Mapping of the CXCR4 Binding Site within Variable Region 3 of the Feline Immunodeficiency Virus Surface Glycoprotein

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Feline immunodeficiency virus (FIV) shares with T-cell tropic strains of human immunodeficiency virus type 1 (HIV-1) the use of the chemokine receptor CXCR4 for cellular entry. In order to map the interaction of the FIV envelope surface unit (SU) with CXCR4, full-length FIV SU-Fc as well as constructs with deletions of extended loop L2, V3, V4, or V5 were produced in stable CHO cell lines. Binding studies were performed using these proteins on 3201 cells (CXCR4hi CD134hi), with or without the CXCR4 inhibitor AMD3100. The findings established that SU binding to CXCR4 specifically requires the V3 region of SU. Synthetic peptides spanning the V3 region as well as a panel of monoclonal antibodies (MAbs) to SU were used to further map the site of CXCR4 interaction. Both the SU V3-specific antibodies and the full-length V3 peptide potently blocked binding of SU to CXCR4 and virus entry. By using a set of nested peptides overlapping a region of SU specifically recognized by CD134-dependent neutralizing V3 MAbs, we showed that the neutralizing epitope and the region required for CXCR4 binding are within the same contiguous nine-amino-acid sequence of V3. Site-directed mutagenesis was used to reveal that serine 393 and tryptophan 394 at the predicted tip of V3 are required to facilitate entry into the target cell via CXCR4.

Although the amino acid sequences are not identical between FIV and HIV, the ability of FIV to bind and utilize both feline and human CXCR4 makes the feline model an attractive venue for development of broad-based entry antagonists.

Feline immunodeficiency virus (FIV) infection in cats is considered a valuable small-animal model of human immunodeficiency virus (HIV) vaccination strategies and drug development, as it is the only nonprimate lentivirus that causes a disease resembling AIDS in its natural host. Although FIV primarily targets CD4+/CD8+ T cells (9, 12, 20), it uses CD134 instead of CD4 as its primary binding receptor (12, 40). CD134 is a member of the tumor necrosis factor receptor superfamily and is specifically expressed on activated CD4+/CD8+ T cells, where it induces antiapoptotic signals to allow for survival and proliferation late in T-cell responses (7). The direct correlation between CD134 upregulation and the preferential targeting of FIV for activated T cells explains the FIV tropism for these cells (12).

Similar to T-cell-tropic HIV type 1 (HIV-1), FIV utilizes CXC chemokine receptor 4 (CXCR4) as an entry receptor for infection (48, 50), after interaction with a primary binding receptor (14). Also, the overall genetic structure of FIV is similar to that of HIV (33, 37, 45), and the FIV envelope gene encodes a surface glycoprotein (SU or gp95) and a transmembrane protein (TM or gp41). The SU protein exhibits considerable amino acid sequence variation, with five consensus major variable (V) regions (V1 to V5) (36, 37). Of these domains, the V3 loop has been identified as a major immunogenic domain in SU by epitope mapping with sera from infected cats (3, 18, 29, 34).

Studies of FIV-PPR interactions with CD134 have identified specific amino acids involved in SU binding in the outermost domain 1 of CD134 (11). Recent studies have indicated the involvement of additional residues in domain 2 in binding of certain other strains of FIV (49). For SU interaction with its entry receptor, CXCR4, involvement of the second extracellular loop of CXCR4 has been reported (5, 47). However, the domains of SU involved in direct interaction with CXCR4 have not been identified.

The purpose of this study was to define the domains of SU that are crucial for the interaction with CXCR4 in order to better understand the mechanism of FIV entry and infection. Analysis of genetic variations in SU proteins of FIV, simian immunodeficiency virus, and HIV-1 has revealed analogies in the locations and distribution of the variable domains V3, V4, and V5 (35). A structural model of FIV SU identified these variable domains as potential exposed loop segments (36). A segment, denoted loop II (L2), in the N-terminal part of FIV SU has also been predicted to fold as an exposed loop (36). As a step toward a better understanding of the molecular interaction between FIV and its target cells, we created SU mutants with deletions of L2, V3, V4, or V5. These mutants were used together with a panel of SU-specific monoclonal antibodies (MAbs) in SU-CXCR4 binding studies. Using nested V3 peptides and site-directed mutagenesis, the CXCR4 binding region of SU was further localized to a discrete stretch of nine amino acids at the predicted tip of the V3 loop, with critical
involvement of serine 393 and tryptophan 394 to facilitate binding and virus entry into the target cell.

