Glycosylation of Immunodominant Linear Epitopes in the Carboxy-Terminal Region of the Caprine Arthritis-Encephalitis Virus Surface Envelope Enhances Vaccine-Induced Type-Specific and Cross- Reactive Neutralizing Antibody Responses

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This study evaluated type-specific and cross-reactive neutralizing antibodies induced by immunization with modified surface glycoproteins (SU) of the 63 isolate of caprine arthritis-encephalitis lentivirus (CAEV-63). Epitope mapping of sera from CAEV-infected goats localized immunodominant linear epitopes in the carboxy terminus of SU. Two modified SU (SU-M and SU-T) and wild-type CAEV-63 SU (SU-W) were produced in vaccinia virus and utilized to evaluate the effects of glycosylation or the deletion of immunodominant linear epitopes on neutralizing antibody responses induced by immunization. SU-M contained two N-linked glycosylation sites inserted into the target epitopes by R539S and E542N mutations. SU-T was truncated at 518A, upstream from the target epitopes, by introduction of termination codons at 519Y and 521Y. Six yearling Saanen goats were immunized subcutaneously with 30 µg of SU-W, SU-M, or SU-T in Quil A adjuvant and boosted at 3, 7, and 16 weeks. SU antibody titers determined by indirect enzyme-linked immunosorbent assay demonstrated anamnestic responses after each boost. Wild-type and modified SU-induced type-specific CAEV-63 neutralizing antibodies and cross-reactive neutralizing antibodies against CAEV-Co, a virus isolate closely related to CAEV-63, and CAEV-1g5, an isolate geographically distinct from CAEV-63, were determined. Immunization with SU-T resulted in altered recognition of SU linear epitopes and a 2.5- to 4.6-fold decrease in neutralizing antibody titers against CAEV-63, CAEV-Co, and CAEV-1g5 compared to titers of SU-W-immunized goats. In contrast, immunization with SU-M resulted in reduced recognition of glycosylated epitopes and a 2.4- to 2.7-fold increase in neutralizing antibody titers compared to titers of SU-W-immunized goats. Thus, the glycosylation of linear immunodominant nonneutralization epitopes, but not epitope deletion, is an effective strategy to enhance neutralizing antibody responses by immunization.

Important research goals in lentivirus vaccine development include defining immune mechanisms and epitopes on viral antigens involved in the control of virus replication and developing immunogens and vaccination strategies to elicit relevant immune responses. Numerous reports indicate that neutralizing antibodies are involved in preventing infection or controlling lentivirus replication (2, 3, 33, 41, 60). Therefore, the induction of neutralizing antibodies by immunization is an important consideration in the development of vaccine strategies.

The identification of human monoclonal antibodies (MAbs) that neutralize primary human immunodeficiency virus type 1 (HIV-1) isolates demonstrates the presence of conserved neutralization epitopes on the gp120 surface envelope (SU) (31, 55). Immunization with soluble gp120 generally elicits antibodies directed primarily to linear epitopes (8, 32, 35, 44, 57), with limited responses to neutralization epitopes (9, 16, 32, 37, 61). The difficulty in eliciting broadly cross-reactive neutralizing antibodies by protein immunization has been attributed to the immunodominance of linear nonneutralizing or weakly neutralizing linear epitopes and the relatively poor immunogenicity or exposure of discontinuous neutralization epitopes (7, 9, 32, 42, 47). This concept is supported by observations that cross-reactive neutralizing antibodies to primary HIV isolates are induced by immunization with either oligomeric HIV SU or monomeric gp120 under conditions that preserve the conformation of SU together with adjuvants that potentiate the immunogenicity of conformational epitopes (15, 31, 36, 46, 51, 53, 54, 58).

Our laboratory is utilizing the caprine arthritis-encephalitis lentivirus (CAEV) model to evaluate immunization strategies to induce cross-reactive neutralizing antibodies by using monomeric SU (10). SU is a primary target of humoral immune responses to CAEV, and infected goats develop high titers of binding antibodies directed to immunodominant nonneutralization epitopes (21, 26). Initial antibody responses to SU are predominately directed to linear epitopes, and maturation of the immune response results in increased reactivity to conformational epitopes (unpublished data), resulting in low titers of generally type-specific neutralizing antibodies in some infected animals (11, 29, 34). A previous study of epitope exposure on CAEV SU suggested that cross-reactive neutralizing antibodies could be induced by immunization with monomeric SU (29). This study showed that recombinant CAEV gp135 SU adsorbs homologous and heterologous neutralizing antibodies in goat sera, indicating that covert cross-reactive neutralization epitopes on virion-associated SU are exposed on soluble monomeric SU. A preliminary immunization trial demonstrated
induction of cross-reactive neutralizing antibodies by multiple immunizations of four goats with purified CAEV SU formulated in Quil A adjuvant (22). However, responses were directed primarily to immunodominant nonneutralization epitopes, neutralizing antibody titers were relatively low compared to titers in CAEV-infected goats (25), and at least one immunized goat developed SU binding antibodies that inhibited virus neutralization.

