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Effects of Natural Sequence Variation on Recognition by Monoclonal Antibodies That Neutralize Simian Immunodeficiency Virus Infectivity

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The determinants of immune recognition by five monoclonal antibodies (KK5, KK9, KK17, Senv7.1, and Senv101.1) that neutralize simian immunodeficiency virus infectivity were analyzed. These five neutralizing monoclonal antibodies were generated to native SIVmac251 envelope glycoprotein expressed by a vaccinia virus recombinant vector. All five recognize conformational or discontinuous epitopes and require native antigen for optimal recognition. These monoclonal antibodies also recognize SIVmac239 gp120, but they do not recognize gp120 of two natural variants of SIVmac239, 1-12 and 8-22, which evolved during the course of persistent infection in vivo (D. P. W. Burns and R. C. Desrosiers, J. Virol. 65:1843–1854, 1991). Recombinant viruses which were constructed by exchanging variable regions between SIVmac239 and variant 1-12 were used to define domains important for recognition. Radioimmunoprecipitation analysis demonstrated that sequence changes in variable regions 4 and 5 (V4/V5) were primarily responsible for the loss of recognition of the 1-12 variant. Site-specific mutants were used to define precise changes that eliminate recognition by these neutralizing antibodies. Changing N-409 to D, deletion of KPKE, and deletion of KEQH in V4 each resulted in loss of recognition by all five monoclonal antibodies. SIVs with these natural sequence changes are still replication competent and viable. Changing A-417 to T or A/N-417/418 to TK in V4 or Q-477 to K in V5 did not alter recognition detectably. These results define specific, naturally occurring sequence changes in V4 of SIVmac that result in loss of recognition by one class of SIVmac neutralizing antibodies.

Antigenic variation during persistent infection has been extensively studied in three lentiviral systems: visna virus, caprine arthritis encephalitis virus, and equine infectious anemia virus (7, 12, 21–23, 25, 26, 31). Animals persistently infected with these ungulate lentiviruses exhibit delayed neutralizing antibody responses against variant viruses which appear during the course of infection. Results derived from these lentiviral systems suggest that the host neutralizing antibody response selects neutralization escape mutants during the course of viral infection and that antigenic variation may contribute to persistent viral replication in chronically infected animals. Sequence changes responsible for resistance of these ungulate lentiviral variants to serum neutralization have not been defined. Furthermore, it is difficult to extrapolate the significance of these findings in ungulate systems to primate lentiviral systems because of the large phylogenetic distance.

Antibodies that neutralize human immunodeficiency virus type 1 (HIV-1) in infected people are largely directed against the envelope glycoprotein gp120, and a number of linear and conformationally determined neutralization epitopes have been identified in this HIV-1-encoded product (for reviews, see references 28 and 35). However, the relative importance of different target epitopes in HIV-1-infected people has not been clearly defined (27, 28, 35, 36). Attempts to demonstrate

the appearance of neutralization-resistant HIV-1 variants in infected humans have been largely hampered by lack of information on the genetic sequence of the initial infecting strain and by the presence of an already complex mix of genotypes in infected humans at the time that samples have been available for study. Nonetheless, several studies have indicated that neutralization-resistant HIV-1 variants emerge during the course of infection (1, 10, 38).

Clear evidence for the emergence of neutralization escape variants has been presented for rhesus monkeys experimentally infected with molecularly cloned simian immunodeficiency virus (SIV) (4). Sequential sera from infected animals showed much higher neutralizing antibody titers to the cloned virus used for infection than to cloned variants obtained 69 and 93 weeks after infection. As an important internal control for the demonstration of immune selection, rhesus monkeys were experimentally infected with cloned variant virus, and reciprocal neutralization tests were performed with variant-specific sera from these experimentally infected monkeys. Each cloned virus was neutralized best by its homologous antiserum. Only 20 amino acid changes in gp120, mostly in discrete variable domains, were responsible for the resistance to cross-neutralization.

We now report that natural sequence variation in variable region 4 (V4) results in loss of recognition by at least one class of conformation-dependent antibodies that neutralize SIVmac infectivity.

MATERIALS AND METHODS

Cells and recombinant viruses. Human CD4⁺ CEMx174 cells were grown in RPMI 1640 medium with 10% fetal calf

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                    10        20        30        40        50        60        70        80
SIVmac239  MGCLGNQLLIAILLLSVYGIYCTLYVTVFYGVPAWRNATIPLFCATKNRDTWGTTQCLPDNGDYSEVALNVTESFDAWNN
SIVmac251  ...................Q.................................S..................L...........E.
SIVmac142  ...................IQ................................D....L...........E.
T69BL1-12  ...................Q...............................M...............
T93V 8-22  ...................Q...............................M...............
T93BL3-18  ...................T...............................M...............
```

V1

```
                    90       100       110       120      | 130       140     | 150      160
SIVmac239  TVTEQAIEDVWQLFETSIKPCVKLSPLCITMRCNKSETDRWGL|TKSITT--TASTTSTTASAKV---DMV|NETSSCIAQDNCTGL
SIVmac251  .........................................  |...S..IT..AP..APV.E.L---...|.........N.....
SIVmac142  .......................................K...|...S..--.....T..TAKS.ETR.I.|....P.VVH......
T69BL1-12  ...........................................|.Q.M..--.....P.M....R.----.T.|...............
T93V 8-22  ...........................................|.Q.M..--.....PP.......---...|...............
```

V2