**MATERIALS AND METHODS**

**Cell lines and virus.** The interleukin-2 (IL-2)-independent feline lymphoma cell line 3201 was obtained from W. Hardy (Stan-Kettering Memorial Hospital, NY). 3201 cells are CXCR4<sup>+</sup> and have a very low or negative phenotype for heparan sulfate proteoglycans and CD134 (16). CHO-K1 cells were obtained from the American Type Culture Collection (Rockville, MD). GFOX cells are Crandell Feline Kidney (CrFK) cells engineered to express CD134 (12). Cat peripheral blood mononuclear cells (PBMCs) were prepared by Ficol-Hypaque Plus (Amer sham Biosciences, Sweden) density gradient purification from heparinized whole feline blood as previously described (26). The primary IL-2-dependent T-cell line 104-C1 is a clone isolated from feline PBMCs by limiting dilution (27). Propagation of the different cell lines was performed as previously described (13, 44).

**FIV-PRR** is a molecularly cloned clade A primary isolate (37).

**Recombinant SU proteins.** Deletion mutants of FIV-PRR SU were generated using PCR-ligation-PCR mutagenesis (1). Briefly, fragments of FIV-PRR SU were joined after amplification in separate PCRs. The PCR primers used for these reactions were designed to contain a blunt-ended restriction enzyme site, and the resultant initial PCR fragments were digested with the corresponding restriction enzymes, gel purified, and ligated. A fraction of each ligation reaction mixture was then used to amplify the ligated DNA by a second PCR, using the 5′ primer for the upstream fragment and the 3′ primer for the downstream fragment. The PCR products were then subcloned into pRS-C-GS-SU-Fc (13), followed by sequencing to confirm the presence of the desired deletion and the absence of random mutations. The PCR products were digested with the restriction enzyme SacI for the generation of the SU-Fc fusion proteins (adhesins) with the desired deletions. Fc (13) or SU-Fc proteins from stable CHO cell supernatants were purified by affinity chromatography over protein A-Sepharose and quantitated as described previously (13). The following amino acids were removed in the SU deletion mutants: delI1, amino acids 249 to 335; delI5, amino acids 378 to 416; delIV, amino acids 463 to 499; and delV5, amino acids 535 to 560). All deletions were constructed by PCR mutagenesis using the QuikChange site-directed mutagenesis strategy (Stratagene, La Jolla, CA) as previously described (13). The following amino acids were removed in the SU deletion mutants: delI1, amino acids 249 to 335; delI5, amino acids 378 to 416; delIV, amino acids 463 to 499; and delV5, amino acids 535 to 560). All deletions were constructed by PCR mutagenesis using the QuikChange site-directed mutagenesis strategy (Stratagene, La Jolla, CA) as previously described (13). The primary IL-2-dependent T-cell line 104-C1 is a clone isolated from feline PBMCs by limiting dilution (27). Propagation of the different cell lines was performed as previously described (13, 44).

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**Western blot analysis.** Purified Fc and Fc-tagged fusion proteins were loaded on an 8 to 16% sodium dodecyl sulfate (SDS)-polyacrylamide gel, and the proteins were subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions and immunoblotted with an antibody specific for human IgG Fc (anti-Fc).

