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Shared Usage of the Chemokine Receptor CXCR4 by the Feline and Human Immunodeficiency Viruses

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Feline immunodeficiency virus (FIV) induces a disease state in the domestic cat that is similar to AIDS in human immunodeficiency virus (HIV)-infected individuals. As with HIV, FIV can be divided into primary and cell culture-adapted isolates. Adaptation of FIV to replicate and form syncytia in the Crandell feline kidney (CrFK) cell line is accompanied by an increase in the net charge of the V3 loop of the envelope glycoprotein, mirroring the changes observed in the V3 loop of HIV gp120 with the switch from a non-syncytium-inducing phenotype to a syncytium-inducing phenotype. These data suggest a common mechanism of infection with FIV and HIV. In this study, we demonstrate that cell culture-adapted strains of FIV are able to use the α-chemokine receptor CXCR4 for cell fusion. Following ectopic expression of human CXCR4 on nonpermissive human cells, the cells are able to fuse with FIV-infected feline cells. Moreover, fusion between FIV-infected feline cells and CXCR4-transfected human cells is inhibited by both anti-CXCR4 and anti-FIV antibodies. cDNAs encoding the feline CXCR4 homolog were cloned from both T-lymphoblastoid and kidney cell lines. Feline CXCR4 displayed 94.9% amino acid sequence identity with human CXCR4 and was found to be expressed widely on cell lines susceptible to infection with cell culture-adapted strains FIV. Ectopic expression of feline CXCR4 on human cells rendered the cells susceptible to FIV-dependent fusion. Moreover, feline CXCR4 was found to be as efficient as human CXCR4 in supporting cell fusion between CD4-expressing murine fibroblast cells and either HIV type 1 (HIV-1) or HIV-2 Env-expressing human cells. Previous studies have demonstrated that feline cells expressing human CD4 are not susceptible to infection with HIV-1; therefore, further restrictions to HIV-1 Env-dependent fusion may exist in feline cells. As feline and human CXCR4 support both FIV- and HIV-dependent cell fusion, these results suggest a close evolutionary link between FIV and HIV and a common mechanism of infection involving an interaction between the virus and a member of the seven-transmembrane domain chemokine receptor family of molecules.

Feline immunodeficiency virus (FIV) infection of the domestic cat induces a disease state characterized by a progressive depletion of CD4$^+$ T lymphocytes (1, 40). FIV infects and causes CD4 down-regulation in CD4$^+$ T cells (57), and CD4$^+$ T cells are the principal reservoir of FIV proviral DNA in the acutely infected cat (12, 21). However, the feline homolog of CD4 does not appear to act as a primary receptor for the virus; antibodies recognizing diverse epitopes on feline CD4 do not inhibit infection with FIV (28, 59), and ectopic expression of feline CD4 on feline fibroblasts does not render them susceptible to infection with lymphotropic isolates of FIV (39). Moreover, FIV infects a range of CD4-negative cell types, including CD8$^+$ T cells, B cells (12, 21), cells of neuronal lineage (18), and monocytes/macrophages (7). Indeed, the principal reservoir of FIV proviral DNA in the chronically infected cat appears to be CD21$^+$ lymphocytes (B cells) and CD8$^+$ T cells (13, 21). Despite extensive studies of the cell tropism of FIV, a primary binding receptor for FIV has not yet been identified. While monoclonal antibodies recognizing the feline homolog of CD9 have been shown to inhibit FIV infection (28), recent studies have suggested that the block to FIV infection in cells treated with anti-CD9 antibody occurs at a postentry stage of the viral life cycle (59), suggesting that CD9 does not function as a primary receptor for the virus.

The primary receptor for human immunodeficiency virus (HIV) is CD4 (11, 32), a member of the immunoglobulin superfamily of molecules. However, expression of CD4 alone on cell types such as the murine fibroblast cell line 3T3 permits binding of the viral envelope glycoprotein gp120 but is insufficient to confer susceptibility to infection with HIV (34), suggesting that infection requires the coexpression of a limiting accessory factor. Recent studies have demonstrated that members of the seven-transmembrane domain superfamily of molecules act as coreceptors for CD4-dependent infection with HIV. T-cell-line-adapted strains of HIV type 1 (HIV-1) were shown to utilize the CXC chemokine receptor CXCR4 (fusin, LESTR) (22), while primary non-syncytium-inducing (NSI) isolates utilized the CC chemokine receptor CCR5 (2, 8, 15, 17, 19). Moreover, the CXC chemokine SDF-1 and the CC chemokines RANTES, MIP-1α, and MIP-1β prevent infection with T-cell-line-adapted and primary NSI strains of HIV-1, respectively (4, 10, 42). The relationship between coreceptor usage and virus strain appears to be complex, and several dual-tropic strains of virus, including primary, syncytium-inducing (SI) strains of HIV-1 (52), that are able to utilize both CC and CXC chemokine receptors as fusion coreceptors have been described (8, 17). Moreover, additional members of the seven-transmembrane domain superfamily, including CCR3 and CCR2b, support CD4-dependent infection by HIV (8, 17).

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Although HIV-1 requires coexpression of both the primary receptor CD4 and a coreceptor such as CXCR4 or CCR5 for infection, recent studies have demonstrated that CD4-independent infection by HIV-2 is mediated by CXCR4 alone (20).

HIV-2 and simian immunodeficiency virus (SIV), but not HIV-1, can infect feline cells expressing human CD4 (35), suggesting that feline cell surface molecules can act as coreceptors for HIV-2 and SIV infection. Similarly, cell culture-adapted strains of FIV can infect and fuse human cells (30, 45). The FIV Env protein is rendered fusogenic in human cells by an increase in the net charge of the V3 loop, typically by a glutamate-to-lysine substitution (51, 55). Similar changes have been observed in the V3 loop of HIV with the switch from an NSI to an SI phenotype (14), suggesting a common underlying mechanism of cell-cell fusion and infection. Moreover, as the V3 loop is a primary determinant of the cell tropism of HIV, SIV, and FIV (26, 29, 31, 49–51, 55) and may determine the nature of the chemokine receptor used by HIV (8), the data implicate feline chemokine receptors as candidate cellular receptors for FIV.

In this study, we investigated the role of the chemokine receptor CXCR4 in infection with FIV. We demonstrate that FIV Env-mediated fusion of human cells by cell culture-adapted strains of FIV is mediated by CXCR4. Furthermore, we cloned the feline homolog of CXCR4 and found that it can support fusion mediated by the HIV-1 and FIV envelope glycoproteins. The data suggest that the usage of chemokine receptors for infection may be a conserved property of lentiviruses and may play a critical role in the immunodeficiency associated with lentivirus infection.