```
                   170       180       190     | 200 |    210       220       230       240
SIVmac239  EQEQMISCKFNMTGLKRDKKKEYNETWYSADLVCEQ|GNNTGNE|SRCYMNHCNTSVIQESCDKHYWDAIRFRYCAPPGYAL
SIVmac251  ............................T.........T......|..S.D..|........................T.............
SIVmac142  ....P.......................................|..S....|................C..D......C.........
T69BL1-12  ............................................|..S....|.....................................
T93V 8-22  ...HI.......................................|..S.D..|.....................................
```

```
                   250       260       270       280       290       300       310┌      320
SIVmac239  LRCNDTNYSGFMPKCSKVVVSSCTRMMETQTSTWFGFNGTRAENRTYIYWHGRDNRTIISLNKYYNLTMKCRRPGNKTVL
SIVmac251  ................................................................................
SIVmac142  ...........N.....................R...............H................
T69BL1-12  ................................................................................
T93V 8-22  ................................................................................
```

V3

```
          ─ cysteine loop ─┐                          | V3 |
                   330       340       350       360       370 | 380      | 390       400
SIVmac239  PVTIMSGLVFHSQPINDRPKQAWCWFGGKWKDAIKEVKQTIVKHPRYTGT|NNTDKINLTAPGG|GDPEVTFMWTNCRGEFL
SIVmac251  ......................................................|.............|.................
SIVmac142  ......A.......V.E.......R...N..E......................|..S........R.|.................
T69BL1-12  ......................................................|....R.......|.................
T93V 8-22  ..............................E.......................|....N.......|.................
```

V4   V5

```
                 | 410       420     |    430       440       450       460       470 | 480 |
SIVmac239  YCKMNW|FLNWVEDRNTANQKPKEQHK|RNYVPCHIRQIINTWHKVGKNVYLPPREGDLTCNSTVTSLIANI|DWIDGNQTNI
SIVmac251  ......|........DVTT.R...R.R|..............................................|..T.......
SIVmac142  ......|........SLTT.....R..|..............................................|N.T.....S.
T69BL1-12  ......|..D.......TK...----.|..............................................|...NE.....
T93V 8-22  ......|..D.......T..----...|..............................................|......K...
```

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                   490       500       510       520
SIVmac239  TMSAEVAELYRLELGDYKLVEITPIGLAPTDVKRYTTGGTSRNKR
SIVmac251  .............................................
SIVmac142  ....................................N........
T69BL1-12  ....................................G........
T93V 8-22  ....................................G........
```
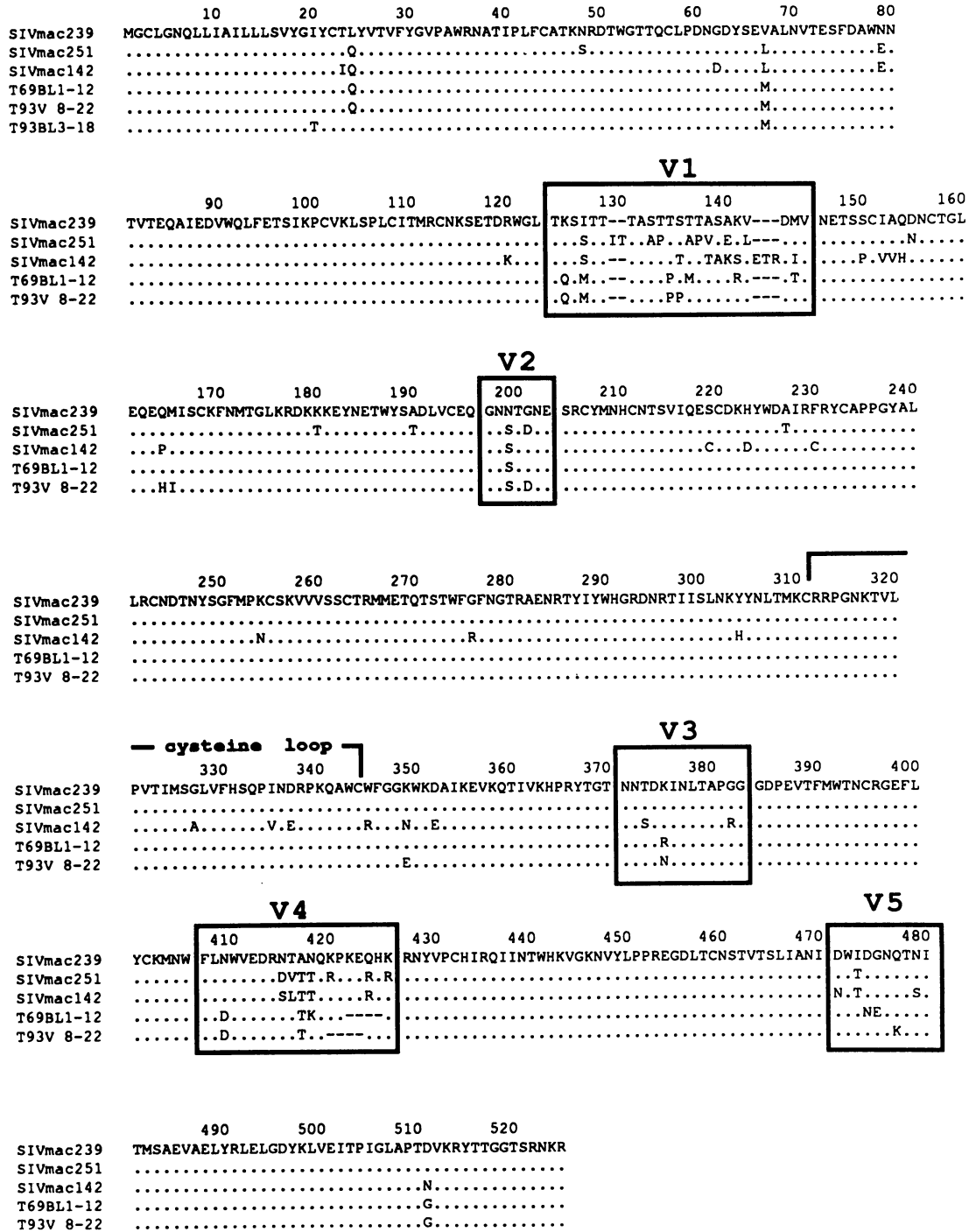
FIG. 1. Amino acid sequence comparisons of gp120s of SIVmac251, SIVmac239 (33), SIVmac142 (6), variant 1-12 (5), and variant 8-22 (5). The sequence of SIVmac251 was derived from the New England Regional Primate Research Center infectious molecular clone (30); its sequence has not been previously published. Dots represent amino acid identity; dashes represent deletions. Variable regions V1 through V5 are boxed. Brackets labeled cysteine loop correspond to the V3 cysteine loop which is variable in HIV-1.

