ability of populations in the southern parts of India, where the Dravidians are the major inhabitants and HTLV-1 is highly prevalent.[10–12] The genetic diversity of the southern Indian populations was estimated to be as high as that of Africans and even higher than that of Europeans and other Asians based on sequence data of mitochondrial DNA and the allele frequency of several genetic loci.[13] Taken together, these findings imply that different lineages of the Dravidians carried different genotypes of subgroup A HTLV-1 when they reached India.

According to this scenario, some of the HTLV-1s in India could have been taken to other HTLV-1-prevalent areas such as the Middle East, South Africa, and the Caribbean basin. The genetic similarities between two of the new Indian isolates (AP15 and TNA) and the Middle Eastern isolates (Fig. 2), as well as the geographic proximity of India and the Middle East, strongly suggest movements of HTLV-1 carriers between these two areas, which is consistent with previous reports.[4] If this is the case, HTLV-1 might have been brought from India to the Middle East on the basis of the higher diversity among HTLV-1s in southern India than among Middle Eastern HTLV-1s (Fig. 2). Our results also show that two of the new isolates (IND001 and IND002) were phylogenetically related to South African and Caribbean HTLV-1s. This raises the possibility that some Indian HTLV-1s were introduced to South Africa and the Caribbean basin, as was previously proposed.[14] This possibility is consistent with the facts that more than a half million Indians migrated to the Caribbean basin as indentured laborers after the abolition of the trans-Atlantic slave trade in the early nineteenth century, and that South Africa served as a waystation during the migration.

After the putative introduction of Indian HTLV-1 to South Africa, some of the migrants may have gone back to India, possibly carrying human immunodeficiency virus type 1 (HIV-1). This is because the seropositivity against HTLV-1 among HIV-1 seropositives was significantly higher than that among HIV seronegatives in southern India.[11] This suggests that HTLV-1 was sexually transmitted among some HIV-1 seropositives in southern India. As Indian HIV likely originated from South Africa, some Indian HTLV-1s may have originated from the same place.

In summary, we speculate that Dravidian speakers originally carried HTLV-1 to southern India. This is supported by the following two points. First, the seven HTLV-1s isolated in southern India in this study are all in subgroup A and are highly heterogeneous. Second, India has the most divergent strain of subgroup A[9] (Fig. 1). Nonetheless, it is unclear how Dravidian speakers originally acquired HTLV-1. With respect to the origin of HTLV-1 of the Dravidians, it is interesting that the sickle cell gene haplotypes in southern India are the same as those in Africa,[15] and that the Dravidian languages have some similarities to those spoken in the Sahel Belt of Africa (from Sudan to Senegal).[16] It also remains unclear why HTLV-1s of Japan and South America are phylogenetically related to those of India. Future phylogenetic analyses of HTLV-1s in the Sahel Belt and South and Central Asia will help to identify the origin of Indian HTLV-1 and elucidate how it was disseminated in Asia.

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Sequence Note

Predominance of Three NF-κB Binding Sites in the Long Terminal Repeat Region of HIV Type 1 Subtype C Isolates from Zambia

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ABSTRACT

Human immunodeficiency virus type-1 (HIV-1) is a leading cause of mortality and morbidity in the world, with almost 46 million people infected globally. HIV-1 subtype C accounts for 55% of these infections. In Zambia, the majority of HIV-1 infections are subtype C. However, to its north most countries have non-subtype C as the most predominant HIV-1 subtype while to its south most of them are predominantly subtype C. The aim of this study was to determine the subtype distribution and to analyze the long terminal repeat (LTR) region of HIV-1 isolates from the northern part of Zambia. We amplified as well as directly sequenced the LTR, *gag*, and *env* regions of 78 HIV-1 peripheral blood samples from adult Zambians. Our results show 95% (74/78) of our isolates were HIV-1 subtype C. Furthermore, of the subtype C samples analyzed across the LTR, 61% (25/41) carried 3 NF-κB signature binding site sequences.

HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1) continues to be among the highest causes of mortality and morbidity in the world. UNAIDS estimates that between 34 and 46 million people are infected with HIV globally. Of these 28 million are in sub-Saharan Africa.[1]

HIV-1 is classified into three groups, namely, M (major), O (outlier), and N (new). Within group M several HIV-1 subtypes, from A through to K, have been described. Of these, subtype C accounts for almost 55% of all global HIV-1 infections and is spreading across the globe. Other major subtypes are subtype A 31%, subtype D 7%, and subtype B 3%; the remaining percentage is covered by the other subtypes.[2]

Zambia is a landlocked country surrounded by eight countries. In the south is Zimbabwe, to the east are Malawi and Mozambique, to the northeast is Tanzania, with most of the northern border shared with Congo DR, and to the west are Namibia, Angola, and Botswana. Botswana, Malawi, and Zimbabwe are predominantly HIV-1 subtype C.[3–5] Angola is known to have subtypes D and G.[6] Congo DR has all known HIV-1 subtypes, although subtype A is the most dominant.[7] Tanzania has predominantly subypes A, C, and D.[8] No clear data are available on subtype distributions in Namibia and Mozambique. From these data it is clear that Zambia is surrounded by countries dominated by HIV-1 subtype C to the south and dominated by a mixture of other HIV-1 subtypes to the north.

Being a landlocked country there is a large volume of human traffic between Zambia and its neighbors for trade as well as importation of goods. In addition to this there is a large influx of refugees from Angola and DR Congo. Thus, these factors should, in theory, greatly influence the HIV-1 subtypes circulating in Zambia. Therefore, in this study we investigated the HIV-1 subtype distribution and the long terminal repeat region

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of samples taken from Lusaka and three provinces in the north of Zambia that share borders with non-subtype C dominant countries.

A total of 78 HIV-1-positive peripheral blood samples were analyzed. Of these, 28 samples were obtained from women attending antenatal clinics in Lusaka urban. The others were 8 from Mansa in Luapula province, 21 from Solwezi in the Northwestern province, and 21 from Mbala and Mpika in the Northern province, collected from patients attending voluntary counseling and testing (VCT) centers. All samples were taken with informed consent in an unlinked anonymous manner.

All DNA extraction, polymerase chain reaction (PCR) amplification, and sequencing of the *env* region were done as described by Handema *et al.*[9] The PCR amplification as well as sequencing for *gag* and LTR was carried out as described by Salminen *et al.*[10] and Gao *et al.*,[11] respectively.

The generated sequences were manually edited and aligned against HIV-1 reference sequences obtained from the Los Alamos laboratory sequence database (WWW.hiv-web.lanl.gov). Unrooted phylogenetic trees were then constructed with the neighbor-joining method using CLUSTAL W. Tree view was used to draw trees for illustration.

We successfully analyzed *gag* and *env* regions of all samples as well as the LTR region of 44 samples from the four different provinces. Upon phylogenetic analysis of the LTR region, all except three samples clustered with subtype C (Fig. 1). Figure 2, 3, and 4 show unrooted phylogenetic trees of *gag* and *env* regions of Luapala, Northern and Northwestern

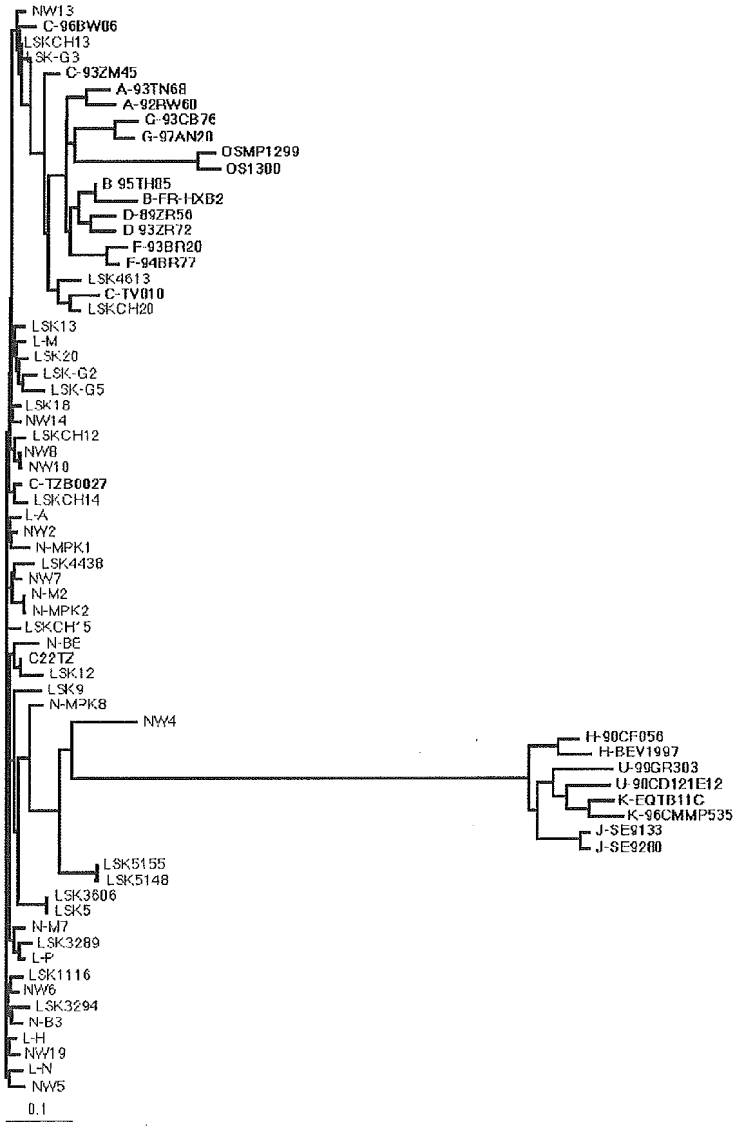

FIG. 1. Unrooted LTR phylogenetic tree of samples from Lusaka, Luapula, Northern and Northwestern provinces.

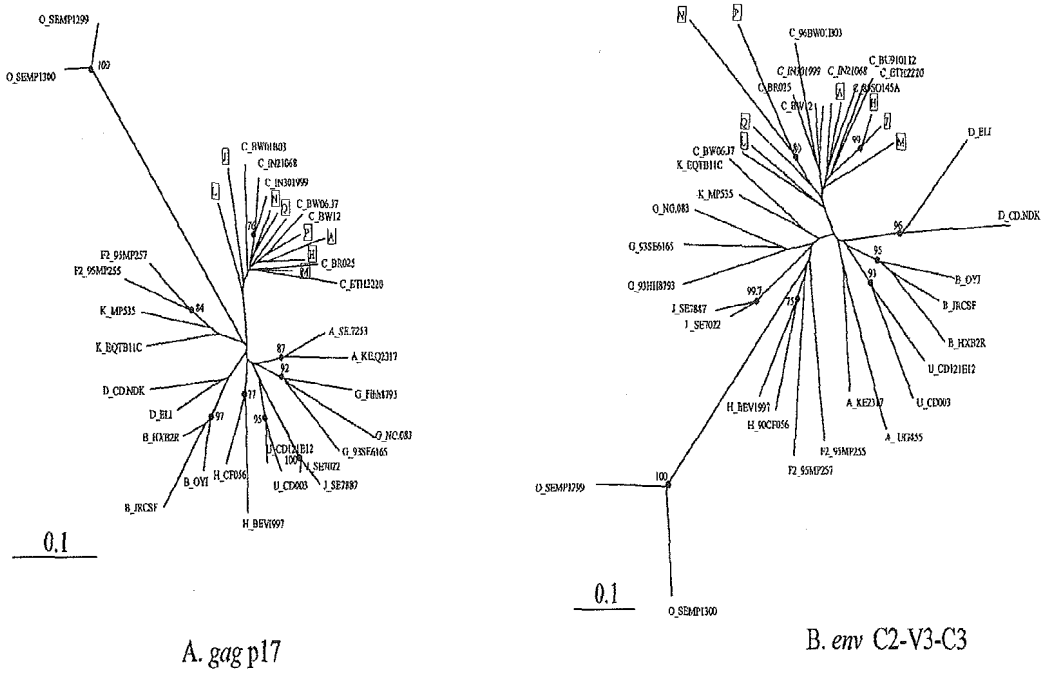

FIG. 2. Unrooted *gag* and *env* phylogenetic trees of samples from Luapula province.

province samples, respectively. All samples except sample 4 of Northwestern province clustered in subtype C in both *gag* and *env* regions as well as the LTR. Sample 4 clustered with subtype C in the *env* region but did not cluster with any specific group in the *gag* and LTR regions.