The present study evaluated SU modifications as a means to diminish responses to immunodominant nonneutralization epitopes and enhance exposure or recognition of less immunoreactive neutralization epitopes. Epitope mapping studies of sera from CAEV-infected goats early in infection confirmed the results of previous reports (5, 56) that most immunodominant linear epitopes of SU are within the carboxy-terminal end. Two strategies were utilized in an attempt to divert immune responses from these epitopes toward neutralization epitopes. One strategy evaluated the effect of introducing N-linked glycosylation sites into targeted immunodominant epitopes. One strategy evaluated the effect of introducing N-linked glycosylation sites into targeted immunodominant epitopes. In addition, we evaluated the effect of deleting 32 amino acids from the carboxy terminus of SU. These epitope manipulations were based on previous studies demonstrating that (i) N-linked glycans reduce the immunogenicity of HIV gp120 epitopes (14, 45, 48) and (ii) under certain conditions, epitope deletion can redirect antibody responses to HIV neutralization epitopes (20, 23).

Immunization of goats with CAEV SU containing glycosylated immunodominant epitopes induced significantly higher titers of type-specific and cross-reactive neutralizing antibodies compared to titers of wild-type SU (SU-W), indicating that glycosylation shifted immune responses from linear epitopes to neutralization epitopes. In contrast, the removal of targeted epitopes shifted antibody responses to linear epitopes within another immunodominant domain of SU and significantly reduced neutralizing antibody responses compared to responses in SU-W. Therefore, we report that masking of carboxy-terminal immunodominant epitopes of CAEV SU by the insertion of glycans, but not deletion of these epitopes, is an effective strategy for directing immune responses to neutralization epitopes.

MATERIALS AND METHODS

Viruses. CAEV-63 and CAEV-Co are closely related independent virus isolates from an Oregon herd (12, 40), distinguished by env gene sequence variability (27, 50) and type-specific neutralizing antibodies (29, 34). CAEV 1g5 (provided by M. McMillan, University of Southern California) was isolated from a California goat by coculture of peripheral blood mononuclear cells with goat synovial membrane (GSM) cells. The amino acid sequence of the 543-amino-acid SU of CAEV-1g5 is 83.6% identical to that of CAEV-63 SU, with approximately one-half of the amino acid changes in the five SU variable regions (I. Hötzeli and W. P. Cheevers, unpublished data). Virus isolates were amplified in GSM cells, and virus infectivity titers were determined by formation of syncytia and expressed as 50% tissue culture infectious doses (TCID_{50}) (24).

Western blots of histidine fusion polypeptides. Western blots of histidine fusion polypeptides corresponding to the SU 2 (72 amino acids), SU 3 (106 amino acids), and SU 4 (84 amino acids) domains of CAEV SU (Fig. 1) were utilized to map immunodominant B-cell epitopes recognized by antibodies present in sera of goats early in CAEV infection. These experiments employed recombinant polypeptides produced in Escherichia coli transformed with previously described pET recombinant plasmids (5) obtained from G. Bertonio, University of Berne. Similar Western blots were employed to evaluate antibody binding to immunodominant domains of SU following immunization with modified SU. Recombinant polypeptides utilized in experiments were quantified by total protein quantification (Pierce bicinchoninic acid [BCA] assay). Western blots utilized 100 ng of recombinant fusion proteins purified on His-Trap columns (Novagen). Blots were reacted with serial dilutions of goat sera followed by...
TABLE 1. SU B-cell epitopes recognized by antibody responses early in CAEV infection

<table>
<thead>
<tr>
<th>Serum source (goat no.)</th>
<th>SU $^{a}$</th>
<th>SU $^{b}$</th>
<th>SU $^{c}$</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
<th>VII</th>
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<tr>
<td>SU 1</td>
<td>&lt;200</td>
<td>&lt;200</td>
<td>800</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>500</td>
<td>1,000</td>
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</tr>
<tr>
<td>SU 2</td>
<td>&lt;100</td>
<td>200</td>
<td>400</td>
<td>&lt;100</td>
<td>100</td>
<td>100</td>
<td>100</td>
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<td>SU 3</td>
<td>&lt;200</td>
<td>&lt;200</td>
<td>800</td>
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<td>500</td>
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<tr>
<td>SU 4</td>
<td>&lt;200</td>
<td>&lt;200</td>
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$^{a}$ Yearling goats 9901, 9902, 9903, and 9904 were inoculated intravenously with 1 x 10$^{10}$ TCID$_{50}$ of molecularly cloned CAEV-Co. Serum for antibody titrations were obtained at 4 to 7 weeks postinfection.

$^{b}$ Serial dilutions of sera were used for determination of antibody titers against histidine fusion proteins SU 2, SU 3, and SU 4 by Western blotting.

$^{c}$ Serial dilutions of sera were used for determination of antibody titers against synthetic peptides derived from the amino acid sequence of SU 4 by using a peptide ELISA.

horseradish peroxidase (HRP)-conjugated rabbit anti-goat immunoglobulin G (IgG) (Kirkegaard & Perry), diluted 1:2,000 and developed with enhanced chemiluminescence reagent. Signals were quantified by densitometry with an IS1000 digital imaging system (Alpha Innotech). Endpoint antibody titers were determined by linear regression analysis of signal intensity plotted against serum dilutions. The percentage of antibody titer was calculated and reported for each serum sample by dividing the antibody titer for each protein by the cumulative titer for all three histidine fusion proteins.

