Retrovirology: Research and Treatment

ORIGINAL RESEARCH

Mapping of Receptor Binding Interactions with the FIV Surface Glycoprotein (SU); Implications Regarding Immune Surveillance and Cellular Targets of Infection

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Abstract: Similar to HIV, FIV uses a two-receptor mechanism to infect $CD4^+$ T cells, the primary target cells in the cat. The T cell activation marker, CD134, serves as a primary binding receptor similar to the role of CD4 for HIV and facilitates interaction with the entry receptor, CXCR4. Heparan sulfate proteoglycans (HSPG) can also act as binding receptors for certain tissue culture adapted FIV and HIV isolates. In the present study, we employed site-directed mutagenesis to investigate the importance of specific residues on the FIV envelope for CD134 and HSPG interactions. We show that certain mutations that disrupt CD134 interactions facilitate HSPG binding by FIV-PPR. In particular, an E407K mutation at the base of the V3 loop knocks out CD134 binding; enhances HSPG binding; and in combination with additional Env mutations E656K and V817I increases entry into $CD134^-$, $CXCR4^+$ target cells by greater than 80-fold over wild type FIV-PPR. The CD134-independent mutant, termed FIV-PPRcr, exhibits a broadened host cell range, but also becomes readily susceptible to CD134-dependent neutralizing monoclonal antibodies. The findings are consistent with the notion that FIV-PPRcr Env has an "open" conformation that readily associates with CXCR4 directly, similar to wild type FIV-PPR Env after CD134 binding. The findings highlight the utility of a two-receptor mechanism that allows FIV V3 residues critical for CXCR4 binding to remain cryptic until reaction occurs with the primary binding receptor, thus thwarting immune surveillance.

Keywords: FIV, V3 loop, SU, CD134, HSPG, CXCR4

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Introduction

Feline immunodeficiency virus (FIV) infection in the domestic cat is characterized by a progressive and irreversible depletion of $CD4^+$ T lymphocytes[1–4] leading to an acquired immunodeficiency syndrome (AIDS)-like syndrome in the host similar to the disease caused by human immunodeficiency virus (HIV)-1.[5] FIV thus provides a valuable experimental model for the development of broad-based anti-lentiviral agents, design of anti-HIV vaccines, and study of lentiviral pathogenesis.[6–9] In respect to receptor usage, FIV utilizes CD134 as a primary binding receptor,[10–12] instead of CD4 as occurs with HIV infection.[10,11] However, FIV shares with T-cell tropic HIV-1strains the use of the chemokine receptor CXCR4 as the entry receptor for infection.[13] Certain FIV isolates also use heparan sulfate proteoglycans (HSPG),[14,15] and DC-SIGN[16] as binding or attachment receptors to facilitate infection, another characteristic shared with HIV.[17,18]

CD134 (OX40) is a type I transmembrane glycoprotein, belonging to the tumor necrosis factor receptor (TNFR) superfamily.[19,20] This family, which also includes CD120a (TNFR α, TNFR I), CD120b (TNFR β, TNFR II), CD27, CD30, CD40, CD95 and CD137,[21–28] is characterized by the presence of three or four cysteine-rich repeat domains within the ectodomain.[21–28] Feline CD134 is predominately expressed on activated $CD4^+$ T cells,[10,29] thus explaining the progressive depletion of $CD4^+$ T cells in FIV infection and resultant AIDS-like disease from FIV infected cats. A panel of FIV primary isolates of diverse subtypes and geographic origins use CD134 as the binding receptor for infection.[12] Primary FIV Env binds and interacts directly with CD134.[10,11] Ectopic expression of feline CD134 in at least certain non-permissive cells renders the cells permissive for binding of both virus[30,11] and soluble envelope glycoprotein (SU).[10,31] Furthermore, pretreatment of virus with soluble CD134 facilitates infection of $CD134^-$, $CXCR4^+$ cells.[32] CD134 expression alone is insufficient to confer susceptibility to infection with FIV; infection always requires the expression of the entry receptor, CXCR4.[13,33,34] Results are consistent with the interpretation that binding of CD134 alters the conformation of FIV SU and promotes high affinity binding to CXCR4.[31]

