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Specific N-Linked and O-Linked Glycosylation Modifications in the Envelope V1 Domain of Simian Immunodeficiency Virus Variants That Evolve in the Host Alter Recognition by Neutralizing Antibodies

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During progression to AIDS in simian immunodeficiency virus (SIV) Mne-infected macaques, viral variants are selected that encode sequences with serine and threonine changes in variable region 1 (V1) of the surface component of the viral envelope protein (Env-SU). Because these serine and threonine amino acid changes are characteristic of sites for O-linked and N-linked glycosylation, we examined whether they were targets for modification by carbohydrates. For this purpose, we used several biochemical methods for analyzing the Env-SU protein encoded by chimeras of SIVMneCL8 and envelope sequences cloned from an SIVMneCL8-infected *Macaca nemestrina* during clinical latency and just after the onset of AIDS. The addition of an N-linked glycan was demonstrated by changes in the electrophoretic mobility of Env-SU, and this was verified by specific glycanase digestions and a detailed analysis of the molecular mass of partially purified Env-SU by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). Molecular mass calculations by MALDI-TOF MS also demonstrated an increased mass, from 102.3 to 103.5 kDa, associated with serine and threonine residues predicted to be O-linked glycosylation sites. Together, these data provide the first direct evidence that the carbohydrate profile of Env-SU is distinct in SIV variants that evolve during infection of the host. Moreover, our studies show that these changes in glycosylation in V1 were directly associated with changes in antigenicity. Specifically, serine and threonine changes in V1 allowed the virus to escape neutralization by macaque sera that contained antibodies that could neutralize the parental virus, SIVMneCL8. The escape from antibody recognition appeared to be influenced by either O-linked or N-linked carbohydrate additions in V1. Moreover, when glycine residues were engineered at the positions where serine and threonine changes evolve in V1 of SIVMneCL8, there was no change in antigenicity compared to SIVMneCL8. This suggests that the amino acids in V1 are not part of the linear epitope recognized by neutralizing antibody. More likely, V1-associated carbohydrates mask the major neutralizing epitope of SIV. These experiments indicate that the selection of novel glycosylation sites in the V1 region of envelope during the course of disease is driven by humoral immune responses.

The lentiviral envelope protein is extensively glycosylated. The surface component (Env-SU) of human immunodeficiency virus type 1 (HIV-1) envelope protein contains over 20 sites that are glycosylated by N-linked carbohydrates (13) and up to 8 sites that are modified by O-linked carbohydrates (4). While N-linked carbohydrates have been suggested to play a role in many of the functions associated with the viral envelope protein, including receptor binding (23, 25), viral assembly (16), syncytium formation (17), and antibody recognition (11, 17), the functional significance of individual glycosylation sites is less clear. The role of O-linked carbohydrates in the functions of HIV-1 Env-SU has yet to be elucidated.

Like HIV-1, the simian immunodeficiency virus (SIV) envelope protein is highly glycosylated, but the functional significance of these modifications is even less understood. Our studies of SIV envelope variation over the course of disease in five *Macaca nemestrina* infected with a clone of SIVMne demonstrated strong selection for variants encoding serine and threonine residues that created motifs that we have hypothesized present novel targets for O-linked and N-linked glycosylation in the V1 variable domain of Env-SU (27, 28). These potential glycosylation sites evolved in viral variants isolated from a number of cell and tissue types, including peripheral blood mononuclear cells, liver, lymph node, and spleen (10). Once selected, viruses encoding these glycosylation motifs were maintained throughout the course of disease and persisted when transmitted to a naive macaque (10, 27, 28). Similarly, selection for viruses with amino acid changes that are predicted to create N-linked glycosylation sites is frequently observed in SIV and other lentiviral infections, but there are, as yet, no data demonstrating that carbohydrate modifications are associated with these sequence changes.

Elsewhere, we showed that chimeras encoding V1 sequences from variants that evolve in SIVMne-infected macaques were not efficiently neutralized by sera that recognized the infecting, parental virus (30). In light of the observation of serine and threonine changes in V1, these data suggested that the glycosylation modifications in V1 may play a role in allowing SIVMne viral variants to escape host neutralizing antibody. Here, we conducted a biochemical analysis of potential glycosylation sites that evolve in V1 during progression to AIDS and demonstrated that the new serine and threonine residues are modified by N-linked and O-linked carbohydrates. These gly-

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cosylation sites are sufficient to allow variant viruses to escape host neutralizing antibody recognition. In addition, we provide evidence that glycosylation induces structural changes in the envelope that mask the major SIV neutralizing epitope.

MATERIALS AND METHODS

Cell lines and viruses. CEMx174 cells (a cell line derived from a fusion of a human T-cell line [CEM] and a human B-cell line [721.174]) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 Units of penicillin per ml, 100 μg of streptomycin per ml, and 1mM glutamine. The human 293T cell line was propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 300 μg of glutamine per ml (complete DMEM). CMMT-CD4-LTR-β-*gal* (sMAGI) indicator cells (9) were grown in complete DMEM plus 0.2 mg of G418 per ml and 50 U of hygromycin (Calbiochem, La Jolla, Calif.) per ml.

Virus was generated by transient transfection of the human 293T cell line with viral plasmid clones by using a transfection kit (Stratagene, La Jolla, Calif.). High-titer virus stocks were made by infecting CEMx174 cells with virus harvested from the 293T cells 2 days after transfection. Virus production was monitored by an assay specific for $p27^{gag}$ antigen (Immunotech, Westbrook, Maine). Viral stocks were generated at times of peak antigen production, typically 2 weeks after infection. Cell-free virus was harvested from the cultures by low-speed centrifugation for 5 min to remove the majority of cells, followed by filtration through a 0.22-μm-pore-size filter. The infectious dose of each viral supernatant was determined by sMAGI assay, as described previously (9).

Cloning of SIV envelope chimera. The CL8-35wkSU and CL8-81wkSU envelope chimeras were described previously (30). These SIVMneCL8-based chimeras contain a 1,153-bp *Nsi*I-*Cla*I fragment (nucleotides 6393 to 7546 of the SIVMneCL8 sequence), which includes sequences encoding the variable regions V1 through V5 of Env-SU derived from variant provirus sequences. CL8-35wkSU encodes the Env-SU of a variant present at 35 weeks postinfection in macaque M87004, and CL8-81wkSU encodes the Env-SU of a variant present at 81 weeks postinfection from the same animal (30). In each case, the Env-SU sequence was inserted into a background of SIVMneCL8.

A series of additional V1 mutants (shown in Fig. 1B) were generated by overlap extension PCR mutagenesis by a method adapted from Ho et al. (19). To create pCL8(35wk-N), we used pCL8-35wkSU as a template in two separate reactions to generate overlapping fragments in which sequence coding for serine-150 was altered to code for a proline residue. The oligonucleotides used as primers and, in brackets, their positions of homology to SIVMneCL8 sequences (non-SIV sequences are shown in italics and positions that represent variation from the template sequence are underlined) are SIV-env1 (5′-*GGTACC*CTCTTTGAGACCTCAATAAA[6357 to 6376]) and SIV-envV1-A (5′-CAAGGACTAATCTCATTGACC [6524 to 6503]) (round 1A) and SIV-envV1-B (5′-ATGAGATTAGTCCTTGTATAA [6508 to 6529]) and SIV-env8 (5′-*ATAGAATTC*CCAATTGGAGTGATCTCTAC [7594 to 7575]) (round 1B). pCL8(81wk-N) was created with the same primers as above except pCL8-81wkSU was used as a template.