Figure 5 shows the unrooted *gag* phylogenetic tree of Lusaka samples. Lusaka *env* sequence results were previously reported in Handema *et al.*[9] All Lusaka samples except 3606, 5148, and 5155 clustered with subtype C in *gag*, *env*, and the LTR. Sample 3606 clustered with subtype G in *env* and *gag* but with subtype C in the LTR. Sample 5148 was subtype C in *gag* and *env* but nonspecific in the LTR. Sample 5155 clustered with C and D in *gag* and *env*, respectively, but with no specific subtype in the LTR.

From our data we note that 95% (74/78) of our samples cluster with subtype C in the LTR, *gag*, and *env* regions. These results are consistent with previous work done by other groups, showing that subtype C is still the predominant subtype in Zambia accounting for about 90% of reported cases, with only a few reported cases of subtypes A, D, G, J, and group O.[9,12]

Samples 4, 3606, and 5155 are probable recombinants as they are C, G, and D, respectively, in the *env*, but other subtypes in the LTR and *gag*. Sample 5148 may also be a recombinant as it is subtype C in *env* and *gag* but nonspecific in LTR.

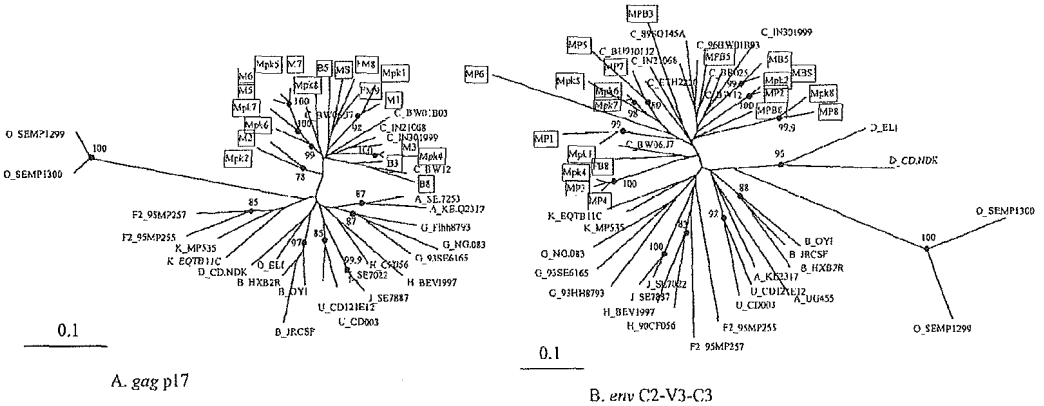

FIG. 3. Unrooted *gag* and *env* phylogenetic trees of samples from Northern province.

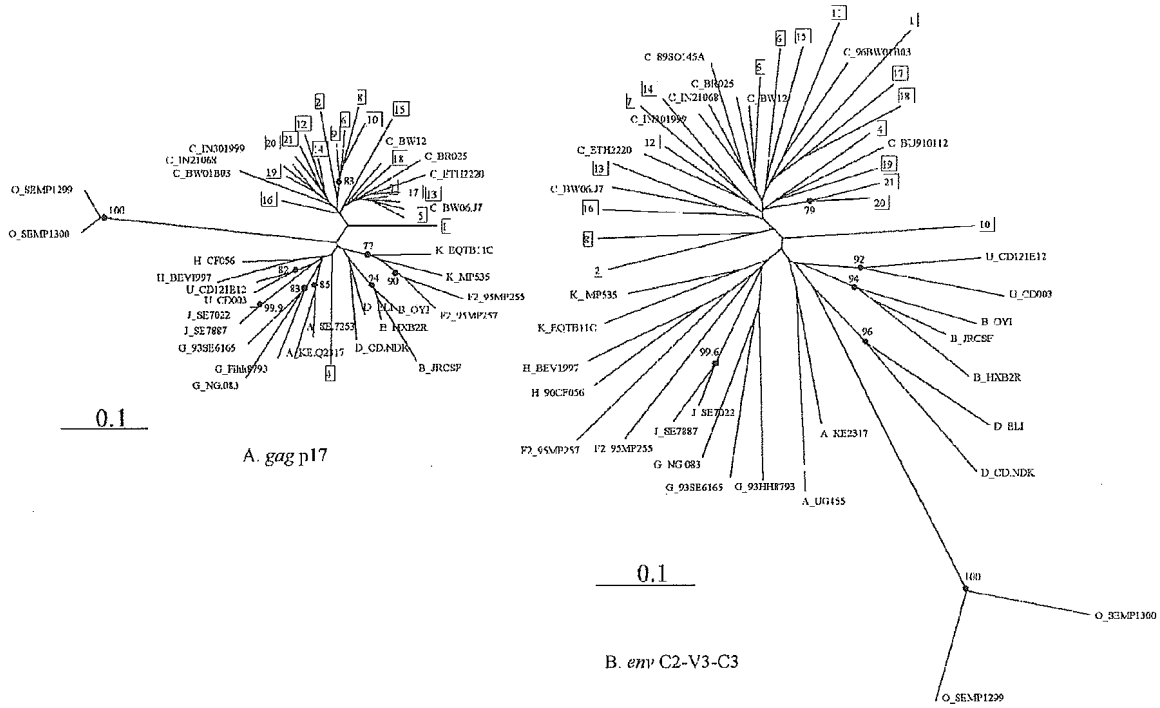

FIG. 4. Unrooted *gag* and *env* phylogenetic trees of samples from Northwestern province.

However, full genome analysis of these four samples is required to fully establish their status.

The subtype differences in various regions of the HIV-1 genome have been well documented. The notable reported subtype differences in the LTR are the presence of three NF-κB binding sites in HIV-1 subtype C as opposed to two in other subtypes.[3,6,14,15]

We therefore proceeded to analyze our samples to determine if the presence of a third NF-κB binding site seen in a majority of subtype C isolates from other countries was also a com-

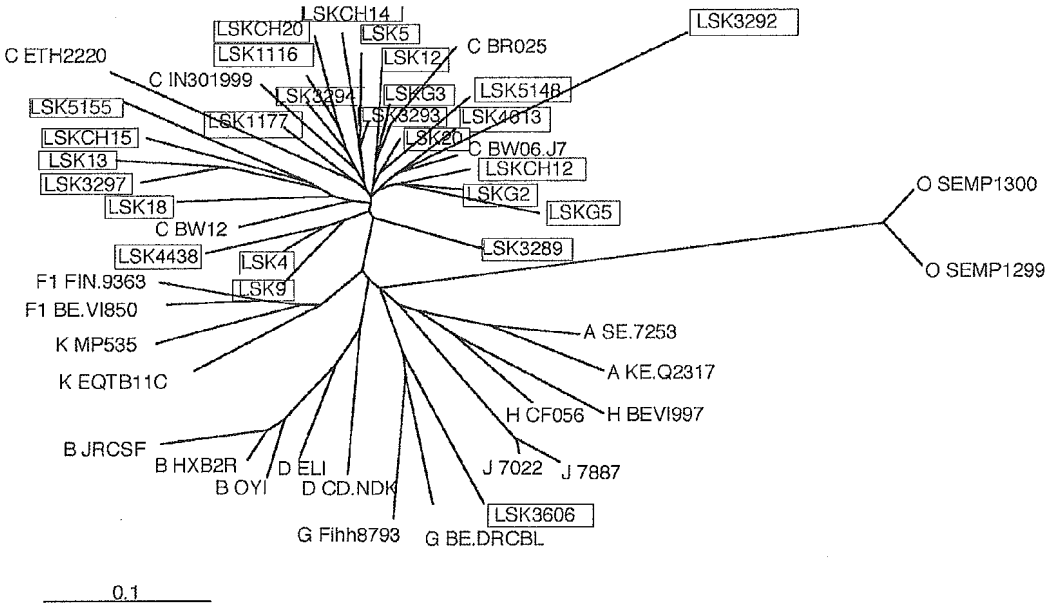

FIG. 5. Unrooted *gag* phylogenetic trees of samples from Lusaka province.

mon feature of the LTR of our samples. We compared our data to the subtype C consensus sequence and isolates of subtype C from South Africa, Botswana, Zimbabwe, and Tanzania. Our data showed that 61% (25/41) of our subtype C isolates had three NF-κB binding site signature sequences of 5′-GGGAC-TTTCC-3′ and 5′-GGGGCGTTCC-3′ (Fig. 6). The other 16, however, still showed NF-κB binding site-"like" signature sequences. Of these, 5 out of the 16 isolates had only single base substitutions opposed to the consensus. Despite this, they still contain sequences identical to those required for NF-κB binding.

Subtype C remains the dominant subtype in Zambia despite the high rate of human mobility and its geographical positioning. Furthermore, there is a reported increase in HIV-1 subtype C in Tanzania[8] where A and D were previously dominant and the southern part of Congo DR where all subtypes are found.[7] From this we can speculate that there is a northward movement of HIV-1 subtype C via Zambia leading to the increase in reported cases in Tanzania and its dominance in the south of Congo DR, due to the higher transmission efficiency of this subtype compared to others.[14] The true reason for this efficiency is still not clear but one possible reason is the presence of the three NF-κB binding sites as opposed to two in all other subtypes. However, more extensive work needs to be done before a definite conclusion can be drawn, as other factors are also important for transmission.

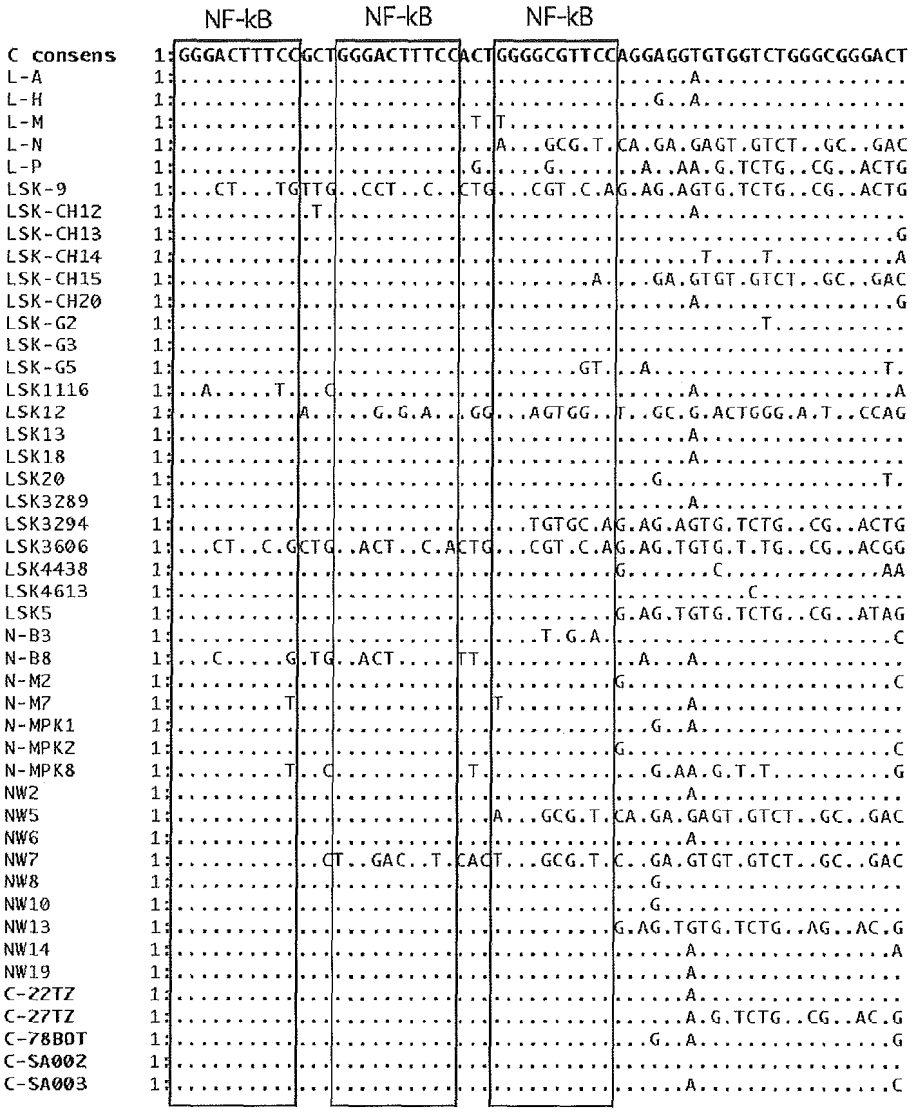

FIG. 6. Alignment figure of NF-κB binding sites in HIV-1 subtype C samples (C-22TZ, C-27TZ, C-78BOT, C-SA002, and C-SA003 are reference samples from Tanzania, Botswana, and South Africa, respectively; accession numbers AF239622, AF239627, AY047310, and AY047311).

