Involvement of the C-Terminal Disulfide-Bonded Loop of Murine Leukemia Virus SU Protein in a Postbinding Step Critical for Viral Entry

Michael D. Burkhart, Paul D’Agostino, Samuel C. Kayman,* and Abraham Pinter*  
Laboratory of Retroviral Biology, Public Health Research Institute, Newark, New Jersey 07103

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Entry of retroviruses into host cells requires fusion of the viral envelope with the cellular or endosomal membrane, a process mediated by the envelope glycoprotein complex (Env) (15, 39). Env is organized in trimeric complexes of heterodimers consisting of surface (SU) and transmembrane (TM) subunits (17, 27). Viral entry is initiated by binding of SU to specific cell surface receptors that, for murine leukemia virus (MuLV), are multitransmembrane proteins that function as transporters (1, 5, 6, 9, 10, 19). The receptor binding activity of MuLV SU has been localized to the N-terminal 230 amino acids (2, 14), and considerable information about this domain was derived from determination of its crystal structure (12). The receptor-binding domain (RBD) is linked via a prolinerich region (PRR) to the C-terminal domain (CTD) of SU, which is joined to TM by a labile disulfide bond (24, 28, 32, 37). Membrane fusion mediated by retroviral Env proteins involves insertion of a hydrophobic, N-terminal region of TM into the target membrane, followed by rearrangement of TM heptad repeats into a six-helix coiled-coil bundle that brings the viral and target membranes into close proximity (38). Recent evidence suggests that following receptor binding, the N terminus of the RBD transmits a signal through the CTD that triggers the fusion activity of TM (3, 4, 20–22). SU and TM in native Env complexes are joined by a metastable disulfide bond (28, 33) that involves the CWLC sequence in the CTD that resembles the CXXC motif found in the active site of thiol-exchange enzymes (32). Although little is known about the structures and mechanisms by which the CTD transduces the signal initiated by receptor binding, it has been suggested that isomerization of this disulfide bond may be an essential step in this process (36, 37).

We have recently identified two neutralization targets in SU, one in the receptor binding pocket of the RBD, and one involving a small region that includes the most C-terminal disulfide-bonded loop of the CTD (designated loop 10) (7). The two CTD-specific rat monoclonal antibodies (MAbs) used in that study, 35/56 (23) and 83A25 (11), have distinct strain specificities. Both of these MAbs neutralize MuLV infectivity by blocking a step prior to viral fusion but after receptor binding (7), and additional definition of these epitopes and characterization of the structure and function of this region were therefore expected to provide new insight into the functions of the CTD during viral infection. In the present study, residues within loop 10 that determined the specificities of these MAbs were identified, a requirement was demonstrated for additional residues outside of loop 10 for reactivity with these MAbs, and mutations at specific sites in loop 10 were identified that differentially affected MuLV Env processing, virion association, and viral fusion.

MATERIALS AND METHODS

Cell lines and antibodies. XC rat sarcoma cells, mouse NIH 3T3 fibroblasts, and human 293 fibroblasts were cultured in complete Dulbecco modified Eagle medium (DMEM [Gibco] supplemented with 10% fetal bovine serum [Sigma], 1% l-glutamine, and 1% penicillin/streptomycin [Gibco]) unless otherwise indicated. 293 cells expressing mCAT-1t (293.mCAT) were provided by James Cunningham and were cultured under G418 selection as described previously (9). The MuLV SU-specific rat MAbs, 35/56 (23) and 83A25 (11), and the human MAbs, 59C9, directed against a conformational epitope involving the receptor binding pocket of MuLV SU (7) were previously described. MAbs were purified from hybridoma cell supernatants using GammaBind G Sepharose or protein A.
Sepharose Fast Flow (Amersham Pharmacia Biotech AB) according to the manufacturer’s recommendations. Goat polyclonal sera raised to Rauscher MuLV SU and capsid (CA) were purchased from Quality Biotech, Camden, N.J. The antithemagglutinin (anti-HA) rat MAB 3F10 and the anti-μc murine MAB 9E10 were obtained from Roche. MAbs and sera were detected in immunological assays with the appropriate species-specific immunoglobulin G or sera conjugated to alkaline phosphatase or fluorescein isothiocyanate (Zymed).

