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Binding of Recombinant Feline Immunodeficiency Virus Surface Glycoprotein to Feline Cells: Role of CXCR4, Cell-Surface Heparans, and an Unidentified Non-CXCR4 Receptor

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To address the role of CXCR4 in the cell-surface attachment of the feline immunodeficiency virus (FIV), a soluble fusion protein, gp95-Fc, consisting of the surface glycoprotein (SU, gp95) of either a primary (PPR) or cell line-adapted (34TF10) FIV strain was fused in frame with the Fc domain of human immunoglobulin G1. The recombinant SU-immunoadhesins were used as probes to investigate the cellular binding of FIV SU. In agreement with the host cell range properties of both viruses, binding of 34TF10 gp95-Fc was observed for all cell lines tested, whereas PPR gp95-Fc bound only to primary feline T cells. 34TF10 gp95-Fc also bound to Jurkat and HeLa cells, consistent with the ability of FIV to use human CXCR4 as a fusion receptor. As expected, 34TF10 gp95-Fc binding to Jurkat cells was blocked by addition of stromal cell-derived factor 1α (SDF-1α), as was binding to the 3201 feline lymphoma cell line. However, SDF-1α, RANTES, macrophage inflammatory protein 1β, and heparin all failed to inhibit the binding of either gp95-Fc to primary T cells, suggesting that a non-CXCR4 receptor is involved in the binding of FIV SU. In this regard, an unidentified 40-kDa protein species from the surface of primary T cells but not Jurkat and 3201 cells specifically coprecipitated with both gp95-Fc. Yet another type of binding of 34TF10 gp95-Fc to either HeLa, Crandel feline leukemia, or G355-5 cells. However, binding was severely impaired in the presence of soluble heparin, as well as after enzymatic removal of surface heparans or on cells deficient in heparan expression. These overall findings suggest that in addition to CXCR4, a non-CXCR4 receptor and cell-surface heparans also play an important role in FIV gp95 cell surface interactions on specific target cells.

The initial stage in infection with human immunodeficiency virus (HIV) is the interaction of the viral envelope (Env) with CD4 and a specific chemokine receptor on the surface of target cells which promote conformational changes that allow fusion of the viral envelope with the plasma membrane (reviewed in references 7, 15, and 81). The major coreceptors used by HIV type 1 (HIV-1) are CXCR4 and CCR5, two members of the superfamily of seven-transmembrane domain G-protein-coupled receptors. Syncytium-inducing or T-cell-tropic (T-tropic) strains of HIV-1 and laboratory-adapted viruses use predominantly CXCR4 (8, 31), whereas non-syncytium-inducing or macrophagotropic strains of HIV-1 use predominantly CCR5 (2, 14, 17, 21, 22). Additional members of the seven-transmembrane superfamily are also able to support infection with HIV-1, HIV-2, and simian immunodeficiency virus (SIV) (18, 24, 25, 30, 42, 48, 55, 64, 71).

The binding of HIV Env with the receptor complex follows a two-step model in which the binding determinants in the interaction of HIV Env with CD4 and the chemokine receptor are contained within gp120, the surface subunit of Env (7, 15, 81). In the first step, gp120 binds CD4, which triggers conformational changes in gp120 that expose or create the coreceptor binding site(s). In the second step, the CD4-gp120 complex interacts with the chemokine receptor, which promotes additional conformational changes that lead to the fusion of the viral and cellular membranes. Although CD4 is required for an efficient interaction between gp120 and the chemokine receptor, some HIV and SIV strains that have gained independence from CD4 can interact directly with their coreceptors on CD4-negative cells (9, 23, 26, 28, 40, 41, 44, 46, 50, 52, 56, 66, 67). While naturally occurring, CD4-independent isolates were reported for HIV-2 and SIV, CD4-independent HIV-1 isolates were observed only after long-term culture of the virus in CD4-positive cells or after adaptation of the virus to replicate in CD4-negative cells (23, 41, 50, 52). Determinants for CD4 independence are dispersed throughout Env in gp120 but also in gp41, the transmembrane subunit (23, 41, 50, 52). These mutations are believed to increase and stabilize the expression of the coreceptor binding site on gp120.