To generate pCL8+N, a virus coding for a new N-linked glycosylation site in V1, pSIVMneCL8 was used as a template for two separate reactions with the primer pairs SIV-env1 and SIV-envV1-C (5′-ACAAGGACTACTCTCATTGACCAC [6524 to 6501]) (round 1A) and SIV-envV1-D (5′-TCAATGAGAGTAGTCCTTGTATAA [6505 to 6528]) and SIV-env7 (round 1B). To generate pCL8-V1(G), pSIVMneCL8 was used as a template for two separate reactions with the primer pairs SIV-env1 and SIV-envV1-E (5′-CCTGTTGGTCCTCCTGTTGGTGCTGTTGTT [6484 to 6455]) (round 1A) and SIV-envV1-F (5′-ACAGGAGGACCAACAGGAGCAGAGGCAATAAAA [6468 to 6500]) and SIV-env7 (round 1B). In all cases, the overlapping fragments from round 1A and round 1B reactions were combined and used as a template for amplification by PCR (round 2) with the primers SIV-env4 (5′-*CTTAAG*TCAAGACGGTGGAGACGTGA [7543 to 7534]) and SIV-env7 (5′-*GACGCTACC*TAAAGCCTTGTGTAAAATTA [6364 to 6383]).

Reaction mixtures contained 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin, 0.4 mM (each) deoxynucleoside triphosphate, 250 ng of each oligonucleotide primer, and 0.5 U of *Taq* polymerase (Perkin-Elmer) in a total volume of 25 μl. Amplification conditions were as follows: for round 1, denaturation (4 min at 94°C) followed by 35 cycles of denaturation (1 min at 94°C), annealing (1 min at 50°C, with the exception of reactions with primers SIV-env7 and SIV-envV1-D, in which annealing was performed for 1 min at 45°C), and extension (2 min at 72°C, with the exception of reactions with primers SIV-env7 and SIV-envV1-F, in which extension was performed for 3 min at 72°C) and for round 2, denaturation (4 min at 94°C) followed by 35 cycles of denaturation (1 min at 94°C), annealing (1 min at 45°C), and extension [2 min at 72°C, with the exception of the second round reaction used to generate pCL8-V1(G), in which extension was for 3 min at 72°C].

Following PCR amplification, mutant envelope sequences were cloned into an M13mp18 vector as described previously (28, 31). Correct clones were verified by sequence analysis of the V1 region. A 244-bp *Nsi*I-*Pst*I fragment (nucleotides 6393 to 6637), which includes the V1 variable region of the envelope, was cloned back into SIVMneCL8 in a three-step cloning protocol. First, a subclone of SIVMneCL8 (p3′CL8 K/N), which spans nucleotides 5239 (a *Kpn*I site) to 8216 (an *Nhe*I site), was digested with *Cla*I and partially digested with *Nsi*I, and a 5.2-kb fragment, which was predicted to have the *Nsi*I cloning site at nucleotide 6392 and the *Cla*I cloning site at nucleotide 7546, was gel purified by standard methods. The M13mp18 clones with PCR-generated mutant envelope genes were also digested with *Nsi*I and *Cla*I, and a 1.1-kb fragment was gel purified. This fragment was ligated into the 5.2-kb *Nsi*I-*Cla*I-digested p3′CL8 K/N. Second, the chimeric *Kpn*I subclone(s) was digested with *Eco*RI and *Pst*I and the 1.4-kb fragment, which included sequences from nucleotides 5239 to 6637, was gel purified. p3′CL8 K/N was digested with *Eco*RI and *Pst*I, and a 4.9-kb fragment, which was predicted to have a unique *Eco*RI cloning site in the multiple cloning site of pUC (5′ of the *Kpn*I site at nucleotide 5239) and a unique *Pst*I cloning site at nucleotide 6637, was gel purified and ligated with the 1.4-kb *Eco*RI-*Pst*I-digested fragment from the chimeric subclone. The full-length viral genome was reconstructed with a unique *Bst*BI site 5′ of the envelope coding region (nucleotide 5343 of SIVMneCL8) and the *Cla*I site at the end of the Env-SU coding sequence (nucleotide 7546). The *Bst*BI-*Cla*I fragment (2.2 kb) was gel purified from the second set of chimeric subclones and ligated to a similarly digested, gel-isolated 14-kb fragment representing the plasmid encoding the entire SIVMneCL8 provirus minus its envelope gene. All clones were verified by restriction site analysis, and the final full-length clones were verified by nucleotide sequence analysis of the envelope gene region spanning the 244-bp *Nsi*I-*Pst*I fragment (nucleotides 6393 to 6637).

SIV envelope immunoprecipitation. CEMx174 cells were infected with high-titer SIV stocks (generated in CEMx174 cells) at a multiplicity of infection of 0.01. During the period of high viral antigen ($p27^{gag}$) production, typically 2 weeks after infection, approximately 5×10^5 SIV-infected CEMx174 cells were cultured for 30 min in methionine (Met)- and cysteine (Cys)-deficient RPMI medium (GIBCO, Gaithersburg, Md.) and then metabolically labeled with 200 μCi of [^{35}S]Met and [^{35}S]Cys (Amersham, Arlington Heights, Ill.) in Met- and Cys-deficient RPMI medium for 3 to 6 h. Cells were lysed in 200 μl of a buffer containing 10 mM Tris (pH 7.5), 50 M KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1% Triton X-100, 1 U of aprotinin per ml, and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). Lysates were precleared with 50 μl of protein A-Sepharose 4B beads (Zymed, South San Francisco, Calif.) overnight with rocking at 4°C. Incorporated radioactivity was determined by precipitation with trichloroacetic acid (TCA). An equal number of TCA-precipitable counts was added to each immunoprecipitation reaction mixture. SIV envelope protein was immunoprecipitated at 4°C in 500 μl of buffer 1 (20 mM Tris [pH 7.5], 50 mM KCl, 400 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM DTT, 20% glycerol, 0.1 U of aprotinin per ml, 0.2 mM PMSF) by incubating a fraction of each lysate sample (typically 10^6 TCA-precipitable counts) with 3 μl of serum from an SIVMne-infected *M. nemestrina* for 3 h and then adding 50 μl of protein A-Sepharose 4B beads and incubating for at least 3 additional h. The immunoprecipitated protein-bead complex was washed four times with buffer 1 and three times with buffer 2 (10 mM Tris [pH 7.5], 100 mM KCl, 0.1 mM EDTA, 20% glycerol, 0.1 U of aprotinin per ml) prior to further manipulation. Glycanase-digested (see below), mock-digested, or undigested immunoprecipitated proteins were suspended in sample loading buffer (60 mM Tris [pH 6.8], 2% sodium dodecyl sulfate [SDS], 10% glycerol, 100 mM DTT, 0.001% bromophenol blue), boiled for 3 min, and then subjected to SDS-polyacrylamide gel electrophoresis (PAGE).