serum. Cloned viruses SIVmac142, SIVmac251, and SIV-mac239 and env variants T69 BL1-12 (variant 1-12) and T93 V8-22 (variant 8-22) have been described previously (5, 6, 19, 30, 33). p239SpSp5', p239SpE3', and p1-12SpE3' are plasmid subclones used to construct recombinant viruses with partially substituted env sequences (4, 33). Four restriction enzyme sites corresponding to nucleotides 6822 (HindIII), 7045 (SpeI), 7758 (MroI), and 8072 (ClaI) were used for the constructions. The

TABLE 1. Summary of the properties of SIVmac envelope MAbs[a]

MAb	Binding to env fragments from E. coli[b]	Binding to peptide	Isotype	Western blot reactivity	Neutralizing activity[c]
Senv7.1	−	−	G1	−	+
Senv101.1	−	−	G1	−	+
KK5	−	−	G2a	−	+
KK9	−	−	G1	−	+
KK17	−	−	G2a	−	+

[a] Derived from the data of Kent et al. (17, 18) and Collignon et al. (8). + or − indicates the presence or absence of significant reactivity.

[b] Fragments corresponding to amino acids 8 to 303, 304 to 492, and 493 to 735 were tested.

[c] Confirmed by a number of laboratories using a variety of different assays (11).

TABLE 2. Relatedness of SIVmac clones

Virus	No. of nonidentical amino acids in gp120				
	SIVmac 251	SIVmac 239	SIVmac 142	Variant 1-12	Variant 8-22
SIVmac251		28	50	37	36
SIVmac239	28		45	20	20
SIVmac142	50	45		52	53
Variant 1-12	37	20	52		17
Variant 8-22	36	20	53	17	

numbering system is that of Regier and Desrosiers (33). V1 is flanked by the HindIII and SpeI sites, and V4 and V5 are flanked by the MroI and ClaI sites (Fig. 1).

MAbs. The monoclonal antibodies (MAbs) Senv7.1, Senv 101.1, KK5, KK9, and KK17 used for these experiments were described previously (8, 17, 18). Briefly, these MAbs were generated by using recombinant vaccinia virus expressing SIVmac251 gp160 native antigen for immunization. BALB/c mice were inoculated intraperitoneally (17) or in the footpads (8). Four weeks later, these mice were boosted intravenously (8) or intraperitoneally (17). Mice were sacrificed, and the spleen cells were fused with the BALB/c myeloma cell line NS0. Hybridomas were screened by enzyme-linked immunosorbent assay and were cloned in soft agar before being inoculated into pristine-primed mice for production of ascitic fluid. The published properties of these MAbs are summarized in Table 1.

Site-specific mutagenesis. All of the mutations except 415/416NT→SL and 409D→N 422–425KEQH were made by using the single-strand phagemid method according to the instructions of Stratagene (La Jolla, Calif.). (In mutation designations, numbers indicate the positions of the amino acids, and arrows point to the resultant amino acids.) All of the primers used for mutagenesis were prepared on a DNA synthesizer (model 8400; Milligen/Biosearch Inc., Burlington, Mass.). For the mutagenesis, the HindIII-HincII DNA fragment (nucleotides 6822 to 8244) of p239SpE3' was subcloned into pBS(−) vector. M13K07 helper phage was used to prepare the single-stranded DNA template. Mutagenic oligonucleotides used for site-specific mutagenesis were as follows: for the change of codon N-409 (here designated 409N) to D in V4, W10 (ATGAATTGGTTTCTAGATTGGGTAGAAG AT); for the change of codon 417A to T in V4, W9 (GAAG ATAGGAATACAACTAACCAGAAGCCA); for the change of codon 417/418AN to TK in V4, W14 (GATAGGAATAC AACTAAACAGAAGCCAAAG); for the deletion of 420–423KPKE in V4, W8 (AATACAGCTAACCAGCAGCATA AAAGGAAT); and for the change of codon 477Q to K in V5, W6 (TGGATTGATGGAAACAAAACTAATATCA). After mutagenesis, a MroI-ClaI fragment containing the V4/V5 region was substituted for the corresponding fragment in p239SpE3'. To create mutant 409D→N 422–425KEQH, which has a variant 1-12 backbone, a 409D-to-N change, and a KEQH insertion, overlap extension PCR using a mutagenic internal primer was performed (13). The two separate amplifications in the first round of PCR were performed with primer pairs W24-W26 and W23-W25, whose sequences are as follows: W24, GTAATTCCTTTTATGCTGTTCCTTTGGCTTC TGTTTAGTTGTATTCCTATCTTCTACCCAATTTAGAA