MATERIALS AND METHODS

Cell lines and cell culture. Cell culture media were obtained from Life Technologies, Paisley, United Kingdom. Adherent cell lines were maintained in Dulbecco's modification of minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (DMEM-10), streptomycin (100 μg/ml), and penicillin (100 IU/ml). The U87, U87.CD4, U87.CXCR4, and U87.CD4.CXCR4 cell lines were kindly provided by Nathaniel Landau, Aaron Diamond Research Center, New York, N.Y. HT11.5 is a $CD4^+$ clone of the human fibrosarcoma cell line HT1080. HT11.5 and HT11.5.CXCR4 (6) were obtained from David Wilkinson, Aaron Diamond Research Center. The U87T4 cells expressing human CXCR4, CCR5, CCR1, CCR2b, and CCR4 were a generous gift from Daniel Littman, Howard Hughes Medical Institute, The Skirball Institute of Biomolecular Medicine, New York University Medical Center. The FIV Env-expressing cell lines were derived from the Ho6T1 clone of the Crandell feline kidney (CrFK) cell line persistently infected with CrFK-adapted strains of FIV-Petaluma (FIV_{PET}) and FIV-Glasgow 8 (FIV_{GL8}) (44). HIV Env-expressing cells were HeLa cells expressing Env from HIV-1_{LAI}, HIV-1_{ADA}, or HIV-2_{ROD} and were a generous gift from M. Alizon, Institut Cochin Génétique et Moléculaire, Paris, France. $HeLa_{LAI}$ cells have been described previously (48).

Plasmid DNAs and viruses. Human CCR5.cDNA3 was obtained from Marc Parmentier, Université Libre de Bruxelles, Brussels, Belgium. Plasmids p478 and p461, in which the *lacZ* gene and human CD4, respectively, are linked to the T7 promoter, were obtained from M. Alizon. Feline CD9-cDNA3 has been described previously (60). The vaccinia virus-T7 recombinant vTF7-3 has been described previously (23). FIV_{GL8} is a primary isolate of FIV propagated in the interleukin-2-dependent feline T-cell line Q201 (57); FIV_{PET} is a cell culture-adapted stock derived from the F14 molecular clone (43) and propagated in CrFK cells.

Antibodies and flow cytometry. The anti-human CXCR4 monoclonal antibody 12G5 (20) was obtained from James A. Hoxie, University of Pennsylvania, Philadelphia. Anti-CD9 monoclonal antibody vpg15 has been described previously (27, 28). Goat anti-RD114 was obtained from Quality Biotech Inc., National Cancer Institute. Anti-FIV polyclonal cat serum Q71 was collected from a specific-pathogen-free cat inoculated with FIV_{PET}. Specificity of the anti-FIV serum was confirmed by immunoblot and immunofluorescence analyses.

Flow cytometric analyses were performed essentially as described previously (60). Briefly, adherent cells were removed from the culture plastic by incubation with EDTA (Life Technologies) and resuspended in phosphate-buffered saline (PBS) supplemented with 1.0% bovine serum albumin and 0.1% sodium azide. The cells were then incubated with either antibody 12G5 or an isotype-matched control for 30 min at 4°C, washed twice by centrifugation, and then incubated for a further 30 min with fluorescein isothiocyanate-conjugated $F(ab')_2$ fragment of goat anti-mouse immunoglobulin G. Samples were washed twice by centrifugation and then analyzed on a Coulter EPICS Elite flow cytometer, 5,000 events being acquired in LIST mode for each sample.

Molecular cloning of a cDNA encoding feline CXCR4. $Poly(A)^+$ mRNA was prepared from the feline T-lymphoblastoid cell line Mya-1 (37) by using a FastTrack 2 kit (Invitrogen B.V., Leek, The Netherlands) and cDNA synthesis by using Copy kit (Invitrogen). Consensus oligonucleotide primers were generated based on the published sequences of the ovine, bovine, and human CXCR4 genes and encompassing the 5′ ATG and the 3′ stop codons (5′-primer CTGGAGA[A/G]CCAGCGGTTACCATGGA; 3′-primer GTCTTAGCTGGAGTGAAAACTTGAAGA). The feline CXCR4 homolog was amplified from both Mya-1 and CrFK cDNAs by using PCR in a Perkin-Elmer model 2400 cycler and AmpliTaq DNA polymerase (Perkin-Elmer), with denaturing for 5 min at 94°C, followed by 35 cycles of 91°C for 1 min, 55°C for 1 min, and 72°C for 3 min, with a final extension of 10 min at 72°C. The feline CXCR4 cDNAs were cloned into the pCRII vector (TA cloning kit; Invitrogen) and sequenced by using Sequenase (Amersham International Ltd.). Amino acid sequence comparisons were performed by using the algorithm of Needleman and Wunsch (GAP and PILEUP [38]), using the Genetics Computer Group package (16). The feline CXCR4 sequence was compared to those of human CXCR4, rat CXCR4, bovine CXCR4, and murine CXCR4 (GenBank accession no. X71635, U54791, M86739, and U59760, respectively). The feline CXCR4 homolog was subcloned into the pcDNA3 and pRep4 eukaryotic expression vectors (Invitrogen) and transfected into the U87 cell line by using the calcium phosphate-DNA coprecipitation technique (24), and stable transfectants were selected by using G418 sulfate (800 μg/ml; Life Technologies) and hygromycin B (50 μg/ml), respectively.

CXCR4 mRNA expression was quantified by Northern blotting using the full-length human CXCR4 cDNA as the probe. Total RNA was prepared from each cell line by using RNAzol B (Biogenesis Ltd., Poole, United Kingdom), and 20 μg of each sample was separated by electrophoresis under denaturing conditions, transferred onto a HybondN membrane (Amersham), and cross-linked by UV irradiation (model XL-1500 UV cross-linker; Spectronics Corporation, Westbury, N.Y.). Equivalent sample loading was confirmed by ethidium bromide staining of a duplicate agarose gel following electrophoresis. Probes were labeled by random priming (High Prime; Amersham) with [α-^{32}P]dCTP (Amersham). A β-actin control probe was prepared by using a 838-bp fragment of the human β-actin cDNA isolated from HeLa mRNA, using human β-actin primers (Clontech Laboratories Inc., Palo Alto, Calif.).