Glycanase digestions. For *N*-glycanase digestions, immunoprecipitated proteins were dissociated from Sepharose 4B beads by boiling the samples in 25 to 100 μl of a buffer containing 20 mM sodium phosphate (pH 8.0), 0.1% SDS, and 50 mM β-mercaptoethanol for 2 min. N-linked carbohydrates were removed with 5 to 20 U of *N*-glycanase (Genzyme, Cambridge, Mass.) per ml in the presence of 0.75% Triton X-100 for 16 to 20 h at 37°C. Control and mock-digested samples were incubated under identical conditions except in the absence of enzyme.

For neuraminidase and *O*-glycanase digestions, immunoprecipitated protein-bead complexes were suspended in 25 to 100 μl of a buffer containing 20 mM sodium phosphate (pH 6.0), 0.1% SDS, and 1 mM calcium acetate. Samples were boiled for 5 min, Triton X-100 and neuraminidase (Genzyme) were added to a final concentration of 0.75% and 200 to 500 mU per ml, respectivley, and the sample was incubated at 37°C for 1 h. *O*-Glycanase digestion was performed by treating the neuraminidase-digested proteins with 40 to 80 mU of *O*-glycanase (Genzyme) per ml for 16 to 20 h at 37°C.

Some immunoprecipitated proteins were digested with *N*-glycanase, neuraminidase, and *O*-glycanase. For these studies, immunoprecipitated proteins were resuspended in 25 to 100 μl of a buffer containing 20 mM sodium phosphate (pH 7.0) with 0.1% SDS, boiled for 5 min, Triton X-100 was added to achieve a concentration of 0.75%, and the sample was then treated with 5 to 20 U of *N*-glycanase per ml for 16 to 20 h at 37°C. Samples were then treated with 200 to 500 mU of neuraminidase per ml for 1 h at 37°C, followed by treatment with 40 to 80 mU of *O*-glycanase per ml for 16 to 20 h at 37°C. Digested and mock-digested immunoprecipitated proteins were suspended in sample loading buffer (60 mM Tris [pH 6.8], 2% SDS, 10% glycerol, 100 mM DTT, 0.001% bromophenol blue) and then subjected to SDS-PAGE.

SIV envelope purification. SIV Env-SU was partially purified by a modification of a protocol described by Gilljam (14). CEMx174 cells were infected with high-titer SIV stocks at a multiplicity of infection of 0.1. To allow virus spread,

infected cells were passaged and expanded for at least 2 weeks, and viral antigen production was monitored by $p27^{gag}$ antigen production. During a period of high viral antigen expression, up to 200 ml of infected CEMx174 cultures were clarified by centrifugation at 7,000 × *g* for 30 min at 4°C. Supernatants were treated with 0.25% Empigen BB (Calbiochem) to remove the envelope glycoprotein, and spun at 24,000 × *g* overnight at 4°C to pellet viral cores. SIV Env-SU was partially purified with a *Galanthus nivalis* GNA lectin (Sigma, St. Louis, Mo.) affinity column. The column matrix was prepared by coupling approximately 2 ml of CNBr-activated Sepharose 4B beads with 2 to 10 mg of GNA lectin in the presence of 0.1 M $NaHCO_3$ (pH 8.3)–0.5 M NaCl overnight at 4°C with rocking. Approximately 3 ml of this matrix was used for each lectin affinity column, and separate columns were used for the purification of different variant proteins. Columns were equilibrated in 0.25% Empigen-BB in 50 mM Tris (pH 8.0) prior to loading the sample. The sample was applied to the column, and then the column was washed with 10 column volumes of 0.25% Empigen-BB in 50 mM Tris (pH 8.0), 50 mM Tris (pH 8.0), 1 M NaCl in 50 mM Tris (pH 8.0), and 50 mM Tris (pH 8.0) in succession. Bound proteins were eluted in 1-ml fractions with 0.5 M methyl-α-D-mannopyraniside (Calbiochem, La Jolla, Calif.)–0.1 U-ml aprotinin–0.2 mM PMSF in 50 mM Tris (pH 8.0); approximately 15 fractions were collected. Positive Env-SU fractions were identified by Coomassie-stained SDS-PAGE and Western blotting (see below) and then were pooled and concentrated with a Centricon-50 spin column (Amicon, Beverly, Mass.). Total protein concentration was determined by Bradford assay with reagents and methods supplied by Pierce (Rockford, Ill.). The concentration of Env-SU in the concentrated fractions was estimated by Western slot blot analysis (see below) with purified SIVmac251 Env-SU as a standard.

Western immunoblot analysis. Protein fractions were electrophoresed on an SDS-7.5% polyacrylamide gel and then electroblotted onto an Immobilon-P membrane (Millipore Corp., Bedford, Mass.). Also, concentrated protein was slot blotted onto nitrocellulose by using a Schleicher and Schuell minifold II slot-blot system (Keene, N.H.). Western and slot blot analysis and enhanced chemiluminescence detection (Amersham) were performed according to the manufacturers' instructions. The primary incubation was performed with a $1:10^4$ dilution of purified immunoglobulin G from a macaque infected with SIVMne in Tris-buffered saline plus 0.5% Tween 20 (T-TBS). The secondary incubation was performed with a 1:5,000 dilution of horseradish peroxidase-conjugated monoclonal goat anti-monkey immunoglobulin G antibody (Nordic Immunology, Capistrano Beach, Calif.) in T-TBS. All washes were in T-TBS at room temperature.

MALDI-TOF MS. The molecular masses of proteins were determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) by using a Voyager Elite Biospectrometry Workstation (PerSeptive Biosystems, Framington, Mass.). Samples were prepared for MALDI-MS analysis as follows: 0.5 μl of matrix solution 1 (0.13 M 2,5-dihydroxybenzoic acid [DHB] and 6 mM 5-methoxysalicylic acid [MSA] in 50% acetone and 50% isopropanol) was applied to the sample stage and allowed to air dry until visible crystals were formed. One microliter of analyte (3 μg of purified SIVmac251 Env-SU or 1.5 to 6.6 μg of partially purified SIVMneCL8 and variant Env-SU) was spotted onto the dried matrix crystals and allowed to air dry. The dried sample was washed with 10 μl of 0.1% trifluoroacetic acid (TFA). One microliter of matrix solution 2 (5 mM DHB, 2.5 mM MSA in 30% acetonitrile, 0.1% TFA) was applied to the sample and allowed to air dry.

Positive-ion mass spectra were acquired with a delayed extraction system (described in reference 33) in reflectron mode. The following instrument parameters were used: 25-kV source potential, 23.8-kV extraction grid potential, and −75-V guide wire potential. Spectra represent the summed average of 50 to 128 laser pulses. Calibration of the mass scale was accomplished with bovine serum albumin (Boehringer Mannheim, Indianapolis, Ind.). The average mass/charge (*m/z*) values of the peaks were determined with the Voyager Elite software package (PerSeptive Biosystems).

