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Induction of antibody-mediated neutralization in SIVmac239 by a naturally acquired V3 mutation

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ABSTRACT

Achieving humoral immunity against human immunodeficiency virus (HIV) is a major obstacle in AIDS vaccine development. Despite eliciting robust humoral responses to HIV, exposed hosts rarely produce broadly neutralizing antibodies. The present study utilizes simian immunodeficiency virus (SIV) to identify viral epitopes that conferred antibody neutralization to clone SIV/17E-CL, an in vivo variant derived from neutralization resistant SIVmac239. Neutralization assays using rhesus macaque monoclonal antibodies were performed on viruses engineered to express single or multiple amino acid mutations. Results identified a single amino acid mutation, P334R, in the carboxy-terminal half of the V3 loop as a critical residue that induced neutralization while retaining normal glycoprotein expression on the surface of the virus. Furthermore, the R334 residue yielded neutralization sensitivity by antibodies recognizing diverse conformational and linear epitopes of gp120, suggesting that neutralization phenotype was a consequence of global structural changes of the envelope protein rather than a specific site epitope.

Introduction

Infection of nonhuman primates with simian immunodeficiency virus (SIV) provides a model for studying immune responses associated with HIV/AIDS in humans (Johnson and Hirsch, 1992). Both cellular and humoral immune responses have been correlated with protective immunity against SIV (Clements et al., 1995; Maecker and Maino, 2003; Paiardini et al., 2008; Sato and Johnson, 2007). Passive protection studies have further demonstrated that antibodies can provide protective immunity when present prior to or immediately preceding HIV or SIV challenge (Haigwood et al., 1996; Lewis et al., 1993; Mascola et al., 1999, 2003, 2000; Nishimura et al., 2002, 2003; Parren et al., 2001; Van Rompay et al., 1998). Unfortunately, these protective antibodies are infrequently observed in exposed hosts and are predominantly directed to intricate, conformationally dependent epitopes of the SIV envelope (*env*) proteins in regions with a propensity for mutation and subsequent immune evasion (Cole et al., 2001; Haigwood et al., 1992; Javaherian et al., 1994, 1992; Sato and Johnson, 2007). Therefore, identifying viral epitopes that play a role in antibody neutralization is important for our understanding of the immunogenic properties of SIV and HIV and will serve to enhance vaccine development.

Some limited HIV and SIV antibody-neutralizing epitopes have been identified within the viral *env* gene (Pantophlet and Burton, 2006; Sato and Johnson, 2007). The HIV and SIV *env* gene produces a polyprotein (gp160) that is extensively modified by post-translational polysaccharide addition and is cleaved by host protease (furin) into two separate glycoproteins, gp120 [surface subunit (SU)] and gp41 [transmembrane subunit (TM)] (Luciw, 1996). On the surface of the virus, complete *env* complexes are comprised of a trimer of noncovalently linked heterodimers of gp120 and gp41. The role of these proteins in virus attachment and entry has been well characterized (Wyatt and Sodroski, 1998). Gp120 is involved with CD4 and co-receptor recognition and binding, while gp41 is responsible for forming the *env* trimer and mediating cell–virus fusion. Less well known is how neutralizing antibody responses to SIV and HIV are directed against the viral epitopes of the *env* proteins. Neutralizing antibody responses against SIV have been demonstrated to be associated with the gp41 cytoplasmic tail, ecto, and transmembrane domains (Bonavia et al., 2005; Overholser et al., 2005; Puffer et al., 2004). Within gp120, V1/V2 (Johnson et al., 2002, 2003; Puffer

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et al., 2004), V3 loop (Means et al., 2001; Palker et al., 1996; Pohlmann et al., 2004), and V4 regions (Choi et al., 1994; Kinsey et al., 1996) have also demonstrated significant roles in affecting virus neutralization by antibodies. Identifying and characterizing these determinants of neutralization in SIV have increased our understanding of the antigenic qualities of envelope proteins. However, more information is required to solve the precise molecular structure and antigenic qualities of these proteins.

The present study was designed to identify the amino acid residues within the *env* gene that contribute to the antibody neutralization phenotype of SIV/17E-CL, a naturally derived clone of neutralization-resistant SIVmac239 that acquired nine amino acid mutations in gp120 (V67M, K141R, T158A, K176N, Q217K, M309I, P334R, K340R, and G382R) (Anderson et al., 1993; Regier and Desrosiers, 1990; Sharma et al., 1992). Our earlier investigations using surface plasmon resonance (SPR) determined that differences in association and dissociation kinetic rates of antibody with SIV/17E-CL *env* proteins were causative for the neutralization phenotype (Steckbeck et al., 2005). However, the region of the viral protein or the epitope(s) involved remained unknown. Here we investigated antibody-mediated neutralization in vitro with viruses engineered to express amino acid residues from SIV/17E-CL gp120 within the SIVmac239 backbone. Results from these studies described a novel V3 epitope that conferred neutralization of SIVmac239 by monoclonal antibodies to both linear and conformational epitopes, suggesting that neutralization phenotype was the consequence of global structural changes of the *env* protein complex.

