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The V1, V2, and V3 Regions of the Human Immunodeficiency Virus Type 1 Envelope Differentially Affect the Viral Phenotype in an Isolate-Dependent Manner

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It is well documented that removal of the V1V2 region or of the V2 loop alone from the envelope glycoprotein of human immunodeficiency virus type 1 (HIV-1) or simian immunodeficiency virus (SIV) increases the susceptibility of these viruses to neutralization by antibodies. The specific role of the V1 loop in defining the neutralization susceptibility of HIV is, however, not well documented. Our current studies indicate that although the V1V2 region is a global modulator of the HIV-1 neutralization susceptibility, the individual roles the V1 and V2 loops have in defining the neutralization susceptibility profile of HIV-1 differ and in some cases are opposite. While deletion of the V2 loop renders the virus more susceptible to neutralization by antibodies that recognize diverse epitopes, in particular certain ones located in the CD4 binding site and the V3 loop, deletion of the V1 loop renders the virus refractory to neutralization, especially by antibodies that recognize CD4-induced epitopes and certain CD4-site binding antibodies. Our current studies also indicate that the relative involvement of the V2 loop of the HIV-1 envelope during virus-cell entry appears to be envelope background dependent. As a result, although deletion of the V2 loop from the clade B, R5-tropic SF162 HIV-1 virus resulted in a virus that was replication competent, the same modification introduced on the background of two other R5-tropic isolates, SF128A (clade B) or SF170 (clade A), abrogated the ability of these envelopes to mediate virus-cell entry.

The human immunodeficiency virus (HIV) envelope glycoprotein can maintain functionality even when large portions of certain variable regions are experimentally eliminated. Elimination of the first and second hypervariable regions (V1 and V2 loops) from the envelope of the X4-tropic HIV-1 virus HxB2 does not abrogate the processing and expression of the modified envelope protein (63), and HxB2 virions expressing such a mutant envelope glycoprotein are capable of replicating in vitro in peripheral blood mononuclear cells (PBMC) (6). Similarly, deletion of 100 amino acids from the V1V2 region from the envelope of the R5-tropic simian immunodeficiency virus SIVmac239 does not abrogate envelope function and the virus expressing this modified envelope replicates both in vitro (20) and in vivo (19). In the case of HXB2, efficient replication in the absence of the V1V2 region requires the introduction of compensatory mutations within gp120, while in the case of SIVmac239, efficient replication requires the appearance of compensatory mutations within the transmembrane gp41 subunit (6, 20). Finally, the individual elimination of the V1 and V2 loops from the envelope of the R5-tropic HIV-1 SF162 virus does not abrogate envelope function and the mutant viruses SF162ΔV1 and SF162Δ2 are capable of replicating in both PBMC and primary macrophages (53). In contrast to what has been reported in the case of SIVmac239 and HxB2, compensatory mutations are not required to occur for the efficient replication of the SF162ΔV1 and SF162ΔV2 viruses.

The neutralization susceptibility of HIV and simian immunodeficiency virus (SIV) is, however, profoundly affected when deletions are introduced within the V1V2 envelope region (6, 20, 53). This is not unexpected since the V1V2 region is positioned within the functional trimeric HIV envelope in a way that overlaps elements of the CD4 binding and coreceptor binding sites on gp120 (21–23, 41, 60–63). In addition, the glycosylation pattern of the V1V2 region also influences the neutralization susceptibility profile of HIV and SIV (8, 28, 36, 40, 45, 47). Despite the extensive investigation of the role that the V2 loop has on defining the overall neutralization susceptibility of HIV to antibodies, very little is know about the role that the V1 loop has in this. It is generally assumed that the V1 loop will act similarly to the V2 loop in protecting the virus from antibody-mediated neutralization.

In the present study we examined the specific roles of the V1 and V2 loops during antibody-mediated neutralization of HIV; whether the SF162 envelope can sustain larger deletions in the V1V2 region than those previously introduced (53) and remain functional; whether it retains functionality when the V3 loop is deleted; and whether envelopes derived from other R5-tropic HIV-1 isolates show the same degree of independence from the V1V2 region for function. Our studies indicate that although the V1V2 region regulates the neutralization susceptibility of HIV to antibodies that recognize diverse epitopes, the specific roles of the V1 and V2 loops in defining the neutralization susceptibility of HIV are often opposite. They also

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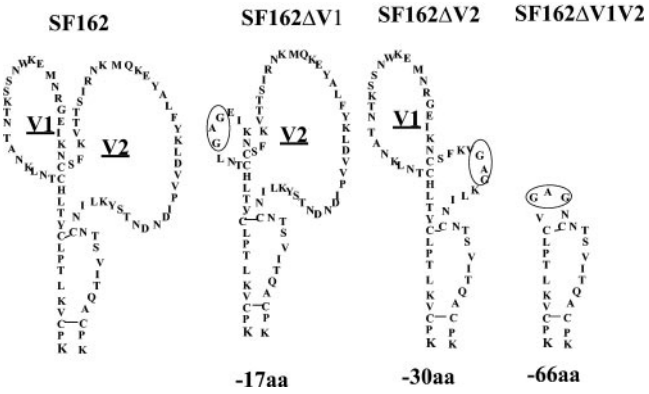

FIG. 1. Amino acid deletions introduced in the V1V2 region of the SF162 envelope. The amino acid composition of the V1V2 region of the SF162, SF162ΔV1, SF162ΔV2, and SF162ΔV1V2 envelopes is shown. The number of amino acids that were deleted to generate the SF162ΔV1, SF162ΔV2, and SF162ΔV1V2 envelopes is indicated. The deleted sequences were replaced by a GAG tripeptide (in the oval).

indicate that the relative importance of the V1V2 region during HIV-cell fusion is isolate dependent. Finally, our results are in agreement with those reported by other groups and indicate that the presence of an intact V3 loop appears to be critical for envelope-mediated HIV-1-cell fusion.

MATERIALS AND METHODS

Cells. Human peripheral blood mononuclear cells were isolated from the blood of healthy donors by Ficol centrifugation. PBMC were resuspended at 3×10^6 cells per ml in RPMI 1640 medium (Cellgro), supplemented with 10% fetal bovine serum (Cellgro), penicillin (100 U/ml; Cellgro), streptomycin (100 μg/ml; Cellgro), glutamine (2 mM; Cellgro), 20 U/ml of recombinant interleukin-2 (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health, from Maurice Gately, Hoffmann-La Roche Inc.) (25) and 3 μg/ml of phytohemagglutinin (Sigma) for 3 days, as previously described (30, 53). The cells were washed in Hanks prior to viral inoculation.

The U87 human astroglioma cell line (N. R. Landau, Salk Institute) expressing various combinations of human receptors (CD4 and CCR5, CD4 and CXCR4, CCR5 alone, and CXCR4 alone) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), and puromycin (1 μg/ml; Cellgro). The U87 cell line expressing CD4 alone (AIDS Research and Reference Reagent Program Catalog, National Institutes of Health) was cultured in DMEM supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM).

CEMx174 5.25 M7 cells (expressing CD4, CCR5, and CXCR4, as well as being stably transduced with an HIV-1 long terminal repeat-green fluorescent protein and HIV-1 long terminal repeat-luciferase reporter construct) were cultured in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), puromycin (0.5 μg/ml), G418 (200 μg/ml; Cellgro), and hygromycin B (200 μg/ml; Cellgro).

Human embryonic kidney (HEK-293T) cells were maintained in DMEM supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM).

Introduction of deletions in the V1V2 region of the SF162, SF128A, and SF170 envelopes. The isolation, molecular cloning, and phenotypic characterization of SF162, SF128A, and SF170 HIV-1 isolates was previously reported (9, 26, 51). SF162 and SF128A are clade B isolates, while SF170 is a clade A isolate. All three viruses use CD4 and CCR5 for entry.

The generation and characterization of SF162 containing a deletion in the V1 loop (SF162ΔV1) or a deletion in the V2 loop (SF162ΔV2) was previously reported (53). SF162ΔV1 contains a 17-amino-acid deletion (from Lys at position 133 to Lys at position 145), and SF162ΔV2 contains a 30-amino-acid deletion (from Thr at position 160 to Tyr at position 189) (Fig. 1). The SF162ΔV1V2 virus was generated by deleting 66 amino acids within the V1V2 region (from Thr at position 127 to Ile at position 192) of the SF162 envelope and replacing the deleted sequence by the GAG tripeptide, as previously described (53). The deletion was introduced into a pUC19 vector expressing the 3′ half of the SF162 genome that contains *tat*, *rev*, *vpu*, *env*, *nef*, and the 3′ long terminal repeat.