Feline immunodeficiency virus (FIV) is the etiologic agent of feline AIDS in the domestic cat (62). One of the most interesting recent findings regarding the similarity between FIV and HIV is that FIV uses the chemokine receptor CXCR4 for efficient infection of target cells, similarly to T-tropic strains of HIV-1 (80). CXCR4 was first shown to be used by FIV isolates adapted for propagation in Crandel feline kidney (CrFK) cells (80); recently, the use of CXCR4 has been extended to primary T-tropic isolates of FIV (27, 68). Primary isolates of FIV (PI FIV) have a cellular tropism restricted to mitogen-activated peripheral blood mononuclear cells (PBMCs), thymocytes, macrophages, and interleukin-2 (IL-2)-dependent T-cell lines, and infection of these primary cells can be efficiently inhibited by stromal cell-derived factor 1α (SDF-1α) or the bicyclam...
AMD3100, two CXCR4 ligands (27, 68). However, PI FIV failed to productively infect CrFK and other nonlymphoid cells but can be adapted to propagate in these cells after several in vitro passages (53, 79). Adapted viruses have retained their parental cellular tropism but have gained the ability to infect CrFK cells as well as other nonlymphoid cells (53, 79). Determinants for the adapted phenotype have been mapped to Env in both the surface and transmembrane subunits (53, 79). The envelope of adapted viruses has also been shown to interact directly with CXCR4 (43), similar to what has been observed for CD4-independent CXCR4-using (X4) HIV isolates (40). It is yet to be determined if CXCR4 acts as a primary receptor or a coreceptor for FIV. However, it is possible that, by analogy with CD4-independent HIV isolates, CrFK-adapted FIV isolates may have gained independence from a CD4-like factor which is required by primary isolates.

To better address the role of CXCR4 and other receptors or coreceptors in the life cycle of FIV, we constructed chimeric fusion proteins between the surface glycoproteins (SU) from primary and CrFK-adapted isolates of FIV and the Fc domain of human immunoglobulin G1 (IgG1). The SU-immunoadhesins were used as tools to investigate the role of CXCR4 and other cell surface component(s) in the binding of FIV SU.

MATERIALS AND METHODS

Cell lines, reagents, and viruses. The Jurkat, CrFK, HeLa, G355-5, CHO-K1, and CHO-derived pgsA745 cell lines were obtained from the American Type Culture Collection (Rockville, Md.). The feline lymphoma cell line 3201 was obtained from W. Hardy. The 104-C1 and MCHS-4 feline primary T-cell lines were isolated by limiting-dilution cloning from feline PBMCs (54). The feline primary T-cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Gemini Bioproducts, Calabasas, Calif.), 2 mM l-glutamine (Sigma), 500 μg of gentamicin (Gemini Bioproducts) per ml, 1 mM sodium pyruvate (Sigma), 10 mM HEPES buffer (Sigma), minimal essential medium-vitamins (Sigma), nonessential amino acids (Sigma), β-mercaptoethanol ( Gibco-BRL, Gaithersburg, Md.), 2.5 μg of concanavalin A (Sigma) per ml, 50 U of human recombinant IL-2 (a gift from Hoffmann-La Roche) per ml, and 50 μg of gentamicin (Gemini Bioproducts) per ml. The Jurkat and 3201 cell lines were maintained in the same medium minus concanavalin A and IL-2. The fibroblastoid and glial cell lines were cultured in Dulbecco modified Eagle medium (DMEM) with the same supplements as described above. The control of the Rous sarcoma virus promoter in pRSC. The GS gene was cloned by PCR amplification from a CHO cDNA library. The sense (5-GGCCGCACGCGCTGACGAAATATTT) and antisense (5-GAGGTTTATATTCCATGGGATCCGCTGGGACC) primers, including HindIII sites (underlined), were designed from the nucleotide sequence of the GS gene (38). The GS gene was cloned by PCR amplification from a CHO cDNA library. The sense (5-GGCCGCACGCGCTGACGAAATATTT) and antisense (5-GAGGTTTATATTCCATGGGATCCGCTGGGACC) primers, including HindIII sites (underlined), were designed from the nucleotide sequence of the GS gene (38). The GS gene was cloned by HindIII and cloned under the control of the Rous sarcoma virus promoter in pRSC.

Expression, amplification, and purification of gp95-Fc fusion protein. For production of stable cell lines expressing Fc and gp95-Fc, 106 CHO-K1 cells were transfected with 10 μg of pRSC-GS-Fc and pRSC-GS-gp95-Fc. At 36 h posttransfection, cells were trypsinized, plated in 96 wells of a microtiter plate, and grown in G-DMEM containing 25 μM methionine sulfonate. After 14 to 21 days, clones surviving the selection were transferred to T25 flasks and grown to confluency. Supernatants were then harvested and incubated with 15 μl of protein A-Trisacryl at 4°C for 12 h. After three washes in phosphate-buffered saline (PBS), the beads were eluted in 25 μl of sodium dodecyl sulfate (SDS) sample buffer and loaded on an SDS–8 to 16% polyacrylamide gel, and the presence of Fc and gp95-Fc proteins was analyzed by Western blotting with antibodies to FIV Env and human Fc. Blots were probed with an enhanced chemiluminescence procedure (Pierce). Recombinant Fc and gp95-Fc proteins were batch purified by affinity chromatography over protein A as specified by the manufacturer (Pierce). The concentration of the purified Fc and gp95-Fc recombinant proteins was determined by using a biocinchoninic acid protein assay (Pierce).

