The “V3” Domain Is a Determinant of Simian Immunodeficiency Virus Cell Tropism

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Thirty-one different mutant forms of simian immunodeficiency virus (SIVmac) were created with changes in the region of *env* corresponding to the V3 domain of HIV-1. Sixteen of these mutants had one amino acid change, 12 had two changes, two had three changes, and one had four changes in the SIVmac “V3” loop. The ability of the mutant viruses to replicate in CEMx174 cells, rhesus monkey peripheral blood mononuclear cells, and rhesus monkey alveolar macrophages was investigated. Ten of the mutant viruses replicated with approximately wild-type kinetics in all three cell types. Of the 31 mutants, 22 were able to replicate in one or more of the cell types. Thus, this region of SIVmac gp120 is quite tolerant to change. Nine of the mutants replicated poorly or not at all in any of the cells tested. The lack of replication competence of some of the mutants was associated with inefficient proteolytic processing of the gp160 precursor. Some mutations had dramatic differential effects in different cell types. For example, changing P to S at position 321 and M to I at position 325 drastically reduced replication in macrophages and CEMx174 cells but had no effect on replication in peripheral blood mononuclear cells. Mutants with altered tropism were blocked at an early stage that includes virus entry into cells. Thus, sequences in SIVmac that correspond to V3 in HIV-1 can affect virus entry and cell tropism in a manner analogous to that of HIV-1 V3.

Human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2) are the etiologic agents of AIDS. The simian immunodeficiency viruses (SIVs) are nonhuman primate lentiviruses that are similar to the HIVs in genomic organization and biological properties (14–16, 21). Infection of macaque monkeys with some SIVs can cause a disease very similar to AIDS in humans, and this provides a useful animal model for the study of HIV pathogenesis and for the testing of vaccine strategies (14–16, 21).

The external glycoprotein of HIV-1, which has about 40% amino acid identity with the SIVmac external glycoprotein, is an important candidate antigen for vaccine testing. Sequence variation within the external glycoproteins of these viruses is nonrandom in a number of significant ways. Most nucleotide substitutions that become fixed with time are nonsynonymous, i.e., they change the amino acid sequence, indicating that selection is occurring in vivo for variant forms (4, 22, 36, 45, 54, 55, 58, 63, 67, 69). Also, sequence changes are not randomly distributed but occur predominantly in discrete variable domains. Variable regions V1, V2, V4, and V5 of SIVmac exactly correspond to those in HIV-1. However, in three separate studies of SIVmac sequence variation, the region in SIVmac corresponding to V3 of HIV-1 was found not to vary significantly (4, 36, 58). More recently, the region corresponding to V3 was found to vary significantly in a portion of SIVmac-infected macaques (42, 43). Furthermore, “V3” sequence variation in animals infected with SIVmac239 seemed to be largely associated with altered cell and tissue tropism (42, 43).

The V3 cysteine loop of HIV-1 is important for fusion of the viral envelope to the host cell membrane subsequent to initial binding of the external glycoprotein to the CD4 molecule on the surface of target cells (1, 13, 19, 20, 25, 59). Sequence variation in HIV-1 V3 can influence cell tropism, including the

tropism for primary macrophages (5–8, 10, 17, 32, 33, 56, 65, 66, 71, 74, 75, 78), and it appears to do so at the level of post-CD4 binding entry events (7, 56).

In the present study, a collection of SIVmac “V3” mutants was constructed and their properties were analyzed. We report that SIV “V3” can affect viral entry and cell tropism in a manner analogous to that of HIV-1 V3.

MATERIALS AND METHODS

Virus. Macrophage-competent variants of the pathogenic SIVmac239 clone called SIVmac239/316ENV*, SIVmac239/316ENV, and SIVmac239/316SU have been described elsewhere (51, 52). SIVmac239/316ENV* and SIVmac239/316SU express a full-sized transmembrane protein (gp41); in SIVmac239/316ENV the transmembrane protein is truncated because of a premature stop codon (51, 52). The *nef* gene was fully open in all of the variants used (40).

For preparation of virus stocks, the 3′ half of the SIV provirus was ligated to the 5′ half of proviral DNA as described previously (39), and full-length proviral DNA was used to transfect COS-1 cells by the DEAE-dextran method described by Cullen (11). At 3, 5, and 7 days posttransfection, virus released into the culture supernatant was harvested, passed through a 0.45-μm-pore-size filter, and stored at –70°C. The p27 antigen levels were determined with a commercial antigen capture kit (Coulter Co., Hialeah, Fla.). The viral stocks were used to infect cells of the human T-B hybrid cell line CEMx174, rhesus macaque peripheral blood lymphocytes (PBM), and alveolar macrophages.