In addition, the interaction between FIV and CD134 is species specific; human CD134 does not initiate FIV infection and thus is not a functional receptor for FIV.[30,11] The species specificity of the FIV/CD134 interaction has facilitated the mapping of the determinants on CD134 that mediate both the binding of soluble Env and viral entry. The binding sites on CD134 for the PPR strain of FIV have been mapped to the first cysteine-rich domain (CRD1);[30] and substitution of CRD1 of human CD134 with that of feline CD134 renders the molecule functional as a receptor for the PPR strain of FIV.[30] It has been reported that additional determinants in the second CRD (CRD2) contribute to binding for some primary strains such as GL8, CPG41, and 0827.[12,35] The regions of the viral envelope glycoprotein (SU) that bind to CD134 remain to be defined but appear to involve non-contiguous regions of the viral glycoprotein.[36,37] Our previous studies revealed that binding of PPRcr (a FIV-PPR isolate adapted to be propagated in CrFK cells) SU to 104-C1 cells ($CD134^{high}$; $HSPG^{low}$; $CXCR4^{low}$) was markedly reduced compared to wild type FIV-PPR SU.[15] One potential explanation for the inability of PPRcr SU to bind to CD134 is that mutations involved in CrFK adaptation might alter the conformation of the Env protein and disrupt conformation-dependent binding to CD134.

In the present study, we have used three different assay approaches to assess the effects of point mutations in Env on receptor interactions, including direct binding by a battery of Env immunoadhesins; facilitation of binding and entry of pseudotyped beta-galactosidase-expressing pseudovirions; and direct analysis of infectivity of the FIV isolates. The study defines residues critical for CD134 interaction and aids in our understanding of virus entry and the relevance of a two-receptor mechanism for virus infection.

Materials and Methods

Cell lines, virus and reagents

CrFK cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD), and the feline glial cell line (G355-5) was kindly provided by Don Blair (National Institutes of Health, Bethesda, MD). These cells are interleukin-2 (IL-2)-independent adherent cells with high heparan sulfate proteoglycan (HSPG) expression; low CXCR4 expression; and are negative for CD134 expression.[14,31] Propagation of the cell lines was performed as previously described.[14]

GFox and (CrFK-fX4) cells are CrFK cells transduced with a murine stem cell retrovirus vector (MIGR1-CD134/CXCR4-GFP)[16] expressing either feline CD134 or feline CXCR4 in tandem with GFP via an internal ribosome entry site linker. GFP-positive cells (transduction efficiency typically > 80%) were sorted by FACS analysis and the sorted cells were selected by G418 and maintained for at least one month to obtain stably expressed cells. The FIV field strain used in the present study, FIV-PPR, is a molecular clone of the clade A San Diego isolate.[38] PPRcr is a FIV-PPR strain obtained after ex vivo passage in the CrFK cell line.[15] FIV-34TF10 is a molecular clone of the FIV Petaluma isolate that had been adapted for growth on CrFK cells.[39] Heparin, AMD3100, heparinase and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). SU2-5 antibody, a CD134-dependent anti-V3 monoclonal antibody,[32] was a gift from Chris K. Grant (Custom Monoclonals International, CA).

Reverse Transcriptase (RT) assay

RT activity was measured as previously described.[14,40,37] Briefly, 50 µL of cell-free supernatant was mixed with 10 µL of lysis buffer (0.75 M KCl, 20 mM dithiothreitol, 0.5% Triton X-100) and incubated at room temperature for 10 minutes. The mixture (40 µL) containing 125 mM Tris-HCl (pH 8.1), 12.5 mM $MgCl_2$, 1.25 µg poly(rA)-poly$(dT)_{12–18}$ (Amersham Biosciences, Piscataway, NJ) and 1.25 µCi of [3H]dTTP (DuPont, Boston, MA) was added to the sample and incubated for 2 h at 37 °C. Activity was quantitated as previously described.[40]

Virus entry assay

pCFIV hybrid vectors pseudotyped with FIV-PPR, PPRcr, 34TF10 envelope (Env) genes or mutants substituted by specific amino acids were co-transfected with a beta-galactosidase (β-gal)-expressing packaging vector in 293T cells.[41] Two days later, viral supernatants were collected and each pseudovirion was assessed for the level of reverse transcriptase (RT) for core expression and also an ELISA assay to detect the expression of Env. Both RT assay and ELISA assay indicated each envelope construct was well expressed. RT activity was further used as an internal quantitation to normalize transfection efficiency and further to ensure input of approximately equal numbers of pseudovirions. Thus, RT values were normalized to 50,000 cpm per infection before performing a single round infection assay in target cells. After 48 h of infection, β-gal activity was measured with the Tropix Galacto-Star chemiluminescent reporter gene assay (Applied Biosystems, Carlsbad, CA) according to the manufacturer's guidelines.