Cytosfluorimetric analysis. Binding of gp95-Fc fusion proteins to the surface of Jurkat, 104-C1, 3201, HeLa, CrFK, G355-5, U87, and CHO cells was analyzed by flow cytometry. Briefly, 106 cells were incubated for 1 h at 4°C with 2.5 μg of gp95-Fc or Fc in PBS–0.1% bovine serum albumin. After two washes in ice-cold PBS, cells were labeled with a 1:100 dilution of fluorescein-conjugated goat anti-human IgG1 Fc antibody (Cappel, Durham, N.C.) for 1 h at 4°C. The cells were washed twice and then analyzed by flow cytometry on a FACScan using CellQuest software (BDIS, San Jose, Calif.). Specificity of SU binding was confirmed by a neutralization assay with a serum from an FIV-infected cat. For inhibition studies, chemokines or heparin were first added to the cells at the indicated concentration for 1 h at 4°C. The cells were then incubated with gp95-Fc recombinant proteins for 1 h, washed, further incubated with the anti-human IgG1 Fc antibody, and prepared for analysis as indicated above. Percent inhibition was calculated by the formula 100 – [(t – c)/(m – c) × 100], where t represents the signal for the test sample, c represents the background signal of mock-infected cells, and m represents the signal obtained for cells infected in the absence of chemokine.

Plasmid construction. SU of the envelopes of FIV-PPR and FIV-34T1F0, extending from the leader sequence to the beginning of the transmembrane region, were subcloned by PCR amplification (see Fig. 2). The sense primers, including NorI sites (underlined), had the sequences 5’-AGAAGATTTTAAATAATTGCGCACCACAATAATTGCGGAC-3’ and 5’-TAGAATAATTATTTGAATTGCGGACCAGAAAGGTCGAC-3’, respectively. The antisense primer, including BamHI sites (underlined), had the sequences 5’-GTATTATATAGTCATAGGATCGCCGTGTC TACTATA-3’ and 5’-AGGTTAGTTATCCATGGTACGCTTGAC AGTAAAGTA-3’ for FPR and 34T1F0, respectively. The amplified DNA product was digested with NorI and BamHI, gel purified, and ligated in frame with the Fc domain of human IgG1 (kindly provided by B. Seed [4]) in pCR3 (Invitrogen, Carlsbad, Calif.). Amplification of the expression of Fc and gp95-Fc protein, the glutamate synthetase (GS) amplification system was used (6). This system uses a vector that has a gene of interest and a GS gene which allows selection and amplification in a glutamine-free environment in the presence of methionine sulfonate, an inhibitor of the GS encoded by the vector and the CHO cells. For this purpose the Fc and gp95-Fc fragments were subcloned under the control of the cytosine DNA virus promoter in pRSC, a mammalian expression vector with two transcriptional units (kindly provided by T. C. Tsang, Arizona Cancer Center, Tucson) (78). The GS gene was cloned by PCR amplification from a CHO cDNA library. The sense (5’-CCCCACACAGCGCTCCGCGTC-3’) and antisense (5’-GATGAACCTAGAAGCTCCGACGCTCAAAG-3’) primers, including HindIII sites (underlined), were designed from the nucleotide sequence of the GS gene (38). The PCR product was digested with HindIII and cloned under the control of the Rous sarcoma virus promoter in pRSC.