**Peptide ELISA.** An enzyme-linked immunosorbent assay (ELISA) utilizing overlapping peptides derived from the sequence of CAEV-63 SU 27 was used to precisely map immunodominant linear epitopes within the SU 4 domain as targets for modification by glycosylation. The peptide ELISA was also used to evaluate shifts in antibody reactivity with SU 4 peptides following the immunization of goats with modified SU. Fourteen-mer peptides with seven amino acid overlaps corresponding to the entire SU 4 domain were synthesized with biotine-SOSG-peptide configurations. The amino acid sequences of the synthetic peptides are shown in Fig. 1. Peptides were synthesized by the Washington State University Laboratories of Bioanalysis and Biotechnology or Genemed Synthesis, Inc. Quadruplicate wells of polystyrene microtiter plates coated with neuraminidase (Pierce) were washed with phosphate-buffered saline (PBS) and coated with 100 μl of peptide (1 mg/ml) for 2 h at room temperature. Plates were washed with PBS-0.1% Tween 20 (PBS-Tween) and blocked with PBS-Tween containing 5% nonfat dry milk for 2 h. After washing with PBS-Tween, wells were incubated for 2 h with 100 μl of goat sera diluted in PBS, washed with PBS-Tween, and reacted for 30 min with HRP-conjugated rabbit anti-goat IgG (Kirkegaard & Perry) diluted 1:4,000 in PBS-Tween. After washing with PBS-Tween, wells were incubated in the dark for 20 min with chromogenic tetramethyl benzidine (Kirkegaard & Perry). Reactions were stopped by the addition of 50 μl of 1% sodium fluoride solution, and the optical density at 620 nm (OD$_{620}$) was measured by using an ELISA plate reader. Uniform binding of the peptides was verified by the reactivity of bound peptides with a 1:2,500 dilution of HRP-conjugated goat antibovine antibody (Kirkegaard & Perry) prior to blocking in milk. The nonspecific background of each assay was determined by using serially diluted preimmune serum for each peptide. Endpoint antibody titers were determined by linear regression analysis of signal intensity plotted against serum dilutions. The percentage of antibody titer was calculated (see Fig. 6A) for each serum sample by dividing the antibody titer for each peptide by the cumulative titer for all four peptides utilized in this assay.

**Construction of pRB21 vaccinia transfer vectors and mutagenesis.** Recombinant vaccinia viruses expressing CAEV-63 SU-W, glycosylated SU-M, and truncated SU-T were produced by using a pV7 variant of vaccinia virus at a multiplicity of infection of 0.1 PFU per cell and incubated at 37°C with serum-free Dulbecco modified Eagle medium. Medium containing soluble SU was collected daily for 4 days. Medium was clarified by centrifugation and filtration (0.22-μm pore-size filter) as previously described (29) and dialyzed overnight against PBS. Triton X-100 was added for a final concentration of 0.1%, and glycoproteins were purified by multiple cycles of chromatography on columns (1 by 10 cm) of lentil lectin-Sepharose (Amersham Pharmacia Biotech) (13). Columns were washed with 10 volumes of PBS containing 0.1 M NaCl, 0.1% Triton X-100, followed by 5 volumes of PBS–10 mM Tris (pH 8.0). Glycoproteins were eluted with 1.0 M methyl-α-D-mannopyranoside (Sigma-Aldrich) in PBS–10 mM Tris (pH 4.5). Fractions were dialyzed against three changes of sterile water and concentrated 20 times by using Spectra/Gel Absorb (Spectrum Laboratories). The yield of recombinant SU (Pierce BCA assay) was estimated to be 2 to 5 mg per roller bottle of rRB vaccinia-infected cells. Glycoproteins utilized in experiments were quantified by total protein quantitation (Pierce BCA assay), and the purity was ≥80% as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by Coomassie blue and silver stains (Bio-Rad). Gel mobility shift assays were used to evaluate apparent molecular mass differences due to additional N-linked glycans (SU-M) or amino acid deletions (SU-T). Reduced SU-W, SU-M, and SU-T preparations were reduced with or without deglycosylation by PNGase F digestion (43) were evaluated by Western blotting with serum from a CAEV SU-immunized goat (22).

**Immunization schedule.** Saanan goats derived from a CAEV-free breeding herd maintained at Washington State University were immunized subcutaneously with 30 μg of recombinant SU-W, SU-T, or SU-M mixed with 2 mg of Quil A saponin (Baker). Proteins were quantified by total protein quantitation (Pierce BCA assay). Six goats were in each immunogen group and designated by number as follows: 01, 08, 12, 13, 18, and 37 for SU-W-immunized goats; 02, 09, 14, 22, 28, and 30 for SU-T-immunized goats; and 31, 32, 36, 38, 39, and 41 for...
SU-M immunized goats. All goats were boosted at 3, 7, and 16 weeks after primary immunizations with the same concentrations of protein and adjuvant.