Cell fusion and antibody blocking assays. FIV-infected CrFK cells were maintained in logarithmic growth phase prior to assay. Target cells (U87, HT11.5, and transfected derivatives) were trypsinized and seeded at 10^4 cells per well of 96-well cell culture plates. The cells were allowed to adhere for 1 h at 37°C, after which the medium was aspirated and replaced with either culture medium supplemented with antibody at the appropriate dilution or culture medium alone. Target cells were incubated for a further 30 min at 37°C before the addition of 10^4 FIV-infected CrFK cells per well. Cells were then incubated for 18 to 24 h at 37°C, and the medium was aspirated; the cells were rinsed once with PBS and then fixed and stained for 10 min with 1% methylene blue–0.25% basic fuchsin in 100% methanol.

Vaccinia virus-T7-based cell fusion assay. cDNAs encoding prospective fusion coreceptors were transfected into 3T3 cells by using DOTAP (Boehringer Mannheim) according to the manufacturer's protocol. Typically, 0.5 μg of each plasmid DNA was added to each well of a six-well culture plate and incubated for 6 h. All of the prospective cofactors were expressed from the cDNA3 vector. The DNA DOTAP mix was then removed and the cells incubated overnight at 37°C in DMEM-10. The transfected cells were then infected with vTF7-3 (multiplicity of infection of approximately 10) for 1.5 h, trypsinized, resuspended in 0.5 ml of DMEM-2.5, and incubated for a further 8 h (expression period). The vTF7-3-infected cells were then plated onto the HIV Env-expressing target cells. Fusion was allowed to proceed for 2 to 2.5 h, and then suspension cells were aspirated, and the adherent cells were rinsed with PBS and then fixed for 10 min with 0.5% glutaraldehyde in PBS. The fixed cells were rinsed a further two times with PBS and then incubated overnight at 4°C with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) substrate solution consisting of 0.5 mg X-Gal per ml in PBS containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM magnesium chloride. Target cells were transfected overnight with T7-*lacZ* (p478) by using a standard calcium phosphate-DNA coprecipitation technique 24 h prior to mixing with the vTF7-3-infected effector cells.

Nucleotide sequence accession number. The sequence of the feline CXCR4 homolog has been deposited in GenBank under accession no. U63558.

RESULTS

CXCR4 supports FIV-dependent fusion between feline and human cells. Previously, we demonstrated that fusion between the human cell line HeLa and FIV-infected cells is inhibited by both the anti-CXCR4 antibody 12G5 and anti-FIV Env monoclonal antibodies (58), suggesting that FIV Env-dependent fusion of human cells was mediated by CXCR4. We therefore examined whether FIV Env-dependent fusion could

TABLE 1. Syncytium formation between FIV Env-expressing cells and cells transfected stably with CXCR4

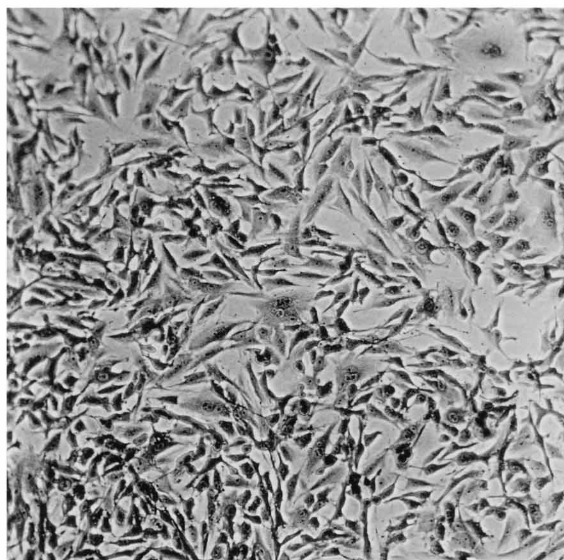
Cells[a]	Syncytium formation[b]					
	U87[c]	U87.CXCR4	U87.CD4	U87.CD4.CXCR4	HT11.5	HT11.5.CXCR4
Uninfected	−	−	−	−	−	−
Infected with:						
FIV_{GL8}	−	+++	−	+++	−	++
FIV_{PET}	−	++	−	++	−	+

[a] Uninfected CrFK cells or CrFK cells infected with FIV_{GL8} or FIV_{PET} were used as the source of FIV Env-expressing cells.
[b] Syncytia were scored as cells with five or more nuclei. Syncytium formation was scored as no syncytium (−) syncytia detected (+), and intermediate (++) and marked (+++) syncytium formation.
[c] Fusion partner.

be reconstituted in human cells that are refractory to fusion by transfection with human CXCR4. Persistently infected CrFK cells were mixed with either HT11.5, U87, or U87.CD4 cells and with derivatives of these cell lines stably expressing human CXCR4 (HT11.5.CXCR4, U87.CXCR4, and U87.CD4.CXCR4). The results are summarized in Table 1. Plating CrFK cells infected with a cell culture-adapted strain of FIV_{GL8} with a 1:1 ratio of HT11.5.CXCR4 resulted in syncytium formation, whereas no syncytia were evident in the control culture (Fig. 1). Similarly, mixing FIV-infected cells with U87.CXCR4 or U87.CD4.CXCR4 resulted in marked syncytium formation, whereas no syncytia were observed in mixtures of FIV-infected cells and U87 or U87.CD4. Syncytium formation was more pronounced in the U87 transfectants than the HT11.5 transfectants. Similar results were obtained when CrFK cells infected with FIV_{PET} were used in place of those infected with FIV_{GL8}. Mixing uninfected CrFK cells with HT11.5.CXCR4, U87.CXCR4, or U87.CD4.CXCR4 did not result in syncytium formation.

Having observed that optimal fusion occurred between FIV_{GL8}-infected CrFK cells and CXCR4-transfected U87.CD4 cells, we next examined whether fusion could be inhibited by anti-CXCR4 antibody. U87.CD4.CXCR4 cells were preincubated with either anti-CXCR4, anti-CD9, or a polyclonal cat serum from a specific-pathogen-free cat infected with FIV prior to mixing with FIV_{GL8}-producing CrFK cells. Syncytium formation was inhibited completely by preincubation with either the anti-FIV serum or anti-CXCR4 antibody, whereas anti-CD9 had no effect on fusion (Fig. 2). Similar findings were observed when the FIV_{GL8}-infected CrFK cells were replaced with FIV_{PET}-infected CrFK cells or when the U87.CD4.CXCR4 cells were replaced with U87.CXCR4 cells (not shown). The data suggest that transfection of U87 cells with human CXCR4 is sufficient to render the cells susceptible to FIV-dependent cell-cell fusion and confirm that cell culture-adapted strains of FIV can utilize CXCR4 to fuse human cells.