TABLE 1. List of primers used to introduce envelope modifications

Primer	Sequence[a]
A	5′-GAGCCAGTAGATCCTAGATTAGAG-3′
B	5′-AGCTGTGTTGAATTACAGTAG-3′
C	5′-ACAATT**TCCAGCTCC**AACACAGAGTGGGG T-3′
D	5′-TGTGTT**GGAGCTGGA**AATTGTAACACCTC A-3′
E	5′-GGGGTACCTGTGTGGAAAGACGCAGAG ACC-3′
F	5′-GCTGTCAGTGCTATTTCCCCCCCATGTGC-3′
G	5′-TAATCT**GCCGGCGCC**GGTCATATTGTAAG-3′
H	5′-ATGACC**GGCGCCGGC**AGATTAATAATTG-3′
I	5′-GGGGCATCATGCTCCTTGGGATGTTGC-3′
J	5′-CCCCTCCTCAATTAAAACTCTGCATTAC-3′
K	5′-CAACCT**TCCAGCTCC**GATATTGAAAGAGC A-3′
L	5′-AATATC**GGAGCTGGA**AGGTTGATAAATTG T-3′
gp160F	5′-GGACCATAGTGTACATAGAATACAG-3′
env9R	5′-GGTCTTAAAGGTACCTGAGGTCTG-3′
ΔV3F	5′-CAATAAT**GGAGCTGGA**GATATAAGACAAGC AC-3′
ΔV3R	5′-TATATC**TCCAGCTCC**ATTATTGTTAGGTCTT G-3′
BamF	5′-CCAGTCCATTAGTGCATGGATCCTTAGCAC TCATCTGGGACG-3′
BamR	5′-CGTCCCAGATGAGTGCTAAGGATCCATGC ACTAATG GACTG-3′
SF162F	5′-GAATTCTGCAACAACTGCTG-3′
ΔV3cF	5′-GCATTTTATGCAACA**GGAGCTGGA**CATTGT AACATTAGTGG-3′
ΔV3cR	5′-CCACTAATGTTACAATG**TCCAGCTCCT**GTTG CATAAAATGC-3′
Nhe162F	5′-GGGATGTTGATGATCTGTGCTAGCGTAGA AAAATTGTGGGTC-3′
Nhe162R	5′-GTGACCCACAATTTTTCTACGCTAGCACAG ATCATCAACATC-3′
Cla160F	5′-GGGCTTTGCTATAACATCGATGGCAAGTG GTC-3′
Cla160R	5′-GACCACTTGCCATCGATGTTATAGCAAAGC CC-3′
gp160R	5′-TCAGCTGGCTCAGCTCGTTTCATTC-3′
D162NF	5′-GAGGATATAATCAGTTTATGGAATCAAAGT CTAAAGCC-3′
D162NR	5′-GGCTTTAGACTTTGATTCCATAAACTGATT ATATCCTC-3′

[a] Nucleotides in bold represent insertions of the GAG tripeptide.

Briefly, a set of two outer primers (A and B) whose amplified product contains the restriction sites NsiI and StuI were synthesized (see Table 1 for primer sequences). Two inner primers (C and D) were designed containing sequences on either side of the region to be deleted and an overlapping region of approximately 20 nucleotides. The deleted sequence was replaced by the GAG tripeptide. First-round PCR was performed with primers A and C, and B and D. In the second round of PCR the two fragments generated in the first round were used as templates for the two outer primers A and B. The product of the second-round PCR was digested with NsiI and StuI and used to replace the corresponding regions in the 3′ pUC19-SF162 plasmid. These PCRs were performed using the Expand High Fidelity PCR system (Roche). This two-round PCR technique was used to introduce several other deletions subsequently described in this paper. From here on, we will only state the inner and outer primers used in this deletion-generating method and the restriction enzymes used for subcloning.

Deletions of 35 and 30 amino acids within the V2 loops of the SF170 (from Thr at position 163 to Tyr at position 197) and SF128A (from Thr at position 167 to

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Tyr at position 196) envelopes were introduced within pUC19 vectors expressing the 3′ halves of the SF170 and SF128A genomes. In the case of SF170, E and F were the two outer primers, and G and H were the inner primers. In the case of SF128A, primers I and J were the outer primers and K and L were the inner primers.

Introduction of deletions in the V3 loop of the SF162 and SF162ΔV2 envelopes. Two different deletions were introduced within the V3 loop domain of the SF162 and SF162ΔV2 envelopes. The ΔV3 version was generated by deleting 21 amino acids from the central region of the V3 loop (from Thr 301 to Gly 321) and the ΔV3c version was generated by deleting 10 amino acids from the carboxy-terminal side of the V3 loop (from Gly 317 to Ala 326) (Fig. 6).

The ΔV3 deletion was first introduced into the pUC19-SF162 3′ and pUC19-SF162ΔV2 3′ constructs, as described above (53). The outer primers used were gp160F and env9R, which amplify all of gp160, producing a fragment containing the restriction sites for Nsi*I* and XhoI. The inner primers used were ΔV3F and ΔV3R, which contain sequences on either side of the region to be deleted and an overlapping region of 22 nucleotides.

The above modification was also introduced within the R7/3-SF162 plasmid, which contains the full-length viral genome of HXB2 with the exception that gp120 and most of gp41 is replaced by the corresponding region from SF162 (27). A BamHI site was introduced into the pUC19-SF162ΔV3 3′ and pUC19-SF162ΔV2ΔV3 3′ constructs (see above), in the gp41 transmembrane region, using primers BamF and BamR and the Quik Change site-directed-mutagenesis kit (Stratagene). The constructs were then digested with BamHI and SalI and the resulting fragments containing either the ΔV3 deletion or the ΔV2ΔV3 deletion were used to replace the corresponding region in the R7/3-SF162 background.

The ΔV3c deletion was generated using as outer primers SF162F and env9R and as inner primers ΔV3cF and ΔV3cR. This modification was introduced into the pUC19-SF162 3′ and pUC19-SF162ΔV2 3′ constructs and in the full-length R7/3-SF162 plasmid.

Virus production. We added 4×10^5 293T cells per well in a six-well dish, and 24 h later they were cotransfected with the 3′ and 5′ pUC19 plasmids (see above) (5 μg each), using DMRIE (Invitrogen), as previously described (30). After 72 h, the supernatant was subjected to centrifugation (5 min at 2,000 rpm at room temperature) and were used to inoculate phytohemagglutinin (PHA)-activated human PBMC for 3 h at 37°C as previously described (30, 53). This virus amplifications step is necessary, because very little virus is produced during cotransfection with the 3′ and 5′ halves of the viral genome. The PBMC were cultured in RPMI medium at a density of 3×10^6 cells per ml. Virus production was monitored by determining the concentration of p24 antigen in the cell supernatant every 3 to 4 days, using an in-house p24 detection enzyme-linked immunosorbent assay (ELISA). Cell supernatants with high p24 content were collected, aliquoted, and stored at −80°C. Subsequently the 50% tissue culture infectious dose ($TCID_{50}$) was determined in human PBMC.

In certain cases, virus was also generated using the full-length R7/3-SF162-derived plasmids (see above). 293T cells were transfected with 5 μg of plasmid. The supernatant was collected, aliquoted, and stored. Because transfection with the full-length viral genome results in a significant production of virus, there is no need to further expand the virus in PBMCs. The $TCID_{50}$ of the virus present in the 293T supernatants was determined using PBMCs as targets.

Generation of pEMC* vectors expressing various gp160 envelope proteins. An NheI and a ClaI site were introduced 5′ and 3′, respectively, of the SF162 gp160 and SF162 ΔV2 envelope sequence in the pUC19-SF162 3′ and pUC19-SF162ΔV2 3′ constructs (see above), using the QuikChange site-directed mutagenesis kit (Stratagene). Primers Nhe 162F and Nhe 162R were used to introduce the NheI site, and primers Cla 160F and Cla 160R were used to introduce the ClaI site. The NheI–ClaI fragment was then cloned into the pEMC* expression vector (42), to generate pEMC*SF162 gp160 and pEMC*SF162ΔV2 gp160.