Viruses and viral neutralization assays. NIH 3T3 cells chronically infected with MuLVs bearing Env substitution mutations were prepared by transfection of CDNA clones of these MuLVs into NIH 3T3 cells and passage of the cells until infection approached 90% as assayed by immunofluorescence (7, 18). These cells were then used as a source of virions for immunofluorescence-based neutralization assays, which were performed as described previously (7, 18). Percent neutralization was calculated as follows: [1 - (percent infection/percent infection of control wells)] × 100. The 50% neutralization dose (ND50) is defined as the concentration of MAb that reduced the number of infected cells by 50% relative to a control infection in the absence of antibodies.

The functional activities of insertion and deletion mutants in Env were quantitated by luciferase assays using lac-expressing virions pseudotyped with mutant Env proteins. These pseudotypes were produced by transecting 293 cells with a mixture of three plasmids (a retroviral vector plasmid expressing luciferase, an expression plasmid for Friend FB29 MuLV Gag/Pol, and an expression vector for the Env protein) using Fugene reagent (Roche Biochemicals) essentially as described previously (7, 35). Forty-eight-hour posttransfection, the cell supernatant was harvested and clarified by filtration through a 0.45-μm syringe filter and used in infectivity assays versus NIH 3T3 cells. The MuLV pseudotypes were normalized by Western blotting for CA levels and used in infectivity assays versus NIH 3T3 cells.

RESULTS

Mapping determinants of the MAB 35/56 and 83A25 epitopes to polymorphic sites in loop 10. The region responsible for the differential reactivity of MAbs 35/56 and 83A25 for AKR and Friend MuLV clone 57 Env was previously mapped to the AKR sequence from this region, using the Friend/AKR(393-426) chimera. Plasmids containing wild-type or mutant proviral DNAs were transfected into NIH 3T3 cells, which single or multiple Friend MuLV-specific residues were placed into the AKR sequence from this region, using the Friend/AKR(393-426) chimera. Plasmids containing wild-type or mutant proviral DNAs were transfected into NIH 3T3 cells, and the spread of infection was monitored by immunofluorescence. All of the substitution mutants were as infectious as the parental virus. The effects of the substitutions on the MAb 35/56 and 83A25 epi-

topes were assessed by the extent of fusion that occurred in the presence of each MAb. The extent of fusion was quantitated by counting the number of nuclei present in syncytia containing more than four nuclei under the microscope.

Cell binding and fusion assays. Fluorescence-activated cell sorting (FACS) assays to detect MuLV binding were performed as described previously (7, 40). Briefly, 293 mCAT cells were detached with trypsin-EDTA, and a total of 5 × 10⁶ cells were incubated with 1 ml of pseudotype MuLV particles at 4°C for 2 h with gentle agitation. Cells were washed with 1 ml of ice-cold 10% fetal bovine serum in PBS (FACS buffer) and resuspended in polyclonal goat anti-Rauscher MuLV SU serum diluted 1:250 in FACS buffer. Cells were incubated for an additional 1 h at 4°C, washed again, and incubated with fluorescein isothiocyanate-conjugated anti-goat immunoglobulin G (Zymed) at 5 μg/ml in FACS buffer. After a 0.5-h incubation at 4°C, cells were washed again, fixed in 4% paraformaldehyde in PBS, and analyzed by flow cytometry using a FacsCalibur flow cytometer (Becton Dickinson) and associated software.

Synctia formation by MuLV pseudotypes on XC cells was assayed as previously described (26). Briefly, XC cell monolayers were incubated with cell culture supernatants containing pseudotyped MuLV particles for 2.5 h of incubation at 37°C. After the cells were washed, fixed with methanol, and stained with cresyl violet, the extent of fusion was quantitated by counting the total number of nuclei present in synctia containing more than four nuclei under the microscope.