ACCA; W23, ACTAAACAGAAGCCAAAGGAACAGCAT AAAAGGAATTAC; W25, TCCCAATTCCAATCGATACA GTTCTGCCAC, and W26, CCTGGAGGAGGAGATCCG GAAGTTACCTTC. The PCR-amplified DNA was purified by Gene Clean (Bio 101 Inc., La Jolla, Calif.), and the second round of PCR was performed with the outer set of oligonucleotide primers, W25-W26. After mutagenesis, a MroI-ClaI fragment containing the V4/V5 region was substituted for the corresponding fragment of variant 1-12. To create mutant 415/416NT→SL, which has a SIVmac239 backbone and SL at positions 415 and 416, overlap extension PCR (13) was again used. The first round of PCR was performed with primers W25-W27 and W26-W28. W27 has the sequence TGGGTA GAAGATAGGAGTCTAGCTAACCAGAAGCCAAAG; W28 has the sequence CTTTGGCTTCTGGTTAGCTAGAC TCCTATCTTCTACCCA. The PCR-amplified DNA was purified by Gene Clean (Bio 101), and the second round of PCR was performed with W25-W26. After mutagenesis, a MroI-ClaI fragment containing the V4/V5 region was substituted for the corresponding fragment of SIVmac239. The mutated subclones that were selected for use were sequenced to verify the desired mutations and the absence of any other changes in viral sequences.

RIPA and precipitation with Sepharose-bound sCD4. CEMx174 cells infected with virus were monitored microscopically for syncytium formation and were metabolically labeled with [35S]methionine and [35S]cysteine around the peak of syncytium formation. Cell-free supernatant was harvested, and the amount of p27 antigen was measured with a Coulter SIV Core Ag Assay Kit (Coulter, Hialeah, Fla.). SIV corresponding to the indicated amount of p27 antigen was lysed with radioimmunoprecipitation assay (RIPA) buffer (1% Triton, 2.5 mM Tris, 150 mM NaCl, 1% deoxycholate, 0.1% sodium dodecyl sulfate), and the radiolabeled gp120 glycoproteins were reacted either with positive sera from SIVmac239-infected rhesus monkeys or with MAbs KK5, KK9, KK17, Senv7.1, and Senv101.1 at 4°C in RIPA buffer. Protein A-Sepharose CL-4B or protein A-Sepharose CL-4B previously bound to rabbit anti-mouse immunoglobulin G (Calbiochem, San Diego, Calif.) was used to precipitate the gp120-MAb complexes. For incubations in which soluble CD4 (sCD4) binding was to be analyzed, Nonidet P-40 (NP-40) buffer (0.1% NP-40 in 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES]–250 mM NaCl) was used instead of RIPA buffer. sCD4 (provided by R. Sweet of SmithKline) was bound to activated CH Sepharose 4B as instructed by the supplier (Pharmacia, Piscataway, N.J.). Sepharose-bound sCD4 was used to precipitate complexes of gp120 and antibody. The proteins were electrophoresed through an 8% polyacrylamide gel, fluorographed, dried, and exposed to X-ray film.
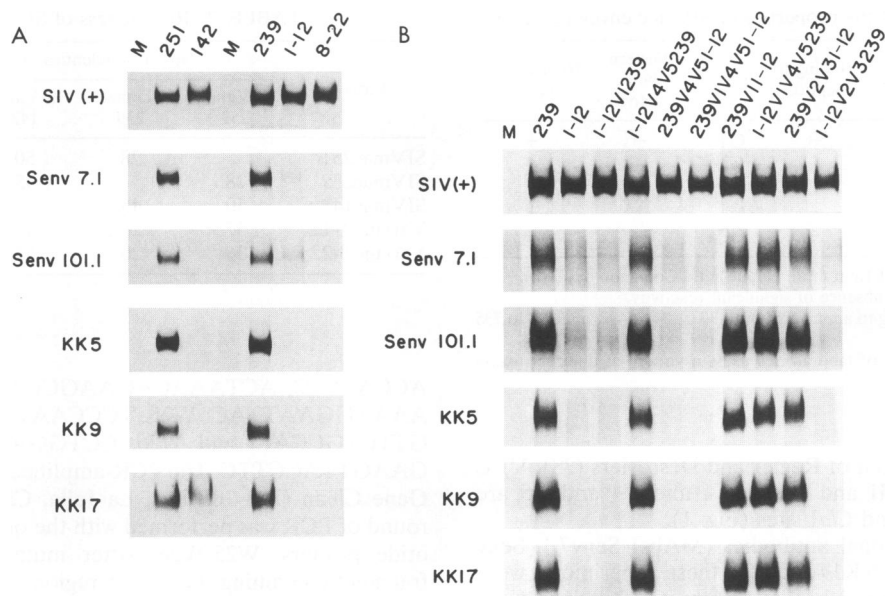
FIG. 2. Immunoprecipitation with MAbs Senv7.1, Senv101.1, KK5, KK9, and KK17. (A) Reactivity of gp120 of cloned variants. (B) Reactivity of gp120 of recombinant derivatives of SIVmac239 and 1-12. SIV(+) indicates sera from a rhesus macaque infected with SIVmac239 as a positive control. CEMx174 cells infected with the indicated cloned or recombinant virus were starved in methionine-cysteine-free RPMI for 1 h and then labeled with [^{35}S]methionine-cysteine for 16 h. ^{35}S-labeled virus was harvested and disrupted with RIPA buffer (see Materials and Methods). SIV equivalent to 100 ng of p27^{gag} (measured by Coulter SIV Core Ag Assay) was used for each lane. Rabbit anti-mouse immunoglobulin G-bound protein A-Sepharose beads were used to precipitate antibody-bound gp120. Bands shown are gp120. M indicates a mock infection, 239 indicates a SIVmac239 infection, and 1-12 indicates infection with variant 1-12. 1-12 V1 239 has a 1-12 backbone with the V1 region replaced by SIVmac239 V1. 1-12 V4V5 239 has 1-12 backbone with the V4/V5 region replaced by SIVmac239 V4/V5. The nomenclature for the other recombinant viruses follows the same pattern.