Results

Synthesis of mutant viruses

The envelope gene of SIV/17E-CL differs from SIVmac239 by 9 amino acids in gp120 (V67M, K141R, T158A, K176N, Q217K, M309I, P334R, K340R, and G382R), see Fig. 1 (Anderson et al., 1993). Single and multiple rounds of site-directed mutagenesis (see Materials and methods) were used to generate amino acid mutations in the gp120 *env* SIVmac239 backbone according to a scheme that represents mutation sets in and around the V1/V2, or V3 regions of SIV/17E-CL (Fig. 1). Viral stocks were generated from recombinant virus constructs in mammalian tissues as described in the Materials and methods section. Synthesis of infectious virus was verified from every engineered construct and yielded viral stock titers ranging from 10^2 to 10^5 $TCID_{50}$ per milliliter (data not shown).

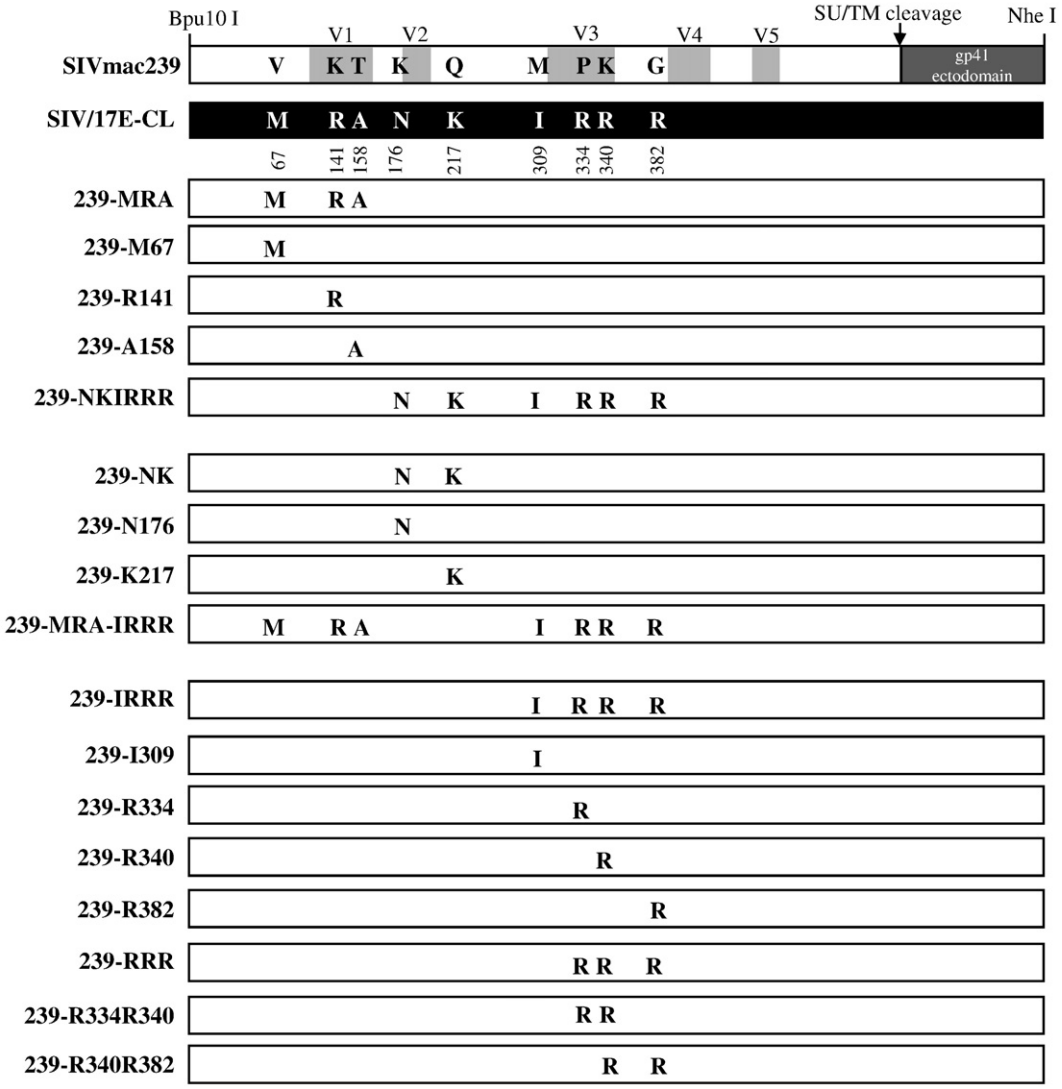

Fig. 1. Design and characterization of SIV mutant viruses. Mutations in SIV/17E-CL are distributed throughout the gp120 *env* gene and cluster around the V1/V2 and V3 regions. The cloning strategy utilizes the Bpu10I restriction site, 5′ to the start codon of gp120, and the *Nhe*1 restriction site on the 3′ end of the ectodomain of gp41. All mutant constructs created infectious virus in vitro that activated LTR-luc expression in the TZM-bl. Viral titer stocks ranged from 10^2 to 10^5 $TCID_{50}$ per milliliter.

Envelope expression in SIV mutants

Previous reports suggest that mutations in SIV gp120 that reduce envelope incorporation into viral membranes and/or increase *env* shedding from the viral membrane result in enhanced sensitivity to antibody-mediated neutralization (Klasse and Moore, 1996; Yuste et al., 2005). We previously demonstrated that SIV/17E-CL *env* expression is similar to SIVmac239 and is therefore not a factor for neutralization (Steckbeck et al., 2005). To determine whether the mutant viruses studied here displayed altered *env* expression, we assessed the quantity of gp120 present in mutant viruses. Fig. 2a demonstrated that with the exception of 239-A158, 239-NK-IRRR, 239-NK, and 239-N176, all mutant viruses expressed levels of gp120 *env* similar to SIVmac239. To measure the level of gp120 *env* in each mutant clone, band densities from duplicate experiments were determined for gp120 *env* and p27*gag* and the ratios were compared to the parental virus, SIVmac239. Using this semiquantitative analysis, four mutant viruses (239-A158, 239-NKIRRR, 239-NK, and 