**RESULTS**

**Construction and expression of FIV SU deletion mutants.** We previously reported an experimental approach using a FIV SU-Fc immunoadhesin (soluble FIV surface glycoprotein fused in frame with the Fc domain of human IgG1) (2) to screen for cell surface receptors involved in FIV binding and infection (11–14, 16, 17). The envelope glycoproteins of FIV, simian immunodeficiency virus, and HIV-1 share only minor sequence similarity in SU. However, there are analogies in the locations and distribution of the SU variable domains V3 to V5 (30, 35–37, 42). To characterize the interaction of FIV with its entry receptor, CXCR4, four FIV SU-Fc constructs with deletions of variable domain V3, V4, or V5 or the predicted loop II were generated and used to produce soluble proteins by establishment of stable CHO cell lines (Fig. 1). Purified wt and deletion mutants of SU were subjected over eight wells, resulting in a dilution series from 1.000 ng/100 μl in the first well to 8 ng/100 μl in the last well. Plates were incubated for 45 min, washed three times, and tap dried. Goat anti-mouse IgG–horseradish peroxidase (Bio-Rad, Hercules, CA) was diluted 1:1,000 in ELISA buffer, and 100 μl/well was added; the plates were incubated for 45 min, washed three times, and tap dried. The substrate (15 ml/well, for 150 μl per well) was used was o-phenylenediamine (Sigma), as follows: 20 μg was dissolved in 15 ml of 50 mM citric acid and 100 mM dibusic sodium phosphate, pH 5.0, and activated with 80 μl per plate hydrogen peroxide (30% solution; Fisher Scientific). The reaction proceeded for 8 min and was then stopped by the addition of 50 μl/2.0 M sulfuric acid. The reaction color was read at 493 nm.

**Peptide synthesis.** Peptides were synthesized on a PTI Symphony multiple peptide synthesizer utilizing standard 9-fluorenylmethoxycarbonyl chemistry. Crude peptide material was purified by high-pressure liquid chromatography, and purity was determined to exceed 90% for all material. The masses of peptides were confirmed by mass spectrometry.

**Infections.** Cells were seeded at 3 × 10<sup>4</sup> cells per well in a 96-well plate and infected for 2 h at room temperature with 20 μl virus stock. Cells were then washed once and incubated in cell medium until day 7, when supernatants were analyzed for virus production by using a micro-RT assay as described below. For analysis of V3 peptide inhibition of infection, cells were preincubated for 30 min with peptides before infection and the incubation time.

**Micro-RT activity assay.** The micro-RT activity assay was performed as previously described (13, 15). Briefly, 50 μl of cell-free supernatant was incubated for 10 min at room temperature with 10 μl of lysis buffer (0.75 M KCl, 20 mM dithiothreitol, 0.5% Triton X-100). Forty microliters of a mixture containing 125 mM Tris-HCl (pH 8.1), 12.5 mM MgCl<sub>2</sub>, 1.25 μg poly(A)-poly(dT)<sub>20-20</sub> (Amersham Biosciences, Piscataway, NJ), and 1.25 μg/ml of [3H]dTPP (DuPont, Boston, MA) was added to the sample, followed by 2 h of incubation at 37°C. RT activity was measured as previously described (15).

**FIG. 1.** Construction (a) and expression (b) of FIV SU-immuno- adhesins with deletions of variable domains. Recombinant deletion mutants of FIV SU-Fc were generated by PCR-ligation-PCR mutagenesis, expressed by stable transfection of CHO-K1 cells, and batch purified from cell supernatants by affinity chromatography over protein A. (b) Samples (500 ng) of SU-Fc (wt and deletion mutants) and Fc protein were subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions and immunoblotted with an antibody specific for human IgG1 Fc (anti-Fc).
jected to SDS-polyacrylamide gel electrophoresis and confirmed to have the expected size by Western blotting with an anti-human IgG1 Fc antibody (Fig. 1b).

Binding of SU to 3201 cells is CXCR4 specific and dependent on V3. 3201 is a feline lymphoma cell line with relatively high expression levels of CXCR4 but lacking CD134 (13). To analyze the binding of SU to CXCR4, we incubated 3201 cells together with the full-length wt SU-Fc immunoadhesin, followed by flow cytometric analysis using a phycoerythrin-conjugated anti-IgG1 Fc antibody. As previously reported (16), SU-Fc bound 3201 cells, and the CXCR4 specificity of this binding was demonstrated by a complete inhibition of binding upon preincubation with the CXCR4 antagonist AMD3100 (Fig. 2a). The lack of binding of Fc to 3201 cells confirmed that cellular binding of the immunoadhesin is entirely attributed to the SU part of the SU-Fc fusion protein (Fig. 2a).