RESULTS

Reactivity of SIVmac envelope gp120. Five MAbs were used for our experiments: Senv7.1, Senv101.1, KK5, KK9, and KK17 (8, 17, 18). These MAbs do not react with peptide or envelope protein expressed in *Escherichia coli*, and they do not react with denatured antigen in Western blots (immunoblots). Env glycoprotein gp120 of SIVmac251 does react well with these MAbs by RIPA and by immunofluorescence tests with infected cells. These properties, summarized in Table 1, indicate that the five MAbs require native antigen for optimal reactivity. These MAbs can neutralize the infectivity of SIVmac251, and they have been classified in the same competition group (8, 11, 17, 18). Antibodies of this type are made by rhesus monkeys as a natural response to SIV infection (24, 34).

The MAbs were tested for reactivity with a number of strains of SIVmac derived from cloned DNA. SIVmac239 was previously derived by animal passage of SIVmac251 (9, 19, 30, 33). SIVmac142 was independently isolated from another rhesus macaque of the same colony at the New England Regional Primate Research Center (6, 9). The gp120s of the cloned SIVmac251 and SIVmac239 used for our studies differ at only 28 of 525 residues in gp120 (95% amino acid identity) (Fig. 1; Table 2). The gp120 of SIVmac142 is more distantly related, with differences at 45 positions compared with gp120 of SIVmac239 (91% amino acid identity) and 50 positions compared with gp120 of SIVmac251 (90% amino acid identity) (Fig. 1; Table 2). Variants 1-12 and 8-22 were derived from rhesus monkeys infected with cloned SIVmac239 (4, 5); these variants differ at only 20 residues in gp120 compared with SIVmac239 (Fig. 1; Table 2).

The gp120s of all of these cloned viruses reacted equally well by RIPA with sera from monkeys infected with SIVmac239 or SIVmac251 (Fig. 2A). The five MAbs all recognized SIVmac239 gp120, but they did not react or reacted poorly with variants 1-12 and 8-22 (Fig. 2A). The gp120 of SIVmac142 was recognized somewhat by KK17, but it did not react significantly with the other four MAbs (Fig. 2A). The results obtained with the five molecularly cloned viruses, summarized in Table 3, indicate that the reactivities of the five neutralizing MAbs are very sensitive to natural sequence variation in gp120.

Variable domains responsible for loss of gp120 recognition. Eight recombinant clones were constructed by using SIVmac239 and variant 1-12 *env* sequences (Fig. 3). All recombinant clones were found to yield virus that was replication competent in CEMx174 cells. Infected cells were labeled with ^{35}S, and the virus produced from these cells was used for RIPA analysis. The gp120s of all recombinant viruses reacted equally

TABLE 3. Reactivities of MAbs with SIVmac gp120 by RIPA

Virus	Reactivity[a]					
	Senv7.1	Senv101.1	KK5	KK9	KK17	SIV+[b]
SIVmac251	+	+	+	+	+	+
SIVmac239	+	+	+	+	+	+
SIVmac142	−	−	−	−	+	+
Variant 1-12	−	−	−	−	−	+
Variant 8-22	−	−	−	−	−	+

[a] No or very weak reactivity, such as that present at some positions in Fig. 2, is indicated by a minus sign.
[b] RIPA results obtained with sera from an SIVmac239-infected rhesus monkey.

	HindIII 6822 SpeI 7045 [V1]	MroI 7758 ClaI 8072 [V4-V5]	Senv7.1	Senv101.1	KK5	KK9	KK17	SIV(+)
SIVmac239			+	+	+	+	+	++
Variant 1-12			-	-	-	-	-	++
1-12 V1 239			-	-	-	-	-	++
1-12 V4V5 239			+	+	+	+	+	++
239 V4V5 1-12			-	-	-	-	-	++
239 V1V4V5 1-12			-	-	-	-	-	++
239 V1 1-12			+	+	+	+	+	++
1-12 V1V4V5 239			+	+	+	+	+	++
239 V2V3 1-12			+	+	+	+	+	++
1-12 V2V3 239			-	-	-	-	-	++

FIG. 3. Composition of recombinant viruses and their reactivities in RIPA. Reactivity was determined from the data shown in Fig. 2 and other repeated experiments which are not shown. The restriction enzyme sites used for recombinant constructions are indicated at the top. The presence or absence of reactivity is indicated by + or −. Very weak reactivity, such as that seen in some positions in Fig. 3B, is indicated by −.

well with sera from SIV-infected monkeys (Fig. 2B). The pattern of reactivity with recombinant viruses strongly indicated that loss of recognition by the MAbs was associated with sequence changes in the V4/V5 region (Fig. 2B and 3).