Cell culture. COS-1 cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. CEMx174 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum and antibiotics.

Rhesus PBM were obtained by banding over sodium diatrizoate-Ficoll (Organon Teknika Corp., Durham, N.C.),

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TABLE 1. Replication of SIVmac "V3" mutants in CEMx174 cells, alveolar macrophages, and rhesus PBMC

SIVmac239 "V3" loop amino acid sequence	Replication of SIVmac mutants in ^a :								
	CEMx174 cells			Macrophages			PBMC		
	ENV*	ENV	SU	ENV*	ENV	SU	ENV*	ENV	SU
311 344									
CRRPGNKTIVLPVTIMSGLVFHSQPIINDRKPQAWC	++	++	++	++	++	++	++	++	++
.....ES.....	-	(-)	-	-	(-)	-	-	-	-
.....N.....	-	-	-	-	-	-	-	-	-
.....A.....	-	-	-	-	-	-	-	-	-
.....F.....	++	++	ND	++	++	ND	++	++	ND
.....I.S.....L.....	-	-	-	-	(-)	-	-	-	-
.....I.....S.....	++	++	++	+	+	+	+	++	++
.....F.....L.....	+	+	+	++	+	++	++	++	++
.....S.....	(-)	(+), d	(+), d	++	++	++	++	++	++
.....S.....I.....	(-)	(-)	-	+	+	+	++	++	++
.....L.....	++	++	ND	++	++	ND	++	++	ND
.....N.....	+	(+), d	(+), d	+	+	(+)	+	(+)	(+), d
.....S.....LL.N.....	-	-	ND	-	-	ND	-	-	ND
.....AN.Y.....	-	-	ND	-	-	ND	-	-	ND
.....L.....	++	++	++	++	++	++	++	++	++
.....I.....	++	++	++	++	++	++	++	++	++
.....I.....F.....	++	++	++	+	+	++	++	++	++
.....K.....L.....	-	-	ND	-	-	ND	-	-	ND
.....Y.....	(-)	(+)	(+)	(+)	(+), d	(+), d	-	(+)	(+)
.....P.F.....	ND	++	++	ND	-	-	ND	(+), d	-
.....R.....	ND	+	-	ND	+	-	ND	-	(-)
.....F.....	++	++	ND	++	++	ND	++	++	ND
.....S.....	++	+	++	++	++	++	++	++	++
.....F.....K.....	-	-	ND	(+), d	+	ND	-	-	ND
.....SG.....	(-)	(-)	(+)	-	-	-	-	-	-
.....F.....	++	++	++	++	++	++	++	++	++
.....L.....K.....	-	(+), d	ND	-	-	ND	-	-	ND
.....L.....V.....	++	++	++	++	+	+	-	-	-
.....KQ.....	++	++	++	++	++	++	++	++	++
.....L.....	++	++	ND	++	++	ND	++	++	ND
.....K.....	++	++	++	++	++	++	++	++	++
.....K.....	++	++	ND	++	++	ND	++	++	ND

^a ENV*, SIVmac239/316ENV*; ENV, SIVmac239/316ENV; SU, SIVmac239/316SU; -, no replication detected; (-), small amounts of p27 (<1 ng) detected; (+), maximal levels of virus production detected were <10% of those of the original clones; ++, maximal levels of virus production detected were 10 to 50% of those of the original clones; ++, virus production comparable to that of the original clones; d, delayed growth kinetics; ND, not done.

treated with 1 µg of phytohemagglutinin per ml for 2 to 3 days, washed, and maintained in RPMI medium containing 20% fetal calf serum, 10% interleukin-2 (Electro-Nucleonics, Inc., Fairfield, N.J.), and antibiotics.

Alveolar macrophages from healthy rhesus macaques that were seronegative for SIV, type D retrovirus, simian T-cell leukemia virus type 1, and simian foamy virus were obtained by bronchoalveolar lavage and maintained in RPMI 1640 medium supplemented with 20% human serum (GIBCO Laboratories, Grand Island, N.Y.), 100 U of penicillin per ml, 100 µg of streptomycin per ml, and 0.25 µg of amphotericin per ml. Culture media were changed two or three times weekly, and aliquots were used to assay p27 antigen levels.