Molecular cloning of feline CXCR4. Having demonstrated that FIV could utilize human CXCR4 to fuse human cells, we next set out to investigate the fusogenic properties of the feline homolog of CXCR4. cDNA clones encoding feline CXCR4 were amplified by PCR from mRNA prepared from either of two cell lines, the interleukin-2-dependent T-cell line Mya-1 and CrFK, and the nucleic acid sequence was determined. The T-cell-derived and CrFK-derived clones of feline CXCR4 differed at a single amino acid (position 244), a predicted isoleucine residue in the CrFK clone being replaced by a valine residue in the Mya-1-derived clone (there is an isoleucine residue at this position of the bovine, human, murine, and rat



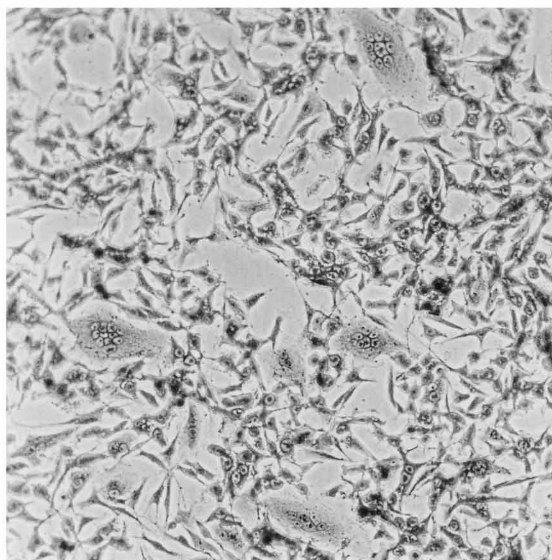


FIG. 1. Transfection of human cells with human CXCR4 confers susceptibility to FIV Env-dependent fusion. FIV_{GL8}-infected CrFK cells were mixed with the human colonic carcinoma cell line HT11.5 or HT11.5 cells transfected with CXCR4. At 18 h postmixing, the cells were fixed, stained, and photographed.

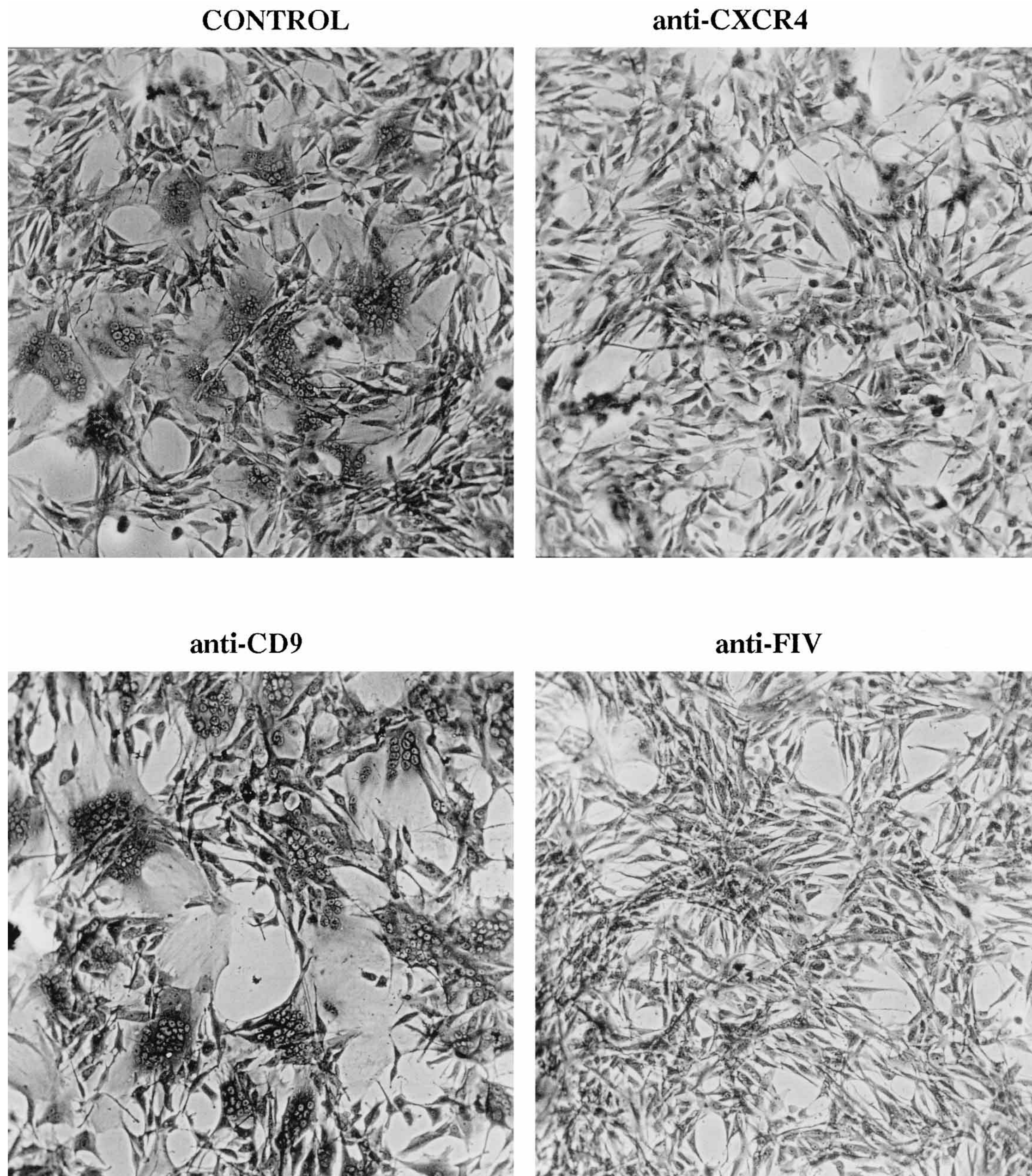


FIG. 2. FIV-dependent cell fusion is inhibited by anti-CXCR4 or anti-FIV antibody. FIV_{GL8}-infected CrFK cells were mixed with U87.CD4 cells stably expressing human CXCR4. Fusion was observed between FIV-infected cells and U87.CD4.CXCR4 cells within 18 h postmixing (control). Incubation of the cells in the presence of either 50 μg of anti-CXCR4 antibody (anti-CXCR4) per ml or a 1/40 dilution of heat-inactivated serum from a specific-pathogen-free cat infected with FIV (anti-FIV) inhibited fusion completely, whereas 50 μg of anti-CD9 antibody per ml had no effect.