Individual amino acid changes responsible for loss of recognition. On the basis of the natural sequence variation in the V4 and V5 regions of the cloned viruses (Fig. 1), seven site-specific mutations were created in SIVmac239. These mutations were designed to test which specific sequences in this region were responsible for loss of recognition by the five MAbs (Table 4). All mutated clones yielded virus that was replication competent in CEMx174 cells. Cells infected with mutant virus were labeled with ^{35}S, and labeled virus contained in the supernatant was used for RIPA analysis. Sera from SIV-infected monkeys reacted equally well with gp120 of all mutant viruses (Fig. 4; Table 4). A change of 409N to D, deletion of KPKE at residues 420 to 423, and deletion of KEQH at residues 422 to 425 each resulted in loss of recog-

nition by all five MAbs (Fig. 4; Table 4). All of these natural sequence variations are in V4. However, changing 417A to T or 417/418AN to TK (which created a potential N-linked glycosylation site) did not alter, or altered only slightly, the
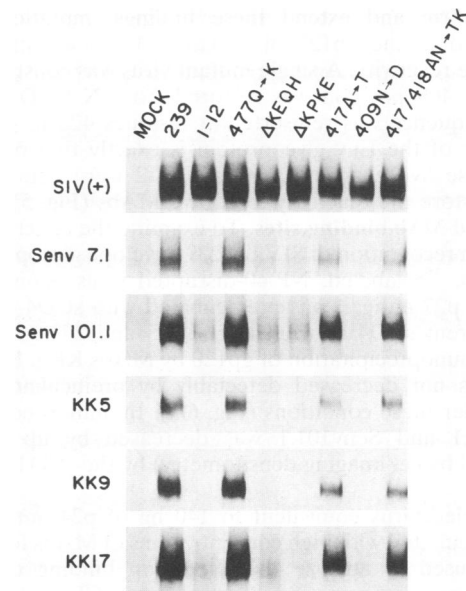

FIG. 4. Immunoprecipitation of site-specific mutants with MAbs. SIV(+) indicates sera from a rhesus macaque infected with SIVmac239 as a positive control. The indicated mutations were made in the V4 or V5 domain of SIVmac239 gp120. CEMx174 cells infected with SIVmac239, variant 1-12, or site-specific mutant virus were starved in methionine-cysteine-free RPMI for 1 h and then labeled with [^{35}S]methionine-cysteine for 16 h. ^{35}S-labeled viruses were harvested and disrupted with RIPA buffer. SIV equivalent to 200 ng of p27^{gag} (measured by Coulter SIV Core Ag Assay) was used for each lane. Rabbit anti-mouse immunoglobulin G-bound protein A-Sepharose beads were used to precipitate MAbs-bound gp120. Bands shown are gp120.

TABLE 4. Reactivities of neutralizing MAbs with mutant gp120s by RIPA

Virus[a]	Reactivity[b]					
	Senv 7.1	Senv 101.1	KK5	KK9	KK17	SIV+[c]
SIVmac239 wild type	+	+	+	+	+	+
Variant 1-12	−	−	−	−	−	+
409N→D (239)	−	−	−	−	−	+
417A→T (239)	+	+	+	+	+	+
417/418AN→TK (239)	+	+	+	+	+	+
Δ420–423KPKE (239)	−	−	−	−	−	+
Δ422–425KEQH (239)	−	−	−	−	−	+
477Q→K (239)	+	+	+	+	+	+
415/416NT→SL (239)	+	+	+	+	+	+
409D→N 422–425KEQH (1-12)	+	+	+	+	+	+

[a] No or very weak reactivity, such as that seen at some positions in Fig. 4, is indicated by a minus sign.

[b] Each strain in parentheses indicates the clonal envelope within which the indicated amino acids were mutated. 239, SIVmac239.

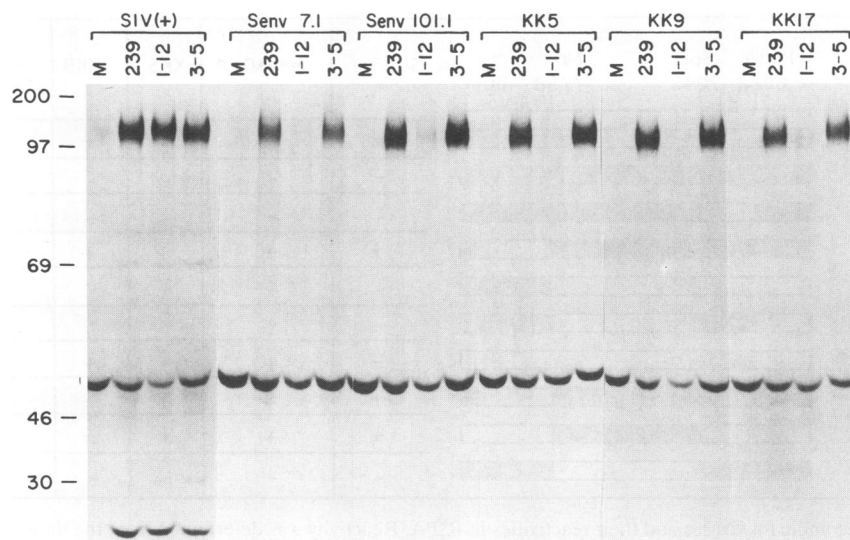[c] RIPA results obtained with sera from a rhesus monkey infected with SIVmac239.