Infections were normalized according to equivalent amounts of p27 antigen. The same virus stocks were used to infect CEMx174 cells, rhesus PBMC, and rhesus alveolar macrophages in parallel.

Site-specific mutagenesis. Oligonucleotide primers were synthesized with a Cyclone DNA synthesizer (Milligen-Bioscience, Burlington, Mass.). Site-directed mutagenesis of the "V3" region was performed by overlap extension PCR (29) using internal primers which were synthesized after each vial of phosphoramidites was "doped" with 1.5% (vol/vol) of each of the other three phosphoramidites. The p239SpE3'/316ENV* plasmid served as the template for PCR, and mutant clones

were isolated and characterized as described elsewhere (41). The PCR products, containing random changes within "V3," were cloned in the 3' half of the proviral p239SpE3'/316ENV* plasmid by using the *Apa*LI and *Bsp*EI sites in the envelope gene. Plasmid DNA was prepared from single colonies, and clones containing DNA migrating at the correct size were analyzed by restriction mapping. For a subset of clones containing amino acid changes within "V3," the entire PCR-derived sequence between the *Apa*LI and *Bsp*EI sites was determined. The *Spe*I-*Bsp*EI fragment of clones containing changes within "V3" was cloned into the 3' halves of the p239SpE3'/316ENV and p239SpE3'/316SU proviral DNAs. The clones used contained no sequence changes outside "V3."

Assay to detect newly synthesized viral DNA. The assay was performed according to the procedure of Mori et al. (51). Virus stocks were treated with 5 µg of DNase I (Worthington Biomedical Corp., Freehold, N.J.) per ml for 1 h at room temperature in the presence of 10 mM MgCl₂. For infection, cells were incubated with the indicated virus stocks for 2 h, washed once with RPMI medium, and then incubated in the media described above for 12 to 16 h at 37°C. Cells were washed twice with phosphate-buffered saline, and the cell pellets were resuspended in 0.3 ml of lysis buffer (4.7 M urea, 1.3% sodium dodecyl sulfate [SDS], 0.23 M NaCl, 6.7 mM Tris-HCl [pH 8.0], 0.67 mM EDTA), extracted twice with

FIG. 1. Locations and biological effects of amino acid changes in the SIVmac "V3" region investigated in this study. *, levels of processing of the gp160 env precursor estimated from the data shown in Fig. 2. The levels are expressed as follows: +++, >80%; ++, 50 to 70%; (+), 10 to 20%; and -, <5%. n.d., not done; LTR, long terminal repeat. Mutants were grouped according to measurements of viral replication in cell culture (Table 1).

phenol-chloroform and once with chloroform, precipitated with ethanol, and resuspended in 25 to 100 μ l of TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]). A 2.5- μ l portion of the DNA was subjected to 30 cycles of amplification (1 min at 94°C, 1 min at 55°C, and 1 min at 72°C with 6 mM MgCl₂) using a thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.) and an ampliTaQ PCR reagent kit. The first primer pair, M97 (5'-AACTCCGTCTTGTCAGGGAAG-3') and M98 (5'-CAACTTCATCCACGTTCAACC-3'), corresponds to SIVmac239 gag gene sequences, and the second pair, M93 (5'-ACACAACGTGTGTTCACTAGC-3') and M94 (5'-CAACTTCATCCACGTTCAACC-3'), corresponds to the human β -globin gene. Primers M93 and M97 were 5' end labeled with ³²P prior to amplification. A 5- μ l portion of each total 25- μ l reaction sample volume was electrophoresed through a 7% polyacrylamide-Tris-borate-EDTA gel for 1.5 h at 200 V. The gels were dried on 3 MM paper and exposed to X-ray film.

Radioimmunoprecipitation. The 3' halves of SIVmac239/316SU and the "V3" mutant clones were modified by inserting the pSR α promoter (70), containing the simian virus 40

early promoter and the R segment and part of the U5 sequence of the long terminal repeat of human T-cell leukemia virus type 1, at the unique *Sph*I site upstream of the *rev* and *env* genes. COS-1 cells were transfected with 2.5 μ g of the modified plasmids. At 48 h posttransfection the cells were labeled with [³⁵S]methionine and [³⁵S]cysteine for 16 h, and cell extracts were obtained as described previously (53). A 100- μ l portion of each lysate plus 400 μ l of lysis buffer (1% Triton X-100, 0.15 M NaCl, 50 mM Tris-HCl [pH 7.4], 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) was incubated with 10 μ l of serum for 1 h at 4°C. Thereafter, 60 μ l of a protein A-Sepharose suspension was added and the solutions were incubated for 30 to 60 min. Pelleted immune complexes were then washed three times in lysis buffer and once in 50 mM NaCl-50 mM Tris-HCl (pH 7.2), resuspended in sample dissociation buffer (125 mM Tris-HCl [pH 6.8], 4% SDS, 20% glycerol, 10% β -mercaptoethanol, 0.2% bromophenol blue), and analyzed on 7.5% polyacrylamide-SDS gels.