CAEV SU ELISA. An indirect ELISA was utilized to determine SU binding antibodies following immunizations. Soluble, lentiviral-produced SU-W released into the medium of CAEV-63-infected GSM cells was purified by MAbs F7-299 affinity chromatography (22, 43). Proteins were quantified by total protein quantification (Pierce BCA assay). Immunol II plates (96-well) were coated overnight at 4°C with 400 ng per well of MAAb-purified SU-W (CAEV-63) in 100 μl of 0.1 M sodium carbonate binding buffer (pH 9.6). Plates were blocked with 10% sterile horse serum at 37°C for 1 h, and duplicate wells were incubated for 1 h with 100 μl of serially diluted goat sera in PBS. Plates were washed with PBS-Tween, and wells were incubated with 100 μl of HRP-conjugated rabbit anti-goat IgG diluted 1:4,000 in PBS with 10% horse serum. Peroxidase conjugates reacted with tetramethyl benzidine were quantified by measuring the OD450 and anti-SU antibody titers were determined as described above for the peptide ELISA. The assay background was determined by using serial dilutions of preimmunization sera. An additional ELISA (cross-binding ELISA) was performed with sera from immunized goats 1 week following the second SU immunization (week 3) and recombinant vaccinia virus-derived antigens. This ELISA utilized recombinant, lentil lectin-purified SU-W, SU-T, and SU-M (200 ng/well) coated on Immunolon II plates according to the method described above. Anti-SU-W, anti-SU-T, and anti-SU-M antibody titers were determined as described above for each immunized goat.

Virus neutralization assay. Sera from SU-immunized goats were evaluated for neutralization of homologous CAEV-63 and heterologous CAEV isolates Co and 1g5 by using an infectivity titer reduction assay based on the formation of syncytia in GSM cells (34). Virus neutralization reactions, performed in triplicate, contained 0.1 ml of heat-inactivated test serum and 0.1 ml of virus at a predetermined infectivity titer. Control virus titers were determined by three replicate infectivity titrations in the presence of preimmunization sera or serum from a CAEV-negative goat. Positive neutralization was defined as a reduction of the virus infectivity titer to less than the 95% confidence interval (CI) of the mean control titer and expressed as percent virus titer reduction. Neutralizing antibody titers were determined by extrapolation of the highest serum dilution that neutralized 50% of the control virus titer.

RESULTS

Identification of target epitopes for SU modification. CAEV gp135 SU is a glycoprotein of 543 to 550 amino acids with 22 cysteine residues that are highly conserved among independent CAEV isolates and CAEV quasispecies from individual animals. CAEV SU contains approximately 23 potential N-linked glycosylation sites that exhibit minor variations in number and location among isolates and five regions of amino acid sequence variability (V1 to V5) that distinguish geographically distinct viruses (27, 50, 56) (Fig. 1). Immunodominant linear B-cell epitopes recognized by field sera and sera from experimentally infected goats were mapped previously by using a random Agt11 expression library of CAEV env supplemented by Western blotting of recombinant histidine fusion proteins (5), as well as peptide-scanning analysis of CAEV SU (56). These studies localized major immunodominant linear epitopes within SU regions, designated SU 2, SU 3, and SU 4 (Fig. 1). These regions of SU are relatively deficient in cysteine residues and potential N-linked glycosylation sites (Fig. 1). In contrast, the relatively complex V4 (particularly the HV2 region of V4) (Fig. 1) and V1 domains are thought to be important regions of SU involved in virus neutralization. Sequence analyses of neutralization variants (1, 19, 52) and site-directed mutagenesis experiments (I. Hötzel and W. P. Cheevers, unpublished data) indicate the presence of V1 and V4 domains that constitute elements of discontinuous neutralization epitopes and/or other amino acid sequences that influence exposure of neutralization epitopes.

Epitope mapping experiments in the present study were designed to determine if anti-SU antibody responses induced initially in CAEV infection are similar to those previously determined with field sera and sera from experimentally infected goats (5, 56). In this study, four yearling goats were infected with CAEV-Co, and epitope mapping experiments were conducted to localize immunodominant linear epitopes of CAEV-63 SU early in infection. Mapped epitopes are targets for deletion or introduction of N-linked glycosylation sites to address the hypothesis that recognition of immunodominant linear nonneutralization and type-specific epitopes results in restricted recognition of conformational cross-reactive neutralization epitopes (14, 22, 39, 57).

Western blots of the histidine fusion proteins SU 2, SU 3, and SU 4 were used to identify immunodominant linear epitopes recognized by four CAEV-infected goats early in infection. Analogous to results previously reported from studies of field sera and experimentally infected goats (5), results reported here demonstrate that SU 4, defined by the carboxy-terminal 62 amino acids of SU (Fig. 1), is the primary immunodominant domain recognized by the initial antibody responses of goats experimentally infected with CAEV (Table 1). To identify the immunodominant linear epitopes in SU 4 early in CAEV infection, eight 14-mer synthetic peptides with seven amino acid overlaps (Fig. 1) were reacted with serial dilutions of goat sera in a peptide ELISA. The highest positive dilution of goat serum for each SU 4 peptide is shown in Table 1. Similar to results previously reported from studies of field
sera from CAEV-infected goats at an unknown duration of infection (56), results reported here demonstrated that peptides VI, VII, and VIII, corresponding to SU 4 amino acids 524I to 550R (Fig. 1), were the most immunoreactive epitopes early in CAEV infection.