Neutralization assays. Serum samples were obtained from four *M. nemestrina*; two of the animals (animals 90027 and 92128) were infected with SIVMneE11S (3), a biological clone from which the molecular clone SIVMneCL8 was derived, and two of the animals (93100 and 92221) were infected with SIVMne (2), the uncloned mixture from which SIVMneE11S was derived. Serum samples were obtained both prior to infection (prebleed) and 5 to 6 months postinoculation. All serum samples were heat inactivated for 30 min at 56°C. sMAGI neutralization assays were performed as described previously (9), with the following modification: cells were added to a 24-well plate 24 h prior to infection at a density of 2.5×10^4 cells per well. Neutralization was scored by comparing the average number of blue infectious centers in the presence of test serum (V_n) with the average number of infectious centers in the absence of serum (V_o). Typically, 100 to 300 infectious virions were used in each infection. Dilutions were plotted against V_n/V_o, and the dilution that reduced V_n to 10% of V_o ($V_n/V_o = 0.1$) was scored as the neutralizing titer, as described by Nara et al. (26). As a control, prebleed serum from all four animals was also tested against each virus.

RESULTS

Carbohydrate analysis of SIV envelope chimera. Glycosylation of SIV Env-SU was analyzed with two chimeras encoding envelope sequences cloned from peripheral blood mononuclear cells of an SIVMneCL8-infected *M. nemestrina* prior to (chimera CL8-35wkSU) and soon after (chimera CL8-81wkSU) the onset of immunodeficiency (30). Both envelope chimeras contain a serine-threonine-rich sequence potentially indicative of O-linked glycosylation and a predicted proline to serine change which creates a new potential site for N-linked glycosylation, relative to the parental virus SIVMneCL8 (Fig. 1A). Chimera CL8-35wkSU has only four predicted amino acid changes relative to the parental clone, SIVMneCL8, and all are to serine or threonine residues and within V1. Compared to SIVMneCL8, chimera CL8-81wkSU has seven amino acid changes in V1, four amino acid changes in the V4 variable region of Env-SU, which lead to the positional shift of a potential N-linked glycosylation site, and an isoleucine to valine change in the V5 variable region (Fig. 1A) (30). As an initial test of whether predicted glycosylation sites in V1 were indeed modified, radiolabelled envelope proteins from infected CEMx174 cells were immunoprecipitated, and their mobility was assessed by SDS-PAGE. By comparison to size standards, SIVMneCL8 Env-SU migrated at approximately 115 to 125 kDa. Env-SU and unprocessed envelope protein from the chimeric viruses CL8-35wkSU and CL8-81wkSU display decreased mobility on SDS-PAGE compared to SIVMneCL8 (Fig. 2, lanes A to C), which is consistent with the hypothesis

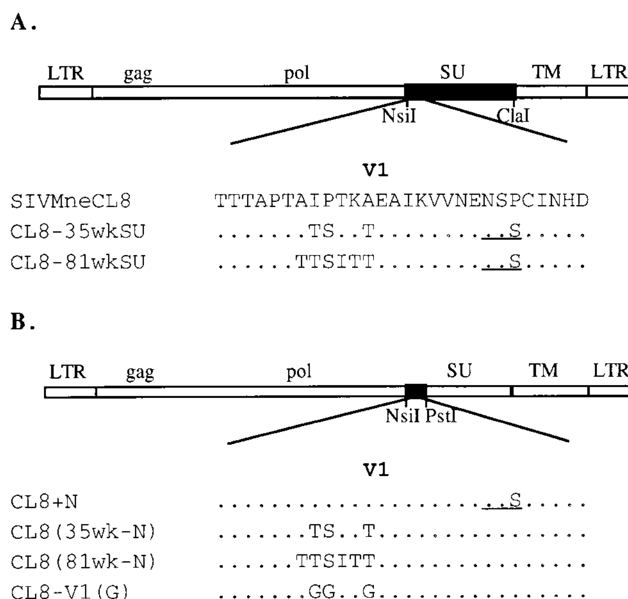

FIG. 1. Predicted amino acid sequence of the V1 region of SIV envelope chimeras. Two schematic diagrams of the structure of the chimeric viruses are shown. In each, the location of the long terminal repeats (LTRs) and the *gag*, *pol*, envelope SU, and transmembrane (TM) reading frames are shown for orientation. (A) CL8-35wkSU and CL8-81wkSU chimeras, which encode 1.1-kb fragments representing the variant envelope genes from 35 weeks and 81 weeks postinfection, respectively, in a background of SIVMneCL8 (30). Below the schematic genome, the amino acid sequence of a portion of the V1 variable region, starting at position 128 of SIVMneCL8 Env-SU, is shown in single-letter code. Predicted amino acid differences in SIV envelope chimeras are shown below. Dots indicate no changes in amino acids, and underlined amino acids indicate consensus sequences for N-linked glycosylation. There are no other differences in amino acid sequences of SIVMneCL8 and CL8-35wkSU (30). The amino acid sequence of the CL8-81wkSU chimera also contains four amino acid differences from the sequence of SIVMneCL8 in the V4 region of the envelope as well as a single amino acid change in V5 (30). (B) Chimeras and mutant viruses that were constructed specifically for this study as described in Materials and Methods. These viral genomes differ from SIVMneCL8 only in the *Nsi*I-*Pst*I fragment encompassing V1, and the sequence changes in this region are indicated below the schematic, as described for panel A.

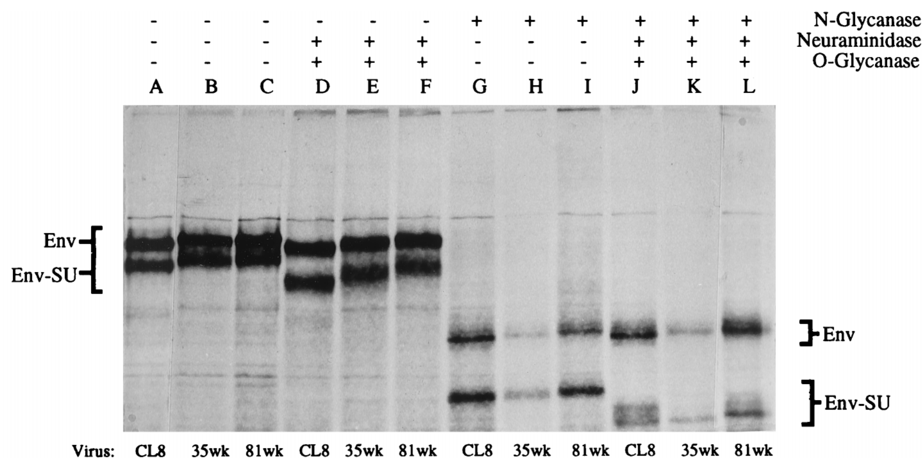

FIG. 2. Electrophoretic mobilities of the envelope proteins encoded by SIVMneCL8 and the CL8-35wkSU and CL8-81wkSU chimeras. Infected CEMx174 cells were labelled with 200 μCi of [^{35}S]Met and [^{35}S]Cys for 6 h, and cells were lysed and then immunoprecipitated with serum from a macaque infected with uncloned SIVMne, as described in Materials and Methods. CEMx174 cells were persistently infected with SIVMneCL8 (lanes A, D, G, and J), CL8-35wkSU (lanes B, E, H, and K), or CL8-81wkSU (lanes C, F, I, and L). Aliquots of protein were untreated, treated with neuraminidase and *O*-glycanase, treated with *N*-glycanase, or treated with *N*-glycanase, neuraminidase, and *O*-glycanase as indicated above the gel. Sample mobility was analyzed via SDS–7.5% PAGE and fluorography. The protein bands corresponding to unprocessed envelope (Env) and the processed Env-SU are indicated. The sizes of Env and Env-SU were calculated from several different SDS-PAGE gels by comparison with molecular weight standards (data not shown).

that envelope proteins of the chimeric viruses were modified by more carbohydrate chains than SIVMneCL8.