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FIV-PPR uses CXCR4 as a coreceptor for entry in primary T cells. The two infectious molecular clones of FIV used in this study were previously reported (54, 63). FIV-PPR was obtained from a PI FIV (63), whereas FIV-34TF10 was cloned after adaptation of a PI FIV to propagate in CrFK cells (76). Both strains could be differentiated by their in vitro growth properties. While 34TF10 has a broad cell range tropism, FIV-PPR could propagate only in PBMCs, as well as IL-2-dependent primary feline T cell lines such as 104-C1 and MCH5-4 (54). Other feline cell lines, such as the lymphoma cell line 3201, the fibroblastoid cell line CrFK, and the glial cell line G355-5, are refractive to productive infection by FIV-PPR (54, 63). All of these cell lines are CXCR4 positive (see Fig. 8), indicating that there is a lack of correlation between CXCR4 expression and susceptibility to FIV-PPR infection. The 3201 cells could be distinguished from the other feline cell lines in that they expressed the highest level of CXCR4 (see Fig. 8). They also failed to be productively infected by FIV-PPR. However, long-term passage of FIV-PPR on these cells resulted in the production of a 3201-adapted FIV with an expanded host cell tropism that included productive infection of CrFK and G355-5 cells (53, 54). Adaptation of FIV-PPR in CrFK cells also resulted in the same expanded host cell tropism (A. de Parseval and J. H. Elder, unpublished data). These observations suggest that wild-type FIV-PPR might use a chemokine receptor different from CXCR4 and that adaptation in 3201 or CrFK cells resulted in a switch to CXCR4 use. We therefore tried to inhibit FIV-PPR infection of 104-C1 cells with SDF-1α, MIP-1β, and RANTES. Figure 1 shows that SDF-1α efficiently inhibited FIV-PPR infection of 104-C1 cells at a dose-dependent manner, whereas the β chemokines slightly increased the level of replication. These results suggest that CXCR4 is involved as a (co)receptor for FIV-PPR in 104-C1 cells, consistent with previous reports regarding the preferential use of CXCR4 by PI FIV (27, 68).

Recombinant SU-immunoadhesins. To analyze the interactions between FIV SU and specific cell surface receptor(s), a recombinant FIV SU-immunoadhesin was generated. Similar immunoadhesins have been previously reported for the SU glycoprotein of avian leukosis viruses (10), dengue virus (13), human herpesvirus 7 (73), and simian foamy virus (39). The FIV SU-immunoadhesins (designated gp95-Fc) consisted of amino acid residues 1 to 603 of FIV-PPR and FIV-34TF10 Env fused in frame to the hinge region of the Fc domain of IgG1 (Fig. 2A). The site corresponding to the disulfide bridge in Fc was preserved in order to maintain the homodimeric form of the recombinant proteins. The recombinant immunoadhesins were stably transfected in CHO cells, expressed at high levels in cell supernatants by using the GS gene amplification system (see Materials and Methods), and batch purified by affinity chromatography with protein A. By SDS-PAGE, gp95-Fc migrated at approximately 130 to 140 kDa under reducing conditions, and well above the 200-kDa marker in the absence of CrFK cells.
β-mercaptoethanol (Fig. 2B), consistent with the formation of disulfide-linked homodimers as reported for other SU-immunoadhesins (10, 13, 39, 73). Both gp95 immunoadhesins were specifically recognized by Western blotting with antibodies to FIV Env and human IgG1 Fc (Fig. 2C).

Binding of FIV gp95-Fc to feline cells correlates with susceptibility to infection. The recombinant immunoadhesins were used as probes to investigate the cellular binding of FIV SU by FACS analysis. Analyses were first performed on total PBMCs, before and after stimulation with concanavalin A and IL-2 (data not shown). As expected, a heterogeneous population of the unstimulated cells bound both SU proteins poorly. After 24 h on IL-2, the population of cells binding the two SU proteins became more homogeneous and the level of binding increased steadily and was nearly identical between both SU, consistent with the outgrowth of the T-cell population (data not shown). These studies demonstrated that the envelope proteins could specifically bind to distinct primary cell populations. However, it was clear that the heterogeneity of this population would preclude any rigorous characterization of receptor binding by either 34TF10 or PPR gp95-Fc. We thus turned to several cell lines, including primary cell lines that had been selected from outgrowth of PBMCs, for further studies (Fig. 3). Binding of Fc, which was used in our experiments as a negative control, was no greater than the background level (secondary antibody alone [data not shown]), indicating that the cellular binding of gp95-Fc was attributable solely to Env determinants. Binding of PPR gp95-Fc was observed only for
the 104-C1 cells. This binding pattern is in good agreement with the host cell range properties of FIV-PPR, suggesting that the block in the replication observed with the 3201, CrFK, and G355-5 cells occurs at the level of cellular binding. Binding of 34TF10 gp95-Fc was observed for all cell lines tested, which reflected the in vitro cellular tropism of FIV-34TF10. Additionally, 34TF10 gp95-Fc also bound to the human Jurkat and HeLa cell lines, consistent with the ability of FIV-34TF10 to use human CXCR4 as a fusion receptor (65, 80).