ACCESSION NUMBERS OF SEQUENCE DATA

All *gag*, *env*, and LTR sequences have been deposited into the DNA Data Bank of Japan (DDBJ) database (accession numbers AB 191525–32, AB191534–63, AB191565–AB191686, and AB192571–2).

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Influence of Glycosylation on the Efficacy of an Env-Based Vaccine against Simian Immunodeficiency Virus SIVmac239 in a Macaque AIDS Model

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The envelope glycoprotein (Env) of human immunodeficiency viruses (HIVs) and simian immunodeficiency viruses (SIVs) is heavily glycosylated, and this feature has been speculated to be a reason for the insufficient immune control of these viruses by their hosts. In a macaque AIDS model, we demonstrated that quintuple deglycosylation in Env altered a pathogenic virus, SIVmac239, into a novel attenuated mutant virus (Δ5G). In Δ5G-infected animals, strong protective immunity against SIVmac239 was elicited. These HIV and SIV studies suggested that an understanding of the role of glycosylation is critical in defining not only the virological properties but also the immunogenicity of Env, suggesting that glycosylation in Env could be modified for the development of effective vaccines. To examine the effect of deglycosylation, we constructed prime-boost vaccines consisting of Env from SIVmac239 and Δ5G and compared their immunogenicities and vaccine efficacies by challenge infection with SIVmac239. Vaccination-induced immune responses differed between the two vaccine groups. Both Env-specific cellular and humoral responses were higher in wild-type (wt)-Env-immunized animals than in Δ5G Env-immunized animals. Following the challenge, viral loads in SIVmac239 Env (wt-Env)-immunized animals were significantly lower than in vector controls, with controlled viral replication in the chronic phase. Unexpectedly, viral loads in Δ5G Env-immunized animals were indistinguishable from those in vector controls. This study demonstrated that the prime-boost Env vaccine was effective against homologous SIVmac239 challenge. Changes in glycosylation affected both cell-mediated and humoral immune responses and vaccine efficacy.

Primate lentiviruses, human immunodeficiency viruses (HIVs), and simian immunodeficiency viruses (SIVs) share common genetic and biological properties. As SIVmac, originally isolated from macaques in primate research centers in the United States, causes AIDS in macaques with remarkable similarities to HIV type 1 (HIV-1) infection in humans, this AIDS monkey model has been utilized to study vaccine development and the pathogenesis of HIV infection (for reviews, see references 10, 14, 17, 43, and 47).

HIV/SIV infection in the host consists of two phases, the primary infection and chronic infection. During the primary infection, extensive viral replication and dissemination of the infection occur. In chronic infection, viral replication continues for a long period, eventually leading to AIDS. Due to the host immune response against the infection, these two phases are separated by a set point at which the viral load reaches its lowest level. The viral loads of the set point and chronic infection are inversely correlated with the control of SIV/HIV infection and predict disease progression (25, 31); however, it remains unclear which host responses determine the viral loads of the set point and chronic infection. Nevertheless, virus-specific immune responses have been implicated in the host's control of the infection. Cellular immunity, such as that shown by cytotoxic T lymphocytes (CTL) and helper T cells, has been reported to correlate with the control of HIV/SIV infection (for reviews, see references 2, 24, 28, and 39). The role of the neutralizing antibody (NAb) in the control of infection and the

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emergence of escape mutants has also been reported previously (7, 16, 51).

Despite these immune responses against HIV/SIV infection, humans and macaques fail to contain the infection due to the virus properties. HIV/SIV infects major target cells, such as $CD4^+$ T cells and macrophages, by binding viral envelope glycoproteins (Env) to cellular surface proteins and CD4 and chemokine receptors (CCR5, CXCR4, or others) on target cells (5, 32). Since viral entry consists of multiple steps (virion binding to these viral receptors, conformational change of Env, and fusion between the virion and the cellular membrane) and the critical parts of Env used in these steps are exposed only during each step, naturally generated antibodies are only partly effective in preventing HIV/SIV infection in their hosts (7, 8). Primary isolates can be neutralized to various degrees by HIV-infected patient serum but not by contemporaneous autologous samples. Consequently, escape mutants against preexisting NAb are selectively replicated (51). Thus, effective NAb is rarely induced in HIV/SIV infection (8, 10). This could partly explain the failure of Env-based vaccine trials against HIV-1 (8, 50).

The heavy glycosylation of Env is a unique feature of HIV/SIV that is distinctive from features of other enveloped viruses and is significantly related to their neutralization-resistant property (8, 29, 44). We therefore assumed that the insufficient immune containment of HIV/SIV might be due to heavy glycosylation in Env and that the removal of some glycans might allow the host to mount a protective immune response against the infection. Thus, we studied the influence of deglycosylation on the replication of SIVmac239 in a T-cell line and created a quintuple deglycosylation mutant of SIVmac239 (Δ5G), which has maximal removal of N-glycans at amino acid residues 79, 146, 171, 460, and 479 in Env and retains a replication capability similar to that of SIVmac239 in phytohemagglutinin-stimulated rhesus peripheral blood mononuclear cells (PBMCs) (36, 40). We then examined the infection of rhesus macaques with Δ5G; although Δ5G was replicated as extensively as SIVmac239 during the primary infection, the subsequent Δ5G infection was restricted to a level less than the detection sensitivity of a plasma viral load assay by 8 weeks postinfection (p.i.), in contrast to high chronic viral replication in SIVmac239 infection. Furthermore, an almost sterilizing immunity against SIVmac239 was induced in Δ5G-infected animals (36). Interestingly, another quintuple-deglycosylation-mutation strain with mutations at amino acid residues 146, 156, 184, 244, and 247 in Env was created (44) and was demonstrated to share common features with Δ5G in viral replication in animals and in functions as an attenuated vaccine (20). Since these two viruses share only one deglycosylation mutation and other mutations distributed differently in surface envelope protein gp120 (SU), these two studies suggest that heavily glycosylated Env determines the pathogenicity of HIV/SIV.

To dissect the mechanism for notable containment of Δ5G infection after primary infection, we hypothesized that the Env of Δ5G, a viral protein that differs from that in SIVmac239, might elicit protective immunity against SIVmac239, because deglycosylation in Env might alter antigenic properties such as B-cell and T-cell epitopes and enhance the protective immunity against SIVmac239. For this purpose, we immunized animals with Env of Δ5G (Δ5G Env) or Env of SIVmac239 (the wild type; wt Env), and examined the effect of these vaccinations against SIVmac239 infection.

MATERIALS AND METHODS

Generation of SU DNA vaccines. DNA vaccine plasmids expressing SIV mac239 SU or Δ5G SU, pJWSUmac239 and pJWSUmacΔ5G, were constructed using the expression vector pJW4303 (45). To produce secreted SU efficiently, the native signal sequence in the SIVmac239 SU gene was replaced with the human tissue plasminogen activator signal in plasmid pJW4303, and a termination codon was created at the cleavage site for SU transmembrane (TM) protein (9). An SIVmac239 SU or Δ5G SU DNA sequence was amplified with a pair of primers, SUmacA (5′-TGT**GCTAGC**TATGTCACAGTCTTTTATGGTGTAC-3′) and SUmacB (5′-CC**AGGATCC**TATTACCTCTTCACATCTGTGGGGG C-3′). The SUmacA primer consisted of nucleotides (nt) 6923 to 6955 of the SIVmac239 sequence (GenBank accession number M33262) and the boldface nucleotides, which were changed to create a NheI site; primer SUmacB consisted of nt 8412 to 8381 and the boldface nucleotides, which were changed to create a BamHI site, and the underlined nucleotides, which generated tandem termination codons. The PCR-amplified fragments were digested with NheI and BamHI and cloned into the NheI- and BamHI-digested eukaryotic expression vector pJW4303 to yield pJWSUmac239 and pJWSUmacΔ5G. These plasmids were prepared using a Plasmid Mega kit (QIAGEN, Tokyo, Japan).

Generation of Env vaccinia vaccines. Recombinant vaccinia viruses expressing Env of SIVmac239 or Δ5G, WRvvmac239 or WRvvΔ5G, respectively, were constructed using a vaccinia virus WR strain (WRvv) as described previously (15). To excise the entire coding region of the *env* gene from the cloned SIV plasmid, BamHI and SmaI sites were introduced by in vitro mutagenesis at 5′- and 3′-end-flanking sites of the *env* gene, respectively. Primer B-6808 (5′-GAA AGAGAAGAAGGATCCCGAAAAAGG-3′) consisted of nt 6796 to 9822 and the underlined mutations of the BamHI site; S-9537 (5′-TATGAATACTCCC GGGAGAAACCC-3′) consisted of nt 9527 to 9550 and the underlined mutations of the SmaI site. DNA fragments containing the *env* gene of SIVmac239 or Δ5G were isolated by digesting the mutated plasmids with BamHI and SmaI and were cloned into the SmaI- and BamHI-digested vaccinia virus vector plasmid pNZ68K2. To transfer the *env* gene from a recombinant plasmid to WRvv, the standard homologous recombination method using CV-1 cells was performed. Env expression in the recombinant vaccinia virus was confirmed by immunoprecipitation. The function of Env was confirmed by CD4- and CCR5-dependent fusion activity. The recombinant Env-expressing vaccinia viruses obtained were propagated and titrated in CV-1cells. The two recombinant viruses were propagated with similar kinetics in CV-1 cells.

Expression of SU-expressing plasmids and Env-expressing vaccinia virus in vitro. CV-1 cells were transfected with equal amounts of the following SU-expressing plasmids: pJWSUmac239, pJWSUmacΔ5G, or the vector pJW4303. Secreted SU metabolically labeled with ^{35}S protein labeling mix (PerkinElmer, Boston, MA) in culture supernatant was concentrated, immunoprecipitated with plasma from SIVmac239-infected monkeys, and then analyzed by sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) as described previously (40). To examine Env-expressing vaccinia viruses, CV-1 cells were infected with WRvvmac239, WRvvΔ5G, or WRvv at a multiplicity of infection of 10, metabolically labeled with ^{35}S protein labeling mix overnight, lysed, immunoprecipitated with plasma from SIVmac239-infected monkeys, and then analyzed by SDS-PAGE as described for the expression of SU-expressing plasmids.

Animals, immunization, and challenge. Twelve juvenile rhesus macaques from Myanmar or Laos that were seronegative for SIV, simian T-cell lymphotropic virus, B virus, and type D retroviruses were used. As the polymorphism of major histocompatibility complex (MHC) genes influenced cellular immune responses against SIV/HIV infection, MHC II haplotypes and alleles of the macaques were determined (data not shown). All animals were housed in individual cages and maintained according to the rules and guidelines for experimental animal welfare stated by the National Institute of Infectious Diseases. As shown in Fig. 1, the 12 animals were divided into three immunization groups of four animals each: the SIVmac239 (wt)-Env immunization group (Mm0005, Mm0007, Mm0010, Mm0012), the Δ5G Env immunization group (Mm0001, Mm0002, Mm0003, Mm0009), and the vector control immunization group (Mm0004, Mm0006, Mm0008, Mm0011). All animals were inoculated with 1 mg of plasmid DNA in 1 ml of saline, one into each quadriceps femoris at 0, 4, and 8 weeks after the initial prime immunization (weeks p.p.). The boost consisted of 5×10^7 PFU of vaccinia virus in 1 ml of phosphate-buffered saline (PBS), administered in two 0.1-ml intradermal inoculations, one into the skin of each femur, and two 0.4-ml inoculations, one into each quadriceps femoris at 21 weeks p.p. All animals were