Friend/AKR (393-426) SU

| RBD | PRR | CTD |
|-----|-----|-----|
|---- |---- |---- |
|---- |---- |---- |
|---- |---- |---- |

Fig. 1. (Top) Diagram of the Friend/AKR (393-426) chimera (SU). (Left) Region responsible for the differential reactivity of MAbs 35/56 and 83A25 for AKR and Friend MuLV clone 57 Env was previously mapped to the AKR sequence from this region, using the Friend/AKR(393-426) chimera. Plasmids containing wild-type or mutant proviral DNAs were transfected into NIH 3T3 cells, and the spread of infection was monitored by immunofluorescence. All of the substitution mutants were as infectious as the parental virus. The effects of the substitutions on the MAb 35/56 and 83A25 epitopes were assessed by the extent of fusion that occurred in the presence of each MAb. The extent of fusion was quantitated by counting the number of nuclei present in synctia containing more than four nuclei under the microscope. (37). The effects of pretreatment with MAb 83A25 (20 μg/ml for 1 h at 4°C) on immunofluorescence reactivities (7).
the polymorphisms at positions 410 and 411 (Table 1). Neutralization and indirect immunofluorescence assay activities of both MAbs were completely abolished by the L411R substitution. MAb 35/56 also did not react with the D410N mutant, while MAb 83A25 retained a low level of reactivity with this mutant. The other individual substitutions had relatively small effects on the reactivities of these MAbs. The T406A substitution reduced neutralization end points threefold for MAb effects on the reactivities of these MAbs. The T406A substitu-

| MuLV          | Sequencea (residues 397 to 411) | Env processing and stability | MAb 35/56 | MAb 83A25 |
|---------------|---------------------------------|-----------------------------|-----------|-----------|
|               |                                  |                             | ND50b     | IF reactivityc | ND50b | IF reactivityc |
| AKR.623       | STGLTPCISTTILDL                  | Normal                      | 0.15      | +           | 0.12  | +           |
| N--L--------  | Normal                          | 0.08                        | +         |             | 0.15  | +           |
| --------A----- | Normal                          | 0.09                        | +         |             | 0.15  | +           |
| --------V----- | Normal                          | 0.47                        | +         |             | 0.88  | +           |
| --------N----- | Normal                          | 0.29                        | +         |             | 0.18  | +           |
| --------L----- | Normal                          | >10                         | -         | >10         | +/-   |             |
| --------R----- | Normal                          | >10                         | -         | >10         | -     |             |
| N--L--------  | Normal                          | 0.20                        | +         |             | 0.43  | +           |
| N--L--------  | Normal                          | 5.00                        | +/-       |             | 0.30  | +           |
| N--L-A-V----- | Normal                          | >10                         | -         | >10         | +/-   |             |
| N--L-A-V-N--  | Normal                          | >10                         | -         | >10         | -     |             |
| Friend clone 57 | N-------L-A-V-V-NR            | Normal                      | >10       | -         | >10       | - |
| Moloney clone 1 | N-------------N--            | Normal                      | >10       | -         | >10       | +/- |
| 4070A         | N-------L-A-V-V-N--            | Normal                      | >10       | -         | 6.50     | +/- |

a Substitutions were introduced into the Fr/AKR (393–426) chimeric Env.

b Concentration of MAb (μg/ml) that reduced infectivity by 50%. >10, no neutralization observed at 10 μg/ml; >10, neutralization below 50% observed.

c Reactivity of MAbs as determined by immunofluorescence (IF). Symbols: +, strong fluorescence; +/-, weak fluorescence; –, no fluorescence.