A non-CXCR4 receptor is involved in the binding of FIV gp95-Fc to primary T cells. We next addressed the question of whether the α chemokine SDF-1α, the natural ligand of CXCR4, could inhibit the binding of gp95-Fc to 104-C1 cells. As shown in Fig. 4, SDF-1α failed to inhibit the binding of either PPR or 34TF10 gp95-Fc to 104-C1 cells, even at a concentration as high as 10 μg/ml (data not shown). Similar results were also obtained with MCH5-4 cells, another feline primary T-cell line (data not shown). Two β chemokines, MIP-1β and RANTES, were also unable to block the binding of gp95-Fc to 104-C1 cells (Fig. 4). RANTES actually increased the binding of both SU-immunoadhesins, a phenomenon previously observed with T-tropic HIV-1 virions (19, 35, 49). These results indicate that binding of FIV SU to primary feline T cells is not exclusively through CXCR4.

Binding of 34TF10 gp95-Fc to Jurkat and 3201 cells is CXCR4 dependent. We next examined whether 34TF10 gp95-Fc used CXCR4 as a binding receptor on Jurkat and 3201 cells. Cells were preincubated with SDF-1α, and gp95-Fc binding was monitored by FACS analysis as described in the legend to Fig. 2. Results are means and standard deviations for triplicate determinations.

FIG. 4. Inhibition of gp95-Fc binding to lymphoid cells. 104-C1, Jurkat, and 3201 cells were pretreated with the indicated chemokines at 0.3 or 1.0 μg/ml for 1 h at 4°C before incubation with gp95-Fc for another hour at 4°C. After washing, gp95-Fc binding was monitored by FACS analysis as described in the legend to Fig. 2. Results are means and standard deviations for triplicate determinations.
inhibit 34TF10 gp95-Fc binding to any of the three cell lines (Fig. 5A), even at concentrations as high as 10 μg/ml (data not shown). The β chemokines were also unable to inhibit gp95-Fc binding (Fig. 5A). Furthermore, gp95-Fc binding was also observed for the CXCR4-negative U87 cell line (data not shown), suggesting that CXCR4 is not required for the cellular binding of 34TF10 gp95-Fc. It is well established that heparan sulfate proteoglycans (HSPGs) serve as facilitators in the cellular attachment of HIV which is both cell type and HIV strain dependent (45, 57, 59, 61, 70, 72). Enzymatic removal of heparan sulfate chains by heparinase reduces HIV attachment and infectivity. These studies have also shown that soluble heparans such as heparin or dextran sulfate inhibited replication of X4 HIV strains by specific binding to the V3 loop of gp120. A similar inhibition mediated by heparin and dextran sulfate was also reported for FIV (77). To elucidate whether HSPGs could also be involved in 34TF10 gp95-Fc binding, we first performed a competition study with heparin. Heparin and 34TF10 gp95-Fc were coincubated with the adherent cell lines, and inhibition of gp95-Fc binding was monitored by FACS analysis. As shown on Fig. 5B, heparin strongly inhibited gp95-Fc binding.

To further investigate the role of cell-surface heparans in mediating gp95-Fc binding, we treated HeLa (Fig. 6A) and CrFK (data not shown) cells with either heparinase I or chondroitinase ABC at a concentration of 10 U/ml for 1 h at 37°C and monitored gp95-Fc binding by FACS analysis. Heparinase and chondroitinase remove specifically the heparan and chondroitin sulfate chains, respectively, from the core of the proteoglycan. The specific enzymatic removal of heparan sulfate chains was assessed with an antibody specific for heparan sulfate chains (29). 34TF10 gp95-Fc efficiently bound to the CXCR4-negative wild-type CHO-K1 cells but failed to bind to pgsA-745 cells (Fig. 6B), suggesting that heparan sulfate chains are necessary for efficient binding of 34TF10 gp95-Fc to the cell membrane.

**Heparin inhibits 34TF10 gp95-Fc-CXCR4 interactions on Jurkat and 3201 cells.** Since HSPGs played an important role in mediating 34TF10 gp95-Fc binding to adherent cells, we also sought to determine their potential role in the binding of FIV gp95-Fc to the lymphoid cells used in this study. Heparin had no effect on the binding of either PPR or 34TF10 gp95-Fc to primary 104-C1 T cells (Fig. 7A), whereas a dose-dependent inhibition in the binding of 34TF10 gp95-Fc was observed for the Jurkat and 3201 cells (Fig. 7A), the two cell lines for which we demonstrated a direct interaction between 34TF10 gp95-Fc and CXCR4 (Fig. 4). To address the issue of whether heparin inhibited an interaction between gp95 and CXCR4 rather than an interaction between gp95 and HSPGs, Jurkat (Fig. 7B) and 3201 (data not shown) cells were treated in the absence or presence of heparinase I (10 U/ml). As shown in Fig. 7B, heparinase treatment had no effect on the binding mediated by heparin. Coincubation of heparin and 34TF10 gp95-Fc resulted in 46 and 40% inhibition of gp95-Fc binding for cells treated in the absence or presence of heparinase, respectively. Inhibition mediated by SDF-1α was also unchanged (Fig. 7B). These results indicate that heparin interferes in the interaction between gp95 and CXCR4, consistent with a recent study in which heparin was shown to interfere with the gp120-CXCR4 association by binding to V3 as well as the coreceptor binding site (58). FACS analysis of the cellular expression of HSPGs and CXCR4 showed that Jurkat and 3201 cells expressed very low level of HSPGs and relatively high levels of CXCR4, whereas for HeLa and CrFK cells the opposite was observed (Fig. 8). Therefore, the preferential binding of 34TF10 gp95-Fc to either CXCR4 or HSPGs on a given cell line could be determined by the relative expression level of both markers.