The data in Table 1 in conjunction with results from previous CAEV epitope mapping studies (5, 56) were used to design two modified CAEV-63 SUs to test the hypothesis that glycosylation or deletion of immunodominant linear epitopes in SU 4 will enhance neutralizing antibody responses following protein immunization. Specifically, N-linked glycosylation sites were inserted by RS39S and E542N mutations for the production of SU-M, and termination codons were inserted at 519Y and 521Y for the production of SU-T. The localization of these mutations in SU 4 is shown in Fig. 1.

Western blotting of SU-M and SU-T. SU-M, SU-T, and SU-W as a control were produced in vaccinia virus and purified and quantified as described in Materials and Methods. Glycosylation of SU-M and truncation of SU-T were evaluated in gel mobility shift assays. Reduced SU with or without deglycosylation with PNGase F was analyzed by Western blotting with serum from a goat immunized with soluble MAb-purified PC (see Results). SU-W, SU-T, and SU-M. Data are the means of three virus infectivity determinations. Bars, ±95% CI.

Exposure of neutralization epitopes on SU-M and SU-T. To ensure exposure of the neutralization epitopes on the modified immunogens, SU-M, SU-T, and SU-W purified by lentil lectin chromatography were tested for adsorption of neutralizing antibodies from goat serum. Results are shown in Fig. 3. Virus-neutralizing serum from CAEV-infected goat 8517 (Fig. 3, serum control [SC]) without the addition of glycoproteins neutralized >90% of the virus input titer (2287 TCID50 of CAEV-63 determined in the presence of nonneutralizing SC from goat 8505). The incubation of neutralizing goat 8517 serum with 30 μg of SU-W, SU-M, or SU-T removed CAEV-63-neutralizing antibodies. The specificity of neutralizing antibody adsorption was determined by two control experiments. (i) A protein control (PC), representing concentrated contaminating proteins in the lentil lectin affinity-purified SU, was derived by lentil lectin chromatography of medium from GSM cells infected with vRBB1 vaccinia virus not containing the env gene sequence. The incubation of 30 μg of PC with goat 8517 serum did not remove CAEV-63-neutralizing antibodies (Fig. 3). (ii) SU-W, SU-M, or SU-T or the PC incubated with negative SC from goat 8505 did not significantly affect the infectivity titer of CAEV-63. The results shown in Fig. 3 indicate that SU 4 modifications did not alter the exposure of CAEV-63-type-specific neutralization epitopes on soluble recombinant SU and that purification of SU by lentil lectin chromatography does not significantly alter exposure of CAEV-63 neutralization epitopes.

Serum antibody titers following immunization with SU-W, SU-T, or SU-M. Figure 4 shows SU antibody titers determined by indirect ELISA of MAb-purified, CAEV-63-produced SU following subcutaneous immunizations of three groups of six goats per group with SU-W, SU-T, or SU-W formulated in Quill A adjuvant. Relatively low SU antibody titers resulting from primary immunizations increased and then declined following booster immunizations at weeks 3, 7, and 16. Five of six goats boosted with SU-M at week 3 had higher anamnestic responses than goats boosted with SU-W or SU-T. This enhanced response in SU-M-immunized goats was not detected following subsequent boosts at week 7 or week 16. Figure 5 shows the mean binding SU-W, SU-M, and SU-T antibody titers at week 4 of all immunized goats within each immunization group against each recombinant vaccinia virus-produced glycoprotein 1 week following the second protein boost (cross-binding ELISA). We attribute the lower overall SU-W antibody titers in this experiment compared to data shown in Fig. 4 to a 50% reduction in the antigen used in the cross-binding ELISA (200 ng/well). Data in Fig. 5 demonstrate three points. (i) The SU-T antigenicity is significantly reduced with regard to the binding of anti-SU antibodies present in sera from SU-W-immunized goats. A similar trend in reduced antigenicity was seen when SU-T was reacted to sera from the SU-M-immunized goats, but this finding was not statistically significant. (ii) There was no detectable altered antigenicity of SU-M in week 4 sera from immunized goats. This observation could be due to variable glycosylation of the inserted glycans within the carboxyl terminus of SU-M; therefore, altered recognition of the glycosylated epitopes cannot be detected at this time point. Conversely, the insertion of glycans could have resulted in the generation of new glycan-dependent epitopes. However, this observation is less likely since glycan determinants of epitopes...
FIG. 4. SU antibody responses in goats immunized with wild-type or modified SU. Six goats per group were immunized subcutaneously with 30 μg of soluble SU-W (A), SU-T (B), or SU-M (C) in 2 mg of Quil A adjuvant and boosted at 3, 7, and 16 weeks (arrows). SU antibody titers for individual immunized goats (designated by numbers) were determined by using serial dilutions of sera reacted against 400 ng of MAb-purified CAEV-63 SU-W by indirect ELISA.
are rare and would not explain a lack of reduced antigenicity of SU-M when reacted with sera from SU-W-immunized goats. (iii) SU-W, SU-T, and SU-M antigens bind antibodies present in sera from SU-T-immunized goats equally, suggesting that immune responses elicited by immunization with SU-T altered recognition of the SU immunodominant domains SU 2, SU 3, and SU 4. Results of this experiment warranted further investigation into the altered antigenicity of modified proteins following additional immunizations.