Using a similar methodology, the pEMC*SF162ΔV1 and pEMC*SF162ΔV1V2 constructs were generated by PCR, amplifying the ΔV1gp160 and ΔV1V2gp160 sequences from the corresponding pUC-3′ plasmids (see above) using primers Nhe 162F and Cla 160R. The pEMC*SF162ΔV3 gp160 and pEMC*SF162ΔV2V3 gp160 constructs were generated by digesting the pUC19 SF162 ΔV3 3′ plasmid with BglII (which cuts in the C2 and V5 regions of gp120), gel purifying (Qiagen) the fragment containing the V3 deletion, and replacing the corresponding region in the pEMC*SF162gp160 and pEMC*SF162ΔV2gp160 constructs. The D-to-N change at amino acid 112 of the envelope (C1 region) was introduced on the pEMC*SF162gp160 and pEMC*ΔV1V2 gp160 constructs using the D162NF and D162NR primers. The generation of expression plasmids for the SF170 and SF128A gp160 envelopes has been previously described (29).

Western blot of envelope glycoproteins. 293T cells were transfected with DNA vectors expressing various gp160 envelope forms, and 48 h later, cell lysates and supernatants were collected, as previously described (30); 1 to 2 μl of cell lysate and 20 to 30 μl of supernatant were resuspended in 30 μl of lysis buffer (50 mM Tris-HCl, 100 mM 2-mercaptoethanol, 2% sodium dodecyl sulfate [SDS], 0.1% bromophenol blue, 20% glycerol) and boiled for 5 min; 15-μl aliquots were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on 10% SDS gels and then transferred to Immobilon P membranes (Millipore). The membranes were incubated with goat-anti-Env2-3 serum antibodies (17) (Chiron; 1:1,000 dilution), and subsequently with protein G-horseradish peroxidase (Bio-Rad; 1:1,000 dilution). Visualization of the gp120 envelope molecules was performed with the use of enhanced chemiluminescence reagents (Amersham).

Generation of single-round competent viruses expressing the SF162-, SF128A-, and SF170-derived envelopes. Luciferase reporter viruses capable of only a single round of replication and expressing various HIV envelope glycoproteins were generated as follows: 3×10^5 293T cells were plated in each well of a six-well plate and 24 h later the cells of each well were cotransfected with 0.1 μg of the vector expressing various gp160 constructs and 2 μg of the pNL-$Luc^+E^-R^-$ construct (N. R. Landau, Salk Institute), using DMRIE. After 6 h, the transfection medium was removed and replaced with fresh medium; 72 h later the cell supernatants were collected, subjected to centrifugation (5 min at 2,000 rpm at room temperature), assayed for p24 content, and frozen in 0.5-ml aliquots at −80°C until further use.

Viral replication kinetics. We added 400 $TCID_{50}$ of each replication-competent virus (or 300 ng p24 in the case of viruses expressing envelopes with deletions in the V3 loop) to quadruplicate wells of a 96-well plate, each containing 2×10^6 PBMC, for 3 h at 37°C. The inoculum was removed and the cells were resuspended in RPMI medium (0.1 ml per well). Half the medium was replaced every 3 to 4 days and tested for viral p24 antigen.

Antibodies and sera. Immunoglobulin G (IgG)-CD4 was obtained from Genentech (7). IgG1b12 monoclonal antibody (MAb) was provided by Denis Burton (Scripps Institute) (4, 38). James Robinson (Tulane University) provided 17b, and Dimitri Dimitrov (National Cancer Institute) provided IgGX5 (34) and M18 (65). 447D, 391-95D, 2909, and 1418 were provided by Susie Zolla-Pazner and Mirek Gorny (New York University) (11, 13, 15, 49). The following MAbs were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health: 2F5, 2G12, and 4E10 from Hermann Katinger (43, 44, 58) and F105 from Marschall Posner (63). HIVIG was obtained from the AIDS Research and Reference Reagent Program, Division of AIDS. Macaque sera were collected from animals chronically infected with $SHIV_{SF162P4}$ (3).

IgGCD4-virion binding. The binding of IgGCD4 to virion-associated envelope molecules was monitored as previously described (55). Briefly, 293T cell supernatants containing single-round competent virions (see above) were subjected to centrifugation (28,000 rpm in an SW28 rotor for 2 h at 4°C) through a 32% sucrose cushion. The viral pellet was resuspended with 0.5 ml cold phosphate-buffered saline, aliquoted, tested for p24 content, and stored frozen until further use. Sucrose-purified virions were incubated with serial dilutions of IgGCD4 for 3 h at 37°C. The virus/IgGCD4 mixture was then centrifuged for 2 h at 4°C and the relative amount of IgGCD4 bound to the viral pellet was determined by ELISA methodology as previously described (55).

Virus neutralization. One day prior to the neutralization assay setup, U87 CD4-CCR5 cells were plated in a 96-well flat-bottomed TC plate (Corning) (7×10^3 cells per well in 0.1 ml of medium). Single-round competent virions (0.5 to 2 ng p24 input) were preincubated with an equal volume of serially diluted MAb for 1.5 h at 37°C in 96-well U-bottomed plates (Corning). For each MAb dilution, quadruplicate wells were used. The cells were treated with 2 μg/ml Polybrene for 30 min at 37°C, the cell supernatant was then replaced with 0.05 ml of fresh medium, and an equal volume of the MAb/virus mixture was added to each well. In separate wells, cells were incubated with virus in the absence of MAb (positive control wells).

In parallel experiments, the viruses were incubated with an irrelevant MAb (1418, an anti-parvovirus antibody). The cells were incubated at 37°C for 72 h, the supernatant was removed, and the cells were then lysed with 0.1 ml 1X cell lysis buffer (cell culture lysis reagent buffer, Promega) and frozen at −80°C for 2 h. Following thawing at room temperature, 0.06 ml from each well were transferred to a Black Enhanced Binding 96-well solid plate, luciferase substrate (Promega) was added to each well (0.1 ml per well), and the relative light units (RLU) associated with each well were recorded with a Fluoroskan Ascent FL (ThermoLabsystems). The percent neutralization was calculated as follows: [(RLU in the positive control wells –RLU in the presence of MAb/RLU in the positive control wells)] × 100. The extent of neutralization recorded in the presence of the irrelevant MAb 1418 is considered due to nonspecific neutralization.

PCR amplification, cloning, and sequencing of the viral envelope during in

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vitro replication in PBMC. Genomic DNA was extracted from 5×10^6 PBMC, infected with either SF162 or SF162ΔV1V2, using the QIAamp DNA blood minikit (Qiagen). The viral envelope was amplified using primers gp160F and gp160R (amplifies the complete viral envelope and 96 bp 5′ to the start codon and 78 bp 3′ to the stop codon) and the Expand High Fidelity polymerase mixture (Roche), according to the manufacturer's instructions. The resulting PCR product was cloned into the pCR3.1 eukaryotic TA bidirectional expression vector (Invitrogen), transformed into chemically competent *Escherichia coli* DH5α cells, and the resulting clones were screened for the presence of inserts. Plasmid DNA was prepared using the Qiagen miniprep kit, and the complete gp160 region was sequenced from 5 to 10 independent clones. The nucleotide sequences were analyzed and aligned using the EditSeq, MegAlign, and SeqMan sequence analysis programs (DNASTAR).

Virus-cell entry assay. The entry potential of viruses expressing the various envelopes was assessed as previously described using U87.CD4.CCR5 cells as targets (30).

RESULTS

Replication of SF162 viruses lacking elements of the V1V2 envelope region. A schematic representation of the various deletions introduced in the V1V2 region of the SF162 envelope is shown in Fig. 1. We previously reported on the generation of SF162ΔV1 (lacking 17 amino acids from the central region of the V1 loop) and SF162ΔV2 (lacking 30 amino acids from the central region of the V2 loop) viruses (53). In the current studies, a third mutant was generated, SF162ΔV1V2, lacking 66 amino acids from the V1V2 region.

Supernatants from 293T cells cotransfected with pUC19 DNA vectors expressing the 3′ and 5′ halves of the SF162ΔV1V2 genome were used to inoculate activated human PBMC. Virus was detectable at the end of the second week of culture (Fig. 2A). PBMC supernatant from day 28 was used to inoculate a second batch of activated PBMC. This time the virus replicated faster and to higher titers. Virus was collected at day 10, aliquoted, stored, and used subsequently for all replication and neutralization assays. Therefore, our stock SF162ΔV1ΔV2 virus was propagated twice in PBMC.