239-N176) displayed significant decreases in gp120 expression (35%, 4.9%, 17%, and 11%, respectively) (Fig. 2b). Interestingly, while 239-A158 displayed reduced gp120 expression, 239-MRA and 239-MRA-IRRR, which also contained the T158A mutation, did not express a reduced gp120 *env* phenotype. So, the significant reduction of *env* by T158A only occurred when T158A was expressed alone (239-A158). Fig. 2b also demonstrated that mutant viruses with the K176N mutation (239-NKIRRR, 239-NK and 239-N176) expressed significantly lower levels of gp120 *env*. Thus, the analysis of gp120 expression showed that *env* content was unperturbed in most mutants and significant variability was limited to few mutants with distinct point mutations.

Lastly, the immunoblot analysis revealed one further observation. Fig. 2a showed that gp120 *env* from mutants with the A158 residue (239-MRA, 239-A158, and 239-MRA-IRRR) resolved at a lower molecular weight than SIVmac239 and mutants with T158. This migration pattern of the *env* proteins would suggest that the T158A mutation resulted in a loss of an N-linked glycosylation residue, as expected by the change in amino acid sequence, N-C-T→N-C-A.

Analysis of V1/V2 region residues for neutralization sensitivity to monoclonal antibodies

Each mutant virus was examined for neutralization sensitivity using the TZM-bl assay (Steckbeck et al., 2005). Neutralization studies were divided into study groups of mutants according to region and are summarized in Fig. 1. Data from V1 region mutants showed that the 239-MRA virus (mutations V67M, K141R and T158A) was not neutralized by MAb at the highest concentration (10 μg/ml) (Table 2). The 239-M67 (V67M) and 239-R141 (K141R) mutants alone were also not sensitive to neutralization (Table 2). However, the 239-A158 mutant (T158A) yielded a clone that was partially sensitive to neutralization by MAbs that recognize epitopes on the carboxy-terminal half of gp120. Four of the eight neutralizing antibodies yielded mean IC_{50} values (0.64–0.037 μg/ml, Table 2) within one log10 deviation from the IC_{50} values observed in SIV/17E-CL (Table 2). Interestingly, the 239-NKIRRR mutant that contains the V2 and V3 mutations and none of the V1 mutations demonstrated a neutralization profile more comparable to SIV/17E-CL (Table 2) with IC_{50} values ranging from 0.39 to 0.003 μg/ml (Table 2). Therefore, the single point mutation T158A in V1 conferred partial neutralization sensitivity in