Altered recognition of linear SU epitopes in immunized goats. Apart from the increased anamnestic response following the first boost with SU-M, there was no difference in the development and decay of SU antibody titers between groups of goats throughout the immunization schedule (Fig. 4). To evaluate the reactivity of mapped immunodominant linear SU epitopes, antibody titers against histidine fusion proteins SU 2, SU 3, and SU 4 were determined for all immunized goats at weeks 3, 4, 9, and 17. These sera corresponded to 3 weeks after primary immunizations with SU-W, SU-T, or SU-M (week 3); at 1 and 4 weeks after the first boost (weeks 4 and 7); at 1, 2, and 9 weeks after the second boost (weeks 8, 9, and 16); and at 1 week after the third boost (week 17). All SU-immunized goats had CAEV-63 neutralizing antibodies at week 17, corresponding to 1 week after the third boost (Fig. 7).

For direct comparison of immunoreactivity to each immunodominant domain, regardless of the overall SU binding antibody titer, the percentage of antibody titer was calculated and reported for each serum sample by dividing the antibody titer for each peptide by the cumulative titer for all four peptides. Results demonstrated a shift in SU-M-induced antibody responses from peptides VII and VIII to peptide VI at week 17 (Fig. 6B). Thus, the 537NRS and 542NLS glycosylations in SU 4 epitopes represented by peptides VII and VIII reduced the immunogenicity of these epitopes and shifted antibody responses to an adjacent SU 4 epitope represented by peptide VI. The reduced immunogenicity of glycosylated epitopes was not statistically significant prior to week 17 (data not shown).

Type-specific and cross-reactive neutralizing antibody responses of goats immunized with SU-W, SU-T, or SU-M. Neutralizing antibody responses against homologous CAEV-63 were assayed at 3 weeks after primary immunizations with SU-W, SU-T, or SU-M (week 3); at 1 and 4 weeks after the first boost (weeks 4 and 7); at 1, 2, and 9 weeks after the second boost (weeks 8, 9, and 16); and at 1 week after the third boost (week 17). All SU-immunized goats had CAEV-63 neutralizing antibodies at week 17, corresponding to 1 week after the third boost (Fig. 7).

Cross-reactive neutralizing antibodies against CAEV-Co and CAEV-1g5 were evaluated at week 17, when all goats were positive for type-specific CAEV-63 neutralizing antibodies. The data in Fig. 8 show that (i) all goats immunized with SU-W, SU-T, or SU-M developed cross-reactive neutralizing antibodies to the closely related Co isolate of CAEV and (ii) all goats except SU-T-immunized goat 28 and SU-M-immunized goat 41 developed cross-reactive neutralizing antibodies to the more distantly related 1g5 isolate of CAEV. Similar experiments demonstrated that most goats with CAEV-63 neutralizing antibodies at week 9 also had cross-reactive neutralizing antibodies to both CAEV-Co and CAEV-1g5 (data not shown). Cross-reactive neutralizing antibody responses were not evaluated earlier than week 9.

Magnitude of type-specific and cross-reactive neutralizing antibody responses induced by SU-W, SU-T, and SU-M. Type-specific and cross-reactive neutralizing antibody titers were determined for week 17 sera from all immunized goats. The effects of serum dilution on the neutralization of CAEV-63, CAEV-Co, and CAEV-1g5 are shown in Fig. 9A, C, and E. Figures 9B, D, and F show derivative neutralizing antibody titers expressed as the means ± 95% CI of serum dilutions that neutralized 50% of each input virus infectious dose. Neutral-
izing antibody titers for SU-T sera were reduced by 2.8- to 4.6-fold compared to titers of SU-W sera \((t\text{test}: P < 0.026\text{ for CAEV-63, } P = 0.027\text{ for CAEV-Co, and } P = 0.004\text{ for CAEV-1g5})\). Thus, deletion of 32 carboxy-terminal amino acids significantly reduced the ability of SU to induce neutralizing antibodies. In contrast, neutralizing antibody titers for SU-M sera were increased by 2.4- to 2.7-fold compared to titers of SU-W sera \((t\text{test}: P = 0.003\text{ for CAEV-63, } P = 0.029\text{ for CAEV-Co, and } P = 0.030\text{ for CAEV-1g5})\). Thus, the addition of N-linked glycosylation sites into carboxy-terminal epitopes significantly enhanced the ability of SU to induce neutralizing antibodies.