In order to localize the SU-CXCR4 interaction, we performed similar binding studies using the L2, V3, V4, and V5 deletion mutants in the presence or absence of a saturating concentration (1.3 μM) of AMD3100 (Fig. 2a). SU-Fc adhesins with deletion of L2, V4, or V5 were functional and retained the capacity to bind 3201 cells, albeit more weakly than full-length wt SU-Fc (Fig. 2a). Binding was totally abolished in the presence of AMD3100, demonstrating CXCR4 specificity. In contrast to the other deletion mutants, the V3-deleted SU failed to bind 3201 cells, indicating that the V3 region is specifically required for interaction of SU with cell surface CXCR4 (Fig. 2a).

Virtually all SU binding observed by fluorescence-activated cell sorting (FACS) analysis of 104-C1 T cells (CD134 hi CXCR4 lo) is via binding to CD134, and binding is not significantly inhibited by AMD3100 (ref 13, 14, 16). Thus, this cell line serves as a venue for measuring CD134 binding by SU, with a negligible contribution from the entry receptor,
TABLE 1. Mapping of binding locations of SU-specific MAbs by ELISA, using full-length or deletion mutant SU proteins

<table>
<thead>
<tr>
<th>MAb</th>
<th>Optical density at 493 nm*</th>
<th>Binding location</th>
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<tr>
<td></td>
<td>wt SU</td>
<td>Δ2</td>
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<td>SU1-5</td>
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</tr>
<tr>
<td>SU2-11</td>
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*Values in bold represent loss of specific antibody reactivity to a given deletion mutant.

CXCR4. Attempts were made to identify the CD134 binding site, using the four deletion mutants described above. In contrast to results with CXCR4<sup>Δ60</sup> 3201 cells, the CD134<sup>Δ60</sup> 104-C1 cells failed to bind any of the deletion mutants (not shown). In addition to binding studies, vectors expressing wt SU and the four deletion mutants were cotransfected with a β-Gal-expressing pCFIV vector in 293T cells in order to generate pseudotyped virus particles expressing each envelope protein. After standardizing the virus supernatants for RT activity, the pseudovirions were used in single-round infections of Gfox cells (CD134<sup>−</sup> CXCR4<sup>−</sup>). Only wt SU facilitated virus entry, consistent with failure of the mutants to bind to CD134 (Fig. 2b). Given the diverse group of deletion mutants, the results indicate that the binding of CD134 is heavily dependent on the overall conformation of SU rather than on a short contiguous epitope as with CXCR4.

Structural mapping of a panel of MAbs to SU. To further characterize the FIV-CXCR4 interaction, we employed the SU deletion proteins and synthetic peptides encompassing the whole or parts of the V3 loop to map the binding locations of SU-specific MAbs generated by immunizing mice with SU-Fc (14). By performing ELISAs using the MAb panel versus full-length or deletion proteins, we were able to assign four MAbs to SU-L2, seven to SU-V3, and one to SU-V4 (Table 1). No reactivity was noted to V5, suggesting that this region may either be nonimmunogenic in mice or reside in an immunologically privileged environment.

Antibodies specific for the central parts of V3 can block SU-CXCR4 interaction. Next, we analyzed the capacity of the FIV-CXCR4 interaction, employed the SU deletion proteins and synthetic peptides encompassing the whole or parts of the V3 loop to map the binding locations of SU-specific MAbs generated by immunizing mice with SU-Fc (14). By performing ELISAs using the MAb panel versus full-length or deletion proteins, we were able to assign four MAbs to SU-L2, seven to SU-V3, and one to SU-V4 (Table 1). No reactivity was noted to V5, suggesting that this region may either be nonimmunogenic in mice or reside in an immunologically privileged environment.