The loop 10 region was expressed in the absence of other SU sequences as a fusion protein in which this loop and a few adjoining residues were flanked by HA and myc epitope tags, and it was expressed as a cell surface protein by linking to a C-terminal transmembrane domain (construct 1 in Fig. 2A). After immunoprecipitation of lysates of cells expressing this construct with an antibody to the myc epitope, a band of the expected size was detected in Western blots by probing with an antibody to the HA epitope tag (Fig. 2B, left panel). The mobility of this band was slightly higher when the sample was analyzed under nonreducing conditions than under reducing conditions, suggesting the presence of the disulfide bond at the base of loop 10. When the anti-myc immunoprecipitate was probed with the 83A25 and 35/56 MAbs, no reactivity was observed (Fig. 2B, right panel), consistent with the lack of immunofluorescent staining of these cells when probed with these MAbs (not shown). These results suggested that loop 10 and the immediate adjoining sequences expressed in this fusion protein were not sufficient for expression of these epitopes. A similar lack of reactivity was observed for a related protein that also contained the additional SU sequences C terminal to loop 10 (not shown), suggesting that residues N terminal to position 397 were required for expression of these epitopes.

Cells expressing either secreted full-length SU or a secreted SU fragment that terminated at position 420, four residues C terminal to loop 10 (constructs 2 and 3 in Fig. 2A), were both positive in immunofluorescent staining assays with the two loop 10-dependent MAbs. The fluorescence intensity of cells expressing the truncated molecule was however lower than that of cells expressing full-length SU (not shown). When the reactivity of these proteins was examined quantitatively by radioimmunoprecipitation, striking differences were observed in the percentage of molecules that expressed these epitopes (Fig.
The C-terminal loop of MuLV SU protein.

To identify which region of the loop was responsible for this processing defect, contiguous three-residue sequences in loop 10 were substituted with GAG (Fig. 3A). Whereas the GAG 404-406 mutant resembled that of the GAG 404-409 mutant in that no SU protein was associated with virions, the GAG 404-409 mutant contained only unprocessed Env precursor even at the latest time points. The absence of any intermediate gPr90 product typically formed upon transport into the Golgi bodies (18) suggested that this loss of residues 404, 405, and/or 406 accounted for the defectiveness of the unprocessed Env precursor even at the latest time points. The absence of any intermediate gPr90 product typically formed upon transport into the Golgi bodies (18) suggested that this loss of residues 404, 405, and/or 406 accounted for the defectiveness of the unprocessed Env precursor even at the latest time points. The absence of any intermediate gPr90 product typically formed upon transport into the Golgi bodies (18) suggested that this loss of residues 404, 405, and/or 406 accounted for the defectiveness of the unprocessed Env precursor even at the latest time points.

Effects of loop 10 mutations on Env function. The potent neutralizing activity and fusion-blocking activity of MAbs 35/56 and 83A25 suggested a role for loop 10 and/or adjacent regions in postbinding function of Env. In an attempt to confirm such a role for this domain and to localize the residues involved, mutations in loop 10 were generated and their effects on infectivity determined using luciferase-encoding viral pseudotypes (Table 2). The general requirement of this loop for Env function was first examined by replacing the 12 central residues of the 12-residue loop of the Fr/AKR(393-426) chimera (positions 405 to 414) with a glycine-alanine-glycine (GAG) tripeptide (Δ loop 10). This mutant was completely defective in the MuLV/luciferase pseudotype assay (Fig. 3A). An analysis of the levels of SU incorporated into virions by radioimmunoprecipitation of labeled virions showed that this lack of infectivity of Δ loop 10 was due to the absence of SU from viral particles (Fig. 3B). The basis of this defect was examined by a pulse-chase analysis (Fig. 3C). After a 30-min pulse, the Env protein of wild-type Fr/AKR(393-426) existed mostly in the form of the gPr80 precursor, with some mature SU also present, while after the 1-h chase period, almost all of the gPr80 was converted to SU, which remained cell associated. In contrast to this, cells producing Δ loop 10 Env contained only unprocessed Env precursor even at the latest time points. The absence of any intermediate gPr90 product typically formed upon transport into the Golgi bodies (18) suggested that this mutant Env was defective in transport from the endoplasmic reticulum, presumably due to improper folding.