**DISCUSSION**

This study tested the hypothesis that the deletion or glycosylation of immunodominant linear epitopes on CAEV SU will redirect antibody responses toward neutralization epitopes following immunization. The rationale for this hypothesis was based on four principles established by previous research. (i) Immunization with monomeric CAEV SU induces cross-reactive neutralizing antibodies; however, neutralizing antibody titers are relatively low due at least in part to neutralization inhibitory antibodies directed to immunodominant nonneutralization epitopes (22). (ii) Immune recognition of discontinuous neutralization epitopes on lentiviral surface proteins is restricted by preferential expansion of B-cell clones responding to immunodominant nonneutralization or type-specific neutralization epitopes (14, 39). (iii) Glycans suppress epitope recognition by immunologic masking (14, 45, 48, 49). (iv) Glycosylation or deletion of epitopes can shift antibody responses to other epitopes (4, 14, 20, 23, 30, 45, 49). Based on these observations, we evaluated type-specific and cross-reactive neutralizing antibody responses induced by immunization with CAEV-63 SU in which carboxy-terminal immunodominant epitopes were either deleted or modified by the introduction of glycosylation sites.

Previous mapping studies of field sera and sera from experimentally infected goats (5, 56) identified linear immunodominant domains of SU, designated SU 2, SU 3, and SU 4 (Fig. 1). Antibody responses to linear SU epitopes predominate early in CAEV infection, and the recognition of discontinuous epitopes dependent on cysteine disulfide bonding increases with maturation of the immune response (unpublished data). To address the hypothesis that recognition of these immunodominant linear nonneutralization or type-specific epitopes results in restricted recognition of conformational cross-reactive neutralization epitopes, we mapped immunodominant linear epitopes recognized early in CAEV infection to identify SU linear epitopes for glycosylation or deletion. Results from our studies are supported by previously reported CAEV B-cell linear epitope mapping studies (5, 56) and identified SU 4 as the principal target of early CAEV-induced antibody responses in infected goats and localized immunodominant epitopes within three overlapping SU 4 peptides corresponding to the 32 carboxy-terminal amino acids of SU (Table 1). Based on these results, two modified SU immunogens were constructed. Recombinant SU-M had R539S and E542N mutations to create glycosylation sites in linear immunodominant epitopes represented in SU 4 peptides VII and VIII (Fig. 1). SU-T was truncated at 518A, upstream from the target epitopes, by the introduction of termination codons at 519Y and 521Y (Fig. 1).

The epitopes targeted for glycosylation correspond in part to variable region V5 of CAEV SU (56), which contains a linear
type-specific epitope (5). However, V5 amino acid sequence variability is not associated with the neutralization phenotype of CAEV isolates (19) and was, therefore, not considered a component of a neutralization epitope. The antibody response of CAEV-infected goat 9903 recognized an immunodominant epitope corresponding to SU 4 peptide III (Fig. 1). This epitope was not selected as a target for mutagenesis because it corresponds to the highly conserved β-strand 25 region of SU involved in the conformational structure of lentiviral SU (17, 18, 28).

The principal significance of our results relates to differential neutralizing antibody responses induced by SU-M and SU-T. Insertion of two N-linked glycosylation sites into carboxy-terminal linear epitopes of SU-M significantly enhanced the induction of neutralizing antibodies following immunization, whereas a 32-amino-acid carboxy-terminal deletion in SU-T significantly reduced the efficiency of SU as an immunogen for the induction of neutralizing antibodies. Enhanced responses by SU-M and reduced responses by SU-T compared to those of SU-W extended to both type-specific and cross-reactive neutralizing antibodies. Therefore, in our model, the masking of immunodominant epitopes of CAEV SU by the
The insertion of N-linked glycans, but not epitope deletion, effectively redirected antibody responses toward neutralization epitopes.

In addition to the effects of modified SU on neutralizing antibody responses, SU modifications also resulted in altered antibody responses to linear epitopes. Western blot analysis of binding antibodies to histidine fusion proteins did not detect a shift in SU-M-immunized goats from glycosylated epitopes in the carboxy terminus of SU 4 to other regions of SU, including the amino-terminal region of SU 4 (Fig. 6A). However, subsequent experiments with synthetic peptides showed that 537NRS and 542NLS glycosylations of epitopes in the carboxy terminus of SU 4 shifted antibody responses to an adjacent unglycosylated SU 4 epitope (Fig. 6B). However, we noted that the reduced immunogenicity of glycosylated epitopes was not statistically significant prior to week 17, corresponding to 1 week after the third boost with SU-M, thus explaining why the altered antigenicity of SU-M was not detected in the cross-binding ELISA of sera from SU-M-immunized goats at week 4 (Fig. 5). This delayed shift in linear epitope recognition may be due to variable glycosylation of the two inserted N-linked glycosylation sites or could be due to immune maturation. The cross-binding ELISA demonstrated no difference in SU binding antibodies in SU-T-immunized goats when reacted to SU-W, SU-T, or SU-M (Fig. 5). Moreover, Western blot analysis of binding antibodies to histidine fusion proteins detected a shift in the response of SU-T-immunized goats to linear immunodominant epitopes in the SU 3 domain (Fig. 6A). These data are consistent with the conclusion that the removal of carboxy-terminal amino acids in the inner domain of SU resulted in enhanced recognition of immunodominant linear epitopes in the outer domain.