Antibodies specific for the central parts of V3 can block SU-CXCR4 interaction. Next, we analyzed the capacity of the characterized MAbs to interfere with the SU-CXCR4 interaction. SU-Fc was preincubated with the various anti-SU MAbs before addition to 3201 cells, followed by analysis of binding by flow cytometry (Fig. 3). Eleven of the 12 characterized SU MAbs inhibited SU binding to CXCR4. Among these, MAbs with binding reactivity specific for V3 were more efficient in interfering with binding of SU to CXCR4 than MAbs recognizing L2 or V4 (Fig. 3a and b). Four of the V3-specific MAbs (SU1-30, SU2-2, SU2-5, and SU2-10) are specific to the E60 region in the center of V3 and are CD134-dependent neutralizing MAbs (14). The V3 E60 region is highly conserved between FIV clade A, B, and C isolates (14) and is the FIV equivalent of a region previously identified as critical for HIV entry (31). Interestingly, MAbs recognizing E60 gave the strongest inhibition of SU binding to CXCR4 (Fig. 3). MAbs SU1-5, SU1-7, and SU1-10 also recognized the V3 peptide and failed to bind SU-Δ3 but had a specificity outside E60 (Table 1 and data not shown). Of these MAbs, SU1-5 and SU1-7 exhibited less influence on SU binding than the MAbs specific for the E60 region (Fig. 3).

Fine mapping of the V3 region required for interaction with CXCR4. Our analyses using SU-Fc deletion mutants and SU-specific MAbs strongly support the involvement of SU V3 in the FIV-CXCR4 interaction. Therefore, the 41-amino-acid V3 loop peptide was assessed for the ability to block SU binding to CXCR4. As analyzed by flow cytometry, preincubation of 3201 cells with the V3 peptide abrogated binding of SU to CXCR4 (Fig. 4a). We next sought to determine the minimal domain within the V3 region needed for SU-CXCR4 interaction by evaluating a panel of nested peptides overlapping the E60 region of FIV SU. The peptides spanning this region were truncated at either the N or carboxyl (C) terminus and exhibited various capacities to interact with SU-CXCR4 binding (Fig. 4b; also see Fig. 6a). All V3 peptides tested displayed a clear dose-dependent inhibition (data not shown), and peptides with an intact N terminus, starting with two conserved (>90%) serines, and with a truncated C terminus gave a much stronger inhibition of SU binding than did peptides with truncations in the N terminus (Fig. 4b; see Fig. 6a). Remarkably, a peptide as short as nine amino acids (SSWKQRNRW), designated N44, was equally potent at blocking SU binding to CXCR4 as the full-length V3 peptide. The C-terminal end of N44 consists of an arginine and a tryptophan that are more than 99% conserved among 227 different FIV isolates (see Fig. 6b and data not shown). Interestingly, truncation of these two amino acids in peptides P26 and P27 abolished most of the inhibitory activity for binding of SU to CXCR4 (Fig. 4b). A peptide corresponding to the C-terminal half of V3 (P28) exhibited no inhibition of SU binding. In summary, these findings verify the significance of the central portion of V3, and in particular the nine-amino-acid long “N44” region, for SU-CXCR4 interaction.

Attempts were also made to utilize both the full-length V3 peptide and N44 peptide to block virus infection ex vivo. Relative RT activity was measured in culture supernatants 7 days after infection with FIV-PPR as a function of peptide addition. A 50% inhibition of infection was observed with the full-length V3 peptide, but the shorter N44 peptide did not block infection in these experiments (Fig. 4c). We surmise that the peptide may have been degraded ex vivo or lacked the avidity required to continuously block virus entry.

The importance of the V3 region of SU for receptor binding is not type specific but is limited to CXCR4. We assessed the blocking activities of the V3 and N44 peptides by analyzing their effects on Fc-tagged SU proteins from genetically divergent FIV subtypes. SU proteins derived from FIV 34TF10 Petaluma (an FIV subgroup A clone) (16, 45) and FIV C36 (19) both bound CXCR4 on 3201 cells (Fig. 5a). The binding of SU-34TF10–Fc or SU-C36–Fc was potently blocked by N44 and V3 peptides in a manner similar to that with FIV-PPR SU (Fig. 5a), indicating that the involvement of the N44 domain...
for CXCR4 interaction is a general feature among different strains of FIV.

As indicated above, FIV SU also binds cell surface CD134 (12, 16, 40). Therefore, the N44 and V3 peptides were tested for the ability to block SU-Fc binding to CD134 expressed on PBMCs (CXCR4+/H11001; CD134+/H11001) and 104-C1 cells (CD134 hi; CXCR4 lo). Only a limited inhibition of SU binding by N44, V3, or AMD3100 was observed for PBMCs (Fig. 5b). Furthermore, there was no effect of either of these peptides or AMD3100 on SU binding to 104-C1 cells (Fig. 5b), again consistent with an independent binding mode for CD134, i.e., V3, in particular its “N44 region,” is required for interaction of SU with the entry receptor CXCR4 but not with the binding receptor CD134.