To examine the specific glycosylation differences between SIVMneCL8 and the variant Env-SU, we treated immunoprecipitated proteins from SIV-infected cells with carbohydrate-cleaving enzymes. Fig. 2, lanes D to F, shows immunoprecipitated cell lysates that were treated with neuraminidase, an enzyme that cleaves terminal sialic acid residues (a requirement for *O*-glycanase activity), followed by *O*-glycanase, an enzyme that cleaves the subset of O-linked sugars in which the serine or threonine residue is attached to a Gal-β(1,3)-GalNAc core disaccharide. While treatment with neuraminidase alone resulted in no significant change in the mobility of Env-SU on SDS-PAGE (data not shown), *O*-glycanase-digested SIVMneCL8 Env-SU migrated at approximately 105 to 110 kDa. Thus, we provide the first demonstration that SIV Env-SU, like HIV-1 Env-SU (4), is O glycosylated. Difference in electrophoretic mobility between variants after neuraminidase–*O*-glycanase treatment may reflect differences in N-linked carbohydrate content. The mobility of *O*-glycanase-treated immunoprecipitated Env-SU varies among the three viruses (Fig. 2, lanes D to F), suggesting that the proteins differ in their extent of N-linked and/or resistant O-linked carbohydrates.

To examine Env-SU modification by N-linked carbohydrates, lysates were treated with *N*-glycanase (Fig. 2, lanes G to I), an enzyme which catalyzes the hydrolysis of all common classes of mammalian N-linked oligosaccharides at the β-aspartylglycosylamine bond between the innermost GlcNAc and the asparagine residue. *N*-Glycanase-digested SIVMneCL8 Env-SU migrates with an apparent molecular mass of approximately 65 to 70 kDa; thus, SIV Env-SU is extensively N-glycosylated, with roughly 40% of the molecular mass of the protein due to N-linked glycosylation. Differences in electrophoretic mobility between variants after *N*-glycanase treatment would suggest differences in O-linked carbohydrate content. As shown here, the mobility differences between the three variant proteins were subtle. The apparent mass of *N*-glycanase-treated Env-SU from CL8-81wkSU is slightly greater than the mass of *N*-glycanase-treated Env-SU from CL8-35wkSU, which, in turn, is slightly greater than the mass of *N*-glycanase-treated SIVMneCL8 Env-SU. While these modest differences may indicate possible differences in O-linked glycosylation between the different Env-SU proteins, analyses of O-linked carbohydrates by gel mobility assays are complicated due to the smaller size of O-linked glycans (4) and the lack of an enzyme that cleaves all O-linked carbohydrates. Moreover, when the lysates were treated with all three carbohydrate-cleaving enzymes (Fig. 2, lanes J to L) the bands corresponding to deglycosylated Env-SU did not comigrate; this could indicate that the peptide backbones of the Env-SU proteins migrate differently or it could reflect the presence of glycanase-resistant oligosaccharides (including, perhaps, the new O-linked sugars in V1). Because the results of O-linked carbohydrate analysis by glycanase digestion and gel mobility assays were not conclusive, additional methods were used to examine this further (see below).

Analysis of V1 chimera. To further examine V1 glycosylation, we mutated V1 to create viruses with either just a potential N-linked or just a potential O-linked glycosylation site changes. As shown in Fig. 1B, chimeras CL8(35wk-N) and CL8(81wk-N) differ from CL8-35wkSU and CL8-81wkSU, respectively, only by the absence of an N-linked glycosylation site in V1; they differ from SIVMneCL8 only by the presence of predicted O-linked glycosylation sites. Chimera CL8+N differs from SIVMneCL8 by the single amino acid change that creates a potential N-linked glycosylation site in V1. Immunoprecipitated lysates from cells infected with these chimeric viruses were analyzed as described above. To specifically examine N-linked carbohydrates in V1, we analyzed viral lysates in pairs, in which the only difference between the predicted V1 amino acid sequence was the presence (or absence) of a potential N-linked glycosylation site. As shown in Fig. 3A, the addition of an N-linked glycosylation site to SIVMneCL8 resulted in a decrease in the mobility of Env-SU (lanes A and B), and the removal of the N-linked glycosylation site from both CL8-35wkSU (lanes C and D) and CL8-81wkSU (lanes E and F) resulted in an increase in the mobility of Env-SU. When the lysates were digested with *N*-glycanase (Fig. 3B) there was no apparent mobility shift between the paired lysates (i.e., the

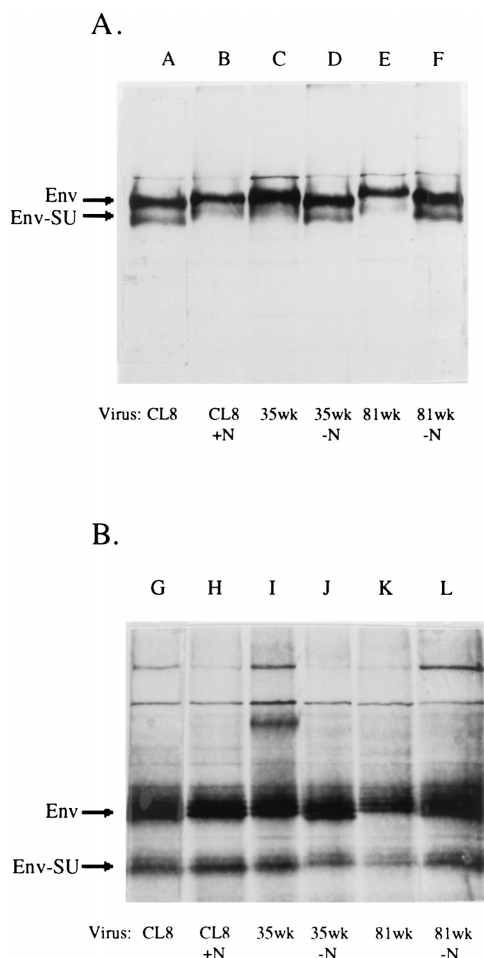

FIG. 3. Electrophoretic mobilities of the envelope proteins encoded by SIVMneCL8 and the variant envelope chimeras. CEMx174 cells infected with SIVMneCL8 (lanes A and G), CL8+N (lanes B and H), CL8-35wkSU (lanes C and I), CL8(35wk-N) (lanes D and J), CL8-81wkSU (lanes E and K), or CL8 (81wk-N) (lanes F and L) were labelled with 200 μCi of [^{35}S]Met and [^{35}S]Cys for 6 h, and cells were lysed and then immunoprecipitated with serum from a macaque infected with uncloned SIVMne as described in Materials and Methods. Aliquots of protein were untreated (A) or treated with *N*-glycanase (B). Sample mobility was analyzed via SDS–7.5% PAGE and fluorography. The protein bands corresponding to unprocessed envelope (Env) and the processed Env-SU are indicated, and they were identified by comparison with molecular weight standards (not shown).

bands corresponding to Env-SU in lanes G and H comigrate, as do the bands corresponding to Env-SU in lanes I and J and lanes K and L), indicating that the differences in mobility seen in the undigested proteins are due to carbohydrate modification of the N-linked glycosylation site in V1. Together, these data suggest that the Env-SU proteins from CL8-35wkSU, CL8-81wkSU, and CL8+N are modified by a carbohydrate addition at the new N-linked glycosylation site in V1.