**PPR and 34TF10 gp95-Fc coprecipitate with a 40-kDa protein species from the surface of feline primary T cells but not from Jurkat or 3201 cells.** Our overall results suggest that neither CXCR4 nor HSPGs interact with PPR or 34TF10...
gp95-Fc on the surface of primary feline T cells, whereas only 34TF10 gp95-Fc interacts with CXCR4 on the surface of Jurkat or 3201 cells and with HSPGs on adherent cells. Successful coprecipitation of immunoadhesins with their specific ligands has been previously reported (1, 10, 33, 34, 74, 82). We therefore supplemented our flow cytometry results with immunoprecipitation assays. Primary feline T cells (104-C1 and MCH5-4), Jurkat cells, and 3201 cells were cell surface biotinylated and used in immunoprecipitation studies with either Fc or gp95-Fc, and coprecipitated complexes were immunoblotted with neutravidin. Figure 9 shows that PPR and 34TF10 gp95-Fc, but not Fc, coprecipitated with a 40-kDa protein species from lysates of primary T cells but not from CXCR4-rich Jurkat or 3201 cells, suggesting that this band is not CXCR4. Coprecipitated complexes were also immunoblotted with antibodies to CXCR4. However, we were unable to detect CXCR4 from Jurkat and 3201 cell lysates coprecipitated with 34TF10 gp95-Fc, even after using three different anti-CXCR4...
antibodies (data not shown). Difficulties in solubilizing CXCR4 have previously been reported (12), which may explain the failure to detect CXCR4 by this approach.

**DISCUSSION**

We report here the cellular binding properties of the SU protein of two divergent infectious molecular clones of FIV by using a recombinant soluble Env protein in which the transmembrane domain was replaced by the Fc domain of human IgG1. These gp95 immunoadhesins were used in FACS analyses to address the role of CXCR4 and other receptors in the binding of FIV SU to different cell lines. Although we observed a common mechanism of binding of FIV SU to primary feline T cells, binding of FIV SU to IL-2-independent T cells and adherent cells was both cell type and SU dependent.

The pattern of gp95-Fc binding to feline cells was consistent with the host cell range properties of both FIV-PPR and FIV-34TF10 (Fig. 3). 34TF10 gp95-Fc binding was observed for all feline cell lines tested, whereas PPR gp95-Fc binding was observed only for primary T-cell lines. This suggests that the inability of FIV-PPR to efficiently replicate in 3201, CrFK, and G355-5 cells is dictated by the first step in the virus life cycle, cellular attachment. This has profound implications for the mechanism by which the virus binds to and enters the target cell. FIV has been shown to use CXCR4 as a receptor for entry. However, certain cells, which are CXCR4 positive, are refractory to productive infection by primary FIV isolates. Here we demonstrate that the limiting step in the efficient replication of FIV-PPR in 3201, CrFK, and G355-5 cells was at