A third batch of activated human PBMC were inoculated with SF162ΔV1ΔV2 and 11 days later, DNA was extracted from the cells and the viral envelope was amplified and sequenced from eight clones. The only change seen in the majority of clones (seven out of eight clones sequenced) was a substitution of Asp by Asn at position 112 within the C1 region of the gp120 subunit. This change created a new potential N-linked glycosylation site at that position. This change could have occurred at any time during this in vitro passage of SF162ΔV1ΔV2.

To examine whether this D-to-N change conferred a greater fusogenic potential to the SF162ΔV1V2 envelope, we compared the ability of the SF162ΔV1V2 and SF162ΔV1V2(D/N) envelopes to mediate virus-cell fusion. We also introduced the D-to-N change on the background of the parental SF162 envelope to examine what effect, if any, this change has on the full-length envelope glycoprotein. Our entry results (Fig. 2B) indicate that no increase in entry was recorded when the D-to-N change was introduced on the SF162ΔV1V2 background. In contrast, a 1 $\log_{10}$ reduction in entry was recorded when the same amino acid change was introduced on the SF162 envelope background. Therefore, we do not believe that the introduction of this N-linked glycosylation site in the C1 region of the SF162ΔV1V2 envelope significantly improved its fusogenic potential.

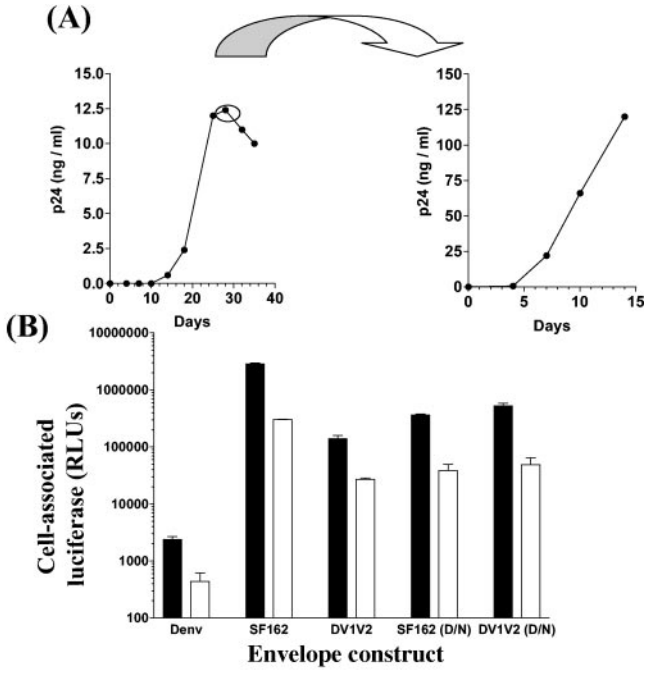

FIG. 2. Generation of the SF162ΔV1V2 virus. (A) Human PBMC were inoculated with supernatant from 293T cells that were transfected with plasmids encoding the SF162ΔV1V2 genome. Supernatant collected at day 28 post-PBMC-inoculation was used to inoculate a new batch of human PBMC. Note the difference in scale of the y-axis between the two graphs. (B) Single-round competent virions expressing the indicated envelopes were incubated with U87-CD4-CCR5 cells, as described in the Materials and Methods and cell-associated luciferase was determined 48 h later. Δenv, Virions that do not express envelope. D/N, aspartic acid at position 112 in the C1 region was replaced by asparagine. Black bars, 1 ng of p24 inoculum. White bars, 0.1 ng of p24 inoculum. Results are from one out of thee independent experiments.

We next compared the replication potentials of infectious virions expressing the SF162, SF162ΔV1, SF162ΔV2, and SF162ΔV1V2 envelopes using activated human PBMC as targets (Fig. 3A). As we previously reported, the SF162ΔV1 virus grows similarly SF162, while the SF162ΔV2 virus grows with slightly delayed replication kinetics. The SF162ΔV1V2 virus replicates with slower kinetics than SF162, but, unexpectedly, with faster kinetics than SF162ΔV2. All three mutant viruses, however, replicate to high titers. The above viral replication kinetics results are supported by results obtained when the fusogenic potentials of the SF162, SF162ΔV1, SF162ΔV2, and SF162ΔV1V2 envelope glycoproteins were compared using single-round competent viruses (Fig. 3B). The SF162ΔV2 envelope was less efficient than the SF162 envelope in mediating virus-cell entry, while the SF162ΔV1V2 envelope was more efficient than the SF162ΔV2 envelope (but less efficient than the SF162ΔV1 envelope) in mediating virus-cell entry.

SF128A and SF170 envelopes lacking the V2 loop do not mediate virus-cell entry. The above results, and those reported by others using distinct HIV-1 or SIV viruses (6, 20), suggest that the envelope glycoproteins of diverse HIV and SIV isolates remain functional despite significant deletions in the V1V2 region. To further examine whether this is a general property of the HIV envelope, we eliminated the central re-

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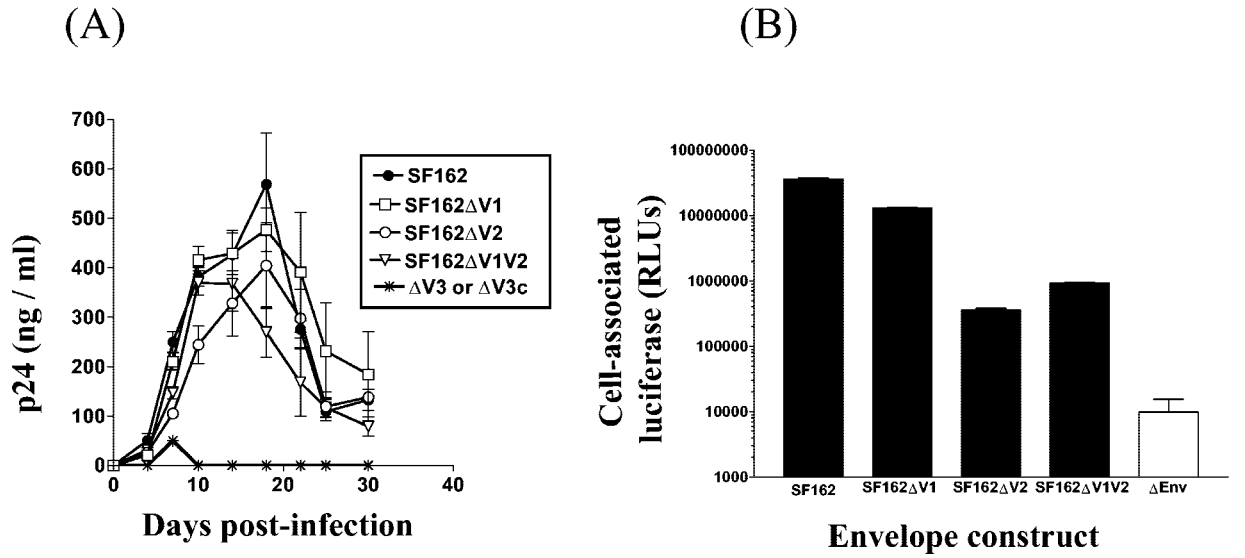

FIG. 3. Human PBMC replication of viruses expressing mutant envelopes. (A) 400 TCID50 of SF162, SF162ΔV1, SF162ΔV2, and SF162ΔV1V2 were used to inoculate PHA-activated human PBMC. The concentration of p24 antigen in the cell supernatant was determined at the indicated time points following inoculation. Values indicate the mean and standard deviation from quadruplicate wells. Experiments were performed at least three times with similar results. In the case of SF162ΔV3 or SF162ΔV3c, supernatants from 293T cells (containing 300 ng of p24) transfected with DNA plasmids expressing the full viral genome were used to inoculate PHA-activated human PBMC. (B) The fusogenic potential of the indicated modified SF162 envelopes was determined using a luciferase-based pseudovirus-cell entry assay, as described in the Materials and Methods section. The viral inocula were standardized based on p24. Δenv, pseudovirions lacking envelope.

gion of the V2 loop from a clade B HIV-1 isolate, SF128A, and from a clade A HIV-1 isolate, SF170, and examined the effect of this modification of SF128A- and SF170-envelope function.

We first examined the expression of these two mutant proteins. We observed that in the case of SF128A, most of the envelope is present in the cell lysate as a gp160 form and very little gp120 is secreted in the cell medium (Fig. 4A). This indicates that deletion of the V2 loop reduced the processing of gp160 into gp120 and gp41, and decreased the strength of the gp41-gp120 association. As a result the gp120 subunit more easily dissociates from the gp41 subunit. Deletion of the V2 loop from the SF170 envelope altered the processing of gp160 even more profoundly and as a result, very little gp120 was present in the cell lysate and supernatant fractions (data not shown).