Mass spectrometry analysis. To obtain a highly accurate estimate of the molecular mass of Env-SU, we analyzed SIV Env-SU by MALDI-TOF MS. MALDI-TOF MS analysis is highly sensitive, with detection limits of femtomoles to picomoles, and yields an accurate estimate of protein molecular mass (33). Based on the properties of this technique, we predicted that MALDI-TOF MS analysis has the potential to distinguish small molecular mass differences, including differences in O-linked glycosylation, between proteins. To assess the feasibility and reproducibility of MALDI-TOF MS analysis we analyzed purified SIVmac251 Env-SU (Advanced Biotechnologies Inc., Columbia, Md.) (Fig. 4A). We detected peaks corresponding to singly charged Env-SU (*m*/*z* ratio, 101,957 Da) and doubly charged Env-SU (*m*/*z* ratio, 51,210 Da). As expected for glycoproteins, the peaks are broad due to glycoform microheterogeneity (18). Thus, the calculated mass represents the average content of Env-SU. Based on eight analyses by MALDI-TOF MS, the estimated molecular mass of SIVmac251 Env-SU is approximately 101,970 +/− 549 Da. This analysis provides the first rigorous calculation of the molecular mass of SIV Env-SU.

SIV Env-SU proteins from SIVMneCL8, CL8-35wkSU, and CL8-(35wk-N) were partially purified from the supernatant of infected CEMx174 cells by GNA lectin affinity chromatography. Based on Western blot analysis and Coomassie staining, the eluted material contained Env-SU as well several predominantly higher-molecular-mass contaminant proteins (data not shown). By estimating the concentration of the partially purified SIVMne Env-SUs in comparison to the purified SIVmac251 control, and determining the concentration of total protein, we estimated that Env-SU constituted approximately 15% of the protein in the pooled column fractions (data not shown). Although contaminant proteins were occasionally detected by MALDI-TOF MS analysis when the machine was in the linear mode, Env-SU was the major protein detected by MALDI-TOF MS analysis when the machine was in the reflectron mode. The lack of signal from contaminating proteins in the reflectron mode may be due to the ion suppression effects that are typically observed in MALDI analysis of high-mass mixtures (5). Representative spectra from MALDI-TOF MS analysis of partially purified Env-SUs from SIVMneCL8 and CL8-(35wk-N) are shown in Fig. 4B and C. In each case, peaks were observed at *m*/*z* values comparable to those observed in the spectra of SIVmac251 Env-SU, suggesting that these peaks do, in fact, represent Env-SU.

The average masses of the Env-SU proteins from all samples were calculated. Our calculation of the mass of each Env-SU protein was based on the average of eight spectra, measured on three independent occasions. According to this analysis, the molecular mass of CL8-(35wk-N) Env-SU was calculated to be 103,464 ± 194 Da, and the molecular mass of SIVMneCL8 Env-SU was calculated to be 102,268 ± 368 Da. These measurements indicate that CL8-(35wk-N) Env-SU is approximately 1,200 Da larger than the Env-SU of SIVMneCL8, suggesting that the potential O-linked glycosylation site(s) in the V1 region of the CL8-(35wk-N) Env-SU is modified by carbohydrate addition. CL8-(35wk-N) differs from SIVMneCL8 at three amino acid positions (all threonine or serine residues), so it is possible that any one or more of the three residues are modified by O-linked carbohydrate addition, but this could not be discerned from this analysis. In addition, MALDI-TOF MS analysis supports our data from mobility shift assays that the novel N-linked glycosylation site in V1 is modified by carbohydrate addition. The molecular mass of Env-SU from CL8-35wkSU was calculated to be 105,655 ± 468 Da, which is 2,200 Da larger than the mass of CL8-(35wk-N) Env-SU, which differs only by the absence of the N-linked glycosylation site. Similar differences in the masses of the variant Env-SU proteins were observed with different protein preparations and with different configuration of the MALDI-TOF MS apparatus (data not shown). Thus, MALDI-TOF MS analysis indicates that both the O-linked and N-linked glycosylation sites in V1 are modified by carbohydrate addition.

Neutralization of SIVMneCL8 variants. Previous studies demonstrated that SIVMneCL8 envelope variation is driven,