FIG. 2. Immunoprecipitation of SIVmac239/316SU env proteins having wild-type or mutant "V3" expressed in COS-1 cells. Proteins in labeled cell extracts were immunoprecipitated with polyclonal antiserum to SIVmac239 (except that in lane 2, for which serum from an uninfected macaque was used) and analyzed by SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. The positions of gp160 and gp120 are indicated. M, marker; lane 1, cells transfected with no DNA; lanes 2 and 3, cells transfected with a plasmid expressing the SIVmac239/316SU envelope; lane 4, 317 K→E, 318 T→S; lane 5, 317 K→N; lane 6, 319 V→A; lane 7, 320 L→F; lane 8, 320 L→I, 323 T→S, 334 P→L; lane 9, 320 L→I, 328 L→S; lane 10, 320 L→F, 325 M→L; lane 11, 321 P→S; lane 12, 321 P→S, 325 M→I; lane 13, 322 V→L; lane 14, 323 T→N; lane 15, 323 T→S, 331 H→L, 332 S→L, 335 I→N; lane 16, 323 T→A, 324 I→N, 326 N→Y; lane 17, 324 I→L; lane 18, 326 S→Y; lane 19, 327 G→R; lane 20, 328 L→S, 329 V→G; lane 21, 330 F→L, 336 I→K; lane 22, 328 L→F; lane 23, 328 L→F, 332 S→K; lane 24, 333 Q→K, 334 P→Q; lane 25, 338 R→K. The SIVmac 239 "V3" sequence has been published by Regier and Desrosiers (61).

RESULTS

Mutagenesis. A random mutagenesis protocol was used to generate a library of "V3" mutations across most of the SIVmac "V3" domain. The 31 different "V3" mutant forms selected for study are shown in Table 1. In this collection of mutants, 22 of the 34 amino acid positions within the SIVmac "V3" domain were changed at least once, with an average of 1.5 amino acid substitutions per clone. Of the 31 mutants, 16 contained a single amino acid change, 12 had two changes, 2 had three changes, and 1 had four changes within the "V3" loop sequence (Table 1). The absence of additional alterations in the PCR-derived portion of each mutagenized plasmid was determined by DNA sequencing.

The "V3" domain is tolerant to change. Of the 31 mutants, 22 were able to replicate appreciably in at least one cell type (Table 1; Fig. 1). Changes in 16 of the 22 amino acid positions mutated in our study did not result in a complete loss of biological activity. However, some of the 22 replication-competent mutants showed considerably reduced and/or cell type-dependent growth activity and were often less cytopathic than viruses containing the original SIVmac239 "V3" sequence. Comparable effects of "V3" changes on viral replication were observed in the backgrounds of the three different variants used in this study (Table 1).

Ten mutants consistently replicated with approximately wild-type kinetics in all three cell types (Table 1; Fig. 1). Changes at position 320 of L to F (320 L→F), 322 V→L, 324 I→L, 325 M→I, 328 L→F, 329 V→F, 333 Q→K + 334 P→Q, 334 P→L, and 338 R→K did not have a significant effect on replication or cytopathicity of the viruses. A change to a positively charged amino acid at position 336 (N→K) close to the C terminus of "V3" enhanced the cytopathicity of the virus in CEMx174 cells. This variant produced less virus in CEMx174 cells than virus harboring the original "V3" sequence because of very rapid cell killing (data not shown). In contrast, two other mutants, containing a 328 L→S change alone or in conjunction with a 320 L→I change, replicated with delayed kinetics (Table 1) and reached higher p27 peak levels than the wild-type viruses did in CEMx174 cells (data not shown). The gp160 env precursors of these viable "V3" mutants were processed with normal efficiency in COS-1 cells (Fig. 1 and 2).