![FIG. 7. Heparin interferes with gp95-Fc binding to CXCR4.](image-url)
a preentry rather than a postentry level, suggesting that a receptor different from CXCR4 is involved in the binding of FIV-PPR SU. This hypothesis was further confirmed by inhibition studies with SDF-1α. While SDF1-α was unable to efficiently block the binding of PPR gp95-Fc to 104-C1 (Fig. 4) and MCH5-4 (data not shown) cells, a dose-dependent inhibition of FIV-PPR replication in 104-C1 cells was observed (Fig. 1). These data demonstrate that FIV-PPR uses CXCR4 as an entry receptor in 104-C1 cells, consistent with previous reports showing that PI FIV use CXCR4. Most importantly, these data strongly suggest that a non-CXCR4 receptor is involved in the binding of PPR gp95 to primary T cells. Immunoprecipitation studies were used to complement our FACS analyses and a 40-kDa protein species distinct from CXCR4 was specifically coprecipitated from the surface of primary T cells but not from the nonpermissive Jurkat and 3201 cells (Fig. 9). Whether this protein is a primary binding receptor for FIV-PPR on primary T cells remains to be determined. Similar results were observed for 34TF10 gp95-Fc on primary T cells, which suggests that different isolates of FIV use a common mechanism of binding to primary T cells. However, FIV-34TF10 has a broader cellular tropism that was reflected by the ability of 34TF10 gp95-Fc to efficiently bind all cell lines tested (Fig. 3). Two mechanisms for the cellular binding of 34TF10 gp95-Fc could be inferred from our results. Our data clearly show that 34TF10 gp95-Fc uses CXCR4 as a primary binding receptor on Jurkat and 3201 IL-2-independent T cells (Fig. 4), whereas HSPGs contribute to the binding of 34TF10 gp95-Fc to adherent cells (Fig. 6). Binding of 34TF10 gp95-Fc to CXCR4 on adherent cells was observed only after enzymatic removal of heparan sulfate chains (Fig. 6A), suggesting that FIV (in this case FIV-34TF10) might be funneled to CXCR4 after initial binding to HSPGs. The low level of binding of 34TF10 gp95-Fc to heparinase-treated cells was unexpected but could be correlated to the low level of expression of CXCR4 on these cells (Fig. 8). The ability of 34TF10 gp95-Fc to bind either HSPGs or CXCR4 could be directly correlated to the level of expression of both markers on the cell surface (Fig. 8). Jurkat and 3201 cells expressed very low levels of heparans but high levels of CXCR4, and 34TF10 gp95-Fc was shown to bind exclusively to CXCR4 on these cells. On the other hand, 34TF10 gp95-Fc binding was predominantly if not exclusively through HSPGs on HeLa and CrFK cells, which expressed high levels of heparans but low levels of CXCR4 (Fig. 8).

Soluble heparans such as heparin and dextran sulfate interact with the V3 loop of T-tropic HIV-1 gp120 without interfering with the binding of gp120 to CD4 (5, 11, 36, 37, 69, 70). This interaction is dependent on the net charge of the V3 loop and is thought to be mediated via electrostatic interactions between the acidic sulfate groups of the heparan sulfate chains and the basic residues contained within the V3 loop. Recently, soluble heparans have also been shown to interact with the conserved coreceptor binding site on gp120 (58). The FIV strains used in our study have a V3 loop with net charges of +4 and +8 for PPR and 34TF10, respectively (Fig. 10). This main difference could explain the specific interaction of 34TF10 gp95-Fc with HSPGs on adherent cells. Our results also corroborate those reported by Moulard et al. (58), since heparin could specifically interfere with the interactions between gp95-Fc and CXCR4 on Jurkat and 3201 cells (Fig. 7).

PI FIV adapted to propagate in CrFK cells have been shown to have a more basic V3 loop, involving in particular the mutation of either one or two glutamate residues (407 and 409) by lysine residues in the V3 loop (60, 75, 76, 79). Adaptation of FIV-PPR for propagation in CrFK cells also resulted in an increase in the net charge of V3 due to a change of glutamate to lysine at position 407 (Fig. 10) (de Perseval and Elder, unpublished). However, adaptation of FIV-PPR in 3201 cells resulted in several point mutations dispersed throughout the entire Env region, but none resulted in an increase in the net charge of V3 (Fig. 10) (53). Interestingly, 3201 cells expressed very low levels of HSPGs whereas CrFK cells express high levels of HSPGs (Fig. 8). Furthermore, X4 HIV isolates passaged in T-cell lines that expressed relatively high levels of HSPGS such as H9 and MT2 cells tend to have more basic V3 loops than those passaged in primary activated T cells which express small amounts of HSPGs (32, 45, 47, 59, 61). Therefore, the increase in the net charge of V3 associated with the CrFK phenotype could result from the adaptation of the virus to HSPGs. FIV-PPR adapted on 3201 cells has the ability to infect cells that were previously refractory for the parental virus (53). Infection of these cells was shown to be mediated through CXCR4 (53). It would therefore be interesting to analyze gp95-CXCR4 and gp95-HSPG interactions with gp95.
immunoadhesins obtained from FIV-PPR adapted to either CrFK or 3201 cells.