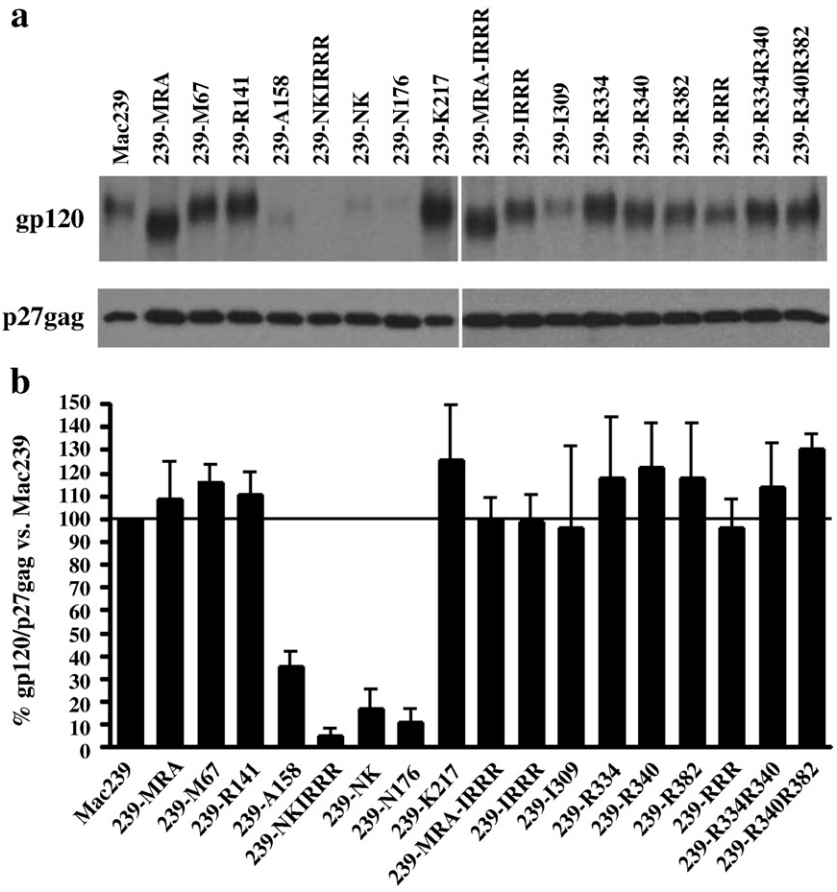

Fig. 2. Analysis of gp120 expression in mutant viruses. (a) Virus supernatants were pelleted, and denatured viral proteins were immunodetected for gp120 *env* (MAb 3.11H) and structural protein p27*gag* (MAb 2F12). (b) Band densities were determined, and the gp120/ p27*gag* ratio of each mutant was calculated. The data reported are the percent change in mean gp120/p27*gag* ratio (±SD) compared to SIVmac239 from two independent experiments.

Table 1
Monoclonal antibody phenotypes: epitope specificity and SIV neutralization.

MAb	Binding group[a]	Putative binding domain[b]	Epitope conformation[c]	Neutralizing antibody titer (μg/ml)[d]	
				SIV/17E-CL	SIVmac239
3.8E	I	N′ terminus	Conformational	>25	>25
3.10A	II	V1	Linear	>25	>25
5.5B	III	V2	Linear	>25	>25
3.11H	IV	V3-cys loop	Linear	0.070	>25
1.11A	V	CCR5 bs	Conformational	0.125	>25
4.7A	V	CCR5 bs	Conformational	0.021	>25
3.11E	VIa	CCR5 bs	Conformational	0.038	>25
3.2C	VIb	V4	Conformational	0.015	>25
E31	VIb	V4	Conformational	0.039	>25
1.10A	VII	V4	Conformational	0.009	>25
C26	VII	V4	Conformational	0.006	>25
3B3	VIII	Unknown	Conformational	>25	>25

[a] Competition groups were previously defined by cross-competition ELISA (Cole et al., 2001).
[b] Putative binding domains are based on previously published data (Cole et al., 2001).
[c] Epitope conformation was previously determined by reducing/nonreducing immunoblot (Cole et al., 2001).
[d] Neutralization antibody titers were previously assayed and reported as the concentration of MAb that inhibited 50% infection (IC_{50}) using the TZM-bl assay (Steckbeck et al., 2005).

only 239-A158, while determinants outside of V1 imparted full neutralization.

Next, mutations in the V2 region were examined. The 239-NK virus containing both the K176N and Q217K mutations was partially sensitive to neutralization (Table 2), albeit to a lesser degree than SIV/17E-CL (Table 2). Only two of the eight neutralizing antibodies displayed IC_{50} values within a log10 deviation of SIV/17E-CL. Further analysis of the V2 mutations was performed with clones 239-N176 (K176N) and 239-K217 (Q217K). 239-N176 was sensitive to antibody neutralization (IC_{50} values, 1.11 to 0.031 μg/ml), while 239-K217 was not neutralized by the antibodies tested (Table 2). Clone 239-MRA-IRRR lacking the V2 region mutations but containing the V1 and V3 mutations was more sensitive to neutralization than the 239-NK virus (Table 2), with IC_{50} values ranging from 0.67 to 0.026 μg/ml. Therefore, the data of the V2 region analysis showed that the K176N mutation was sufficient to confer partial neutralization in SIV/17E-CL, but that the V3 region contained factors for neutralization as well.