To identify which region of the loop was responsible for this processing defect, contiguous three-residue sequences in loop 10 were substituted with GAG (Fig. 3A). Whereas the GAG 407-409, 410-412, and 413-15 substitution mutants all induced levels of luciferase activity similar to those induced by the control Env, the GAG 404-406 mutant possessed no activity. The defect of the GAG 404-406 mutant resembled that of the Δ loop 10 mutant in that no SU protein was associated with virions (Fig. 3B) and lysates of transfected cells contained only the unprocessed Env precursor (Fig. 3C). This suggested that the loss of residues 404, 405, and/or 406 accounted for the defectiveness of Δ loop 10.
To further pinpoint the contribution of these residues to the defectiveness of these mutants, individual substitutions were prepared at each of the first three positions of the loop. 404G is found in other natural sequences, and 404L and 406A are present in the Friend MuLV Env, and thus, it was presumed that these substitutions would retain function. I404A, S405A, and T406G mutants were generated and the resulting pseudovirions analyzed for Env incorporation levels and functional activity (Table 2 and Fig. 3). The I404A mutant possessed wild-type levels of infectivity and a normal level of SU in virions. The T406G mutant also possessed the normal amount of SU and TM and that additive effects of changes at residues 405 and 406 contributed to the defectiveness of these mutants, individual substitutions were prepared at each of the first three positions of the loop. 404G is found in other natural sequences, and 404L and 406A are present in the Friend MuLV Env, and thus, it was presumed that these substitutions would retain function. I404A, S405A, and T406G mutants were generated and the resulting pseudovirions analyzed for Env incorporation levels and functional activity (Table 2 and Fig. 3). The I404A mutant possessed wild-type levels of infectivity and a normal level of SU in virions. The T406G mutant also possessed the normal amount of SU and TM and that additive effects of changes at residues 405 and 406 contributed to the defectiveness of the GAG 404-406 mutant.

**Effects of loop 10 mutations on epitope expression.** The effects of the mutations in loop 10 described above on the expression of the epitopes recognized by MAb 35/56 and 83A25 were examined by immunofluorescence assays on transfected cells and, for infectious mutants, by neutralization assays (Table 2). The Δ loop 10 mutant and the first three GAG substitutions, encompassing residues 404 to 412, were not reactive with either MAb 35/56 or 83A25. This was consistent with the contributions of residues in the region of positions 404 to 411 to these epitopes (Table 1). The GAG(413-415) substitution mutant retained reactivity with both MAbs and was considerably more sensitive to neutralization by these two MAbs, with ND50, 35- to 45-fold lower than that for the wild-type Env. The sensitivity of this mutant to neutralization by the RBD-specific MAb 59C9 was similar to that of wild-type Env, suggesting that the substitution of these residues did not cause a general hypersensitivity to neutralization but rather induced an increased affinity or accessibility specifically for MAbs.

![Image](http://jvi.asm.org/)
83A25 and 35/56. The S405A mutation possessed the same (for MAb 35/56) or slightly higher (for MAb 83A25) neutralization sensitivity as the wild type, indicating that despite the functional defects of this mutation, it did not affect the structure or accessibility of these epitopes. However, in contrast to the natural I404L polymorphism, the I404A mutation resulted in a significant decrease (three- to eightfold) in neutralization sensitivity to both MABs, and the T406G substitution resulted in a complete loss of neutralization by both MABs, again in contrast to the natural T406A polymorphism that had only a small effect on neutralization (Table 1). These results indicated subtle contributions of these positions to the formation of these epitopes.