Deletion of the V2 loop from the SF128A envelope did not alter the overall conformation of that protein, as it retained its ability to bind to CD4 (Fig. 4B). However, neither the SF128AΔV2 and SF170ΔV2 envelopes were capable of mediating virus-cell fusion (Fig. 5). Therefore, although the position and extent of the V2 deletion introduced on the SF128A and SF170 backgrounds were similar to those previously introduced on the SF162 envelope, they differentially affected the functionality of these three envelopes. As a result, we were unable to generate replication-competent SF128A and SF170 viruses with deletions in their V2 loops despite repeated attempts. We want to emphasize, however, the fact that we did not maintain the cell cultures for more than 40 days. It is possible that during this period, low but undetectable viral replication was occurring and that if we had waited longer, compensatory mutations may have been introduced in gp120 or gp41 as other have previously reported (6, 20), in which

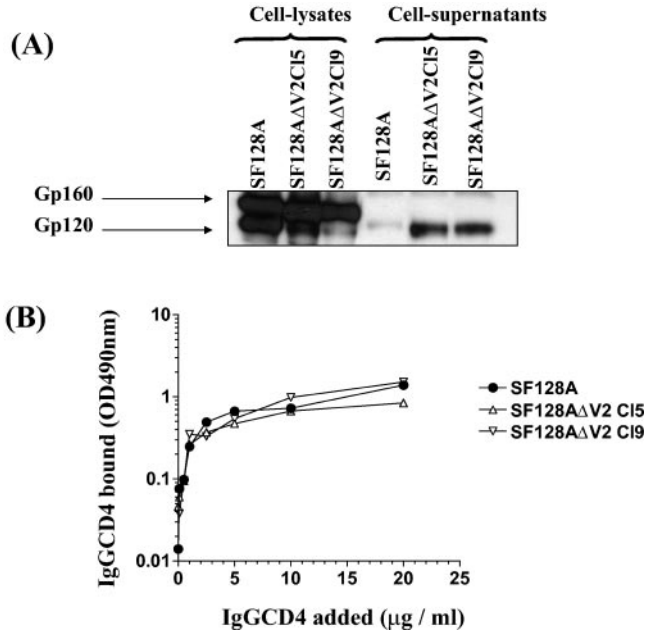

FIG. 4. Processing and CD4 binding potential of SF128A-derived envelopes lacking the V2 loop. (A) 293T cells were transfected with DNA vectors expressing wither the SF128A or SF128AΔV2 (two clones, 5 and 9) gp160 envelopes. The presence of gp160 and gp120 in the cell-lysates or cell-supernatants was determined as described in the Materials and Methods section. (B) Single-round competent virions expressing the SF128A or SF128AΔV2 envelopes were purified through sucrose and then incubated with the indicated concentrations of IgGCD4. Virion-associated IgGCD4 was determined as described in the Materials and Methods section.

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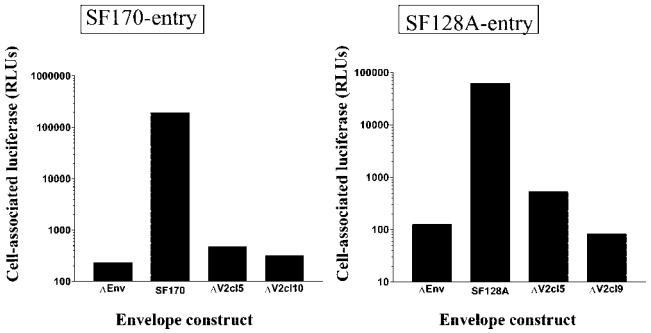

FIG. 5. Fusion potential of SF170 or SF128A-derived envelopes. Single-round competent virions (5 ng of p24 equivalent per well of a 96 well plate) expressing the indicated envelopes derived either from SF170 or SF128A were incubated with U87-CD4-CCR5 cells for 72 h and the cell-associated luciferase was determined. Data are the average of triplicate wells, and two independent clones each of SF170ΔV2 and SF128AΔV2 were tested in parallel with similar results.

case, highly replication-competent SF128A and SF170 viruses lacking the V2 loop may have been generated. Also, our efforts focused on human PBMC. It is possible that SF128AΔV2 and SF170ΔV2 virus may replicate more efficiently in certain established T-cell lines.

SF162 envelope that lacks elements of the V3 loop is not functional. The ability of the SF162 envelope to maintain its functional structure despite the introduction of significant deletions within the V1V2 region led us to investigate whether this viral protein remains functional when other variable domains are eliminated. To this end we introduced two types of deletions in the V3 loop (Fig. 6A). One construct (ΔV3) lacks 21 out of the 35 amino acids from the central region of the V3 loop, but the base of the V3 loop is intact. The second construct (ΔV3c) lacks 10 amino acids from the carboxy terminal region of the V3 loop, and thus the crown and the amino-terminal side of the V3 loop remain intact. The same modifications were introduced on the background of the ΔV2 envelope (ΔV2V3 and ΔV2V3c constructs, respectively).

We were unsuccessful in generating replication-competent viruses expressing the above V3 loop-lacking envelopes in PBMC. The inability of the SF162ΔV3 or SF162ΔV2ΔV3 viruses to replicate in PBMC could be due to defects in their abilities to bind to and/or fuse with target cell membranes. We did not attempt to generate replication competent viruses in established T cells. We examined the binding of IgGCD4 to virions expressing the WT and the V3 loop deletions envelopes (Fig. 6B). Although not all binding curves reached saturation, it appears that deletion of the V3 loop (either the ΔV3 or the ΔV3c construct) from the full-length SF162 envelope reduced

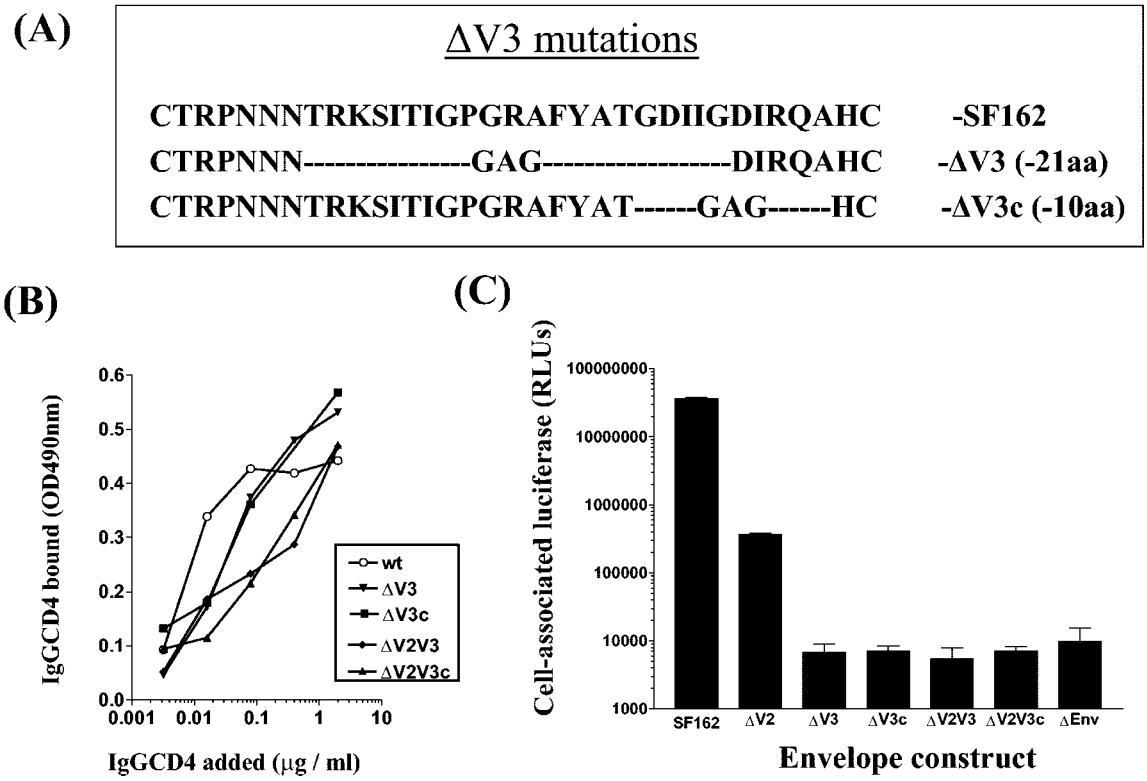

FIG. 6. CD4 binding and fusogenic potential of SF162 envelopes with deletions in the V3 loop. (A) The indicated deletions were introduced within the V3 loop of the SF162 envelope. The deleted sequences were replaced by the GAG tripeptide. (B) The binding of IgGCD4 to SF162 and SF162ΔV2 virions with and without deletions in the V3 loop was determined by monitoring the binding of IgGCD4 to virions. (C) The cell fusion potential of SF162 and SF162ΔV2 envelopes lacking elements of the V3 loop was assessed using U87-CD4-CCR5 as target cells. Δenv, pseudovirions lacking envelope were used as negative controls. The values represent the average and standard deviations from quadruplicate wells from a single experiment. Experiments were performed at least three times with similar results.