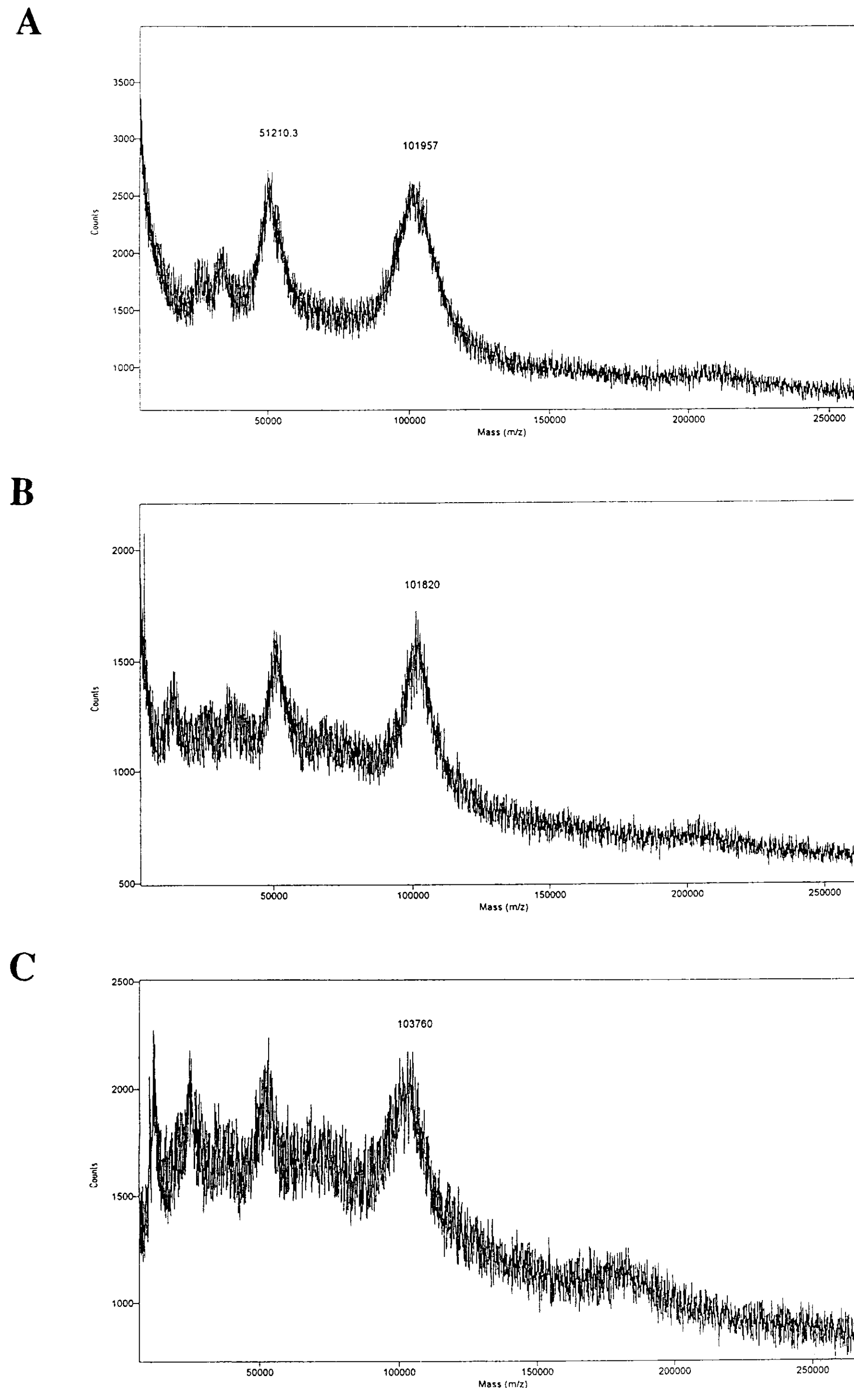

FIG. 4. MALDI-TOF MS analysis of Env-SU samples. (A) Purified SIVmac251 Env-SU (1 μl of analyte containing 3 μg of Env-SU). (B) Partially purified cell extracts from CEMx174 cells infected with SIVMneCL8. One microliter of analyte, which was approximately 0.25 μg of SIVMneCL8 Env-SU (based on Western blot analysis and comparison to the purified SIVmac251 protein), was analyzed. (C) Partially purified cell extracts from CEMx174 cells containing CL8(35wk-N) Env-SU (1 μl of analyte containing approximately 1 μg of Env-SU). *m/z* ratios are plotted versus relative intensities (counts). Each spectrum contains multiple peaks, which represent differentially charged Env-SUs. Indicated above each peak is the calculated mass, as determined by the Voyager Elite analysis software package (PerSeptive Biosystems).

at least in part, in response to host neutralizing antibody (30). These data suggest that the addition of glycosylation sites in V1 may mediate shifts in viral antigenicity and, consequently, immune recognition. For this reason, we examined the ability of V1 chimeras containing either the N-linked or the O-linked glycosylation sites to escape antibody neutralization using the sMAGI neutralization assay (9), an assay that exploits the ability of the sMAGI cell line to quantitatively detect infectious SIV. SIVMne chimera were tested against sera isolated approximately 6 months postinoculation from four infected *M. nemestrina*, two of which (animals 90027 and 92128) were infected with SIVMneE11S (3), a biological clone from

TABLE 1. Neutralization titers of sera against SIVMneCL8 and V1 chimeras

Virus or chimera	Neutralization titer of sera from animals infected with[a]:			
	SIVMneE11S		Uncloned SIVMne	
	90027	92128	93100	92221
SIVMneCL8	1:16	1:32	1:128	1:50
CL8-35wkSU	<1:4	<1:4	<1:4	<1:4
CL8-81wkSU	<1:4	<1:4	<1:4	<1:4
CL8(35wk-N)	<1:4	<1:4	<1:4	<1:4
CL(81wk-N)	<1:4	<1:4	1:40	1:10
CL8+N	<1:4	1:40	<1:4	<1:4
CL8-V1(G)	1:32	1:10	>1:128	1:70

[a] Data are 90% neutralization titers, as described in the text. Serum samples were isolated from animals at 5 to 6 months postinoculation.

which the molecular clone SIVMneCL8 was derived, and two of which (animals 93100 and 92221) were infected with SIVMne (2), the uncloned complex mixture from which SIVMneE11S and SIVMneCL8 were derived. For these studies, SIVMneE11S and SIVMneCL8 can be considered homologous viruses because the envelope sequence of SIVMneE11S is essentially identical to that of SIVMneCL8 (31a). In all cases prebleed sera from all four animals failed to efficiently neutralize any of the SIV isolates tested (90% neutralization titer of < 1:4), demonstrating that neutralizing antibodies are specific to SIV (data not shown).

SIVMneCL8 was neutralized by sera from all four of the macaques tested, at 90% neutralization titers ranging from 1:16 to 1:128 (Table 1). As has been demonstrated previously (30), CL8-35wkSU and CL8-81wkSU were not efficiently neutralized (>90%) by any of the sera tested (titer of < 1:4). Similarly, CL8(35wk-N) was not neutralized by any of the sera tested, indicating that the three amino acid changes leading to a new O-linked glycosylation site(s) in V1 are sufficient to allow for escape from neutralizing antibody recognition. The other two V1 chimeric viruses vary in their ability to respond to the panel of sera that we tested. CL8(81wk-N) was neutralized by sera from the two animals infected with uncloned SIVMne (at titers of 1:10 and 1:40) but not by sera from the two animals infected with SIVMneE11S. The stock of uncloned SIVMne is a complex mixture of virus, and it is possible that this mixture may contain a viral variant that that is antigenically similar to CL8(81wk-N). CL8+N was neutralized by serum from one SIVMneE11S-infected animals (at a titer of 1:40) but not by sera from the other three animals. Thus, our data indicate that either the novel O-linked or N-linked glycosylation site in the V1 region of envelope allows viruses to escape neutralization from most, but not all, of the serum samples tested.

To distinguish whether the ability to escape neutralization is mediated directly by the new glycosylation sites or whether any amino acid changes in this region would allow the virus to escape the humoral immune response, we created an additional site-directed mutant, CL8-V1(G) (Fig. 1B). In this construct, we substituted three glycine residues for the three amino acid positions (alanine-134, isoleucine-135, and alanine-139) that evolved in vivo to serine and threonine residues in CL8-35wkSU. Substitution of the small, polar amino acid glycine for three hydrophobic residues in the hypervariable region of V1 is predicted to radically alter the primary sequence and possibly the structure of the V1 loop without affecting glycosylation. The CL8-V1(G) virus was generated by transient transfection of 293T cells with cloned virus and is replication competent in CEMx174 cells, as measured by increasing p27^{gag} antigen levels after infection (data not shown). Despite these amino acid changes in V1, CL8-V1(G) was neutralized by sera from all four animals (Table 1) at relatively high titers (similar or slightly higher than the neutralizing titer of SIVMneCL8). These results suggest that specific amino acid changes in this region of V1 do not alter a linear B-cell epitope recognized by macaque sera. They also suggest that amino acid changes in V1 do not necessarily lead to changes in the neutralizing activity of antibody. Thus, our results are consistent with a model whereby additional postranslational modification by carbohydrates in V1 induces structural changes in the envelope that mask the major neutralizing epitope.