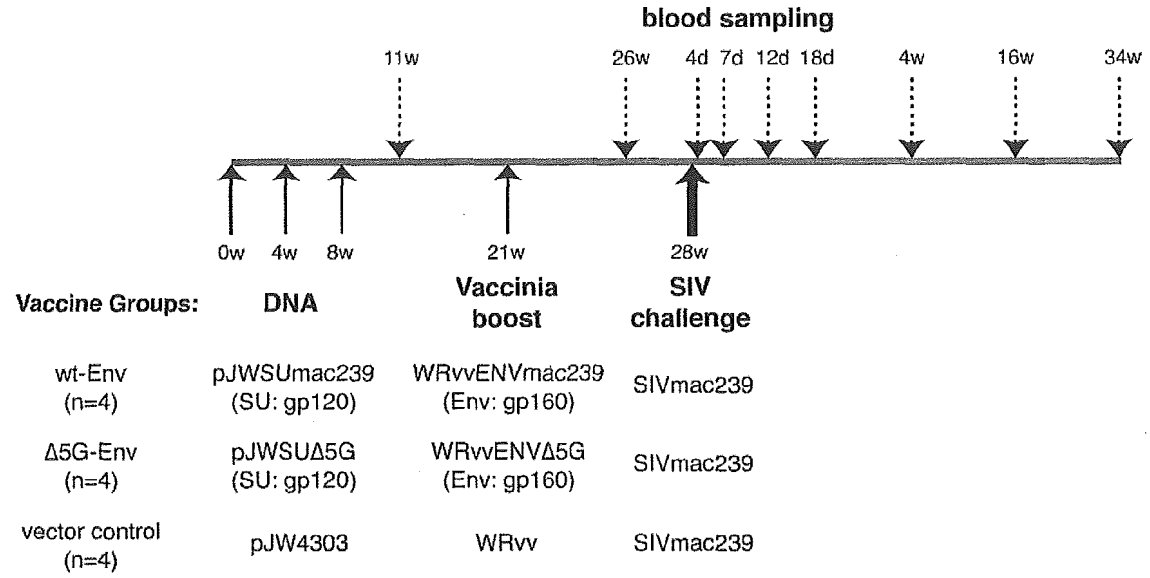

FIG. 1. Outline of immunization, challenge infection, and blood sampling. Twelve juvenile rhesus macaques were divided into three immunization groups of four animals each: the wt-Env immunization group (Mm0005, Mm0007, Mm0010, and Mm0012), the Δ5G Env immunization group (Mm0001, Mm0002, Mm0003, and Mm0009), and the vector control immunization group (Mm0004, Mm0006, Mm0008, and Mm0011). Animals were inoculated with a DNA vaccine (pJWSUmac239 for the wt-Env vaccine group, pJWSUΔ5G for the Δ5G Env vaccine group, and pJW4303 for the vector control group) at 0, 4, and 8 weeks p.p. The boost vaccine consisted of vaccinia virus (WRvvENVmac239 for the wt-Env vaccine group, WRvvENVΔ5G for the Δ5G Env vaccine group, and the WR strain for the vector control group) administered at 21 weeks p.p. All animals were challenged with 10 $TCID_{50}$ of SIVmac239 intravenously at 28 weeks p.p. w, weeks; d, day.

challenged with 10 50% tissue culture infective doses ($TCID_{50}$) of SIVmac239 intravenously at 28 weeks p.p.

Viral load measurement. To monitor SIV infection, the plasma viral load was measured by the real-time-PCR method described previously (36). Viral RNA was isolated from plasma from the infected animals using a commercial viral-RNA isolation kit (PE Applied Biosystems, Urayasu, Japan). SIV *gag* RNA was amplified and quantified using a commercial RNA reverse transcription (RT)-PCR kit (TaqMan EZ RT-PCR; PE Applied Biosystems) with the two *gag* primers, namely, the forward primer 1224F (5′-AATGCAGAGCCCCAAGAA GAC-3′), the reverse primer 1326R (5′-GGACCAAGGCCTAAAAAACCC-3′), and TaqMan probe 1272T (6-carboxyfluorescein-5′-ACCATGTTATGGCC AAATGCCCAGAC-3′-6-carboxymethylrhodamine). Purified viral RNA (10 μl) was reverse transcribed and amplified in a MicroAmp optical 96-well reaction plate (PE Applied Biosystems) according to the manufacturer's instructions and with the following thermal cycle conditions: 1 cycle of three sequential incubations (50°C for 2 min, 60°C for 30 min, and 95°C for 5 min) and then 50 cycles of amplification (95°C for 5 s, 62°C for 30 s) in a 7000 Prism sequence detection system (PE Applied Biosystems). In vitro RNA transcripts were quantified by optical density at 260 nm (OD_{260}) measurement and branched DNA assay for SIV viral RNA (Bayer Diagnostics, Tarrytown, N.Y.). RNA equivalent to 10 to 10^7 copies per reaction was used as the standard for each assay. The detection sensitivity of plasma viral RNA using this method was 1,000 copies/ml.

Flow cytometry. CD4 depletion was monitored by measuring the percentage of $CD4^+$ T cells, memory cells (CD29 high $CD4^+$) T cells (48) in PBMCs. PBMC samples were purified from a citrate anticoagulant containing blood using standard Ficoll-Hypaque gradient centrifugation. For flow cytometry, 2×10^5 PBMCs were reacted with fluorescein isothiocyanate or phycoerythrin-labeled antibodies (anti-human CD4, Nu-Th/I [Nichirei, Tokyo, Japan]; anti-human CD8, Leu2a [Becton Dickinson, San Jose, CA]; anti-human CD29, 4B4 [Coulter, Miami, FL]; anti-monkey CD3, FN-18 [Biosource, Camarillo, CA]; and anti-human CD20, Leu16 [Becton Dickinson, San Jose, CA]) as previously described (36, 37, 48).

Peptides. Overlapping peptides were synthesized by Emory University, Microchemical Facility, Winship Cancer Center (Atlanta, GA.). All SIVmac239 viral proteins except Env, Gag, Pol, Vif, Vpr, Vpx, Tat, Rev, and Nef were covered by consecutive 20-mer peptides overlapped by 12 amino acids. Env of SIVmac239 was covered by 72 consecutive 25-mer peptides overlapped by 13 amino acids. Peptides were dissolved in PBS with 10% dimethyl sulfoxide (Sigma Chemical, St. Louis, Mo.).

rSeV. Recombinant Sendai viruses (rSeV) expressing SIVmac239 Gag, SU, or Δ5G SU were used to infect herpesvirus papio-transformed B-lymphoblastoid cell lines (B-LCLs) to prepare autologous B-LCLs presenting these viral antigens. rSeV Gag expressing unprocessed SIVmac239 Gag and p55 (22, 23) and rSeV SU and rSeV/Δ5G SU expressing wt SU and Δ5G SU were constructed as described previously (52) and were also used to infect autologous B-LCLs.

Anti-SIV ELISA. A 1:100 dilution of each plasma sample in PBS (pH 7.4) containing a blocking reagent (Dainippon Seiyaku, Osaka, Japan) was assayed for SIV-specific antibody by using a standard enzyme-linked immunosorbent assay (ELISA) technique with 96-well plates precoated with SIVmac239 virion lysate. The OD_{492} was measured using a microplate reader (range of absorbance with linearity, 0 to 3.0; Tecan Japan, Tokyo, Japan) and utilized as a relative measurement of the antibody titer.

ELISPOT assay. Virus-specific $CD4^+$ T cells and $CD8^+$ T cells in PBMCs were measured using a monkey γ-IFN ELISPOT assay kit (U-CyTech, Utrecht, The Netherlands).

Cryopreserved PBMCs were thawed and cultured overnight in R-10 medium (RPMI 1640 [Sigma] supplemented with 10% heat-inactivated, defined fetal bovine serum [HyClone, Logan, Utah], 55 μM 2-mercaptoethanol, 50 U/ml penicillin, and 50 μg/ml streptomycin). PBMCs were subjected to the depletion of $CD4^+$ cells with magnet beads coated with anti-human CD4 Ab (Dynal ASA, Oslo, Norway) or subjected to the depletion of $CD8^+$ cells with magnet beads coated with anti-human CD8 Ab (Miltenyi Biotec, Bergisch Gladbach, Germany). Depletion of $CD4^+$ or $CD8^+$ cells from PBMCs was confirmed by flow cytometry. Using this depletion method, more than 95% of $CD4^+$ or CD8 cells were removed from PBMCs. These PBMCs were used for ELISPOT assay for virus-specific $CD8^+$ T cells and virus-specific $CD4^+$ T cells. Virus-specific stimulation of T cells was performed with autologous B-LCLs pulsed with pooled peptides for Pol, Vif, Vpx, Vpr, Tat, Rev, and Nef or B-LCLs infected with an rSeV for Gag, wt Env, and Δ5G Env. B-LCLs were incubated with pooled peptides corresponding to each viral protein at a final concentration of 2 μg/ml or infected with rSeV at a multiplicity of infection of 10 at 37°C overnight. Peptide-pulsed or infected B-LCLs were inactivated with long-wave UV irradiation (19) in the presence of 10 μg/ml psoralen (Sigma) for 10 min at a distance of 3.5 cm from a UV light, washed three times with R-10, and then used as stimulators in an ELISPOT assay. $CD4^+$ or $CD8^+$ cell-depleted PBMCs were cultured with these stimulators in an anti-γ-IFN Ab-coated ELISPOT plate (U-CyTech) overnight according to the protocol for the kit. Spots on the ELISPOT plate were imaged using an Olympus model SZX12 microscope

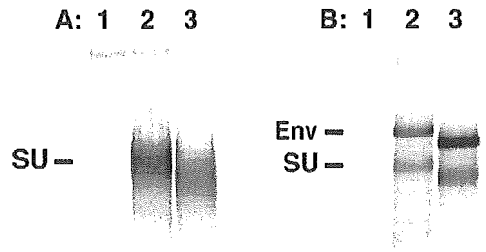

FIG. 2. Expression of SU and Env by SU-expressing DNA vaccines and Env-expressing vaccinia viruses. A: SU secreted in supernatant from CV-1 cells transfected with SU-expressing plasmids. Lane 1, pJW4303 vector; lane 2, pJWSUmac239; lane 3, pJWSUmacΔ5G. B: Env in cell lysates of CV-1 cells infected with recombinant vaccinia viruses. Lane 1, WRvv; lane 2, WRvvmac239; lane 3, WRvvΔ5G.

(Olympus, Tokyo, Japan) equipped with a digital camera, PDMCIe/OL (Polaroid, Cambridge, MA), and analyzed using a personal computer with MAC SCOPE version 2.61 (Mitani Corporation, Toyama, Japan). The results were calculated as numbers of spot-forming cells (SFC) per million PBMCs after subtraction of the background.

Neutralization assay. The original protocol of this neutralization assay was reported by Means et al. (29). Plasma that was heat inactivated at 56°C for 30 min was serially diluted and incubated with a fixed concentration of SIVmac239, Δ5G, or a macrophage-tropic SIV, 239/envMERT, at room temperature for 1 h. CEMx174/SIVLTR-SEAP cells were added to the mixture and then incubated at 37°C for 3 days. Secreted alkaline phosphatase activity in the culture supernatant was measured using a Phospha-Light System (Applied Biosystems). Chemiluminescence was detected with a Wallac Microbeta plate reader.

Statistical analysis. Statistical analysis was based on the Mann-Whitney test and performed using GraphPad Prism 4.0 software.

RESULTS

Experimental design. We adopted a DNA prime-vaccinia virus boost regimen to immunize rhesus macaques with wt Env or Δ5G Env as shown in Fig. 1. Twelve macaques were immunized at 0, 4, and 8 weeks after the initial prime immunization (weeks p.p.) with one of three different DNA expression plasmids (n = 4): pJWSUmac239 expressing SU of SIVmac239, pJWSUΔ5G expressing SU of Δ5G, or the vector pJW4303. At 21 weeks p.p., all animals were boosted with recombinant WR vaccinia viruses expressing the respective Env proteins: vaccinia virus expressing Env of SIVmac239, vaccinia virus expressing Env of Δ5G, or vaccinia virus (Fig. 1).

Expression of SU DNA plasmids and Env vaccinia viruses in vitro and in animals. Although Δ5G replicated similarly to wild-type SIVmac239 in animals (36), quintuple deglycosylation might affect the expression of SU in a plasmid vector and the expression of Env in the vaccinia virus vector. Thus, we examined the expression of these vaccines in CV-1 cells. SU expressions in the wild-type plasmid (pJWSUmac239) and in the deglycosylated SU plasmid (pJWSUmacΔ5G) were at similar levels (Fig. 2A). The expression and processing of Env in the wild type (WRvvENVmac239) and in the deglycosylated Env mutant vaccinia virus (WRvvENVΔ5G) were also at similar levels (Fig. 2B). The reduced molecular size of the proteins due to deglycosylation was confirmed by PAGE (Fig. 2). As the amount of secreted SU in the supernatant by DNA transfection was comparable to that of Env in the cell lysate from CV-1 cells infected with WRvvEnv, a high expression of SU was achieved in a *rev*-independent manner by the pJW403 expression plasmid as described previously (9).

The expression of Env vaccines in the immunized animals was indirectly estimated by Env-specific antibody responses measured by a peptide ELISA using overlapping Env peptides. Env peptide-specific Ab was detected from 11 weeks p.p. after immunization with DNA vaccines, whereas there was no significant difference in the titers and the specificity of the responses between the two vaccine groups (data not shown), suggesting similar amounts of Env expressed in animals immunized with either Env vaccine. To examine the protective effect of the Env vaccines, all animals were challenged with 10 $TCID_{50}$ of SIVmac239 intravenously at 28 weeks p.p.