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its relative affinity for IgGCD4 (Fig. 6B). The simultaneous deletion of the V2 and V3 loops from the SF162 envelope decreased even further its relative affinity for IgGCD4. Similar observations were made by others when deletions in the V1, V2, and V3 loops were introduced on the background of the HxB2 envelope (63). However, despite the fact that the SF162 and SF162ΔV2 envelope glycoproteins lacking the V3 loop could still bind CD4, they could not support virus-cell fusion (Fig. 6C). Overall, these results indicate that deletion of the V3 loop from the SF162 and SF162ΔV2 envelopes decreased their CD4 binding potentials and abolished their abilities to mediate virus-cell fusion.

Neutralization susceptibility of SF162 viruses with deletions in the V1V2 envelope region. The V1V2 region of the HIV envelope glycoprotein is positioned in a way that masks elements of the CD4 and coreceptor binding sites (23, 60, 61). As a result, important phenotypic properties of HIV, such as its fusogenic and replication abilities, coreceptor utilization, and neutralization susceptibility, are modulated by the length and glycosylation pattern of the V1V2 region (2, 12, 16, 18, 21, 22, 28, 32, 33, 35, 37, 41, 50, 54, 56, 64).

We previously reported that the partial deletion of the V2 loop from the SF162 envelope renders the virus significantly more susceptible to neutralization by heterologous clade B and non-clade B sera (53). Here we compared in more detail the neutralization susceptibility of SF162 viruses lacking the V1 and V2 loops, either individually or in combination, using various well-defined MAbs (Fig. 7). We used MAbs that bind to CD4 binding site elements, the V3 loop, epitopes that are CD4 induced (CD4i), and the gp41 glycoprotein.

Deletion of the V2 loop enhanced the neutralization susceptibility of the virus to antibodies that bind to distinct envelope regions. SF162ΔV2 was more susceptible than SF162 to neutralization by IgGCD4 and F105 (bind to the CD4 binding site) (Fig. 7A), Fab M18 (whose epitope has not yet been defined, but it is not CD4 induced) (Fig. 7A), 447D and 391-95D (both bind to the V3 loop) (Fig. 7B), 17b, and X5 (both bind to CD4i epitopes) (Fig. 7B). In contrast, SF162ΔV2 was less susceptible than SF162 to neutralization by IgG1b12 (Fig. 7A). This MAb binds to an epitope overlapping the CD4 binding site, but its binding is affected by the composition of the V2 loop (31). Interestingly, removal of the V2 loop enhanced the neutralization susceptibility of SF162 to the anti-gp41 MAbs 2F5 and 4E10 (Fig. 7C).

A very different neutralization susceptibility profile was recorded when the V1 loop was eliminated. Although SF162ΔV1 was as susceptible as SF162 to neutralization by MAb IgG1b12, it was less susceptible to neutralization by IgGCD4 and, significantly less susceptible to neutralization by F105 and Fab M18. Also, SF162ΔV1 was significantly more resistant than SF162 to neutralization by antibodies that recognize CD4i epitopes, such as MAbs 17b and X5, and by anti-V3 loop MAbs 447D and 391-95D. Finally, in contrast to what we observed when the V2 loop was eliminated, deletion of the V1 loop did not alter the neutralization susceptibility of the SF162 virus to the anti-gp41 MAbs 2F5 and 4E10.

Interestingly, the SF162ΔV1V2 virus was more susceptible than SF162ΔV1 to neutralization by antibodies that bind to CD4i epitopes but less so than the SF162ΔV2 virus, and highly susceptible to neutralization by anti-V3 loop MAbs. Also, the SF162ΔV1V2 virus was highly susceptible to IgGCD4 (as susceptible as the SF162ΔV2 virus) and more susceptible to F105 neutralization than SF162, but less susceptible than SF162ΔV2. SF162ΔV1V2 virus was more resistant to neutralization by IgG1b12 compared to SF162. Finally, although the SF162ΔV1V2 virus was more susceptible than SF162 or SF162ΔV1 to neutralization by 2F5 and 4E10, it was less susceptible to neutralization than SF162ΔV2.

The neutralization susceptibility of all four viruses to 2G12 (Fig. 7C) was similar. This antibody binds to sugar molecules on N-linked glycosylation sites present in the C3 and V4 regions of gp120 (5, 48). Thus, removal of the V1V2 region from the SF162 envelope does not appear to alter significantly the overall three dimensional association of the remaining envelope regions in a way that disrupts the 2G12 epitope.

MAb 2909 (Fig. 7A) is a recently isolated antibody from an HIV-infected patient whose epitope is conformational and highly dependent on the quaternary association of the V2 loop, V3 loop, and CD4 binding site (14). This MAb very efficiently neutralized the SF162 and SF162ΔV1 viruses, but did not block the replication of viruses whose envelope lacked the V2 loop (SF162ΔV2 and SF162ΔV1V2). As expected, all viruses were resistant to neutralization to an irrelevant MAb, 1418 (anti-parvovirus antibody) (Fig. 7C).

We also compared the neutralization susceptibility of these viruses against heterologous human serum IgG (HIVIG) and sera collected from rhesus macaques infected with a SHIV ($SHIV_{SF162P4}$) that expresses an envelope that is homologous to HIV-1SF162 (Fig. 7D) (3). All sera neutralized the viruses lacking the V2 loop significantly more potently than the parental SF162 virus, in accordance with our previous observations (53). The heterologous human serum IgG, however, neutralized SF162 and SF162ΔV1 with similar efficiency, while the two macaque sera neutralized the SF162ΔV1 virus more efficiently than SF162.

DISCUSSION

We previously reported that the individual elimination of the V1 or V2 loop from the SF162 envelope does not abrogate the potential of that viral protein to support virus-cell fusion (53, 54). Those results were in agreement with observations made by others on the background of HIV-1 HxB2 and SIVmac239 (6, 20). Collectively, the above studies suggested that the HIV-1 and SIV envelopes could mediate virus-cell fusion in the absence of the V1 and V2 loops. Our current results indicate that the role of the V1V2 region in HIV envelope function is isolate dependent. The partial elimination of the central region of the V2 loop from SF128A (clade B) or SF170 (clade A), both R5-tropic HIV-1 clade B isolates, prevented the replication of these isolates in human PBMC. The inability of the SF170ΔV2 envelope to mediate virus-cell fusion is most likely related to the inefficient processing of this mutant envelope. In contrast, it is not yet clear why the SF128AΔV2 envelope does not mediate efficient virus-cell fusion. One possibility is that the highly labile association of gp120 with gp41 on the SF128AΔV2 envelope background renders the protein less capable of undergoing the proper CD4-induced conformational changes that lead to virus-cell fusion.