Analysis of V3 region residues for neutralization sensitivity to monoclonal antibodies

Lastly, we evaluated mutations in and around the V3 region (M309I, P334R, K340R, and G382R) for neutralization sensitivity. Mutant clone 239-IRRR, expressing all four point mutations around V3, showed IC_{50} values equivalent to SIV/17E-CL, ranging from 0.31 to 0.006 μg/ml (Table 3). To elucidate the critical neutralizing mutations in V3, additional single point mutants were generated and assayed for neutralization. The 239-I309 (M309I), 239-R340 (K340R), and 239-R382 (G382R) mutants were resistant to neutralization by all MAbs tested (Table 3). Of these clones, only 239-R334 (P334R) demonstrated a neutralization phenotype equivalent to 239-IRRR and SIV/17E-CL (Table 3). To determine the contribution of the neighboring mutations that also introduced an arginine (R) in and around V3 (K340R and G382R), combination mutant clones were assayed for neutralization. The data from clones 239-RRR, 239-R334R340, and 239-R340R382 reinforced the observation of the P334R mutation as the neutralizing determinant, as only those clones containing the R334 residue (239-RRR and 239-R334R340) yielded a neutralization phenotype equal to 239-IRRR (Table 3). Furthermore, all mutant clones assayed that contained P334R (239-NKIRRR, 239-MRA-IRRR, 239-IRRR, 239-R334, 239-RRR, and 239-R334R340) were neutralization sensitive (Tables 2 and 3) with IC_{50} values for MAbs that were equivalent to SIV/17E-CL.

Discussion

This study analyzed the antibody neutralization phenotypes resulting from single and multiple point mutations in SIVmac239 gp120 representing mutations derived from SIV/17E-CL. Using a broad panel of rhesus MAbs with linear and conformational epitopes to multiple domains throughout SIV gp120, we sought to identify the mutation(s) responsible for inducing antibody mediated neutralization seen in SIV/17E-CL. The first mutation identified, T158A, only influenced neutralization when expressed alone, as seen in virus 239-A158 (Table 2). Interestingly, the mutation of threonine 158 to alanine removes a potential N-linked glycosylation site from SIVmac239 (g6) by disrupting

Table 2
Neutralization phenotype resulting from V1 and V2 mutations.[a]

Monoclonal antibody	Binding domain	Mac239	17E-CL	V1					V2			
				239-MRA	239-M67	239-R141	239-A158	239-NKIRRR	239-NK	239-N176	239-K217	239-MRA-IRRR
3.8E	N′ terminus	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10
3.10A	V1	>10	>10	>10	>10	>10	>10	6.43 (0.81)	>10	>10	>10	>10
5.5B	V2	>10	>10	>10	>10	>10	>10	1.18 (0.45)	>10	>10	>10	>10
3.11H	V3-cys loop	>10	0.046 (0.02)	>10	>10	8.30 (2.4)	0.510 (0.41)	**0.028 (0.02)**	1.01 (0.43)	1.11 (1.1)	>10	**0.260 (0.18)**
1.11A	CCR5 bs	>10	0.075 (0.05)	>10	>10	>10	**0.143 (0.14)**	**0.039 (0.01)**	**0.215 (0.01)**	0.857 (0.90)	>10	**0.673 (0.31)**
4.7A	CCR5 bs	>10	0.016 (0.01)	>10	>10	>10	0.300 (0.27)	**0.017 (0.01)**	0.530 (0.32)	0.233 (0.15)	>10	0.300 (0.16)
3.11E	CCR5 bs	>10	0.024 (0.02)	>10	>10	8.65 (1.9)	0.640 (0.65)	**0.010 (0.01)**	1.24 (1.1)	**0.154 (0.19)**	>10	**0.220 (0.07)**
3.2C	V4	>10	0.022 (0.01)	>10	>10	>10	0.225 (0.22)	**0.029 (0.01)**	0.273 (0.16)	0.331 (0.29)	>10	**0.133 (0.07)**
E31	V4	>10	0.029 (0.02)	>10	>10	>10	**0.175 (0.02)**	**0.023 (0.01)**	**0.195 (0.02)**	**0.042 (0.01)**	>10	0.520 (0.28)
1.10A	V4	>10	0.005 (0.004)	>10	>10	5.25 (3.7)	**0.026 (0.02)**	**0.002 (0.001)**	0.084 (0.01)	**0.029 (0.03)**	5.49 (4.1)	**0.033 (0.01)**
C26	V4	>10	0.004 (0.003)	>10	>10	>10	**0.037 (0.003)**	**0.003 (0.001)**	0.089 (0.03)	**0.031 (0.04)**	>10	**0.026 (0.01)**
3B3	Unknown	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10

Values in bold type represent mean IC_{50} values that do not exceed 1 log10 variance from the IC_{50} of SIV/17E-CL.
[a] IC_{50} neutralization titers are reported as the mean concentration of MAb (μg/ml) (±SD) from at least two independent experiments.