Cellular immune responses elicited by Env vaccines. The DNA prime-vaccinia virus boost regimen has been used in many studies, has successfully induced a high frequency of virus-specific $CD8^+$ T cells in macaques, and has conferred protective immunity against chimeric simian/human immunodeficiency virus (SHIV) (3, 27, 45). We therefore examined the vaccine-induced Env-specific T-cell responses by IFN-γ ELISPOT assay. Since deglycosylation in Env might change T-cell epitopes in SIVmac239, we measured the wt-SU and Δ5G SU-specific T-cell response by using autologous B-LCLs infected with recombinant Sendai viruses expressing either wt SU and/or Δ5G SU, respectively.

Although there was a tendency for more ELISPOT-positive cells to be observed by homologous SU than heterologous SU, comparable results were obtained by both assays (Fig. 3A and B). As vaccinated animals were challenged with SIVmac239, the results from the wt-SU assay were subsequently used to assess the SU-specific immune response. Immunization with the DNA vaccine induced only marginal SU-specific $CD8^+$ T cells or $CD4^+$ T cells at 11 weeks p.p.; however, boost immunization with recombinant WR vaccinia virus significantly increased SU-specific $CD8^+$ T cells and $CD4^+$ T cells in PBMCs at 26 weeks p.p. (Fig. 3A, B, and C). Notably, SIVmac239 Env (wt Env) induced twofold more SU-specific CD8 T cells (mean, 770 SFC per million PBMCs; range, 540 to 880) responding to wt SU than Δ5G Env (mean, 320; range, 110 to 400) (P = 0.029) (Fig. 3A and C). Similarly, twofold more SU-specific $CD4^+$ T cells were observed in wt-Env vaccinees (mean, 1,260; range, 840 to 1,710) than in Δ5G Env vaccinees (mean, 680; range, 150 to 1,260) at 26 weeks p.p. (P = 0.11) (Fig. 3B and C). Thus, a twofold-greater number of both SU-specific $CD4^+$ T cells and $CD8^+$ T cells were induced in SIVmac239 Env vaccinees than in Δ5G Env vaccinees at 26 weeks p.p. In vector controls, only negligible SU-specific $CD4^+$ T cells and $CD8^+$ T cells were detected in PBMCs at 26 weeks p.p. (Fig. 3A and B).

Humoral immune response elicited with Env vaccines. The anti-Env Ab titer was examined by SIVmac239 virion lysate ELISA. Anti-SIV Ab was detected in both wt-Env vaccinees and Δ5G Env vaccinees after an rVV boost (Fig. 4) (26 weeks p.p.). Anti-SIV Ab titers were comparable between the two vaccine groups.

Next, we examined the NAb against either SIVmac239, Δ5G, or a macrophage-tropic mutant, 239env/MERT (33, 35), in the two vaccine groups. Macrophage-tropic SIVs were highly susceptible to neutralization by plasma from most SIV-infected macaques (29), whereas SIVmac239 was highly resistant to neutralization as were most clinical isolates of HIV-1

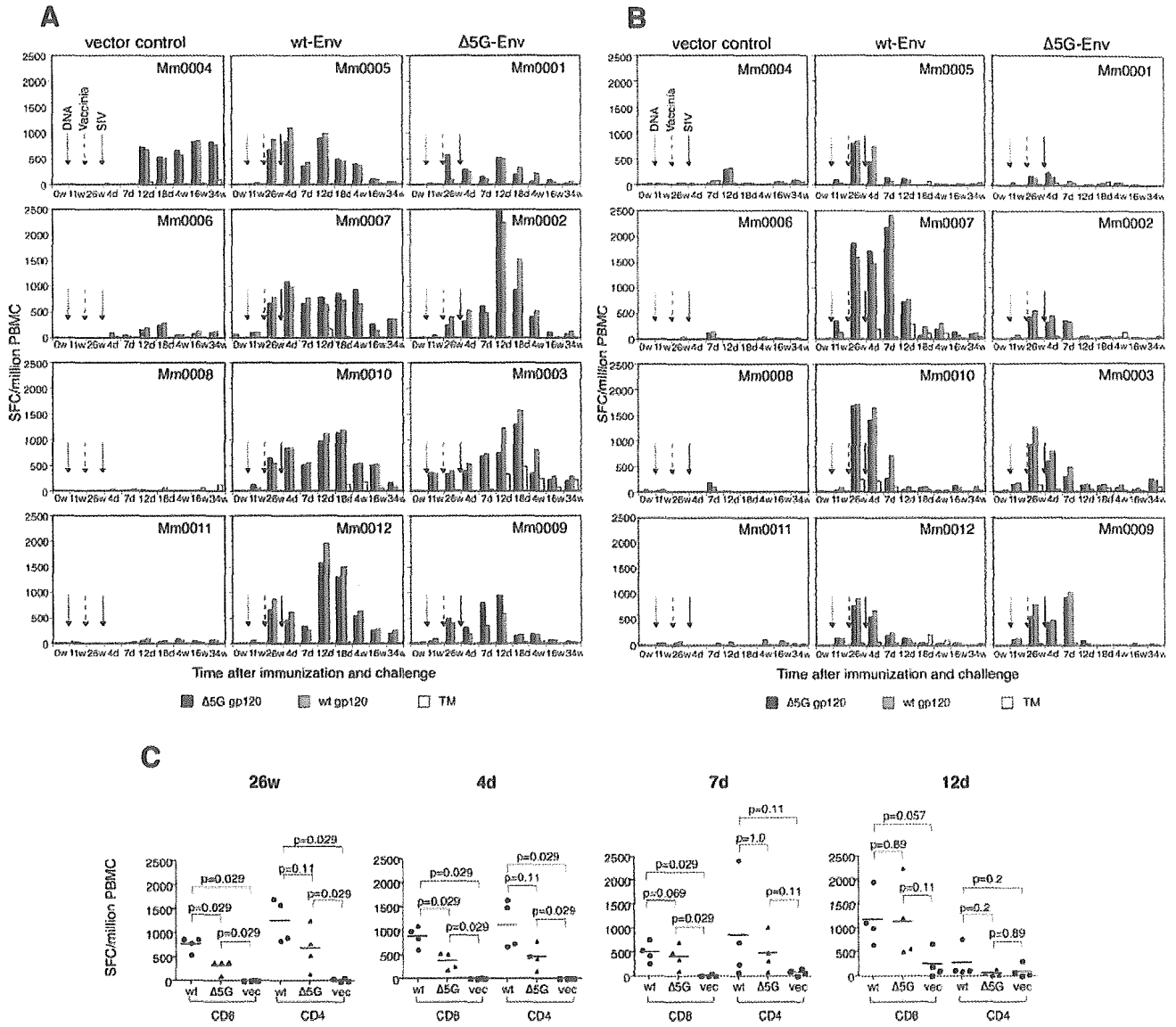

FIG. 3. Env-specific $CD4^+$ T-cell and $CD8^+$ T-cell responses in 12 macaques. A: Env-specific $CD8^+$ T cells in PBMCs were measured by ELISPOT assay for IFN-γ in three groups. B: Env-specific $CD4^+$ T cells in PBMCs were measured by ELISPOT assay for IFN-γ in three groups. ELISPOT results are colored as follows: Δ5G SU-specific T cells (red), wt-SU-specific T cells (green), and TM-specific T cells (yellow). Arrows with a dotted line, arrows with broken line, and arrows with a solid line indicate the time of the third DNA vaccination at 8 weeks p.p., the time of the vaccine boost at 21 weeks p.p., and the time of SIVmac239 challenge at 28 weeks p.p., respectively. C: Comparison of SU-specific $CD8^+$ T cells and $CD4^+$ T cells in PBMCs among the wt-Env vaccine group, the Δ5G Env vaccine group, and the vector control group at 26 weeks p.p. and 4, 7, and 12 days p.i. The numbers of SFC responding to SIVmac239 SU were used to compare the effects of the two vaccines. w, weeks; d, days.

(21, 29, 30). Plasma at 26 weeks p.p. from all immunized animals failed to neutralize not only SIVmac239 but also a multiple-deglycosylation-mutation strain, Δ5G (Table 1); in contrast, these plasma specimens did neutralize 239env/MERT. Furthermore, a marked difference was observed between the two vaccine groups. The NAb titer in the wt-Env vaccine group was eightfold higher than in the Δ5G Env vaccine group (Table 1). The difference of this immune response between the two vaccine groups was significant ($P = 0.029$).

SIV replication in Env-immunized animals. As described above, wt-Env vaccine and Δ5G Env vaccine induced different magnitudes of virus-specific cellular and humoral immunity in macaques. To examine the effect of the two vaccines, we challenged the vaccinated animals with SIVmac239. Viral loads in vector controls were mostly consistent with our previous results with SIVmac239-infected rhesus macaques (36, 48). The mean peak viral load at 2 weeks p.i. was 1.4×10^7 copies/ml, with a range of 0.5×10^7 to 2.2×10^7 copies/ml. Viral loads in chronic infection diverged into two patterns (Fig. 5A). Subsequent to the set point at 20 weeks p.i., the viral loads in three animals increased more than 10^4 copies/ml. In contrast, viral loads in one animal (Mm0011) remained as low as 1,000 copies/ml up to 45 weeks p.i.

Compared with the vector controls, viral loads in wt-Env

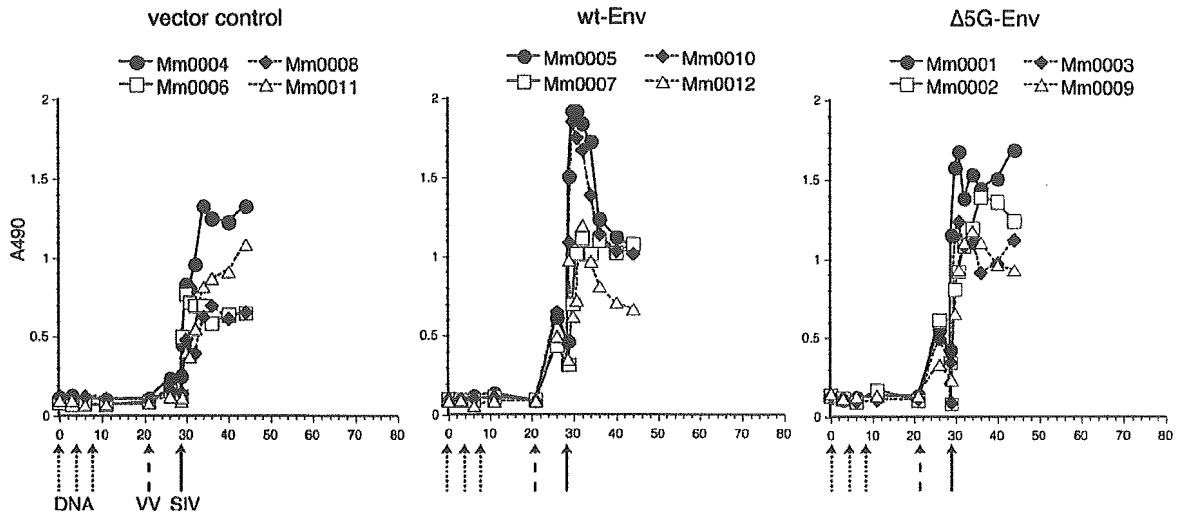

FIG. 4. Humoral immune response during immunization and after challenge infection. The OD_{492} was used as a relative measurement of anti-SIV ELISA antibody titer.

vaccinees were markedly reduced (Fig. 5B). Peak viral loads at 2 weeks p.i. (mean, 1×10^6 copies/ml; range, 0.8×10^6 to 1.2×10^6 copies/ml) were 1-log lower than those in the vector controls. Furthermore, viral loads decreased to as low as 1,000 copies/ml by 8 to 20 weeks p.i., remaining low until autopsy at 45 weeks p.i.

Unexpectedly, viral loads in the Δ5G Env vaccine group resembled those in vector controls (Fig. 5C). Peak viral loads (mean, 2.4×10^6 copies/ml; range, 0.9×10^6 to 4.2×10^6 copies/ml) were slightly lower than those in vector controls. Set points and viral loads in the chronic phase were similar to those of vector controls.

In summary, as shown by the mean viral loads in primary and chronic infection (Fig. 5D) and statistical analysis (Fig. 5E), the effects of vaccination differed between the wt-Env vaccine and Δ5G Env vaccine. In the effect on primary infection (up to 6 weeks p.i.), wt-Env vaccination decreased viral loads more extensively and significantly than Δ5G Env vaccination (P = 0.029 versus P = 0.057); however, in chronic infection (viral loads after 8 weeks p.i.), significant reductions in viral loads compared with those in vector controls were seen only in the wt-Env vaccine group and not the Δ5G Env vaccine group (Fig. 5E). Collectively, wt-Env vaccination induced significantly effective immunity to control SIVmac239 infection, whereas Δ5G Env vaccination induced a marginal effect seen only in primary and not in chronic infection.