CXCR4 sequences). The predicted amino acid sequence of feline CXCR4 displayed 94.9% identity to both human CXCR4 (Fig. 3) and bovine CXCR4 and 90.5% identity to murine CXCR4. The majority of the differences in predicted amino acid sequence between feline and human CXCR4 are located in the N terminus and the second extracellular loop, while single amino acid changes were observed in the first and third extracellular loops. The amino terminus of feline CXCR4 is a single aspartate residue (amino acid 15 of feline CXCR4) longer than human CXCR4, a feature that it shares with bovine CXCR4. There is a single predicted site for N-linked glycosylation in feline CXCR4 at amino acid 11 of the N terminus. Interestingly, the predicted site for N-linked glycosylation present at amino acid 177 of human CXCR4 is unique to human CXCR4 and is absent from feline, macaque, bovine, rat, or murine CXCR4.

The expression of CXCR4 mRNA in a range of feline cell lines was examined by Northern blotting (Fig. 4). The quality of each RNA sample was confirmed by hybridization with a β-actin probe (not shown). CXCR4 mRNA expression was detected in the feline cell lines 3201, CrFK, and Mya-1, which are susceptible to infection with cell culture-adapted strains of FIV. CXCR4 expression was not detected in the cell line AH927, which is refractory to infection with FIV (Table 2).

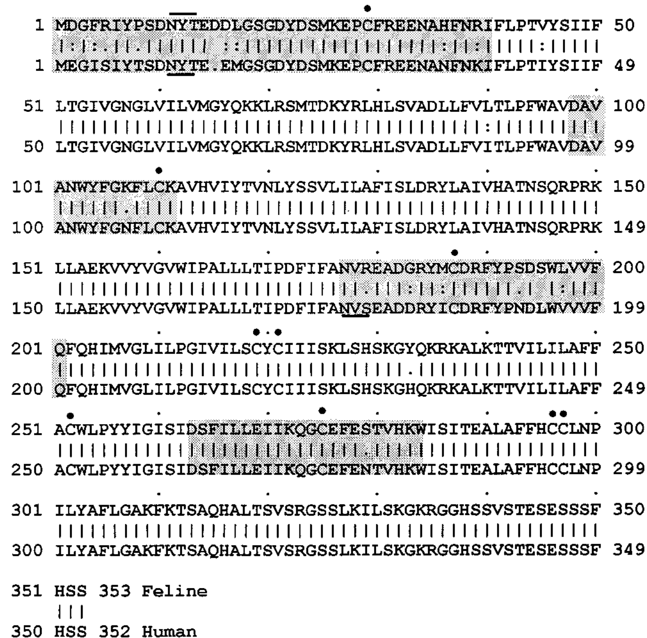


FIG. 3. Comparison of the predicted amino acid sequences of feline and human CXCR4. Extracellular domains are shaded, dots represent conserved cysteine residues, and predicted sites for N-linked glycosylation are marked by horizontal bars. The characters |, :, and . denote degrees of amino acid identity as defined in the blosum62 scoring matrix (25), where | denotes an identical residue, : denotes an amino acid whose comparison value is greater than or equal to the average positive nonidentical comparison value in the scoring matrix, and . denotes a value in the matrix greater than or equal to 1.

Significant levels of CXCR4 mRNA were detected in the human cell line HeLa but were not detected in the murine fibroblast cell line 3T3, in agreement with previous reports (22). The failure to detect CXCR4 expression by Northern blotting in the AH927 cell line may suggest that the resistance of this cell line to infection with cell culture-adapted strains of FIV may be linked to the low level of CXCR4 expression. CXCR4 mRNA expression did not correlate with susceptibility to infection with a primary isolate of the FIV_{GL8} strain of FIV (Table 2, $FIV_{GL8\text{-}PBMC}$).

Feline CXCR4 expression confers susceptibility to FIV-dependent fusion. Having cloned cDNAs encoding the feline homolog of CXCR4, we examined whether it could act as a coreceptor for fusion. The human cell line U87 was transfected stably with either feline CXCR4 or feline CD9 and screened for ability to support FIV-dependent cell fusion (Table 3). Marked syncytium formation was observed when FIV_{GL8}-infected CrFK cells were mixed with either U87 expressing feline CXCR4 or human CXCR4 but not with U87, U87 expressing

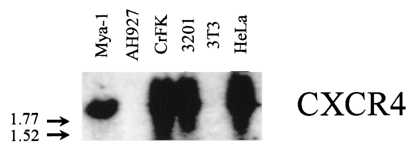


FIG. 4. CXCR4 mRNA expression in feline cell lines correlates with susceptibility to infection with cell culture-adapted strains of FIV. Aliquots of 20 μg of total RNA from the feline cell lines Mya-1, AH927, CrFK, and 3201, the human cell line HeLa, or the murine cell line 3T3 were reverse transcribed, transferred to a nylon membrane, and probed with an [α-^{32}P]dCTP-labeled CXCR4 probe. Approximate locations of 1.77- and 1.52-kb RNA markers are indicated by arrows.

TABLE 2. Comparison of CXCR4 mRNA expression and susceptibility to infection with a cell culture-adapted isolate ($FIV_{PET\text{-}CrFK}$) and a primary isolate ($FIV_{GL8\text{-}PBMC}$)

Assay for:	Result of analysis					
	Feline				Human (HeLa)	Murine (3T3)
	3201	CrFK	Mya-1	AH927		
CXCR4[a]	+	+	+	−	+	−
$FIV_{PET\text{-}CrFK}$[b]	+	+	+	−	+[c]	−
$FIV_{GL8\text{-}PBMC}$[b]	−	−	+	−	−	−

[a] Northern blotting analysis.
[b] Susceptibility to infection as described previously (28, 56); −, nonsusceptible; +, susceptible.
[c] Viral entry but not productive infection supported.