Table 3
Neutralization phenotype resulting from V3 mutations.[a]

Monoclonal antibody	Binding domain	Mac239	17E-CL	V3							
				239-IRRR	239-I309	239-R334	239-R340	239-R382	239-RRR	239-R334R340	239-R340R382
3.8E	N′ terminus	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10
3.10A	V1	>10	>10	3.58 (0.39)	>10	6.43 (2.8)	>10	>10	7.13 (4.9)	>10	>10
5.5B	V2	>10	>10	9.08 (1.85)	>10	5.58 (4.7)	8.90 (2.2)	>10	>10	>10	>10
3.11H	V3-cys loop	>10	0.046 (0.02)	**0.313 (0.38)**	>10	**0.111 (0.06)**	8.98 (2.0)	9.36 (1.1)	**0.086 (0.06)**	**0.435 (0.50)**	>10
1.11A	CCR5 bs	>10	0.075 (0.05)	**0.228 (0.24)**	>10	**0.110 (0.07)**	>10	>10	**0.069 (0.03)**	**0.200 (0.12)**	>10
4.7A	CCR5 bs	>10	0.016 (0.01)	**0.056 (0.07)**	>10	**0.051 (0.03)**	>10	>10	**0.042 (0.02)**	**0.075 (0.02)**	>10
3.11E	CCR5 bs	>10	0.024 (0.02)	**0.059 (0.07)**	>10	**0.062 (0.04)**	7.85 (4.3)	7.68 (2.7)	**0.050 (0.02)**	**0.072 (0.05)**	>10
3.2C	V4	>10	0.022 (0.01)	**0.014 (0.02)**	>10	**0.048 (0.03)**	7.10 (3.6)	7.76 (4.4)	**0.016 (0.01)**	**0.060 (0.04)**	>10
E31	V4	>10	0.029 (0.02)	**0.010 (0.13)**	>10	**0.101 (0.03)**	>10	>10	**0.161 (0.15)**	**0.050 (0.01)**	>10
1.10A	V4	>10	0.005 (0.004)	**0.005 (0.004)**	>10	**0.004 (0.002)**	6.73 (2.8)	7.08 (3.6)	**0.004 (0.002)**	**0.023 (0.03)**	7.80 (3.8)
C26	V4	>10	0.004 (0.003)	**0.006 (0.007)**	>10	**0.004 (0.004)**	7.85 (4.3)	9.06 (1.6)	**0.006 (0.002)**	**0.010 (0.01)**	>10
3B3	Unknown	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10

Values in bold type represent mean IC_{50} values that do not exceed 1 log10 variance from the IC_{50} of SIV/17E-CL.
[a] IC_{50} neutralization titers are reported as the mean concentration of MAb (μg/ml) (±S.D.) from at least two independent experiments.

the N-linked glycosylation signal (Asn-X-Ser/Thr), N-C-T→N-C-A. Previous research suggests that the carbohydrates around V1/V2 and V3 shield epitopes on *env* because removal of glycosylation sites in SIV often enhances antibody neutralization (Chackerian, Rudensey, and Overbaugh, 1997; Cole et al., 2004; Reitter and Desrosiers, 1998; Reitter et al., 1998). In our study, the T158A Δg6 mutation induced a neutralizing phenotype when expressed alone (239-A158) but showed no effect when other mutations were expressed in *cis*, 239-MRA (V67M, K141R, and T158A). A more significant observation of the 239-A158 (T158A) mutant was a significant reduction in gp120 *env*/p27*gag* ratio compared to SIVmac239 (Fig. 2b). Reduced envelope content in the virion has been correlated with neutralization sensitivity of HIV and SIV (Klasse and Moore, 1996; Yuste et al., 2005). Therefore, reduced *env* protein expression would explain the neutralization sensitivity of the 239-A158 clone, yet does not dictate the neutralization phenotype of SIV/17E-CL, which does not have lowered *env* expression.