No three-dimensional analyses have been performed with FIV SU. However, JPRED analysis (8) of FIV SU indicates that FIV V3 has a similar secondary structure to that of HIV V3 (23). Notably, the SSWKQRNRW amino acids of N44 are located at the predicted tip of V3, between two beta sheets (Fig. 6c). A similar motif has been identified in HIV V3 in the crystal structure of HIV-1 SU (23).
To further define residues critical for CXCR4 binding, site-directed mutagenesis was performed on the N-terminal portion of the N44 peptide region, with particular emphasis on the relatively conserved Ser392-Ser393-Trp394 sequence highlighted as important from the above studies. These three residues were mutated, individually or as a group, to alanine in the context of SU, and pseudovirions were then prepared as described above in order to test the effects of these mutations on virus entry. As shown in Fig. 7, Ser392 tolerated the change, but the Ser393Ala or Trp394Ala substitution or substitution of all three residues abrogated the ability of the mutant SU to facilitate virus entry into Gfox cells.

**DISCUSSION**

To characterize the interplay between FIV and its entry receptor, CXCR4, we generated deletion mutants of the FIV SU protein and analyzed their binding characteristics. These studies suggested that the V3 loop of SU contains the main determinants for FIV SU binding to CXCR4. Given the lower level of total binding of mutants relative to that of wt SU, the L2 and V4 loops may also contribute, directly or indirectly, to the interaction with CXCR4. However, these regions appear to be much less critical than V3. Next, a panel of SU MAbs was mapped based on their binding locations. These characterized MAbs were then employed to further study the SU-CXCR4 interaction. Binding studies demonstrated that MAbs specific for the V3 region of SU were more efficient than L2- or V4-specific MAbs in blocking the SU binding to CXCR4. Furthermore, the most efficient inhibition of SU binding was primarily displayed by MAbs recognizing the central part of the V3 loop. A set of peptides overlapping this region was analyzed for the ability to block SU binding to CXCR4. By this approach, the chemokine receptor binding region of SU was narrowed down to nine amino acids corresponding to the predicted tip of the V3 loop. Further mutagenesis studies demonstrated particular involvement of residues Ser393 and Trp394 in the SU-CXCR4 interaction.
Although the consensus sequences between the conserved cysteine residues of the FIV and T-cell-tropic HIV-1 V3 domains display a low degree of homology, V3 domains from both viruses are positively charged (13, 21). Furthermore, a JPRED analysis predicted the secondary structure of FIV V3 to display a high degree of similarity to that of the HIV V3 domain.

**FIG. 5.** (a) Binding of SU from FIV strains 34TF10 Petaluma and C36 to CXCR4 is blocked by preincubation with N44 and V3 peptides. (b) Analysis of the effects of peptides on SU binding to PBMCs and 104-C1 cells. A peptide corresponding to a region of the feline leukemia virus (FELV) transmembrane region was used as a negative control. Results are representative of three independent determinations.

**FIG. 6.** (a) Sequences of a panel of V3 peptides and summary of the ability of the peptides to interfere with SU binding to CXCR4. (b) Consensus sequence and amino acid percent frequency of the “N44 domain.” (c) JPRED prediction of the secondary structure of V3. E, beta strand; -, indicates coil. Amino acids corresponding to the “N44 domain” are underlined.

**FIG. 7.** Site-directed mutagenesis of highly conserved amino acids at the predicted tip of the V3 loop of FIV SU. Residue Ser392, Ser393, or Trp394 or all three amino acids were replaced with alanine, and β-Gal-expressing pseudovirions were prepared to display each mutant SU, as described in Materials and Methods. Supernatants from transfected 293T cells containing each pseudovirus were assessed for RT activity, normalized, and then used to infect Gfox cells as detailed in the legend to Fig. 2. An alanine at Ser392 was tolerated, but substitution of alanine for either Ser393 or Trp394 abrogated the ability of SU to facilitate CXCR4 binding and virus entry.

