immunochemicals). A cocktail of MAbs 4E10, 2F5, 2G12, and b12 at 1 μg/ml were used as primary antibodies (modified from (Moore et al., 2006)) and IRDye800DX-conjugated goat-anti-human IgG at a dilution of 1:8000 was used as a secondary antibody (Rockland Immunochemicals). To determine p24 levels, mouse anti-p24 (cat. no. 4121; NIH AIDS Research and Reference Reagent Program) was used as a primary antibody and IRDye800DX-conjugated goat-anti-mouse IgG was used as a secondary antibody (Rockland Immunochemicals). Western blotting and protein quantification was performed using the Odyssey infrared imagining system (LI-COR Biosciences), which has a large dynamic detection range.

Percent cleavage was calculated as amount of gp120 signal/total amount of Env signal (both gp160 and gp120) × 100. The fold-difference between an envelope variant compared to the wild type parental Env Q461.e2 was defined as the amount of gp120 for that variant Env/amount of gp120 for the wild type, Q461e2.

SPR analyses of the interactions between Fab or Fv 4E10 against peptide

Protein/peptide interaction analyses by surface plasmon resonance (SPR) were conducted at 25 °C in HBS-EP buffer (Biacore AB) on a Biacore T100 system. For analyses of the binding of 4E10 Fv and Fab analytes to peptides corresponding to the 4E10 epitope in clade A Q461e2 *env* variant (wild type and mutants; Table 3), peptide at 10 to 50 μg/ml in 10 mM sodium acetate (pH 5.0) was covalently immobilized on a CM5 research-grade sensor chip (Biacore AB) by standard amine coupling chemistry following the manufacturer's protocols; reference flow cells were left blank. Four C-terminal lysine residues were added to each peptide sequence to aid coupling and match peptides used in prior studies (Cardoso et al., 2007; Xu et al., 2010). Immobilization of ~200 to 270 response units (RU) resulted in optimal responses in subsequent analyses. Different concentrations of 4E10 Fv or Fab analytes were injected in randomized duplicate runs, at a flow rate of 10 μl/min for 180 s to reach equilibrium, followed by a 120 second dissociation phase. Regeneration of the sensor chip was achieved by injection of 10 mM glycine (pH = 1.5) at a flow rate of 20 μl/min for 6 s followed by HBS-EP buffer stabilization for 180 s. Sensorgrams obtained from SPR measurements were blank-corrected by the double-referencing method (Myszka, 1999). Equilibrium dissociation constants (K_D) were estimated by plotting steady-state binding levels of 4E10 Fv or Fab analytes on sensorchip-coupled epitope peptides against analyte concentrations with BIAevaluation 2.0 software (Biacore AB). Other anti-HIV-1 MAbs were also tested for binding to immobilized Q461e2 peptides, with no measurable responses for 800 nM Fab 447 or 800 nM Fab b12 analytes, showing the specificity of the 4E10 responses (data not shown).

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