Next, a K176N mutation amino-terminal to V2 was observed to induce monoclonal antibody mediated neutralization in vitro, albeit to a lesser degree than SIV/17E-CL (Tables 1 and 2). As reported previously, V1/V2 plays a role in neutralization, given that mutations and deletions of V1/V2 induce a neutralizing phenotype (Johnson et al., 2002, 2003; Puffer et al., 2004). Neutralization sensitivity has been reported for SIVmac316 that expresses a mutation at amino acid 176 in gp120 (Johnson et al., 2003; Means et al., 2001). Furthermore, the MERT cluster of mutations (V67**M**, K176**E**, G382**R** K573**T**) is implicated in the macrophage tropic phenotype associated with SIVmac316 (Mori et al., 1992). Macrophage tropism is often associated with antibody neutralization sensitivity in SIV (Means et al., 2001; Puffer et al., 2002; Rudensey et al., 1998). Indeed, SIV/17E-CL is macrophage tropic (Mankowski et al., 1997); however, the V67M and G382R mutations had no effect on neutralization sensitivity in this study (Tables 2 and 3). More importantly the K176N mutation yielded significant reductions in envelope expression (Fig. 2). As may be the case for the T158A mutation, the atypically lowered expression of the glycoproteins on the viral membrane of the SIV K176N mutant induced the observed antibody neutralization phenotype.

In this study, the most significant changes in antibody mediated neutralization were observed in the carboxy-terminal portion of the V3 loop at residue 334 (Table 3). All mutants assayed that contained R334 (239-NKIRRR, 239-MRA-IRRR, 239-IRRR, 239-R334, 239-RRR, and 239-R334R340) were neutralized by MAbs at concentrations equivalent to SIV/17E-CL while retaining normal protein expression of *env* (Fig. 2). Although impressive, it is not so surprising given the documented importance that HIV and SIV V3 has in regard to viral tropism and neutralization phenotypes (Hartley et al., 2005). Evidence thus far suggests that the V3 loop may be split into two domains each with a different role in determining phenotype. The amino-terminal portion is suggested to directly interact with neutralizing antibodies, while the carboxy-terminal portion is weakly or non-immunogenic and may interact with other domains of the gp120 monomer (Benichou et al., 1993; Huang et al., 2005; McBride et al., 1993; Miller et al., 1992; Palker et al., 1996; Pohlmann et al., 2004; Rosen et al., 2005; Rosen et al., 2008). Changes in the carboxy-terminal domain of V3 are suspected to influence phenotype via structural changes of the individual monomers or the complete trimer (Huang et al., 2005; Rosen et al., 2008; Rosen et al., 2006). Our data support this theory in part because the neutralizing phenotype obtained by the P334R mutation was apparent not only for a V3-binding antibody (3.11H) but for the entire panel of MAbs recognizing both linear and conformational epitopes clustered throughout the gp120 molecule (Table 3).

One particular model in HIV could explain structure changes associated with R334. The "11/25" rule states that in HIV-1-negative/neutral charges at residues 11 or 25 in the V3 loop confer CCR5 co-receptor usage and macrophage tropism, while positive charges yield CXCR4 co-receptor usage and T-lymphocyte or dual tropism phenotype (De Jong et al., 1992; Fouchier et al., 1992; Resch et al., 2001). The corresponding amino acids in T-lymphotrophic SIVmac239 V3 are 11 (P306) and 25 (P334). Interestingly, Rosen et al. (2008) recently demonstrated that the 25th amino acid in HIV-1 V3, residue 322, displays electrostatic interaction with residue 440 in the C4 domain. The 440 residue in C4 was mapped as the second amino acid following a conserved YAPP domain, which is also present in SIVmac as a YLPP domain. Interestingly, the second amino acid carboxy-terminal to this domain in SIV is a negatively charge glutamic acid E454. If SIV structure is homologous to HIV-1, the R334 and E454 would theoretically interact with electrostatic attraction and yield structure and phenotype of macrophage tropic viruses. Indeed, studies of SIV V3 mutations found that changing P334 to uncharged amino acids leucine or glutamine did not confer macrophage tropism in SIVmac239 (Kirchhoff et al., 1994; Pohlmann et al., 2004), yet SIV/17E-CL (R334) is a macrophage tropic virus (Mankowski et al., 1997). Therefore, it is feasible that the 11/25 rule for positivity applies to HIV-1 but could more broadly reflect the necessity for position specific electrostatic interactions between V3 and other regions of SIV gp120, such as V4.

This study identified the P334R mutation in the C′-terminal portion of the V3 loop to be the main determinant of neutralization in SIV/17E-CL. Since this residue affected neutralization by MAbs recognizing diverse conformational and linear epitopes in gp120 and that the V3 region has been observed to be associated with viral tropism and neutralization phenotypes, it is likely that the neutralization-sensitive phenotype of SIV/17E-CL is a result of the structural changes of the envelope protein elicited by the P334R mutation. Given the currently limited data describing V3 structure within the context of gp41/gp120 trimers, this report supports the importance of further studies on the V3 region to assist in developing effective therapeutics and preventative strategies against HIV AIDS.