Changes close to the tip of the SIVmac "V3" can reduce viral replication. Some amino acid changes close to the tip of the "V3" loop (323 T→N, 326 S→Y, 326 S→P + 328 L→F, 327 G→R, and 328 L→F + 332 S→K) reduced viral replication considerably in all three cell types (Table 1). The gp160 env precursors of these mutants were shown to be efficiently

processed (Fig. 1 and 2), with the exception of mutant 326 S→P + 328 L→F which was not investigated. In contrast to the original SIVmac239/316ENV*, SIVmac239/316ENV, and SIVmac239/316SU, these SIVmac "V3" variants caused little or no cytopathic effect or cell death in CEMx174 cells. Considerably less syncytium formation between the envelope-expressing COS-1 cells and the CD4 receptor-bearing CEMx174 cells was observed when these cells were compared with cells transfected with *env* sequences that allow normal viral replication (data not shown).

Some "V3" changes severely impair viral replication. Of the 31 mutants, 9 were not able to replicate detectably in any of the cell types used (Table 1; Fig. 1). The lack of infectivity was often associated with inefficient proteolytic processing of the gp160 env precursor (Fig. 1 and 2). Interestingly, the gp160 of one triple "V3" mutant (320 L→I + 323 T→S + 334 P→L) had about the same electrophoretic mobility as the original gp160 and was efficiently processed, but the gp120 showed clearly increased electrophoretic mobility (Fig. 2, lane 8). Two other nonviable mutants had a change (330 F→L) in the highly conserved bulky amino acid close to the tip of the loop (Table 1). Changes around the potential N-linked glycosylation site (NXT or NXS) close to the N terminus of the "V3" loop (NKT at positions 316 to 318 to NES or NNT), as well as a single V→A change at position 319, resulted in severely impaired replication (Table 1) and inefficient proteolytic processing (Fig. 2, lanes 4 through 6). In two of these mutants, the positively charged Lys-317 was changed to a polar Asn or a negatively charged Glu (Table 1). Most other inactive "V3" mutant viruses contained multiple amino acid changes throughout the "V3" loop (Table 1; Fig. 1) and produced inefficiently processed gp160 env precursors (Fig. 2, lanes 15 and 16).

Certain "V3" changes have differential effects in different cell types. Most changes within "V3" had comparable effects on viral replication and cytopathicity in CEMx174 cells, rhesus PBMC, and rhesus alveolar macrophages (Table 1). However, some mutations had dramatic differential effects on replication in different cell types. For example, a change of P→S at position 321 considerably reduced the ability of the virus to replicate in CEMx174 cells but had little if any influence on replication in PBMC or macrophages (Table 1; Fig. 3). Moreover, compared with those observed with the wild-type virus, minimal cytopathic effects were observed in CEMx174 cells with this mutant. An M→I mutation at position 325 had no significant influence on viral replication on its own (Table 1; Fig. 3). However, in conjunction with the P→S change at position 321, viral replication in macrophages was greatly

FIG. 3. Replication of SIVmac239/316SU and "V3" mutants in rhesus PBMC (A), CEMx174 cells (B), and rhesus alveolar macrophages (C). Virus stocks were prepared by transient expression in transfected COS-1 cells, and virus containing 5 ng of p27 antigen was used for infection. Virus production was monitored by assays of p27 in culture supernatants at the indicated numbers of days postinfection. Similar results were obtained after infection with the same "V3" loop alterations in the SIVmac239/316ENV and SIVmac239/316ENV* backgrounds.

reduced, but it remained unaltered in PBMC (Table 1; Fig. 3). Moreover, the 321 P→S + 325 M→I mutant did not replicate at all in CEMx174 cells (Fig. 3). These results were independently confirmed by using the variants SIVmac239/316ENV, SIVmac239/316ENV*, and SIVmac239/316SU (Table 1).

Certain other "V3" amino acid changes also caused differential effects on SIVmac cell tropism. One double mutant (332 S→L + 337 D→V) replicated with approximately wild-type kinetics in CEMx174 cells but was much less active in PBMC and macrophages (Table 1; Fig. 4). Similar results were obtained with another double mutant (326 S→P + 328 L→F) which replicated with slightly delayed kinetics in CEMx174 cells but did not replicate detectably in PBMC or macrophages after infection with 1 ng of p27 from SIVmac239/316ENV (data not shown) or 5 ng of p27 from SIVmac239/316SU (Fig. 4). Infection with higher doses of these two double mutants (100 ng of p27 from cell-free CEMx174 culture supernatant)

did result in some replication, but it was relatively inefficient (Fig. 4).