CD4, or U87 expressing feline CD9. The data confirm that feline CXCR4 acts a coreceptor for FIV Env-dependent fusion. As human CXCR4 supported FIV Env-dependent fusion, we next examined whether other human chemokine receptors were able to support fusion. FIV-infected CrFK cells were plated with a panel of U87-T4 cells stably transfected with human CCR1, CCR2b, CCR4, CCR5, and CXCR4 and examined for cell-cell fusion. While syncytia were observed between the CXCR4-expressing U87-T4 cells and CrFK cells infected with FIV_{GL8} or FIV_{PET} (data not shown), syncytia were not detected in the U87-T4 cells expressing human CCR1, CCR2b, CCR3, CCR4, or CCR5. CXCR4-expressing U87 and U87-T4 cells were infected with 1,000 50% tissue culture infective doses of either CRFK-adapted FIV_{PET} or a primary isolate of FIV_{GL8}, and culture supernatants were monitored for p24 production by enzyme-linked immunosorbent assay. Despite prolonged culture (up to 1 month), FIV p24 was not detected in the culture supernatants from the cells (data not shown), suggesting that CXCR4 expression alone on U87 cells is not sufficient to confer susceptibility to productive infection with FIV.

Feline CXCR4 functions as a coreceptor for HIV Env-mediated fusion. HIV-2 and SIV have been shown to infect and fuse feline cells expressing human CD4 (46), suggesting that a feline molecule can substitute for human CXCR4 in CD4-dependent cell-cell fusion. To address whether feline CXCR4 could function as a fusion coreceptor for HIV Env-mediated fusion, feline CXCR4 was subcloned into the cDNA3 vector and compared with human CXCR4 in an HIV Env-mediated fusion assay. HeLa cells expressing Env from a CXCR4-dependent strain of HIV-1 (LAI), a CCR5-dependent strain of HIV-1 (ADA), and a strain of HIV-2 (ROD) known to infect feline cells expressing human CD4 were mixed with 3T3 cells which had been transiently transfected with human CD4 and either human CXCR4, feline CXCR4, or human CCR5 and

TABLE 3. FIV-infected CrFK cells fuse with human cell line U87 transfected with feline CXCR4[a]

Virus	Syncytium formation				
	Control	Human CD4	Human CXCR4	Feline CD9	Feline CXCR4
None	−	−	−	−	−
FIV_{GL8}	−	−	+++	−	+++
FIV_{PET}	−	−	+	−	+

[a] U87 cells stably transfected with human CD4, human CXCR4, feline CD9, or feline CXCR4 were mixed with either uninfected CrFK cells (none), FIV_{GL8}-infected CrFK cells, or FIV_{PET}-infected CrFK cells in 96-well plates. Wells were scored for the presence of syncytia as for Table 1.

infected subsequently with vTF7-3. Control 3T3 cells were transfected with the CD4 expression plasmid alone. Following a 2-h incubation at 37°C, fusion was observed between the 3T3 cells coexpressing human CD4 and CXCR4 and HeLa-LAI or HeLa-ROD but not HeLa-ADA, confirming previous findings that CXCR4 is a coreceptor for CD4-dependent fusion by the LAI and ROD envelope glycoproteins (17, 52, 53). In contrast, HeLa-ADA cells fused with 3T3 cells transfected with CCR5 but not those transfected with CXCR4 (as reported previously [8]). HeLa-ROD cells fused efficiently with 3T3 cells expressing either human or feline CXCR4 or human CCR5. Surprisingly, feline CXCR4 supported fusion mediated by both HIV-1 and HIV-2 envelopes. As HIV-2 infects feline cells expressing human CD4, we predict that feline CXCR4 should support HIV-2 envelope-mediated fusion; however, HIV-1 does not infect feline cells expressing human CD4 unless they are transfected with human CXCR4 (3), suggesting either that feline CXCR4 expressed in feline cells behaves differently from feline CXCR4 expressed in murine cells or that HIV-2 utilizes an alternate receptor to CXCR4 on feline cells.

The efficiency of feline CXCR4 as a fusion coreceptor was evaluated further by using the colorimetric β-galactosidase chlorophenol red-β-galactopyranoside (CPRG) assay as described previously (41). HeLa-LAI, HeLa-ADA, or HeLa-ROD cells transfected with the T7-*lacZ* expression plasmid p478 were mixed with 3T3 cells that had been cotransfected with human CD4 and the panel of chemokine receptor plasmids and subsequently infected for 8 h with vTF7-3. Human and feline CXCR4 mediated CD4-dependent fusion between 3T3 cells and HeLa-LAI efficiently (Fig. 5). Compared to human CXCR4, feline CXCR4 proved an efficient cofactor for fusion mediated by T-cell-line-adapted LAI envelope glycoprotein. Transfection of CCR5 and CD4 or of CD4 alone into 3T3 cells did not support fusion with the LAI Env-expressing cells. Human CCR5 supported fusion between 3T3 and HeLa-ADA, in agreement with previous studies on the receptor usage of this primary NSI envelope (8, 15, 19). The ROD envelope glycoprotein utilized both human and feline CXCR4 and CCR5 for CD4-dependent fusion with similar efficiencies. The pattern of chemokine receptor usage by the LAI, ADA, and ROD envelope-expressing cells in the 3T3 assay system was highly reproducible.

Having demonstrated that feline CXCR4 supported CD4-dependent fusion by HIV Env, we then examined whether feline CXCR4 was recognized by the anti-human CXCR4 antibody 12G5. U87 cells stably transfected with feline or human CXCR4 and which supported FIV Env-dependent fusion (described in Table 3) were transfected with plasmid p461 encoding human CD4 under the T7 promoter, infected with vTF7-3, and assayed for ability to support fusion mediated by either LAI or ADA Env by using the CPRG assay as described above. The human and feline CXCR4 stable transfectants both supported fusion with HeLa cells expressing LAI Env but not ADA Env, confirming that the cells expressed CXCR4 (Fig. 6A). Cells were analyzed in parallel for 12G5 reactivity (Fig. 6B). While 65.3% of the human CXCR4 transfectants were recognized by antibody 12G5 (control, 1.9%), only 3.5% of the feline CXCR4 transfectants were recognized by the 12G5 antibody (control, 1.4%), suggesting that the epitope recognized by antibody 12G5 is not conserved between human and feline CXCR4.