Effects of insertions in the N-terminal region of loop 10 on Env function. There is a stretch of three Ser/Thr residues at positions 405 to 407 near the N terminus of AKR loop 10. In the course of producing the NLV variant (Table 1), an extraneous serine residue was serendipitously introduced adjacent to Ser405, resulting in the NLV S405+S insertion mutant. This mutant Env was partially attenuated in the luciferase assay (Fig. 3A and Table 3). Pseudoviral particles generated with this Env mutant contained a normal amount of SU (Fig. 3B), indicating that the decreased activity was not due to defects in expression, processing, or association of SU with particles. In contrast to the NLV Env, the NLV S405+S mutant was not recognized by MAB 83A25 (Table 3), consistent with the sensitivity of this epitope to other changes near the N terminus of loop 10.

The basis for the defectiveness of this mutant was further examined by measuring the extent of binding of virions pseudotyped with Friend MuLV clone 57, NLV, or NLV S405+S Env to 293.mCAT-1 cells by flow cytometry (Fig. 4A, left panels). Equivalent concentrations of the control and S405+S mutant particles displayed roughly equal fluorescent intensities, indicating no apparent disruption in the binding activity of this mutant Env to its receptor. On the other hand, the S405+S mutant was severely defective for fusion of XC cells relative to the Friend MuLV wild-type and NLV controls (Fig. 4A, right panels); no syncytia were observed in wells that received the S405+S mutant, while control viral pseudotypes yielded an average of 72 (± 10) (for Friend MuLV clone 57) and 81 (± 11) (for NLV) fused nuclei per grid area. The more apparent decrease in binding activity with MAb 83A25, further confirming the importance of positions 405 and 406 to the MAB 83A25 epitope. Insertion of a serine or threonine at the adjacent position strongly affected activity with MAb 83A25, further confirming the importance of positions 405 and 406 to the MAB 83A25 epitope. Insertion of Thr instead of Ser slightly increased the level of attenuation, while converting the original Ser to Thr resulted in a highly defective Env, and the mutant with Thr at both positions was completely inactive in the luciferase assay (Table 3). All of the insertion mutants were also defective in syncytium assays (not shown). Binding assays to cells expressing murine cationic amino acid transporter (MCAT) indicated that even the highly defective mutants retained normal binding activity, and an analysis of the protein content of these virions by Western blotting indicated that the defective Env proteins all underwent normal processing and were incorporated at normal levels into viral particles (Fig. 4B). The most defective S405T+S mutant. In contrast to these effects for the insertion mutants, the Ser deletion mutant exhibited essentially normal function. Thus, while S405 could be deleted without affecting function, substitution by a threonine together with insertion of a serine or threonine at the adjacent position strongly affected function at a postbinding step, with the level of activity decreasing with increasing size of the substituted and inserted amino acids.

Neither mutation of loop 10 nor its binding by neutralizing MAB prevented denaturation-induced isomerization of the intersubunit disulfide bond. SU and TM are present in virions as a disulfide-bonded heterodimer that dissociates into its subunits by isomerization of the intersubunit disulfide bond upon solubilization of virions or treatment with denaturants (24, 32, 33, 36, 37). This isomerization is blocked by prior treatment with the thiol alkylating agent NEM (33) and involves a cysteine present in the CWLC motif at positions 312 to 315 of the CTD domain (32). The possibility that the functional effects seen upon antibody binding or mutagenesis of loop 10 were
related to this disulfide bond rearrangement was investigated for the Friend MuLV NLV Env and its fusion-defective derivative bearing the S405T+S mutation (Fig. 5). As expected, solubilization with NP-40 induced isomerization of essentially all of the NLV SU to free SU, and this isomerization was blocked by pretreatment with NEM. A similar effect was seen upon denaturation with urea, although in this case isomerization was less complete. Prebinding of MAb 83A25 to NLV virions at neutralizing concentrations did not inhibit dissociation to SU under either condition, indicating that binding of this MAb did not interfere with isomerization of the disulfide bond. Similarly, for the defective S405T+S Env mutant, the SU-TM disulfide-bonded complex was also present after treatment with NEM, and the dissociation of this complex to free SU occurred as efficiently following treatment with either NP-40 or urea for this mutant as for the parental NLV Env. These results did not support a role for interference with isomerization of the intersubunit disulfide bond for either the neutralizing activity of 83A25 or the lack of function of the S405T+S Env mutant.