Effect of "V3" mutations on virus entry. To examine the ability of "V3" mutant viruses to enter cells, 10 ng of p27-containing SIVmac239/316ENV and mutants 317 K→N and 326 S→Y produced after transfection of COS-1 cells was incubated with CEMx174 cells. At 16 h postexposure, the amounts of newly synthesized viral DNA were estimated by PCR. The two mutants showed no (317 K→N) or very little (326 S→Y) replication in CEMx174 cells (Table 1; Fig. 5A). Much smaller amounts of newly synthesized viral DNA were detected with the 317 K→N and 326 S→Y mutants than with the wild-type virus (Fig. 5B). Independent experiments using the 317 K→E + 318 T→S, 319 V→A, and 327 G→R mutants produced similar results (data not shown).

The double mutant 321 P→S + 325 M→I did not replicate efficiently in CEMx174 cells or in macrophages, but it replicated like wild-type virus in PBMC (Fig. 3). After the incubation of cells with this double mutant, newly synthesized viral DNA could be detected in PBMC but not in CEMx174 cells or alveolar macrophages (Fig. 6). Moreover, the single 321 P→S change which selectively reduced viral replication in CEMx174 cells (Fig. 3B) also selectively reduced the amount of viral DNA detectable in CEMx174 cells after 16 h of incubation (Fig. 6).

Effects of "V3" sequence changes on processing of the gp160 env precursor. The gp160 env precursors of efficiently replicating "V3" mutants and those mutants which were severely impaired in replication because of amino acid changes close to the tip of the "V3" loop were efficiently processed in COS-1 cells (Fig. 1 and 2). In contrast, amino acid changes near the N-linked glycosylation site close to the N terminus of the loop

FIG. 4. Replication of SIVmac239/316SU and "V3" mutants in rhesus PBMC (A), CEMx174 cells (B), and rhesus alveolar macrophages (C). As indicated in the figure, the cells were infected with virus containing 5 ng of p27 antigen derived from COS-1 cells or 100 ng of p27 derived from CEMx174 cells.

published in the Los Alamos HIV sequence data base (54). It differs at only six positions from the consensus of 22 HIV-2 "V3" sequences (Fig. 7). Most amino acids which differed between the HIV-2 consensus sequence and the SIV consensus sequence were variable among the different HIV-2 isolates or represented conservative changes (Fig. 7B). In the present study, single amino acid changes at six different positions in the SIVmac "V3" loop severely impaired viral replication. Amino acids at these six positions are identical in the SIVmac-SIVsmm and HIV-2 consensus sequences and are 97% conserved among the 22 HIV-2 isolates and the 10 SIV isolates (Fig. 7A and B). In contrast, only 9 of the 13 positions which were more tolerant to change are identical in the HIV-2 and SIVmac-SIVsmm consensus sequences. These amino acids are only 81% conserved among the 22 HIV-2 "V3" sequences. The degree of amino acid conservation within the entire HIV-2 "V3" loop is 90% (Fig. 7B). Thus, the ability to tolerate change in the present study agrees quite well with the presence of natural sequence variation in this region.

Although the V3 loop of HIV-1 is highly variable and is highly divergent compared with the SIVmac "V3" loop, some general features are conserved even between SIVmac and HIV-1. The V3 loop sequence of HIV and the "V3" loop sequence of SIV have about the same length and contain more basic than acidic amino acids, resulting in a net positive charge (Fig. 7C). A potential N-linked glycosylation site on the N-terminal side, an aromatic amino acid (F) close to the tip, and sequences at the N and C termini of the loop show conserved features (Fig. 7C). Moreover, charged amino acids are often present at similar positions. It is quite surprising that the consensus "V3" loops of the distantly related SIVmac and SIVagm groups differ at only six amino acid positions (Fig. 7C).

(317 K→E + 318 T→S, 317 K→N, and 319 V→A) or multiple changes across the "V3" loop which severely impaired replication led to inefficient proteolytic processing of the gp160 env precursor (Fig. 1 and 2). These results suggest that some "V3" changes may influence the folding of the gp160 precursor, resulting in inefficient or altered proteolytic processing. The gp120, but not the gp160, of a triple "V3" mutant (320 L→I + 323 T→S + 334 P→L) showed increased electrophoretic mobility (Fig. 2, lane 8).