The fact that the introduction of the same modification on three different HIV envelope backgrounds differentially af-

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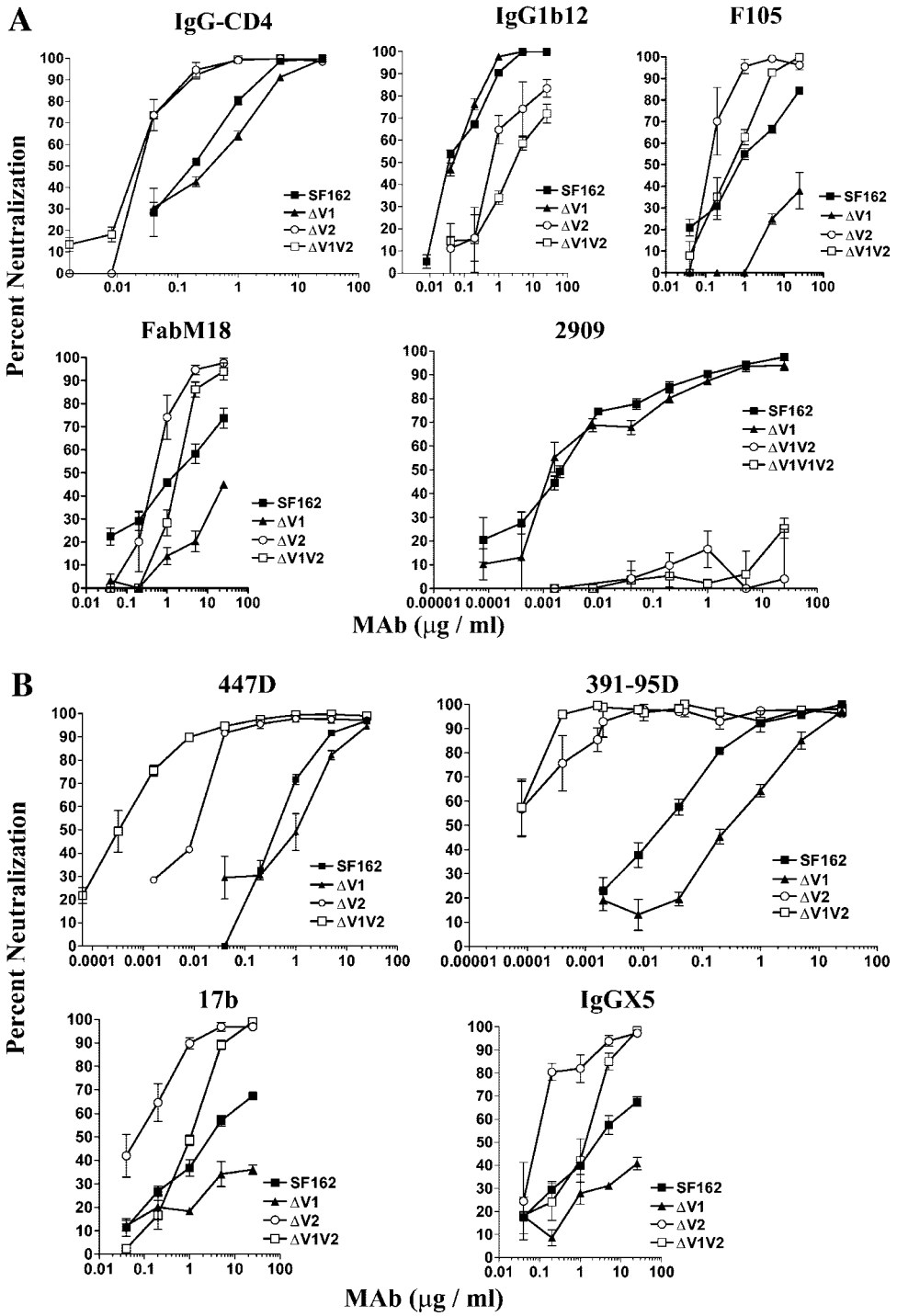

FIG. 7. Neutralization susceptibility of SF162, SF162ΔV1, SF162ΔV2, and SF162ΔV1V2 viruses. The neutralization susceptibility of the indicated viruses against a panel of MAbs and sera was determined using luciferase-expressing single-round replication-competent viruses and U87-CD4-CCR5 cells as targets. Values indicate the average and standard deviation from quadruplicate wells. Each neutralization assay was repeated at least two independent times. (A) IgGCD4, IgG1b12, F105, and M18 bind to the CD4 binding site, while 2909 recognizes a complex epitope. (B) 447D and 391-95D bind to the V3 loop, while 17b andX5 bind to CD4 induced epitopes. (C) 2F5 and 4E10 bind to the extracellular region of gp41, while 2G12 binds to a complex carbohydrate epitope; 1418 is a non-anti-HIV MAb used as a negative control. (D) Sera from macaques C640 and A141 were isolated from animals infected with $SHIV_{SF162P4}$, while HIVIG is heterologous human serum IgG.

fected the functionality of these envelopes indicates that the role of the V2 loop on HIV envelope function is background dependent. The envelopes of SF162, SF128A, and SF170 support a CCR5-dependent virus-cell fusion and most likely the overall organization of these envelope proteins is common. However, significant differences must exist in the particular orientation of the V2 loop within the trimeric form of these three envelopes that differentially affect their processing, sta-

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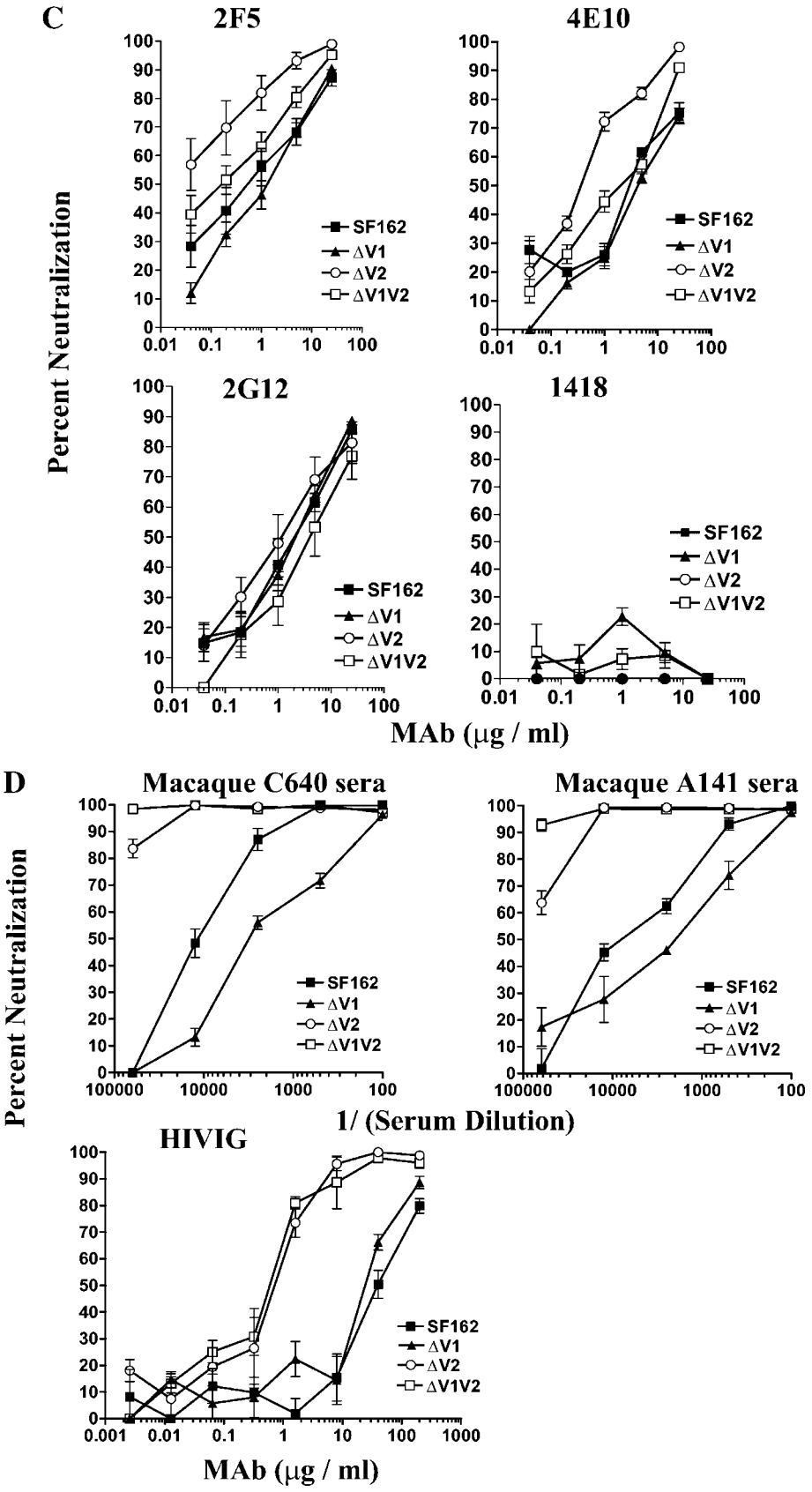

FIG. 7—*Continued*.