TABLE 1. Neutralizing-antibody titers in the vaccinated macaques at 26 weeks p.p.

Vaccine	Animal	Neutralizing-antibody titer[a]			Mean[b]
		SIVmac239	Δ5G	239/envMERT	
wt-Env	Mm0005	<20	<20	800	400
	Mm0007	<20	<20	400	
	Mm0010	<20	<20	400	
	Mm0012	<20	<20	200	
Δ5G-Env	Mm0001	<20	<20	100	50
	Mm0002	<20	<20	20	
	Mm0003	<20	<20	100	
	Mm0009	<20	<20	50	

[a] Reciprocal of the dilution of plasma giving 50% inhibition of SIV replication.
[b] The difference in NAb levels between the two vaccine groups was significant (P = 0.0029).

CD4$^+$ T-cell subsets in PBMCs. CD4 cell depletion is a primary manifestation indicating immune disorder in HIV/SIV infection. As CD4 depletion results from HIV/SIV infection in lymphatic tissue, it correlates with the extent of viral replication. Accordingly, viral loads were correlated mostly with CD4 depletion (Fig. 5 and 6A). Despite fluctuations due to immunizations and the challenge infection, the percentage of CD4$^+$ T cells in wt-Env-immunized animals in the chronic phase recovered to the levels at the initiation of the experiment. By contrast, in vector controls and Δ5G Env vaccinees, the percentage of CD4$^+$ T cells decreased in the chronic phase. Among them, an extensive decrease in CD4$^+$ T cells occurred in animals with high viral loads in the chronic phase (Mm0001, Mm0008, and Mm0009) (Fig. 5 and 6A). However, in the other animals, the levels of CD4$^+$ T cells remained as before the challenge (Mm0003, Mm0011).

A subset of CD4$^+$ CD29 high cells, approximately corresponding to memory CD4$^+$ T cells, is useful for diagnosing a deterioration in the immune function in animals with AIDS (26, 38, 48). Although this parameter usually correlates with the percentage of CD4$^+$ T cells, remarkable differences were noted between two Env vaccine groups after the challenge infection. First, all animals in the wt-Env vaccine group showed an increased percentage of this subset in the chronic phase (Fig. 6B). Second, three of the Δ5G Env vaccinees had a marked decrease after the challenge infection (Mm0001, Mm0002 and Mm0009), whereas the remaining animal (Mm0003) showed an increased percentage of this subset. In

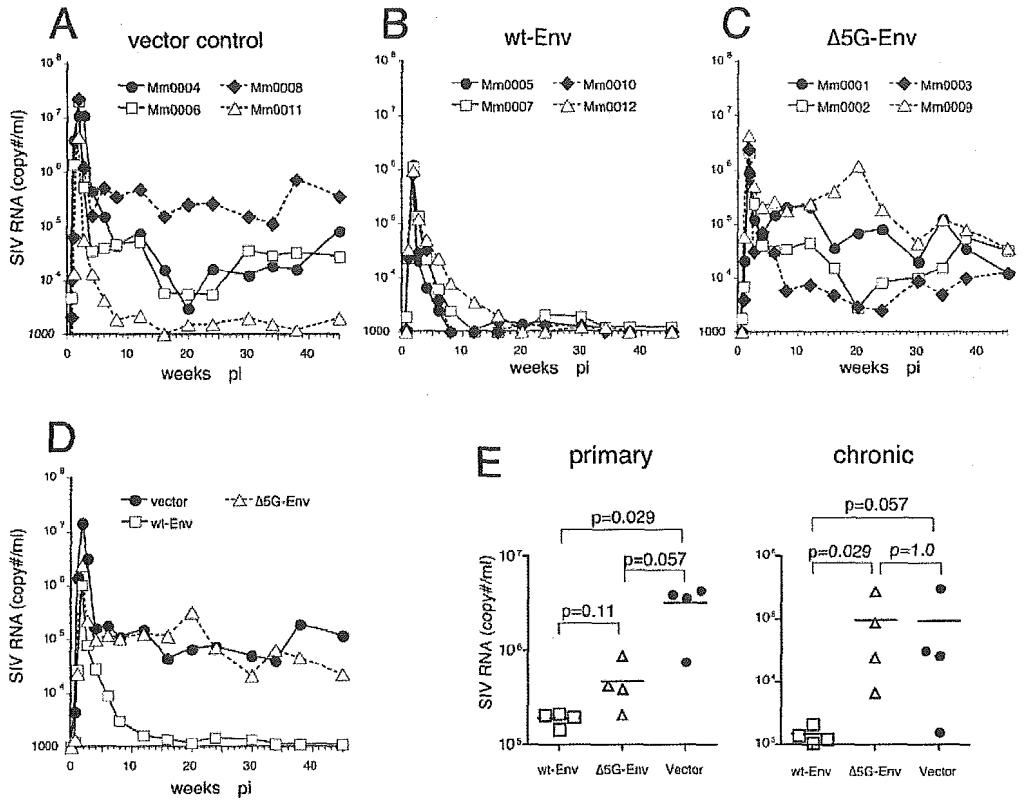

FIG. 5. Plasma viral loads after SIVmac239 challenge infection. Plasma viral load was measured by real-time PCR with a detection limit of 1,000 copies/ml. A: wt-Env vaccine group; B: Δ5G Env vaccine group; C: vector controls; D: comparison of viral loads among three groups; E: comparison of viral loads during the primary infection (5 days to 6 weeks p.i.) and chronic infection (8 weeks to 45 weeks p.i.) among three groups. Viral load was determined by averaging over a period of time.

vector controls, this subset remained in the range before the challenge infection in all animals but one (Fig. 6B).

Env-specific-T-cell immunity after the challenge infection. The magnitude of Env-specific T cells after the challenge infection is assumed to be influenced not only by vaccination but also by viral replication. Namely, SU-specific T cells at 4 days p.i. and those at 12 days p.i. were likely influenced by the former and the latter respectively. The magnitudes of SU-specific $CD4^+$ T cells and $CD8^+$ T cells at 4 days p.i. were comparable to those before challenge at 26 weeks p.p. (Fig. 3A and B); therefore, twofold-more SU-specific $CD8^+$ T cells and $CD4^+$ T cells were present in wt-Env vaccinees than in Δ5G Env vaccinees up to 4 days p.i. (Fig. 3C). However, this difference in the magnitudes of SU-specific $CD8^+$ T and $CD4^+$ T cells was not sustained at 7 and 12 days p.i. (Fig. 3C). Present with robust viral replication in primary infection, SU-specific $CD4^+$ T cells immediately decreased to an undetectable level at 12 days p.i. In contrast, SU-specific $CD8^+$ T cells increased (Fig. 3A and B). Subsequently, SU-specific $CD8^+$ T cells gradually decreased to very low or undetectable levels by 34 weeks p.i. (Fig. 3A). Thus, vaccine-induced SU-specific $CD8^+$ T and $CD4^+$ T cells were sustained only for a short period of time after challenge infection in both Env vaccine groups.

SIV-specific T-cell immunity after challenge infection. Despite an Env vaccination, robust SIV infection occurred shortly after the challenge infection (Fig. 5B and C). Consequently, SIV-specific $CD8^+$ T cells and $CD4^+$ T cells were elicited not only in vector controls but also in Env vaccine groups (Fig. 7A and B). To examine the effect of these SIV-specific T cells on the control of SIV infection, all animals were divided into SIV infection-controlled (controlled) and SIV infection-uncontrolled (uncontrolled) animals. Viral loads in chronic infection and the percentage of $CD4^+$ cells in PBMCs were used to classify the animals as controlled or uncontrolled (Fig. 6A). All animals in the wt-Env vaccine group, Mm00011 in vector controls, and Mm0003 in the Δ5G Env vaccine group were grouped as control animals. The remaining animals, Mm0004, Mm0006, and Mm0008 in vector controls and Mm0001, Mm0002, and Mm0009 in the Δ5G Env vaccine group were grouped as uncontrolled animals. Notably, SIV-specific $CD4^+$ T cells as well as the percentage of $CD4^+$ CD29H cells remained high in the chronic phase in controlled animals (Fig. 7B and 6B, respectively).

Although overall SIV-specific $CD8^+$ T cells were high in Env-vaccinated controlled animals, such correlation was not seen in vector controls grouped as uncontrolled animals (Fig. 7A). Therefore, to examine the relevance of virus-specific T cells to the control of SIV infection, the magnitudes of every viral-protein-specific T cell in controlled and uncontrolled animals were compared. As shown in Fig. 7C, Gag-specific $CD8^+$ T cells and $CD4^+$ T cells, and Tat/Rev-specific $CD4^+$ T cells

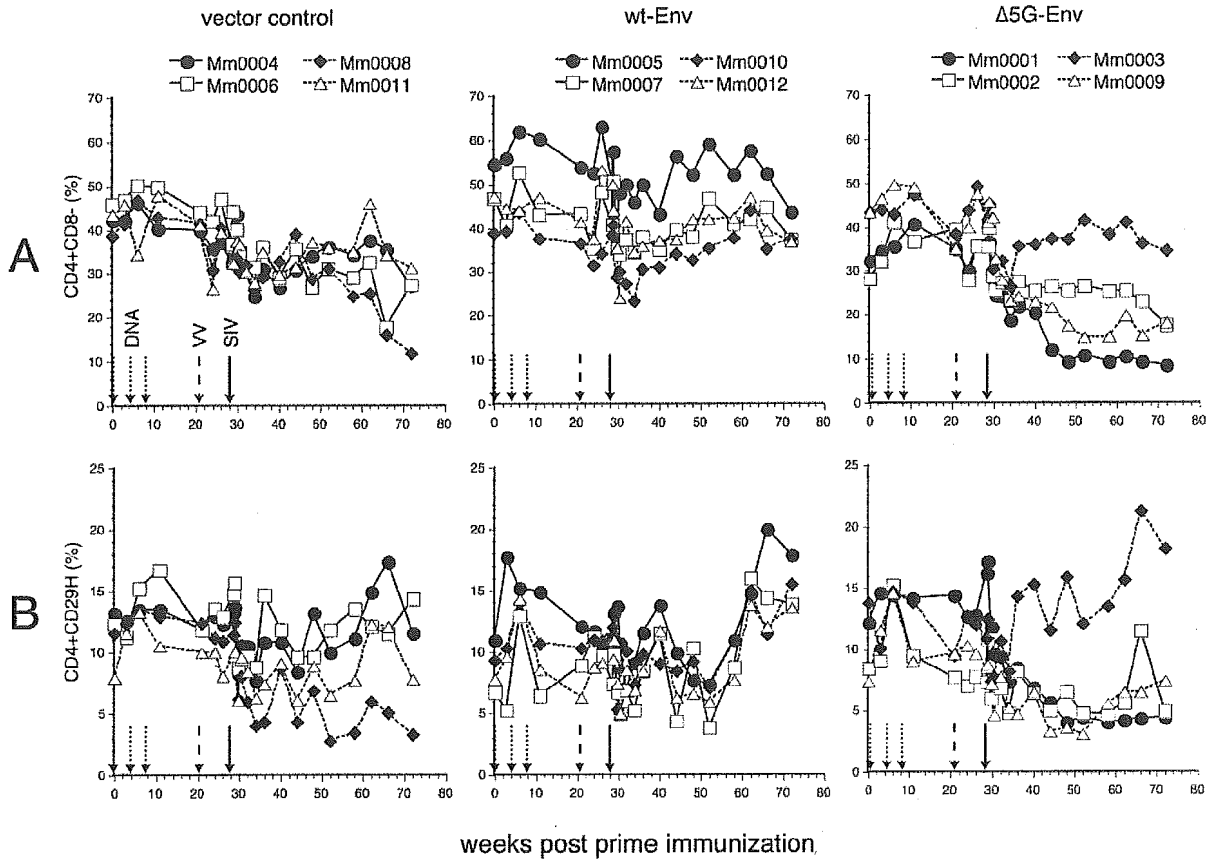

FIG. 6. $CD4^+$ T cells in PBMCs from rhesus macaques during immunization and after the challenge infection. A: Percentage of $CD4^+$ T cells in PBMCs; B: percentage of $CD4^+$ CD29high T cells in PBMCs.

were induced, with statistical significance ($P < 0.05$), in the control animals.