Comparison with "V3" sequences in a data base. The SIVmac239 "V3" sequence mutated in this study is identical to the consensus sequence of 10 SIVmac and SIVsmm sequences

FIG. 5. (A) Replication of SIVmac239/316ENV and mutants containing a 317 K→N or a 326 S→Y change in CEMx174 cells. Virus containing 1 ng of p27 antigen was used for the infections. (B) Entry of "V3" mutant viruses into CEMx174 cells. Virus containing 10 ng of p27 antigen derived from the supernatant of transfected COS-1 cells was used for the infections. +, the virus stock was heat inactivated at 65°C for 1 h prior to incubation with CEMx174 cells; -, the virus stock was not heat inactivated. Lanes 7 to 11, SIV standards corresponding to 200, 40, 10, 2, and 0 copies of p239SpSp5' plasmid DNA diluted in genomic DNA from uninfected CEMx174 cells, respectively; lane 12, water control.

DISCUSSION

Although the SU gp120 proteins of HIV-1 and SIVmac share only about 40% amino acid identity, a number of conserved features of their frameworks are apparent. The numbers and locations of cysteine residues are in general well conserved between the SU glycoproteins of these viruses, suggesting a similarity in the folded structure (31). Both proteins are extensively glycosylated and contain interspersed regions of variable and constant domains. Variable regions V1, V2, V4, and V5 exactly correspond in the two viruses (4, 69). The D residue within a conserved GGDPE sequence within C4 of HIV-1 gp120 has been shown by Olshevsky et al. (57) to be important for contacting CD4; Morrison et al. (53) have shown that the corresponding residue within SIVmac gp120 is similarly important for binding CD4. Independent groups using quite different methodologic approaches have shown that amino acid residues in C4 cooperate with amino acids to the

FIG. 6. Entry of SIVmac239/316ENV and site-specific "V3" mutants into rhesus PBMC (A), CEMx174 cells (B), and rhesus alveolar macrophages (C). Virus containing 100 ng of p27 derived from the supernatant of infected PBMC was used for infection. The mutations within "V3" are indicated above the lane numbers in panel A. + and - are defined as in the legend to Fig. 5. Lanes 9, uninfected cells. (A) Lanes 10 to 15, 400, 100, 25, 6.25, and 0 ng of DNA from uninfected CEMx174 cells in the reaction, respectively. (B and C) Lanes 10 to 14, 1,000, 200, 40, 10, and 2 copies per reaction tube, respectively (see legend to Fig. 5); lanes 15, water controls. The reactions for the amplification of gag and β-globin sequences were run separately for panel C to enhance the sensitivity of PCR.

left of the tip of "V3" in the process of virus entry into the cell for both SIVmac and HIV-1 (50, 53, 77).

The functional equivalence of the V3 and V3-corresponding regions of these viruses has previously been called into question. The V3 cysteine loop of HIV-1 shows extensive inter- and intrapatient variation and is an important target of neutralizing-antibody responses (8, 22-24, 26, 28, 30, 34, 37, 44-47, 54, 55, 60, 62, 63, 67-69, 73). Sequence variation in V3 of HIV-1 has also been shown to be a major determinant of macrophage and cell line tropism (5-8, 10, 17, 27, 32, 33, 56, 65, 66, 71, 74, 75, 78), apparently acting at the level of virus entry into the cell (7, 56). Initial studies with SIVmac yielded results for "V3" variation that were not quite in line with the findings with HIV-1. Three different groups that charted sequence variation in SIVmac env following experimental infection with cloned virus failed to find significant variation in the region corresponding to HIV-1 V3 (4, 36, 58). Also, antibodies raised against SIVmac251/239 "V3" failed to neutralize viral infectivity (35, 38, 48). Major physiological sequence changes

FIG. 7. Comparison of primate lentivirus "V3" consensus sequences. (A) SIVmac239 "V3" sequence. Changes which allowed replication with near wild-type efficiency in at least one of the three cell types used are indicated above the sequence; changes which severely impaired the ability of the virus to replicate are indicated below the SIVmac239 "V3" sequence. (B) Variability in the "V3" loop sequences of 21 HIV-2 and 10 SIVmac and SIVsmm sequences derived from the Los Alamos HIV sequence data base (54). Subscript numbers refer to the frequencies of occurrence (percent) at variable locations. (C) Comparison of the SIVmac-SIVsmm, HIV-2, SIVagm, and HIV-1 "V3" consensus sequences (54). Charged amino acids, the potential N-linked glycosylation site (NXT/S), and a conserved F residue close to the tip of the loop are enclosed in boxes.

responsible for imparting high replicative capacity of SIVmac in macrophages mapped to the envelope but were located outside "V3" (52) and functioned at some level subsequent to virus entry into the cell (51).