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bility, and fusogenic potential. These results are relevant in better understanding the relationship between HIV envelope structure and function and potentially in the design of relevant HIV envelope-based immunogens. Our current results suggest that SF162 envelope-derived immunogens lacking elements of the V1V2 region may adopt a structure and organization that is close to those of the functional virion-associated envelope trimers (52), but this may not be the case of SF128A and SF170 envelope-derived immunogens. Whether and how such structural differences will affect the immunogenic properties of such envelopes remain to be determined.

Replication competent HIV-1 viruses that lack the V3 loop have not yet been reported. This suggests that the V3 loop is required for HIV-1 replication. In accordance with previous studies conducted with different HIV envelopes (61), our current results indicate that the SF162 envelope is rendered fusion incompetent when amino acid deletions are introduced in its V3 loop. These deletions decrease the ability of the SF162 envelope to bind CD4 and eliminate its ability to mediate virus-cell fusion, probably due to inefficiencies during the post-CD4 binding-induced envelope conformational changes. Alternatively, the absence of the V3 loop may prevent the binding of the envelope to CCR5 molecules (10, 57, 59).

Interestingly, removal of the V3 loop from the background of an HIV-2 virus, HIV-2/NIHz, termed VCP, does not abrogate its ability to replicate (A. Bertolotti-Ciarlet G. Lin, M. Biscone, B. Haggarty, J. Romano, R. W. Doms, and J. A. Hoxie, 11th Conference on Retroviruses and Opportunistic Infections, 2004). It would be interesting to determine whether this is a general property of HIV-2 isolates or unique to this particular isolate. The former possibility would suggest that the overall structural organization of the HIV-1 and HIV-2 envelopes differs significantly.

The V1V2 region is known to play a major role in defining the overall neutralization phenotype of HIV and SIV (6, 20, 33, 35, 41). However, our results indicate that the V1 and V2 loops differentially affect the neutralization susceptibility of HIV-1. The individual deletion of the V2 loop enhanced the neutralization susceptibility of SF162 to almost all MAbs tested here, irrespective of the epitopes they recognize. It is therefore possible that the overall position of the V2 loop within the trimeric envelope regulates the exposure of multiple, diverse epitopes. Two exceptions were noted. First, deletion of the V2 loop did not alter the viral neutralization susceptibility 2G12. Second, deletion of the V2 loop (individually or in combination with deletion of the V1 loop) decreased the viral neutralization susceptibility to IgG1b12. These results are not entirely unexpected, since the binding of 2G12 to the HIV envelope glycoprotein depends on the presence of N-linked glycosylation sites in C3 and V4 (5, 48), and the binding of IgG1b12 is affected by the amino acid composition, and glycosylation pattern, of the V2 loop (31, 39).

The increased susceptibility of the SF162ΔV2 and SF162ΔV1V2 viruses to IgGCD4 neutralization could be due to an increase in the exposure of the CD4 binding site upon deletion of the V2 loop. We previously reported that the binding of IgGCD4 to intact SF162 and SF162ΔV2 virions was similar (54). We caution however that the assays used to monitor the binding of antibodies to intact virions are far from being ideal, and may not be sensitive enough to report on small differences in the relative exposure of the CD4 binding site that may have significant implications on the neutralization susceptibility of the virus to IgGCD4. Additionally, if deletion of the V2 loop resulted in the increased exposure of the CD4 binding site, this did not in turn result in a higher entry potential of the SF162ΔV2 virus compared to SF162.

In part, the greater neutralization susceptibility of SF162ΔV2 to MAbs that recognize CD4i epitopes could be related to delays in the kinetics of post-CD4 binding steps required for HIV-cell fusion, as suggested by our virus-cell fusion results. Such delays in the fusion reaction may increase the length of time during which neutralization epitopes become optimally exposed (and accessible to MAb binding) following or during the binding of the viral envelope glycoprotein to cellular CD4 molecules.

Some of the MAbs we used here, neutralize HIV with differential ability depending on whether the target cells are PBMC or cell lines (1). We previously reported on the neutralization susceptibilities of SF162, SF162ΔV2, and SF162ΔV1 using PBMC as target cells and a limited number of MAbs (53). In those studies, we recorded an increased susceptibility of SF162ΔV2 to neutralization by anti-CD4 binding antibodies similar to what we observed here. In general, SF162 is less susceptible to neutralization by antibodies such as 17b, X10, or M18 compared to antibodies that recognize the CD4 binding site. The resistance of SF162 to neutralization by 17b was even more pronounced when PBMC were used as targets (53). Because of this, we were previously unable to record any differences in the neutralization susceptibility of SF162 and SF162ΔV1 by CD4i antibodies using PBMC as targets.

In accordance with our previous observations (53), deletion of the V2 loop individually or in combination with the V1 loop rendered the virus significantly more susceptible to neutralization by heterologous or homologous sera. This most likely suggests that such modifications increase the exposure of multiple conserved neutralization epitopes on SF162. Interestingly, although the heterologous human HIVIG neutralized SF162 and SF162ΔV1 with similar potency, the two homologous macaque sera tested neutralized SF162ΔV1 more efficiently than SF162. Most likely, the homologous sera contain higher titers of anti-V3 specific antibodies than the heterologous sera.

Our results indicate that the V1 and V2 loops have opposing roles in defining the neutralization susceptibility of HIV-1. In contrast to what we recorded in the case of SF162ΔV2, the individual deletion of the V1 loop rendered the virus (SF162ΔV1) resistant to neutralization by antibodies that recognize CD4i epitopes, by certain CD4 binding site antibodies, and by the anti-V3 loop MAbs we tested. It is possible that when the V1 loop is removed, the remaining envelope regions are repositioned in a way that more effectively mask CD4i epitopes, such as those involved in HIV envelope-CCR5 binding. Such a repositioning may alter the exposure of elements of the bridging sheet, which connects the inner and outer domains of gp120 and participates in envelope-coreceptor binding (23, 46, 60). These structural alterations do not however decrease the ability of the SF162ΔV1 envelope to mediate virus-cell fusion, and the SF162ΔV1 virus replicates as efficiently as SF162.

The simultaneous elimination of the V1 and V2 loops resulted in a virus, SF162ΔV1V2, that displayed a neutralization susceptibility profile that depending on the MAb used, was either similar to that of SF162ΔV2 (in the case of anti-CD4

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binding site and anti-V3 loop MAbs) or intermediate between that of SF162 and SF172ΔV2 (in the case of anti-CD4i MAbs). The fact that elimination of the V2 loop from the SF162ΔV1 background only partially restores the neutralization susceptibility of the virus to antibodies that bind to CD4i epitopes suggests that one of the regions that are repositioned upon deletion of the V1 loop is the V2 loop. However, other regions are also repositioned to prevent the binding of antibodies to CD4i epitopes. One such region could be the V3 loop. A repositioning of the V3 loop following deletion of the V1 loop may also affect the exposure of V3 loop epitopes, in accordance with our observation that the SF162ΔV1 virus was less susceptible to neutralization by certain anti-V3 loop antibodies than SF162. The repositioning of the V1, V2, and V3 loops (and potentially other variable domains) within a gp120/gp41 monomer (24) may very well alter the interaction of these envelope regions within the trimer, the target of neutralizing antibodies.

It is also important to note that the V2 loop influences the neutralization susceptibility of SF162 to certain anti-gp41 antibodies. Deletion of the V2 loop, and to a lesser extent of both the V1 and V2 loops, but not of the V1 loop alone, resulted in a greater neutralization susceptibility of SF162 to 2F5 and 4E10. We previously reported that elimination of certain N-linked glycosylation sites from the C2, C4, and V5 regions of gp120, enhances the neutralization susceptibility of SF162 to 2F5 (30). Thus, the accessibility of the membrane proximal domain of gp41, which contains conserved neutralization epitopes (1), is influenced by the positioning of the V2 loop and by the extent of gp120 glycosylation.

In summary, our studies indicate that the V2 loop is indispensable for the proper function of the viral envelope in some HIV isolates but not others, which suggests a potentially distinct positioning of the V1 and V2 regions on the surface of diverse HIV-1 isolates. Our results also suggest that because the V1 and V2 loops have contrasting roles in defining the neutralization susceptibility of HIV-1, a more detailed analysis of the way that these two envelope regions interact is required to better understand the mechanism of HIV evasion of neutralizing antibodies. Hopefully, such studies will also assist the development of HIV envelope-based immunogens capable of eliciting broadly reactive neutralizing antibodies.

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