DISCUSSION

During SIV infection of macaques and HIV-1 infection in humans, there is a propensity for amino acid changes to predicted glycosylation sites in the viral envelope protein, but there has previously been no direct evidence that glycosylation changes are associated with these sequence changes. Furthermore, it is unknown how changes in individual carbohydrate moieties influence envelope protein function. In the course of our studies of SIV variation in infected *M. nemestrina*, we observed that there was strong selection for viral variants with new potential N-linked and O-linked glycosylation sites in the V1 variable region of envelope prior to, or in conjunction with, the onset of immunodeficiency (27). On the basis of these results, and the observations that these sites were conserved over the course of disease and upon transmission (10, 27, 28), we hypothesized that the introduction of new glycosylation sites in V1 leads to important functional changes in the virus. In the present study, we provide direct evidence that the predicted N-linked and O-linked glycosylation sites in envelope are modified by carbohydrate addition, and we determined that glycosylation of these sites mediated shifts in viral antigenicity.

Enzymatic deglycosylation of SIV envelope glycoproteins and analysis by gel electrophoresis confirmed that SIV Env-SU is extensively modified by the addition of N-linked carbohydrates and provided the first evidence for the presence of O-linked carbohydrates on this protein. By this analysis, we estimated that N-linked carbohydrates constitute roughly 40% of the molecular weight of Env-SU and O-linked carbohydrates constitute roughly 10 to 15% of the molecular weight of Env-SU. Our mass spectrometry studies also allowed us to provide a more precise estimate of the size of SIV Env-SU. Based on MALDI-TOF MS analyses, we calculated that the molecular mass of SIV Env-SU is 100 to 105 kDa, depending on the carbohydrate and sequence composition of the variant.

Because our interest was in defining biochemical and functional changes in viruses that evolve during progression to disease, in this study we focused on representative envelope proteins from variants that evolved during the course of infection. In addition, we constructed pairs of chimeric viruses which differed from their parent by the presence or absence of naturally occurring, predicted N-linked and O-linked glycosylation sites in V1. Mobility shift assays and glycanase digestions suggested that an N-linked glycosylation sequence motif that evolves in the V1 domain of variants that are selected prior to development of simian AIDS is modified by carbohydrate addition. In addition, by MALDI-TOF MS analysis, we determined that the addition of this N-linked glycosylation site in V1 leads to an increase in the size of the protein by approximately 2 kDa. Together, these data provide the first demonstration that predicted amino acid changes that evolve in the

SIV or HIV envelope alter the carbohydrate composition of Env-SU. These results may have broad implications given the extensive heterogeneity of potential glycosylation sites among SIV and HIV variants.

We have hypothesized that the serine- and threonine-rich cluster that evolves in a hypervariable region of V1 of SIV Env-SU creates a new site for O-linked glycosylation (27). However, this model was somewhat speculative because there is no characteristic amino acid motif that is highly predictive of modification by O-linked glycosylation. In the present study, analysis of a variant with serine-threonine changes in the hypervariable domain of V1 relative to the parental virus provides direct support for our hypothesis. The addition of three serine or threonine residues leads to a shift in the size of Env-SU from 102.3 to 103.5 kDa, as determined by the highly sensitive method of MALDI-TOF MS. Although we cannot rule out the possibility that the increase in mass is due to an indirect effect of the serine-threonine changes at distal glycosylation sites, the shift in mass is consistent with the addition of O-linked rather than N-linked sugars. Thus, these data provide strong evidence that viruses with O-linked carbohydrate changes are selected during the course of SIVMne infection.

SIVMneCL8 variants containing changes in the V1 variable region are resistant to neutralization by sera that effectively neutralized SIVMneCL8 (30). In the studies presented here, we showed that the new glycosylation sites in V1 mediate this shift in antigenicity. A variant which contains only three amino acid changes from SIVMneCL8, leading to the introduction of an O-linked glycosylation site, was not neutralized by sera from any of the four animals who had neutralizing antibodies directed to SIVMneCL8. Additional SIVMneCL8 variants that encoded an additional N-linked or O-linked glycosylation site(s) in V1 escaped neutralization against some, but not all, of the serum samples we tested. These results suggest that individually the O-linked or the N-linked glycosylation site in V1 may confer some resistance to neutralizing antibodies against SIVMneCL8. We hypothesize, then, that O-linked and N-linked glycosylation sites coevolved in V1 to enhance resistance against host neutralizing antibody.

The fact that serine and threonine changes in V1 have such a pronounced effect on the ability of the virus to be neutralized by host antibodies suggests two possibilities (i) that neutralizing antibodies directly recognize epitopes in V1 or (ii) that carbohydrate modifications lead to conformational changes that mask a major neutralizing epitope in another region of the envelope. Our studies show that changes in the primary sequence of SIVMneCL8 V1 do not, in themselves, affect neutralizing antibody recognition. Neutralization sensitivity of the virus is altered specifically when there are changes to serine and threonine but not when there are changes to glycine at the same positions in V1. These data support the second model and indicate that glycosylation is required for neutralization escape. Furthermore, our data suggest that V1 is not a linear B-cell epitope.

Studies of SIVmac have defined several domains in the envelope that appear to function as either linear or conformational epitopes (7). By reacting synthetic peptides corresponding to regions of Env-SU with anti-SU monoclonal antibodies that have neutralizing activity, SIVmac linear neutralization epitopes were identified upstream of the V2 variable region and within the V4 region (1, 21). However, it is not clear if these epitopes are actually recognized by macaque neutralizing antibodies. In studies of SIV envelope variation in rhesus macaques infected with SIVmac239, several groups have demonstrated that at least some of the genetic variants that arise during the course of disease are resistant to neutralization by antisera that can neutralize SIVmac239 (6, 22). The precise B-cell epitopes of SIV that are recognized by sera from infected macaques are not known, but sequences in V2, V3, and V4 have all been implicated (22, 35). Kinsey et al. demonstrated that the ability to evade neutralizing antibody mapped to the V4 variable region of SIVmac239 Env-SU; within this domain were amino acid differences that could lead to a N-linked carbohydrate change (22). In light of our data demonstrating a role of Env-SU glycosylation in SIV neutralization escape, we speculate that the potential N-linked glycosylation site in V4 of SIVmac239 variants is modified by carbohydrates so as to alter some conformational epitope affected by V1 carbohydrate changes. Given the structural effects of carbohydrates, it is possible that glycosylation modifications in several domains within the envelope may affect neutralizing antibody recognition in vivo.

A major difference between SIV and HIV-1 is that V3, the major neutralization epitope of HIV-1 (15, 24, 29, 32), remains fairly conserved throughout the course of SIV infection (8, 20, 28). However, other regions of the HIV-1 envelope have been recognized as containing important neutralization epitopes, including the V1-V2 domain and the CD4 binding domain (reviewed in reference 7). There is some evidence that potential glycosylation sites in the V1-V2 region of HIV-1 Env-SU may affect envelope structure and, consequently, neutralization epitope presentation (12, 34, 36). Taken together with our data, these results suggest that glycosylation of the V1-V2 region may play a major role in epitope presentation in the envelope proteins of HIV-1 and SIV. Moreover, the data presented here provide the first direct demonstration that viruses that are selected during SIV or HIV infection acquire both O-linked and N-linked glycans that allow them to escape the host humoral immune response. Thus, in designing vaccines and immunotherapies, it will be important to consider the role that carbohydrate-associated structural changes in the SIV and HIV-1 envelopes may play in viral escape.

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