The inability of SDF-1α to inhibit 34TF10 gp95-Fc binding to HeLa or CrFK cells (Fig. 5A) was unexpected, since FIV gp95 from another CrFK-adapted strain, FIV-F14, has been reported by Hosie and coworkers to compete with SDF-1α for binding to CXCR4 on CXCR4-transfected U87 cells (43). Here we found that 34TF10 gp95-Fc bound efficiently to the CXCR4-negative parental U87 cells through HSPGs (data not shown). FIV-14 and FIV-34TF10 have very similar amino acid sequences, since they were obtained from the same FIV primary isolate which was adapted for propagation in CrFK cells (60, 76). However, FIV-F14 as a V3 loop with a net charge of +6, compared to +8 for FIV-34TF10 (Fig. 10), and Hosie et al. (43) used a monomeric form of gp95 whereas our gp95 immunoadhesin is in a dimeric form (Fig. 2B). Recent modelings of oligomeric gp120 suggest that electrostatic properties are stronger for oligomeric gp120 than monomeric gp120, and are influenced by the net charge of V3 (51, 58). These reports confirmed an earlier study by Roderiguez et al. (70) in which monomeric gp120 from an X4 HIV isolate failed to interact with cell-surface heparans, whereas oligomeric gp120 from the same isolate bound specifically. Therefore, the decrease in both the electrostatic properties and the net charge of FIV-F14 gp95 V3 loop compared to 34TF10 gp95-Fc could explain the inability of F14 gp95 to interact with cell-surface heparans and its specific interaction with CXCR4.

The distinct host cell range properties and the preferential use of CXCR4 by primary as well as CrFK-adapted FIV isolates, combined with our current findings, support the possibility that a primary receptor could be required by FIV, similar to CD4 for HIV, a hypothesis already suggested by others (68, 81). CXCR4 expression in vitro does not correlate with susceptibility to infection with PI FIV (54). Several CXCR4-positive cell lines are refractory to productive infection by PI FIV. These observations are supported by our results, which suggest that PPR gp95-Fc failed to bind directly and specifically to CXCR4 (Fig. 4). However, CXCR4 is implicated, since replication of FIV-PPR (Fig. 1) and other PI FIV (27, 68) is inhibited by SDF-1α or AMD3100. This suggests that cellular attachment of PI FIV might follow a two-step model similar to the case for HIV. In a first step, gp95 would bind to a CD4-like factor that would induce conformational rearrangements in gp95; in a second step, gp95 would interact with CXCR4. This hypothesis would explain the inability of PPR gp95-Fc to bind directly to CXCR4 (Fig. 4). The fact that 34TF10 gp95-Fc interacted directly with CXCR4 on Jurkat and 3201 cells (Fig. 4) could be a result of its adaptation to propagate in CrFK cells similar to HIV isolates adapted to grow in CD4-negative cells. Consistent with the current opinion on HIV cellular binding and entry events (20), the differences we observed in the cellular binding of PPR and 34TF10 gp95-Fc suggest that binding affinity may be influenced by (i) the conformation of the SU in question, (ii) the lack or presence of a CD4-like factor that would induce conformational changes in SU to allow binding to CXCR4, (iii) the conformational heterogeneity of CXCR4, or (iv) some combination of the above.

Our study further addresses the issue of a candidate primary cell-surface receptor for FIV. gp95-Fc but not Fe specifically coprecipitated a 40-kDa protein from the surface of primary feline T cells but not from either Jurkat or 3201 cells (Fig. 9). Interestingly, SDF-1α was unable to inhibit the binding of 34TF10 gp95-Fc to primary feline T cells (Fig. 4), and we showed that 34TF10 gp95-Fc also coprecipitated this 40-kDa protein from primary T cells (Fig. 9). These findings suggest a preferential high-affinity binding of 34TF10 gp95-Fc for this protein rather than to CXCR4 as is the case for CD4-independent HIV variants which have retained the property of high-affinity binding to CD4 (20). Jurkat and 3201 cells do not express this gp95-Fc binding protein, or, in the case of the Jurkat cells, the human counterpart might not be specific for FIV. Adaptation of FIV-34TF10 to propagate in CrFK cells might have allowed the virus to gain independence from that 40-kDa protein. This would explain the ability of 34TF10 gp95-Fc to bind directly to CXCR4 on Jurkat and 3201 cells (Fig. 4). We were unable to show a specific coprecipitation of 34TF10 gp95-Fc with CXCR4 from Jurkat and 3201 cell lysates (data not shown). However, difficulties in solubilizing CXCR4 have been reported (12). The use of different detergent combinations might help us to successfully coprecipitate 34TF10 gp95-Fc with CXCR4. To definitively address the question of whether this gp95-Fc binding protein is a putative primary binding receptor for FIV, we are currently purifying the protein and attempting to isolate the gene encoding this protein by expression cloning.

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REFERENCES


\[\text{FIG. 10. Amino acid sequences and overall charge of FIV V3 loop. Residues differing between the clones are indicated. Amino acids (positions 407 and 409) that are important for CrFK tropism are boxed.}\]