The different recognition patterns of immunodominant SU domains identified by sera from CAEV-infected goats early in infection compared to late in infection (reference 5 and unpublished data) and following multiple immunizations with 30 µg of SU-W and SU-M are attributed to antigen dose, repeated antigenic stimulation, and maturation of humoral immune responses. It is possible that glycosylation of additional linear nonneutralization epitopes in the outer SU 3 domain of SU could result in even greater increases in the immunogenicity of the neutralization determinants of SU, provided that glycans do not detrimentally alter the conformation of SU.

These results and previous studies of HIV-1 SU suggest that the alteration of neutralizing antibody responses by modified SU may be related to the effects of modifications on immune recognition of linear epitopes in the outer domain. Several studies have been published to evaluate the role of specific variable loop deletions on the exposure of HIV-1 neutralization epitopes (4, 20, 23, 30). To our knowledge, deletion mutants of HIV-1 SU analogous to CAEV SU-T have not been analyzed. However, similar to the present results for SU-T, loop deletions in HIV-1 SU redirected antibody responses to epitopes that mapped primarily to conserved regions and either eliminated or reduced neutralizing antibody responses. One interpretation of the SU-T results is that the carboxy terminus of CAEV SU contains elements of the neutralization epitopes. However, this possibility is considered unlikely since SU-T adsorbed CAEV-63 neutralizing antibodies from goat serum, demonstrating that SU-T retains at least type-specific
neutralization epitopes or the correct conformation for the retention of these neutralization epitopes (Fig. 3). In this regard, we note that assessments of epitope antigenicity by using soluble proteins in vitro do not necessarily predict epitope exposure or immunogenicity in vivo. Our results show that deletion of carboxy-terminal amino acids in the inner proximal domain of SU enhanced the focus of antibody responses to linear epitopes that map to putative β-strand regions of the outer domain (18), with a consequent reduction of neutralizing antibody responses. Therefore, similar to previous results of loop deletions in HIV-1 SU, modifications of CAEV SU that favor the enhanced recognition of linear epitopes provide an

FIG. 9. Type-specific and cross-reactive neutralizing antibody titers following SU immunizations. Neutralizing antibody titers were determined by using neutralization-positive sera at week 17. Data in panels A, C, and E represent the effects of serum dilutions on the neutralization of CAEV-63, CAEV-Co, or CAEV-1g5. Data represent the mean percent reduction (± standard error of the mean) of control virus titer by sera from each immunized goat determined in triplicate. Control virus titers determined in triplicate in the presence of serum from uninfected control goat 8505 were 470 ± 54 TCID₅₀ (95% CI) for CAEV-63, 523 ± 92 TCID₅₀ for CAEV-Co, and 284 ± 53 TCID₅₀ for CAEV-1g5. Data in the panels B, D, and F represent mean neutralizing antibody titers (± 95% CI) for each group of immunized goats determined by extrapolation of the highest serum dilution that neutralized 50% of the control virus titer.
neutralizing antibodies in the inner domain of SU-M enhanced CAEV neutralizing antibody responses without enhancing the immunogenicity of linear epitopes in the outer domain. These results suggest that the effective refocusing of antibody responses toward neutralization epitopes requires the suppression of responses to immunodominant linear epitopes in the inner domain without a consequent increase in the immunogenicity of other linear epitopes, particularly those within the outer domain. This interpretation is supported by previous studies in which various strategies were evaluated to enhance the exposure and immunogenicity of neutralization epitopes on HIV-1 by glycosylation of nonneutralizing or type-specific neutralization epitopes (14, 45, 48, 49). In one study (14), glycosylation of a linear epitope in V3 of a T-cell-adapted strain of HIV-1 shifted the neutralizing antibody response to a V1 epitope with a somewhat broader specificity for neutralization of other T-cell-adapted strains. In another study, which evaluated the binding of antibodies to primary HIV-1 isolates, the hyperglycosylation of selected variable loops and core domains of the SU of HIV-1 JR-FL eliminated binding of a panel of nonneutralizing and weakly neutralizing antibodies but retained low affinity binding of the broadly neutralizing MAb b12 (45).

In summary, the immunization of goats with CAEV SU containing glycosylated immunodominant epitopes induced significantly higher titers of type-specific and cross-reactive neutralizing antibodies compared to titers of SU-W, indicating that glycosylation shifted immune responses from linear epitopes to neutralization epitopes. In contrast, the removal of carboxy-terminal epitopes shifted antibody responses to linear epitopes within another immunodominant domain of SU, resulting in lower neutralizing antibody titers compared to titers of SU-W. Therefore, masking of carboxy-terminal immunodominant epitopes of CAEV SU, but not deletion of these epitopes, is an effective strategy for directing immune responses to neutralization epitopes.

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