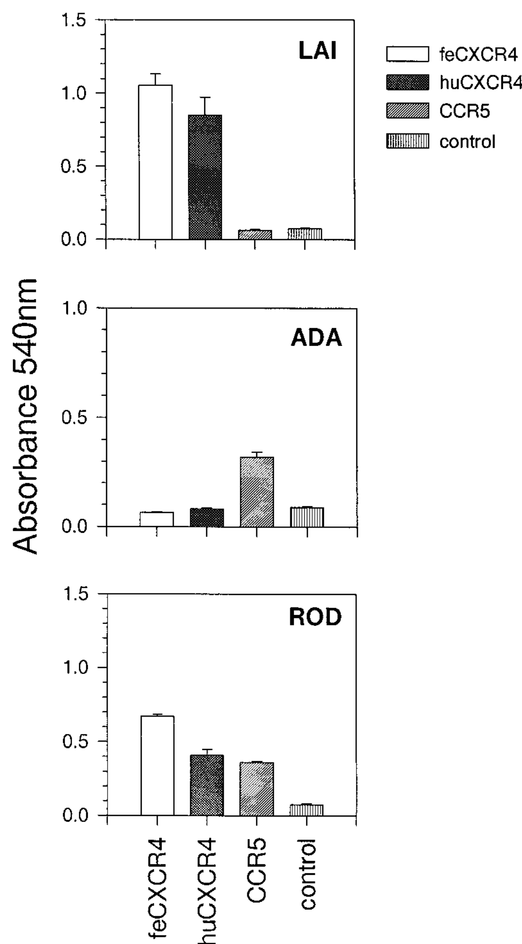


FIG. 5. Quantitative comparison of the degree of HIV-Env-dependent fusion mediated by feline and human CXCR4. The CPRG assay was performed essentially as described previously (41). NIH 3T3 cells were cotransfected with human CD4 and either feline CXCR4 (feCXCR4), human CXCR4 (huCXCR4), or human CCR5. Control cells were transfected with CD4 alone. Following overnight incubation, the cells were infected with vTF7-3 (multiplicity of infection of 10) for 8 h and then mixed with HeLa cells infected with HIV strain LAI, ADA, or ROD and plated in 96-well plates. Following a 2-h incubation, the cells were lysed, and 50 μl of lysate was added to 50 μl of CPRG substrate and incubated overnight in the dark at room temperature. Fusion was quantified by measuring the absorbance at 540 nm. Each point represents the mean of three replicates and is typical of three separate experiments.

DISCUSSION

In this study, we present evidence of shared chemokine receptor usage between primate and feline lentiviruses. FIV Env-dependent fusion of human cells is mediated by the chemokine receptor CXCR4 and can be inhibited by the anti-CXCR4 antibody 12G5 (58). Human cells transfected with human CXCR4 fuse with FIV-infected cells, whereas cells transfected with CD4 or mock transfected do not. cDNAs encoding feline CXCR4 were generated from two independent sources and were shown to display a high degree of homology with human CXCR4. Feline CXCR4-transfected U87 cells supported FIV Env-dependent fusion, whereas feline CD9- or human CD4-transfected cells did not support fusion. Moreover, U87-T4 cells expressing the human β-chemokine receptor CCR1, CCR2b, CCR3, CCR4, or CCR5 failed to support FIV-Env-dependent fusion. CXCR4 expression on U87 cells alone was insufficient to confer susceptibility to productive infection with cell-free virus despite rendering these cells susceptible to fusion with FIV-infected cells. Feline CXCR4 supported CD4-dependent fusion of human cells expressing HIV-1 envelope glycoprotein from CXCR4-dependent strains

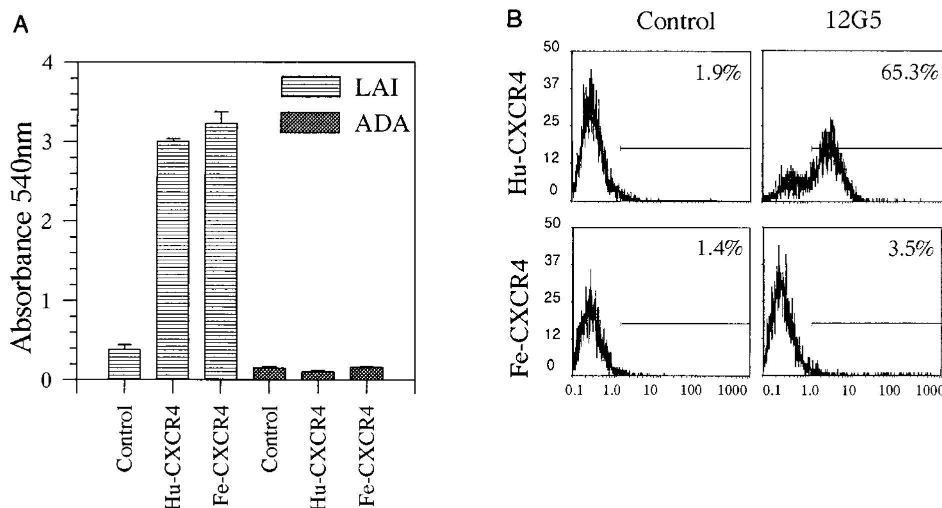


FIG. 6. Feline CXCR4 is not recognized by the anti-human CXCR4 antibody 12G5. (A) U87 cells stably transfected with feline or human CXCR4 (fe- or hu-CXCR4) were transfected transiently with the human CD4 expression plasmid p461, infected with vTF7-3, and assayed for ability to support fusion with HeLa cells expressing LAI or ADA Env, using the CPRG assay as for Fig. 5. (B) Cells were processed in parallel for flow cytometry, using monoclonal antibody 12G5 or an isotype-matched control antibody.

of HIV-1 (HIV_{LAI}) and HIV-2 ($HIV\text{-}2_{ROD}$) but not with cells expressing envelope glycoprotein from a primary NSI strain of HIV-1 (HIV_{ADA}).