Although the consensus sequences between the conserved cysteine residues of the FIV and T-cell-tropic HIV-1 V3 domains display a low degree of homology, V3 domains from both viruses are positively charged (13, 21). Furthermore, a JPRED analysis predicted the secondary structure of FIV V3 to display a high degree of similarity to that of the HIV V3 domain.
loop. Both V3 regions are conserved in length (HIV V3 is 35 residues long, and FIV V3 is 41 residues long), and they exhibit a relatively conserved centrally located tip flanked by two beta-sheets (23, 26) (Fig. 6b and c). The consensus sequence of the HIV V3 tip is comprised of the amino acid sequence GPGG or GPGQ. Similarly, the amino acids in the “N44 region” at the tip of FIV V3 are also fairly conserved, with six of the amino acids having a frequency of 90% or higher in over 200 HIV isolates (Fig. 6b and data not shown). The V3 loop of HIV gp120 is the major determinant for coreceptor usage and hence cell tropism (4, 6, 22, 25, 32, 43). In particular, the amino acid sequence at the tip/crown of HIV V3 is important for coreceptor usage (6, 32). In this region, basic residues and conserved hydrophobic residues have been shown to contribute to CXCR4 binding (4). Interestingly, the amino acid sequence in the FIV V3 region corresponding to N44 is also rich in hydrophobic and basic residues, including two tryptophans (98 and 100% conserved) and two arginines that are 73% and 99% conserved.

The “N44 domain” in FIV V3 is located outside the lysine and glutamic acid residues at positions 407 and 409 that have been implicated in the affinity of SU for heparan sulfate proteoglycans and in CrFK cell tropism for tissue culture-adapted FIV (16, 41, 46). Furthermore, the amino acids of the “N44 domain” correspond to the central section of peptides that have been reported to both absorb neutralizing activity in pooled cat sera and induce neutralizing Abs in cats (29, 38). Together with our data, these findings suggest that FIV neutralizing Abs recognize the CXCR4 binding part of SU or epitopes in close proximity to this domain.

As noted previously (12), and in contrast to SU binding to CXCR4(94–102) 3201 cells, the majority of SU binding to either PBMCs or 104-C1 T cells occurs via CD134. Barely detectable contributions from direct binding to CXCR4 are evident by FACS analysis, as shown by the relative insensitivity of binding to the latter cells in the presence of the CXCR4 antagonist AMD3100 (Fig. 5). The present studies show that the V3 peptides did not block SU binding to either PBMCs or 104-C1 cells, which indicates that binding of SU to CD134 either occurs via regions other than V3 or is more conformation dependent, such that short peptides fail to block the CD134-SU interaction. In keeping with this observation, studies using HIV SU deletion mutants have demonstrated that V3 is not directly involved in CD4-SU interactions (51). Furthermore, none of the diverse deletion mutants employed here were able to facilitate virus entry when expressed on pseudovirions, consistent with the interpretation that there is a conformation-dependent binding of CD134 to noncontiguous regions of SU.

CXCR4-independent binding of the virus to CD134 on total PBMCs and 104-C1 cells may explain the low success of the V3 peptides in competing for virus infection ex vivo. FIV will bind to these cells via CD134 in the presence of peptides capable of blocking CXCR4 binding. Therefore, when peptide exchange/uptake occurs and/or when new CXCR4 entry receptors are generated, the virus is able to bind CXCR4 and enter the cell. However, it has been reported previously for HIV V3 peptides that circularized peptides can compete successfully for virus entry, whereas linear forms cannot (39). Consistent with this observation, the FIV full-length V3 peptide that demonstrated inhibitory effects on virus replication ex vivo had N- and C-terminal cysteine residues and thus could cyclize, whereas the shorter N44 peptide which failed to inhibit virus replication did not.

FIV and primate lentiviruses share the same model of cell entry, with a sequential interaction of SU with a binding receptor followed by binding and entry via a chemokine entry receptor. These binding steps are critical for viral entry and therefore offer potential targets for therapeutic or prophylactic intervention. The studies reported here have defined the residues critical for CXCR4 binding, which will aid in the use of the FIV-cat system as a small-animal model for development of inhibitors relevant to the treatment of HIV/AIDS.

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