**DISCUSSION**

This study localized determinants of two potent neutralization epitopes to loop 10 in the CTD of MuLV SU and demonstrated effects for this loop on both processing and function of Env. The earlier demonstration that loop 10-specific MAbs inhibited fusion but not receptor binding (7) suggested a role for this region in viral fusion. This conclusion was supported in the present study by the demonstration of attenuated infectivity and severe defects in syncytium-inducing activity of mutants bearing serine or threonine insertions near the N terminus of loop 10, despite normal processing and receptor binding activity of these mutants (Fig. 4). These results suggested that the insertion mutations in the loop interfered with the same function blocked by binding of the loop 10-directed neutralizing MAbs. A role for the first three positions of loop 10 in regulating maturation of the Env precursor was shown by the defective processing of the GAG 404-406 substitution mutant, and an additional role for the second position of the loop in the stable association between SU and TM was suggested by the increased shedding and decreased incorporation of SU into virions for the S405A mutant (Fig. 3B and C).

The most critical determinants in loop 10 for the epitope specificity of MAbs 35/56 and 83A25 were positions 410 and 411. Substituting Arg411 in the AKR sequence by Leu resulted...
in the complete loss of reactivity with both MAbs, while conversion of Asp410 to Asn resulted in complete loss of reactivity with MAb 35/56 and a significant reduction in the affinity and neutralizing activity of MAb 83A25. An Asn at position 410 introduces a signal for N-linked glycosylation (13, 18), and the presence of this glycan may contribute to the inhibition of binding of these MAbs. Earlier studies have reported that MAb 35/56 was highly type specific for endogenous MuLV isolates represented by the AKR strain (23, 25, 31), while MAb 83A25 was more broadly reactive with MuLVs, including ecotropic Moloney MuLV and amphotropic 4070A MuLV (8, 11, 16). Asp410 is present in the Akv provirus locus and in endogenous MuLVs produced from expression of this locus, while Asn410 is in the consensus sequence for exogenous MuLV Env proteins. The weak reactivity of MAb 83A25 with the Asn410 substitution mutant in the AKR-derived loop 10 was consistent with its relatively low reactivity with Moloney MuLV and 4070A Env proteins in both immunofluorescence and neutralization assays (Table 1).

Additional positions in the N-terminal half of the loop also contributed to the reactivity of these epitopes but in more subtle ways. The reactivity of both MAbs was completely lost upon replacement of the Thr406 by Gly, while substituting an Ala at this position or mutating the Ile at position 404 to Ala resulted in relatively small increases in ND50 for these MAbs (Tables 1 and 2). Mutations of other polymorphic residues in this region (Ser 397 to Asn, Ile404 to Leu, and Ile408 to Val) had little or no effect on either MAb when introduced individually but had a significant impact on the activity of MAb 35/56, but not 83A25, when combined (the NLV substitution in Table 1). Adding the Thr406-to-Ala substitution to the other three changes resulted in complete loss of MAb 35/56 reactivity and strongly attenuated the neutralization activity of MAb 83A25. Thus, the greater strain specificity of MAb 35/56 over 83A25 was strongly attenuated by the neutralization antibody to this region indirectly disrupts the Env function of the proximal structure.

Areas for further study that can help distinguish these possibilities include additional identification of determinants.
within and outside of loop 10 that mediate its effects on fusion, including the identification of regions in SU and/or TM that interact with this region, and more complete determination of the structures of the MAB 35/56 and 83A25 epitopes. A powerful way of obtaining such information would be by determining the crystal structure of complexes between SU and loop 10-directed MAbs. Isolation and characterization of mutants that are resistant to loop 10-directed MAbs and functional revertants of defective loop 10 mutants would also provide useful insights into these questions.

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