The feline CXCR4 homolog displays a high degree of homology to human and bovine CXCR4, with interspecies variation in the amino acid sequence clustering predominantly around the amino-terminal region and the second large extracellular loop. It is of interest that the anti-human CXCR4 antibody 12G5 fails to react with feline CXCR4-expressing feline cells. As the 12G5 antibody inhibits both CD4-dependent and CD4-independent HIV infection and cell fusion (20), the feline CXCR4 clone should provide a useful tool with which to map the 12G5 binding site and consequently regions of CXCR4 critical for infection and fusion with HIV. Indeed, in preliminary experiments, we have found that a chimeric CXCR4 molecule containing the second and third loops of human CXCR4 but the N terminus and first loop of feline CXCR4 is recognized by antibody 12G5 whereas the reverse chimera containing the second and third loops of feline CXCR4 is not, indicating that the second loop of CXCR4 may comprise all or part of the 12G5 binding site (54). Recent studies using chimeric rat-human CXCR4 molecules have also suggested that the 12G5 binding site maps to the second extracellular loop (5). In this study, we have demonstrated that feline CXCR4 can support HIV Env-dependent fusion between the murine cell line 3T3 and the human cell line HeLa. As CXCR4 supports both FIV and HIV gp120-mediated fusion, then some conservation of structure and function between HIV and FIV gp120 is indicated.

Previous studies have demonstrated that some strains of HIV-2 are able to infect feline cells expressing human CD4 (9). As these strains of HIV-2 are able to use CXCR4 as a cofactor for infection and fusion (20), the data implicate feline CXCR4 as the cofactor in feline cells. In the absence of reagents specific for feline CXCR4, we have demonstrated that feline CXCR4 is capable of supporting CD4-dependent fusion by HIV envelope glycoprotein. Intriguingly, feline CXCR4 supported fusion mediated by both HIV-1 and HIV-2 envelope glycoproteins. It is possible that the vaccinia virus-T7-enhanced expression system amplifies weak interactions not observed in vivo and that the interaction between HIV-1 envelope glycoprotein and feline CXCR4 in feline cells is insufficient to support infection with HIV-1, but that a sufficiently strong interaction exists between HIV-2 envelope glycoprotein and feline CXCR4 to support infection of feline cells expressing human CD4. Alternatively, feline CXCR4 expressed on murine cells may be processed differently from feline CXCR4 expressed on feline cells, and only the form expressed on murine cells is fusogenic for HIV-1 Env. As transfection of CCC-CD4 cells with human CXCR4 renders them susceptible to infection with HIV-1 (3), feline cells are clearly capable of expressing fusion-competent CXCR4. However, differential posttranslational modification in cells from different species may affect the conformation of the chemokine receptor when expressed ectopically and subsequently may provide conflicting data regarding cofactor function for HIV infection. Accordingly, the anti-CXCR4 antibody 12G5 failed to block fusion mediated by the LAI envelope in U87.CD4 cells transfected with CXCR4 (36), suggesting that CXCR4 exists in antigenically and functionally distinct forms depending on the cell type in which it is expressed. We have shown previously that human CD9 (a member of the four-transmembrane domain family of molecules) expressed on murine cells adopts an antigenic conformation different from that of human CD9 expressed on human cells (60).

The envelope glycoprotein of cell culture (CrFK)-adapted strains of FIV is fusogenic in human cells, and the switch to this SI phenotype is accompanied by mutations in the FIV V3 loop which increase the net charge of the loop (51, 55). These changes mirror changes in the V3 loop of HIV with the switch from an NSI to an SI phenotype (14). As T-cell-line-adapted SI strains of HIV show preferential usage of CXCR4 as a cofactor for fusion (22), the finding that cell culture-adapted strains of FIV utilize CXCR4 to fuse human cells suggests a common mechanism of infection and conservation of function despite significant evolutionary divergence at the level of DNA sequence and genomic organization. These findings may provide an insight into the mechanism by which FIV and HIV induce similar disease states in their natural hosts, in spite of the finding that FIV does not utilize the CD4 molecule as a pri-

mary binding receptor. Indeed, FIV has a broad cell tropism in vivo, infecting not only $CD4^+$ T cells but also $CD8^+$ T cells and B cells (12, 21). Originally, such findings were thought to be critical differences between FIV and HIV; however, recent studies have demonstrated that SIV, like FIV, infects $CD8^+$ T cells in vivo (13), and $CD8^+$ lymphocytes are frequently infected in HIV-1-infected individuals (33). It is has been suggested that the use of CD4 as a high-affinity binding receptor for HIV is a relatively recent development in the evolution of HIV and that the CD4-independent infection observed for HIV-2 and SIV reflects a more primitive route of infection. As recent studies have demonstrated that CD4-independent infection with HIV-2 can be mediated by CXCR4 alone (20) and that a glutamine-to-lysine (Q310K) change in the V3 loop of HIV-2 enhances a CD4-independent phenotype (47), future studies should investigate whether FIV utilizes chemokine receptors alone for infection or whether infection in vivo requires coexpression of a high affinity receptor such as CD4. Indeed, it is conceivable that a chemokine receptor such as CXCR4 is the ancestral primary receptor for FIV and HIV-2 and that adaptation to the use of CD4 has occurred relatively recently in the evolution of the primate lentiviruses. We have found that while CXCR4 expression confers susceptibility to FIV-specific cell fusion, the CXCR4-expressing cells were not susceptible to productive infection with cell-free virus. Previous studies have demonstrated that a postentry block to FIV replication exists in human cells (30), it is possible that FIV infection of CXCR4-transfected human cells is blocked at a postentry stage of the viral life cycle. Future studies should address whether CXCR4 is the primary receptor for FIV or a fusogenic cofactor.

The virus strains used in this study were cell culture-adapted strains of FIV, analogous to T-cell-line-adapted strains of HIV such as LAI. The finding that these strains of FIV utilize CXCR4 but not CCR5 is in good agreement with the current data on infection with SI strains of HIV. However, the question arises as to whether primary isolates of FIV also utilize CXCR4 for infection or whether the feline homologs of other chemokine receptors such as CCR5 are utilized by primary isolates of the virus. Indeed, until reagents specific for feline chemokine receptors are available, this will remain a difficult problem to address. As CXCR4-expressing human cells will fuse with FIV-infected feline cells yet CXCR4-expressing hamster cells will not (data not shown), the presence of an accessory molecule in human cells involved in FIV infection is implicated. The identification of CXCR4 as a fusion factor for FIV should enable the characterization of such an accessory molecule in that cDNA expression libraries cotransfected with CXCR4 into murine cells should render the cells permissive to infection and fusion.

Our findings suggest that FIV and HIV share a common mechanism of infection involving an interaction between the virus and a member of the seven-transmembrane domain superfamily of molecules. As FIV does not appear to use CD4 as a primary receptor for infection yet induces an immunodeficiency in the cat similar to AIDS in humans, then the interaction between the virus and the chemokine receptor is implicated as a critical determinant of the pathogenesis of AIDS.

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