Materials and methods

Cells

293T cells and SIV-permissive TZM-bl cells (Wei et al., 2002), which express CD4, CCR5, CXCR4, and HIV/SIV-inducible firefly luciferase, were maintained in Dulbecco's modified Eagle's medium (DMEM) media (Gibco-Invotrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA).

Construction of mutant proviruses

Previously characterized provirus constructs SIVmac239 (Regier and Desrosiers, 1990) and SIVmac17E-CL (Anderson et al., 1993) were digested with *Nhe*1 and *Bpu*10I to isolate the encoding region of gp120 and a portion of the ectodomain of gp41 (Fig. 1). This region was ligated with homologous restriction sites into a CMV promoter expression vector, TR600 (Bower et al., 2004), using T4 DNA ligase (New England Biolabs, Ipswich, MA) and recombinant DNA was synthesized in DH5α competent cells. The wild-type gp120 *env* sequences were mutagenized using the QuickChange XL Site Directed Mutagenesis kit (Stratagene, La Jolla, CA) according to manufacturer's protocol and sequence specific primers. The mutant *env* constructs were ligated back into the SIV proviral backbone using *Nhe*1 and *Bpu*10I restriction sites and T4 DNA ligase and propagated in Stbl2 competent cells (Invitrogen, Carlsbad, CA). Transfection grade plasmids clones were verified by DNA sequencing at the University of Pittsburgh Genomics and Proteomics Core laboratory (Pittsburgh, PA) using an ABI 3730xl DNA analyzer (Applied Biosystems, Foster City, CA).

Production of viruses

SIVmac239, SIV/17E-CL, and mutant proviral DNA were transiently transfected as previously described (Bower et al., 2004). Briefly, 8 μg of proviral DNA was conjugated with 24 μl Lipofectamine 2000 (Invitrogen) in Opti-MEM media (Invitrogen) and incubated with $6–7\times10^6$ 293T cells for 5 h at 37 °C. The media was replaced with 10 ml of fresh Opti-MEM and the cultures were incubated an additional 48 h at 37 °C. Cell free supernatants were harvested and frozen at −80°C prior to experimental analysis. Virus titer was determined by serially diluting viral supernatants in DMEM media and culturing with TZM-bl cells (10^4 cells per well) in 96-well plates (BD-Falcon, San Jose, CA). Following an incubation of 48 h, supernatants were removed and the cells were lysed with 50 μl cell lysis buffer (Promega, Madison, WI) for 15 min at room temperature. Lysates (40 μl) were transferred to white luminescence plates (USA Scientific, Ocala, Fl) and assayed for luciferase production on a Lmax II luminometer (Molecular Devices, Union City, CA) with 25 μl of Luciferase Assay Substrate (Promega) per well. The $TCID_{50}$ was determined using the Reed and Meunch method (Poli and Fauci, 2004), with a positive cutoff value 2.5 times that of the negative samples.

Virus neutralization determination

MAbs (Table 1) were serially diluted 5-fold in DMEM containing 10% FBS in sterile 96-well V-bottom plates (Sarstedt, Newton, NC) for a final assay concentration range of 10–0.00013 μg/ml. Virus was diluted in DMEM with 10% FBS to yield an MOI of 0.01, mixed with diluted MAb, and pre-incubated 1 h at 37 °C. The virus–MAb mixture was added to TZM-bl cells (10^4 cells per well) in 96-well plates (BD-Falcon) and cultured in the presence of 20 μg/ml DEAE-Dextran (Amersham Biosciences, Piscataway, NJ) for 48 h. SIV infection was assayed by luciferase production as described above. Neutralizing titers are reported as the IC_{50}, the MAb concentration (μg/ml) that neutralized 50% of the virus infection, using the method of Karber (1931). All values represent the averages of at least two independent experiments assayed in duplicate.

Western blot analysis

To quantitate envelope content of each SIV mutant, viral supernatants were pelleted over a 10% glycerol cushion at $19{,}000\times g$ for 1.5 h at 4 °C, denatured by boiling in reducing buffer and resolved by SDS-PAGE using Tris–HCl 5 and 12.5% gels (Bio-Rad, Hercules, CA). Viral proteins were transferred to polyvinylidine difluoride membrane and probed for gp120 *env* using a MAb directed against a linear epitope in V3 not affected by the mutations in this study [3.11H (Cole et al., 2001)] and p27*gag* using MAb 2F12 (NIH AIDS Research and Reference Reagent Program). Band densities of gp120 *env* and p27*gag* were determined using ImageJ software (National Institutes of Health, Bethesda, MD). Each immunoblot contained a positive control of SIVmac239, thus allowing for calculations of the gp120/p27*gag* ratio for each mutant virus in relation to the parental strain SIVmac239.

Competing interests

The authors declare that they have no competing interests.

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