DISCUSSION

The heavily glycosylated structure of Env has been considered a main cause of chronically persistent viral replication and the pathogenicity of HIV/SIV, primarily because it potentially interferes with the development of the host immune response associated with protective immune functions, such as NAb and CTL (10, 36, 44). This characteristic constitutes the primary reason for the difficulty of developing effective vaccines. We therefore examined the efficacy of a deglycosylated-Env vaccine and compared it with the wt-Env vaccine. This study showed that quintuple deglycosylation neither improved the immunogenicity of the wt-Env vaccine nor elicited NAb against SIVmac239. This was in contrast to what occurred with Δ5G infection in rhesus macaques, because the host response elicited by Δ5G infection not only contained Δ5G infection but also protected the animals from SIVmac239 challenge infection (36). This study therefore suggested that an almost sterilizing immunity against SIVmac239 induced in Δ5G-infected animals could not be explained by the immunogenicity of Δ5G Env; instead, it is likely associated with the property of Δ5G as an attenuated virus. In fact, Δ5G was more neutralization-sensitive than SIVmac239 (36). Alternatively, the immunogenic property of Env in Δ5G could not successfully be duplicated by immunization with a Δ5G Env DNA prime-vaccinia virus boost regimen. Therefore, another immunization regimen might be able to elicit the protective immune response induced by Δ5G infection.

The Env vaccine is superior to other vaccines containing other viral proteins with respect to the induction of NAb; however, both the Δ5G Env vaccine and the wt-Env vaccine could not induce detectable NAb against either SIVmac239 or Δ5G. Instead, the wt-Env vaccine induced higher NAb against macrophage-tropic SIV than the Δ5G Env vaccine. Notably, this parameter most significantly correlated with the efficacies of the two Env vaccines. As Ab neutralized the macrophage-tropic variant 239/envMERT, which has only four separate amino acid substitutions distributed in *env* of SVmac239 (34), it might recognize unknown epitopes conserved between SIVmac239 and 239/envMERT. On the other hand, Δ5G Env may not sufficiently present this epitope due to mutations. Regarding the role of nonneutralizing Ab for the control of SIVmac239 infection, it is assumed that, as the neutralization assay did not necessarily reflect in vivo conditions, such nonneutralizing Ab with potential virus-binding ability may interfere with SIVmac239 infection in animals. Alternatively, Ab

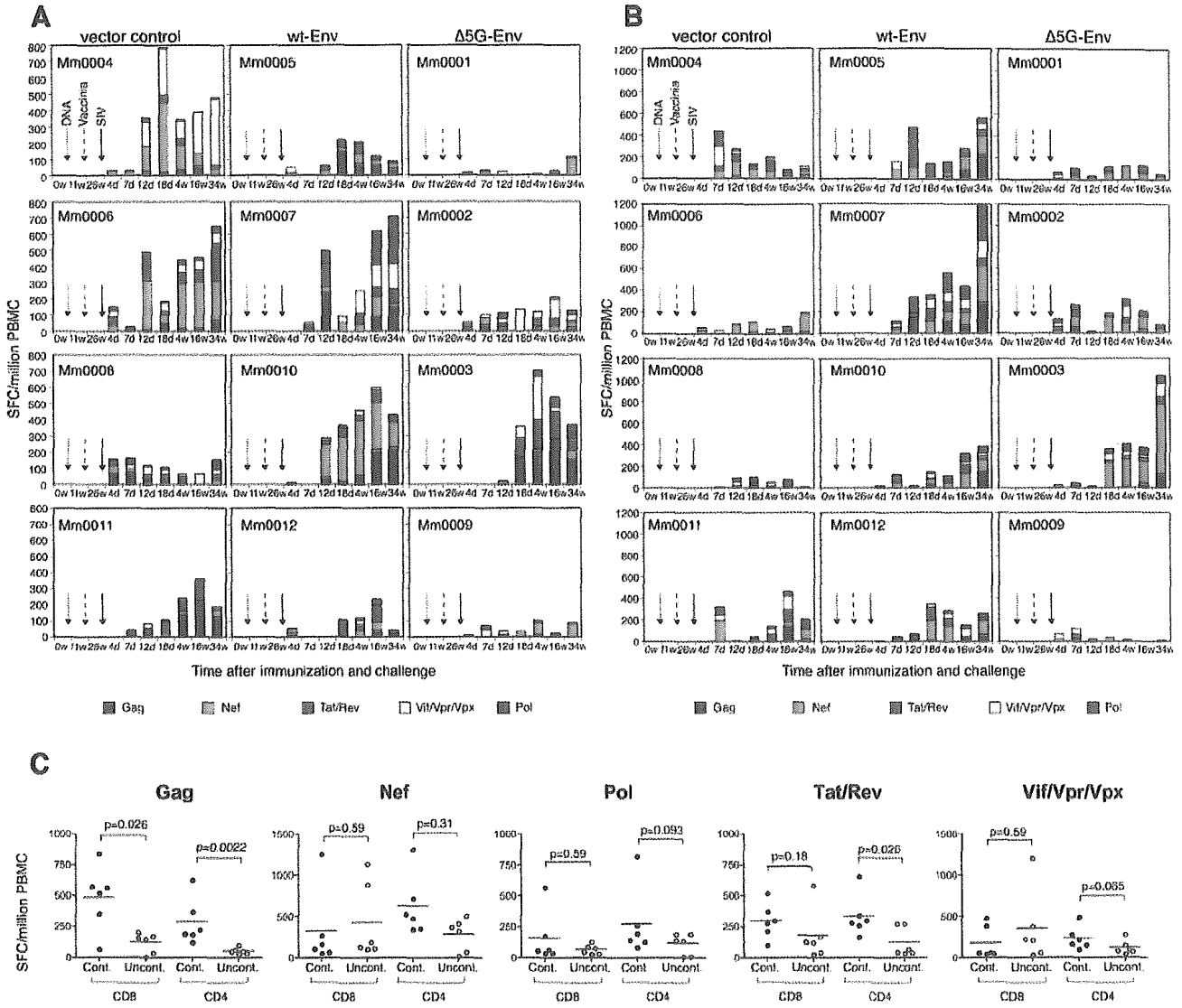

FIG. 7. SIV-specific $CD8^+$ T-cell and $CD4^+$ T-cell responses in 12 animals. A: SIV viral-protein-specific $CD8^+$ T cells in PBMCs were measured by ELISPOT assay for IFN-γ in three groups: vector controls, wt-Env vaccine group, and Δ5G Env vaccines. B: SIV viral-protein-specific $CD4^+$ T cells in PBMCs were measured by ELISPOT assay for IFN-γ in three groups. ELISPOT results of individual SIV proteins are colored as follows: Gag (red), Nef (green), Tat/Rev (blue), Vif/Vpr/Vpx (yellow), and Pol (pink). C: Comparison of cumulated $CD8^+$ T cells or $CD4^+$ T cells specific to the viral proteins Gag, Pol, Nef, Tat/Rev, and Vif/Vpr/VpX between SIV infection-controlled and uncontrolled animals. w, weeks; d, days.

might play a role in other effector functions, such as antibody-dependent cell-mediated cytotoxicity to eliminate the infected cells. The antibody-mediated enhancement of viral antigen processing and cross presentation is also a mechanism potentially related to the control of SIV infection in vivo (49).

Reduced immunogenicity in the Δ5G Env vaccine was also noted in cellular immunity. The levels of stimulation of antigen-specific $CD8^+$ T cells and $CD4^+$ T cells are MHC I and MHC II dependent, respectively. As the macaques in this study have different MHC haplotypes (data not shown), the magnitude and breadth of SIV-specific T cells should vary among the animals. Nevertheless, the magnitude of SU-specific $CD8^+$ T cells and $CD4^+$ T cells in PBMCs was greater in the wt-Env vaccine group than in the Δ5G Env vaccine group. Although the expression of SU by expressing plasmids and that of Env by the vaccinia virus vector elicited by either the wt-Env vaccine or Δ5G Env vaccine were indistinguishable in cultured cells (Fig. 2), wt-Env might persist longer than Δ5G Env in vaccinated animals. T-cell epitopes in the wt-Env vaccine might therefore be more efficiently presented on MHC molecules in antigen-presenting cells than in the Δ5G Env vaccine. Differences in glycosylation levels might also affect some processes in antigen-presenting cells associated with the presentation of T-cell epitopes in Env.

Taking all results together, Env glycosylation might affect the presentation of B-cell epitopes and T-cell epitopes required for Ab-mediated and T-cell-mediated immunities related to the control of SIV infection.

As seen in viral loads and SU-specific T cell levels after challenge infection (Fig. 3 and 5), the effect of vaccination was limited. That seemed related to the development of escape mutants. Therefore, distinctive cellular immune responses after the challenge infection were also implicated in the control of SIVmac239 replication. The magnitude of virus-specific $CD8^+$ T cells did not always correlate with the suppression of viral replication as reported previously (1, 6), particularly in vector controls (Fig. 5 and 7A); however, selected epitope-specific CTL responses might be associated with infection control. Gag-specific CTLs are such candidates, because a high magnitude of Gag-specific $CD8^+$ T cells was significantly elicited in five control animals (Fig. 7C). The magnitude of Gag- or Tat/Rev-specific $CD4^+$ T cells was statistically correlated with infection control (Fig. 7C). This may simply indicate a lower depletion of virus-specific $CD4^+$ T cells in animals with lower viral loads as reported previously (11). Alternatively, these virus-specific $CD4^+$ T cells may play an important role in protective immunity (39). Taken together, these results implicated the dominant role of selected epitope-specific $CD4^+$ T cells and $CD8^+$ T cells for the control of SIVmac239 infection.

The challenge virus that should be used has been an important issue in AIDS vaccine studies (8, 10, 12). Many studies have reported impressive efficacy in a pathogenic-SHIV macaque model (3, 4, 45, 46); however, pathogenic SHIVs use CXCR4 as a coreceptor, whereas the majority of clinical isolates of HIV-1 use CCR5 (13, 27). Therefore, the challenge virus for an AIDS vaccine study should be an R5 virus, such as SIV (10). Consistent with this concern, a DNA prime–modified-vaccinia virus Ankara boost regimen, inducing broad SIV-specific T-cell responses, reduced the initial viral replication but did not prevent disease progression against SIVmac239 challenge (18). Thus, vaccine studies using pathogenic SHIV should be reevaluated by using an R5 virus (10).

Matano et al. reported that a DNA prime-Sendai virus boost regimen induced the CTL-based control of SIVmac239 in rhesus macaques (27). This study demonstrated that a DNA prime-vaccinia virus WR boost regimen expressing only Env controlled the chronic infection of SIVmac239 in rhesus macaques. The relatively lower viral loads in macaques from Myanmar or Laos than in those of Indian origin might contribute to the control of SIVmac239 infection. Nevertheless, it is important that these two studies demonstrated the efficacies of the two vaccine regimens against highly pathogenic SIVmac239. In earlier studies, other R5 SIVs were used as a challenge virus for an efficacy study of vaccine candidates. An Env-based vaccine in vaccinia virus vector priming and subunit protein boosting protected cynomologous macaques against homologous SIVmne clone E11S (42). In recombinant modified vaccinia virus, Ankara viruses expressing Gag-Pol and/or Env exhibited vaccine efficacy because of reduced viremia and the increased survival of rhesus macaques infected with uncloned SIVsmE660 (41). Accordingly, the efficacy of vaccine candidates might be influenced by the experimental conditions. Thus, well-defined animal models with detailed virological, immunological, and genetic information and suitable challenge viruses are required for the evaluation of vaccine candidates and the development of an AIDS vaccine.

This study demonstrated the importance of Env as a component of the AIDS vaccine, and Env-specific $CD8^+$ and $CD4^+$ T cells and nonneutralizing Env-specific Ab were suggested as protective immunity components. Quintuple deglycosylation in Env reduced vaccine efficacy and Env-specific immune responses. Env may therefore be comprised of appropriate antigenic properties to elicit humoral and cellular immune responses required for protective immunity against homologous or allele-specific target SIV/HIV. These properties could be modified by the alteration of glycosylation.

In conclusion, although Env is an important immunogen for the AIDS vaccine, Env properties, including glycosylation, should be carefully considered to design vaccines specific to the targeted viruses.