FIG. 5. Immunoprecipitation of mutant 3-5 with MAbs. The 3-5 mutation is 409D→N and 422-425KEQH inserted into the 1-12 variant gp120 backbone. SIV(+) indicates sera from a monkey infected with SIVmac239 as a positive control. CEMx174 cells infected with SIVs were starved in methionine-cysteine-free RPMI for 1 h and then labeled with [^{35}S]methionine-cysteine for 16 h. SIV equivalent to the 200 ng of p27^{gag} (measured by Coulter SIV Core Ag Assay) was used for each lane. Lanes: M, mock infection; 239, infection by SIVmac239; 1-12, infection by variant 1-12; 3-5, infection by virus with the 3-5 mutation described above. Sizes are indicated in kilodaltons.

recognition by the MAbs by RIPA (Fig. 4; Table 4). Changing 477Q to K in V5 did not significantly alter the recognition by the five MAbs in RIPA (Fig. 4; Table 4).

To confirm and extend these findings, mutations were constructed in the gp120 of variant 1-12 in an attempt to restore the reactivity. A single mutant virus was constructed in which the 409 position was restored from N to D and the KEQH sequence was reinserted at residues 422 to 425. The remainder of the Env glycoprotein is exactly that of variant 1-12. These five changes in variant 1-12 were able to completely restore the reactivity to all five MAbs (Fig. 5).

CD4 and MAb binding sites. To examine the effect of sCD4 binding on recognition of SIVmac239 envelope glycoprotein by the MAbs, ^{35}S-labeled, NP-40-disrupted virus equivalent to 140 ng of p27 antigen was preincubated with sCD4 for 16 h. Two different sCD4 concentrations, 2.5 and 20 μg/ml, were used. Immunoprecipitation of gp120 by MAbs KK5, KK9, and KK17 was not decreased detectably by preincubation with sCD4 under these conditions (Fig. 6A). Immunoprecipitation by Senv7.1 and Senv101.1 was decreased by up to 35% (measured by gel imaging densitometry) by the sCD4 preincubation.

^{35}S-labeled virus equivalent to 140 ng of p24 antigen was also preincubated with high concentrations of MAbs for 16 h at 4°C and used to analyze the effects on binding of sCD4. Following preincubation, Sepharose-bound sCD4 was used to precipitate gp120. Binding of sCD4 to the SIVmac239 envelope was not affected by the MAb prebinding (Fig. 6B). This result indicates that the binding domains of the MAbs and sCD4 are not overlapping.

DISCUSSION

Sequence variations that accumulate in *env* with time of SIV infection result from selective forces operating in vivo (5). Sequence changes in *env* become fixed predominantly in discrete variable domains, and within these variable domains, a

remarkably high percentage of nucleotide substitutions are nonsynonymous (5, 16, 32). Thus, amino acid changes in the discrete variable domains provide selective advantage to mutant virus. Neutralizing antibodies appear to be one of the selective forces, since one result of the sequence variation is escape from ongoing neutralizing antibody responses (4). However, it is not known to what extent other selective forces, such as cell type and tissue tropism, may be influencing the fixation of amino acid substitutions. Furthermore, it is not known which variable domains are primarily responsible for the escape from neutralization.

In this report, we have shown that natural sequence variation in SIV V4 can result in escape from neutralization by at least one class of neutralizing antibodies. The neutralizing antibodies that were used were raised against native *env* antigen by immunization of mice with a vaccinia virus recombinant (8, 17, 18). Most of the neutralizing antibodies that were identified from these studies, including the five used here, required native antigen for optimal recognition (8, 17, 18). These findings suggest that neutralizing antibodies to SIVmac251 that recognize discontinuous or conformational epitopes may predominate over those that recognize linear epitopes, consistent with previous studies (15). The five MAbs used in the present study are representative of a single, major, cross-competition group (8, 11, 14, 18), and antibodies of this type have been shown to appear in rhesus monkeys as a natural response to SIV infection (24, 34). Sequences in V4 appear to be uniformly important for recognition by this major class of SIVmac neutralizing antibody.