Other observations, and the findings described in this report, suggest that the differences between SIVmac "V3" and HIV-1 V3 may not be fundamental and may be more apparent than real. Kodama et al. have recently shown that some infected rhesus monkeys do display significant sequence variation in "V3" (42, 43). Variability in V3 of HIV-1 is also seen in some, but not all, infected humans and chimpanzees (49, 67, 76). Sequence divergence in "V3" of experimentally infected rhesus monkeys seems to be largely associated with changes in cell type and tissue tropism (42, 43). The "V3" loop of HIV-2, which is closely related to SIVmac (54), varies to a similar degree as the V3 loop of HIV-1 (3), can elicit neutralizing antibodies (2), and is important for the fusion step (18). In some studies of HIV-1 cell tropism, only a small portion of the capacity to replicate in macrophages was imparted by V3 sequences (56, 66). In the current study, we have shown that SIVmac "V3" is tolerant to change, functions in virus entry into cells, and can determine cell tropism.

The "V3" domains of SIVmac, SIVsmm, and HIV-2 are surprisingly quite homologous to the "V3" domain of SIVagm, but they show little sequence homology to the V3 domain of HIV-1 (Fig. 7C) (54). However, the N and C termini of the loop and certain other features, such as a potential N-linked glycosylation site at the N terminus, a net positive charge, and

an aromatic amino acid close to the tip, appear to be conserved between HIV-1 and SIVmac, SIVsmm, and HIV-2.

Changes in, or close to, the potential N-linked glycosylation site in the "V3" loop of SIVmac impaired the ability of the virus to replicate and resulted in inefficient proteolytic processing of the gp160 env precursor. For HIV-1, an influence of sequence alterations in V3 on the efficiency of gp160 processing has not been described previously. However, studies on the HIV-1 V3 loop were performed with mutants containing changes in the conserved tip of the loop (1, 12, 59) or various deletions in V3 (72). We also found that changes close to the tip of SIVmac "V3" severely reduced viral replication but had little or no effect on gp160 processing. Additional data are necessary to clarify if certain regions of the V3 loop of HIV-1 and the "V3" loop of SIVmac are of similar importance for proper folding and processing of the gp160 env precursor.

The "V3" domains of SIV and HIV-1 are positively charged (Fig. 7C) (54). Charged amino acids in V3 are often present at similar positions relatively close to the N and C termini (Fig. 7C). The introduction of positively charged amino acids at different positions to the left and right of the tip of V3 changed the non-syncytium-inducing phenotype of HIV-1 to the syncytium-inducing phenotype (8, 12, 17, 44). We found that the removal of a positively charged amino acid residue at position 317 of the SIVmac "V3" loop impaired viral replication, whereas the introduction of a positively charged amino acid at position 336 increased the cytopathicity of the virus in CEMx174 cells. This suggests that appropriately located posi-

tively charged amino acids in the "V3" domain are important for the cytopathicity of both SIVmac and HIV-1.

A bulky amino acid located on the carboxyl side of the crown of the "V3" loop is conserved among all primate lentiviruses (9, 54). A change of this residue from F to L at position 330 in SIVmac severely impaired the ability of the virus to replicate. It has been suggested that proteolytic cleavage of "V3" may be important for virus entry and that the conserved bulky amino acid may represent a potential site for proteolytic activity (9, 64). Such potential cleavage sites are theoretically present in all SIVs and HIVs (9). A role for proteolytic cleavage at this site in virus entry has not, however, been demonstrated.

The present study and recent studies of Kodama et al. (42, 43) have shown that "V3" can be an important determinant of SIVmac cell tropism and that it acts at the level of early events including virus entry into the cell. The changes 321 P→S and 325 M→I within "V3" of SIVmac were sufficient to reduce viral replication and entry into macrophages about 100-fold. These changes did not influence the ability of virus to enter or replicate in rhesus PBMC. These observations appear analogous to results of previous studies of V3 determinants of HIV-1 macrophage tropism. SIVmac239 has a "V3" sequence that allows efficient entry into macrophages, but sequence changes in *env* outside "V3," acting at a level subsequent to viral entry, are needed for a high level of replication in macrophages (51, 52). A role for envelope sequences outside V3 for HIV-1 replication in macrophages can be surmised from previous reports (7, 56, 66, 74, 75).

In summary, we have shown that SIVmac "V3" is tolerant to change and important for virus entry and that it can determine cell tropism, all of which are analogous to HIV-1 V3 characteristics. The effects of virus strain, gp120 backbone, and "V3" sequence on the ability of "V3" to serve as a target of neutralizing antibody are in need of further clarification, both for HIV-1 and for SIVmac.

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