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Role of the Specific Amino Acid Sequence of the Membrane-Spanning Domain of Human Immunodeficiency Virus Type 1 in Membrane Fusion

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Fusion between cell and virus membranes mediated by gp41 initiates the life cycle of human immunodeficiency virus type 1. In contrast to the many studies that have elucidated the structure-function relationship of the ectodomain, the study of the membrane-spanning domain (MSD) has been rather limited. In particular, the role that the MSD's specific amino acid sequences may have in membrane fusion as well as other gp41 functions is not well understood. The MSD of gp41 contains well-conserved glycine residues that form the GXXXG motif (G, glycine; X, other amino acid residues), a motif often found at the helix-helix interface of membrane spanning α-helices. Here we examined the role that the specific amino acid sequence of the gp41 MSD has in gp41 function, particularly in membrane fusion, by making two types of MSD mutants: (i) glycine substitution mutants in which glycine residues of the MSD were mutated to alanine or leucine residues, and (ii) replacement mutants in which the entire MSD was replaced with one derived from glycophorin A or from vesicular stomatitis virus G. The substitution of glycines did not affect gp41 function. MSD-replacement mutants, however, showed severely impaired fusion activity. The assay using the Env expression vector revealed defects in membrane fusion after CD4 binding steps in the MSD-replacement mutants. In addition, the change in Env processing was noted for MSD-replacement mutants. These results suggest that the MSD of gp41 has a relatively wide but not unlimited tolerance for mutations and plays a critical role in membrane fusion as well as in other steps of Env biogenesis.

The envelope glycoprotein (Env) of human immunodeficiency virus type 1 (HIV-1) plays a critical role in the entry process in the viral life cycle. Env is synthesized as a precursor, gp160, and then processed into a heterodimer consisting of gp120 and gp41. Interaction of gp120 with CD4 and chemokine receptors triggers the membrane fusion process.

The gp41 is believed to play a central role in the fusion process during postreceptor binding. It is divided into three subunits or domains: the extracellular, membrane-spanning, and cytoplasmic domains. The contribution of the extracellular domain to membrane fusion has been well documented. It contains conserved heptad repeats preceded by the fusion peptide and is thought to undergo conformational changes during membrane fusion to form a trimeric coiled-coil, commonly observed in the envelope proteins of viruses such as the influenza virus, the Moloney murine leukemia virus, and the Ebola virus (4–6, 11, 20, 37, 43, 44).

The cytoplasmic domain is relatively long compared with those of other simple animal retroviral envelope proteins and bears two well-conserved amphipathic helices called LLP1 and LLP2 (10, 25, 40). The cytoplasmic domain is important to intracellular trafficking as well as to the efficient incorporation of Env onto the budding viral particle (9, 32, 46). The cytoplasmic domain may affect fusion activity of Env (13, 27).

The membrane-spanning domain (MSD) of gp41 anchors Env on the lipid bilayer, and its amino acid sequences are highly conserved among independent isolates of HIV-1. Several studies have indicated that the MSD is involved in membrane fusion—the glycosylphosphatidylinositol-anchored Env of HIV-1, which lacks the MSD and the cytoplasmic domain, could not induce syncytia (35, 42). However, whether specific amino acid sequences in the MSD are required for its function in HIV-1 replication remains controversial. For example, the previous study showed that substituting a leucine residue for the conserved arginine residue within the MSD resulted in a replication-incompetent virus (31). Substituting isoleucine for the same arginine residue, however, did not affect gp41 function (42). Furthermore, fusion activity was retained in a mutant in which the entire MSD and cytoplasmic domain were replaced with those of CD4 (41). Wilk et al. have reported a replication-competent recombinant HIV-1 in which a CD22 MSD replaced the gp41 MSD (45). Because there is no apparent sequence homology among the MSDs of gp41, CD4, and CD22, these results suggest that gp41 function may not require a specific sequence in the MSD or, alternatively, that some as-yet-undetermined characteristic of CD4 and CD22 MSDs might compensate for the naturally occurring sequence. One feature common to the MSDs of gp41, CD4, and CD22 is the presence of several glycine residues (Fig. 1A). A glycine residue is not a rarity in the MSDs of membrane proteins (8, 39).

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A

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                    GXXXG
          HXB2:   yikLFIMIVGGLVGLRIVFAVLSIVnrv
CONSENSUS_A-A1:   yikIFIMIVGGLIGLRIVFAVLSIInrv
   CONSENSUS_B:   yikIFIMIVGGLVGLRIVFAVLSIVnrv
HIV-1 CONSENSUS_C:   yikIFIMIVGGLIGLRIIFAVLSIVnrv
   CONSENSUS_D:   yikIFIMIVGGLIGLRIVFAVLSLVnrv
   CONSENSUS_O:   yikIAIIIVGALIGVRVVMIVLNLVrni
          GpA:   epeITLIIFGVMAGVIGTILLISYGIrrl
        VSV-G: kssIASFFFIIGLIIGLFLVLRVGIHLCIklk
        HCVE1:       gahWGVLAGIAYFSMVGNWAKVLVVLLLFAGVdae

         CD22:   grrVAVGLGSCLAILILAICGLklq
          CD4:   vqpMALIVLGGVAGLLLFIGLGIFFCVrcr
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B

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Glycine substitution mutants
     WT: yikLFIMIVGGLVGLRIVFAVLSIVnrv
   690A: yikLFIMIVAGLVGLRIVFAVLSIVnrv
   690L: yikLFIMIVLGLVGLRIVFAVLSIVnrv
   691A: yikLFIMIVGALVGLRIVFAVLSIVnrv
   691L: yikLFIMIVGLLVGLRIVFAVLSIVnrv
   694A: yikLFIMIVGGLVALRIVFAVLSIVnrv
   694L: yikLFIMIVGGLVLLRIVFAVLSIVnrv
     3A: yikLFIMIVAALVALRIVFAVLSIVnrv

MSD replacement mutants
  MSD GpA: yikITLIIFGVMAGVIGTILLISYGIrv
MSD GpAG83I: yikITLIIFGVMAIVIGTILLISYGIrv
  MSD VSV-G: yikSSIASFFFIIGLIIGLFLVLRVGIHLCIrv
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FIG. 1. Amino acid sequences of the MSD of HIV-1 isolates and several membrane proteins. (A) Amino acid sequences of the predicted MSD of HXB2, HIV-1 isolates, and several membrane proteins are shown. The sequence of HIV-1 isolates was according to HIV Sequence Compendium 2001 (17). The MSDs of CD4 and CD22 that could replace the MSD of gp41 without affecting gp41's function are also shown (41, 45). The capital letters indicate the amino acid sequences within the predicted MSD. The small letters indicate the amino acid sequences surrounding the MSD. Glycine residues in the GXXXG motif are underlined. (B) Amino acid sequences of MSD mutants. The mutated portions are underlined.

With respect to several viral membrane proteins, glycine residues are more often found in the MSDs of envelope proteins than in the MSDs of nonenvelope proteins (8). Although the importance of glycine residues in Env-mediated membrane fusion has been studied with viruses such as vesicular stomatitis virus (VSV) and the influenza virus (8, 23), the role of glycine residues in the MSD of gp41 has not been investigated.

In the gp41 MSD of HIV-1, the glycine residues form the GXXXG motif (G, glycine; X, other amino acid residues). This motif is often observed in transmembrane α-helices and is believed to stabilize helix-helix interactions of membrane proteins (12, 34). In the case of glycophorin A (GpA) MSD, this motif is critical for homodimerization (26). A recent study showed that the transmembrane domains of hepatitis C virus E1 and E2 envelope glycoproteins are required in heterodimerization and that E1 also has the GXXXG motif in its MSD (29). The above-mentioned CD4 also contains the GXXXG motif. Thus, the maintenance of fusion activity in gp41 mutants having MSDs that were replaced with those of CD4 or CD22 might depend on the presence of glycine residues in the CD4 and CD22 MSDs (41, 45). To investigate whether the specific amino acid sequence in the MSD of gp41 is required for fusion activity, we mutated glycine residues or replaced the entire MSD of gp41 with heterologous MSDs and analyzed the effect that these mutations had on gp41 function. We found that the latter heterologous replacement enhanced the processing of gp160. The fusion activity of gp41 was severely impaired. Our analysis of the membrane fusion process of these mutants revealed that the defects are manifested in the postreceptor binding steps preceding lipid mixing and possibly in the steps after the initial pore formation.

MATERIALS AND METHODS

Construction of plasmids. The 1.2-kb NheI-BamHI fragment covering the *env* portion of the modified HXB2, HXB2RU3ΔN (*vpr*$^+$, *vpu*$^+$, *nef*$^+$, and one NheI site within the vector were deleted), was subcloned into pGEM7zf(+) (Promega, Madison, Wis.) as a target for mutagenesis. To generate each glycine substitution (Gly^{sub}) mutant, site-directed mutagenesis was performed using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, Calif.) with complementary oligonucleotide pairs (for 690A, CATAATGATAGTAGCAGGCTTGGT AGGT and ACCTACCAAGCCTGCTACTATCATTATG; for 690L, CATAA TGATAGTACTAGGCTTGGTAGGT and ACCTACCAAGCCTAGTACTAT CATTATG; for 691A, ATGATAGTAGGAGCCTTGGTAGGTTTA and TAA ACCTACCAAGGCTCCTACTATCAT; for 691L, ATGATAGTAGGACTCT TGGTAGGTTTA and TAAACCTACCAAGAGTCCTACTATCAT; for 694A, AGGAGGCTTGGTAGCTTTAAGAATAGTTTTTG and CAAAAACTATT CTTAAAGCTACCAAGCCTCCT; and for 694L, AGGAGGCTTGGTACTTT TAAGAATAGTTTTTG and CAAAAACTATTCTTAAAAGTACCAAGCCT CCT), generating the substitution of an alanine or a leucine residue for a glycine residue. The PCR was performed using Pfu turbo (Stratagene). The 3A mutant was created by site-directed mutagenesis using a complementary oligonucleotide pair (CATAATGATAGTAGCCGCCTTGGTAGCCTTAAGAATAGTTTTTG and CAAAAACTATTCTTAAGGCTACCAAGGCGGCTACTATCATTATG).

To generate the MSD-replacement mutants, megaprimers that were produced by PCR that targeted the MSD portion of VSV-G or GpA as a template were used as mutagenesis oligonucleotides (for MSD GpA, CAAATTGGCTGTGG TATATAAAAATCACCCTGATCATC and GAATATCCCTGCCTAACTCT GATGCCGTAGCTGAT; for MSD VSV-G, CAAATTGGCTGTGGTATATA AAAAGCTCTATTGCCTC and GAATATCCCTGCCTAACTCTAATGCAA AGATGGATAC). MSD GpAG83I was produced by site-directed mutagenesis using a subclone of MSD GpA as a template with a primer set (CATCTTCGG CGTGATGGCCATCGTGATCGGCACCATCCTG and CAGGATGGTGCC GATCACGATGGCCATCACGCCGAAGATG). Following mutagenesis, the 1.2-kb NheI-BamHI fragments were sequenced and cloned back into the pSP65HXB2RU3ΔN plasmid. After the cloning back, the entire NheI-BamHI portion together with the junction was verified by sequencing.

An Env expression vector, pElucEnv, was used to express *env* genes in this study. Although the details of its construction will be described elsewhere, it is a derivative of pSP65HXB2RU3ΔN and lacks the *gag* and *pol* portions of the provirus. The *rev* function was provided by *rev* cDNA cloned into the *nef* region. The original *rev* gene was inactivated by mutating its initiation codon. A BsiWI site was introduced near the initiation codon of the original *rev* gene by this mutagenesis. Because this vector contains an enhanced green fluorescent protein (EGFP)-firefly luciferase expression module outside the provirus, the transfected cells fluoresce green, allowing us to estimate transfection efficiency by measuring firefly luciferase activity. To generate each Env expression vector, the NheI-BamHI fragment of the pSP65HXB2RU3ΔN wild type (WT), MSD GpA, MSD GpAG83I, or MSD VSV-G was cloned into pElucEnv. As a negative control, pElucEnv EnvKO was produced. The *env* gene of pElucEnv EnvKO has a stop codon after its 25th codon. The 2.7-kb SalI-BamHI fragment covering the *env* portion of the pElucEnv WT was subcloned into pGEM3zf(+) (Promega) as a target for the mutagenesis. The site-directed mutagenesis used to create the stop codon was performed with a specific primer set (CTCCTTGGGATGTTGTAG ATCTGTAGTCTACA and TGTAGCACTACAGATCTACAACATCCCAAG GAG). The BsiWI-BamHI fragment of the subclone was sequenced and cloned back into pElucEnv WT.

A reporter vector, pTM3hRL, which has a reporter gene, renilla luciferase, under the control of the T7 promoter, was generated from the pTM3luci vector (1) by replacing the firefly luciferase gene with the humanized renilla luciferase gene. The renilla luciferase gene was amplified from phRL-CMV vector (Promega) by PCR with a primer set (CGACTCACTATAGGCTAGCC and GCT CGAGGCGGCCGCTCTAGAATTAC), cloned into pCR4Blunt-TOPO (Invitrogen, Carlsbad, Calif.), and sequenced before cloning. To generate the T7 RNA-polymerase (T7 RNApol) expression vector, pCMMP T7RNApoliresGFP, the gene encoding T7 RNA polymerase, was PCR amplified from pVR-T7-1 (1)