Several neutralization epitopes in the envelope of SIVmac have been previously reported. Some of the MAbs identified by Kent et al. (18) and Benichou et al. (2) that neutralize SIVmac infectivity react with peptides corresponding to amino acids 170 to 190 of gp120. A weak type-specific neutralization determinant was identified in a variable region of the transmembrane protein of SIVmac (20). Torres et al. (37), using peptides to elicit antibodies, and Collignon et al. (8), using
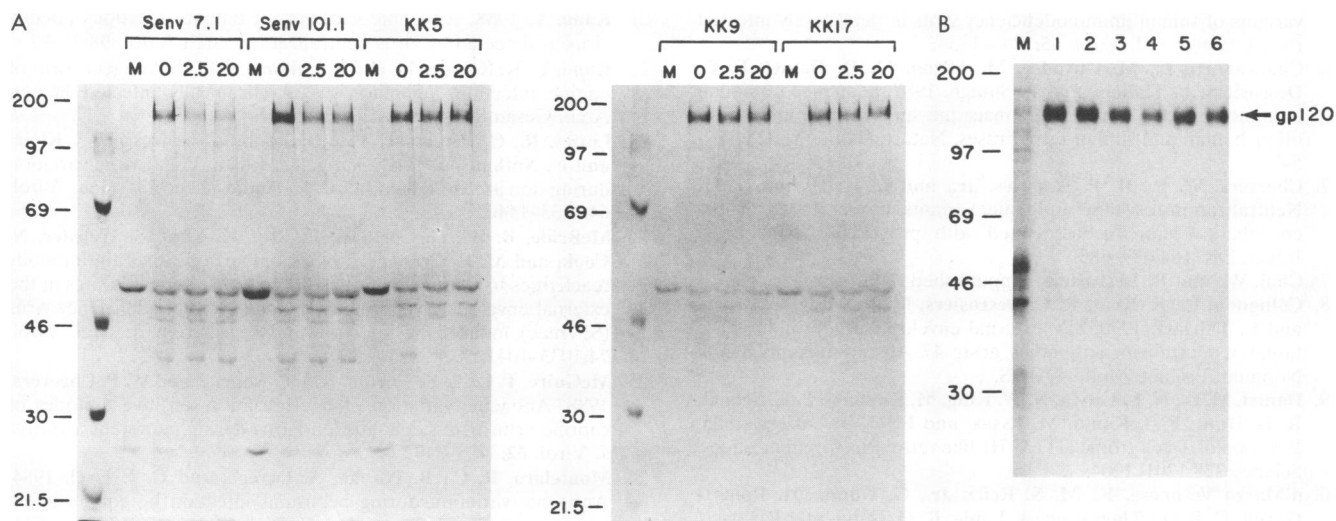
FIG. 6. (A) Immunoprecipitation of SIVmac239 gp120 prebound with sCD4. ^{35}S-labeled SIV equivalent to 140 ng of p27^{gag} (measured by Coulter SIV Core Ag Assay) was disrupted with NP-40 buffer and preincubated with sCD4 (2.5 or 20 μg/ml) for 16 h at 4°C, and gp120 was immunoprecipitated with MAbs. (B) Precipitation by sCD4-Sepharose of SIVmac239 gp120 prebound with MAb. Lanes: M, mock-infected CEMx174 cell supernatant; 1, 239 only, precipitated with sCD4-bound Sepharose beads; 2 to 6, SIVmac239 prebound with MAbs Senv7.1, Senv101.1, KK5, KK9, and KK17, respectively, and then precipitated with sCD4-bound Sepharose beads. gp120 bands are indicated. Sizes are indicated in kilodaltons.

peptide to block the neutralizing activity of polyclonal sera, demonstrated that amino acids 410 to 430 in V4 of gp120 contain a neutralization epitope. The 409 and 420–425 determinants described in the current report are within or near this linear epitope described previously. Although extremely sensitive to sequence changes in V4, the five neutralizing antibodies used in this study do not react to any large extent with synthetic peptides, with E. coli-produced subfragments, or with denatured protein on Western blots, and thus they recognize conformational or discontinuous epitopes.

How do these results on the important role of V4 for recognition by one class of SIVmac neutralizing antibody relate to sequence requirements for neutralization of HIV-1? Unfortunately, little work has been done on the effects of natural sequence variation in HIV-1 V4 on recognition by conformation-sensitive MAbs that neutralize HIV (29). Berkower et al. (3) have reported a major, conformation-dependent HIV-1 neutralization determinant mapping to a region of gp120 (residues 342 to 511) that includes V4. It will be important to learn whether natural sequence variation in V4 of HIV-1 may similarly result in loss of recognition by some HIV-1 neutralizing antibodies.

The lack of reactivity of cloned SIVmac142 and other recombinants suggests that sequence changes outside of V4 may also result in escape from recognition by these five MAbs. Comparison of the sequences in V4 of SIVmac142 with V4 sequences of clones that do react (SIVmac251, SIVmac239, and mutants 417A→T, 417/418AN→TK, and 415/416NT→SL) would predict no sequence changes in V4 of SIVmac142 that should obviate recognition by the five MAbs (Fig. 2; Table 4). Indeed, when the V4/V5 region of SIVmac142 was substituted in SIVmac239, positive reactivity was observed with all five MAbs (7a). Similarly, exchange of V1 sequences did not alter the pattern of recognition. SIVmac142 contains three amino acid changes at positions 327, 335, and 337 within the cysteine loop corresponding to V3 of HIV-1 (Fig. 2), and these could possibly be responsible for the loss of recognition. Kent et al. (16a) have recently found that some SIVmac neutralization

escape mutants generated in vitro to this class of antibody result from changes in V3, and Javaherian et al. (14) have presented evidence for conformationally determined SIV neutralizing activity dependent upon appropriate interaction of C-terminal sequences with V3.

The genetic approach that we have used is a powerful one for studying neutralizing antibodies that recognize complex conformational determinants. Because it utilizes the effects of natural sequence variation, it allows dissection of the mechanisms involved in escape from immune surveillance. Continued use of this approach should lead to a more detailed understanding of which variable domains are responsible for escape from different classes of neutralizing antibodies.

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