Construction of mutant plasmids

Mutations were introduced into a cytomegalovirus-FIV hybrid vector (pCFIV)[41] using the Quick-Change site-directed mutagenesis strategy (Stratagene, La Jolla, CA) as recommended by the manufacturer. The presence of the desired mutations and the absence of any other mutations were confirmed by DNA sequencing.

Recombinant SU proteins

Expression plasmids encoding SU of FIV-PPR and FIV-PPRcr were constructed and used for production of stable CHO-K1 cell lines, as previously described.[14,40] Single colonies with high expression of desired Fc-tagged proteins were selected and SU-Fc fusion proteins (adhesins) were purified as described[36] and quantified using a human IgG ELISA quantitation kit (Bethyl Laboratories, Inc, Montgomery, TX). Finally, relative quantitation of proteins was confirmed by western blot analysis, as previously described.[36]

Flow cytometry analysis

Binding of SU-Fc adhesins or Fc (negative control) to the surfaces of CrFK, GFox and CrFK-fX4 cells were detected using a phycoerythrin-conjugated goat anti-human IgG1 Fc antibody (MP Biomedicals, Aurora, OH) and analyzed by flow cytometry, using FLOWJO software (Tree Star, San Carlos, CA). Briefly, 1×10^5 cells were detached in EBSS containing 5 mM EDTA, washed once, and resuspended in binding buffer (EBSS containing 0.1% BSA), then PPR or PPRcr SU-Fc (500 ng) was added to cells and incubated at 25 °C for 45 min. After washing, cells were labeled with a 1:1000 dilution of PE-conjugated goat anti-human IgG1 antibody for 35 min. SU-Fc binding was monitored by FACS analysis. CXCR4-specific binding was confirmed by pre-treatment of cells with the CXCR4 antagonist, AMD3100, at 1 µg/mL for 30 min, followed by the

addition of SU-Fc adhesins. HSPG-specific binding was confirmed by co-incubation with SU-Fc adhesins plus heparin (10 µg/mL). Percent inhibition was calculated by the formula $100 - [(t\text{-}c)/(m\text{-}c) \times 100]$, where *t* represents the signal for the test sample; *c* represents the background signal in the absence of SU-Fc; and *m* represents the signal obtained for SU-Fc in the absence of peptides or inhibitors.

Heparinase treatment

For FACS assays, parental CrFK, GFox and CrFK-fX4 cells were detached in EBSS containing 5 mM EDTA, washed once, and resuspended in heparinase buffer (EBSS containing 2 mM $CaCl_2$ and 0.1% BSA) in the absence or presence of 10 U of heparinase per mL. After 30 min incubation at room temperature, cells were washed twice and resuspended in binding buffer and utilized in FACS analysis as described above.

Virus infection assay

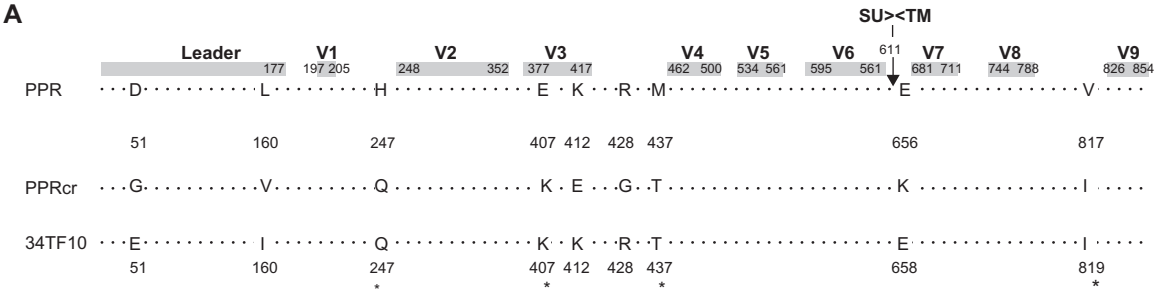
Viruses with RT values above 100 K cpm were used in all infection assays. 2×10^4 cells were seeded in a 12-well plate and 100 µL of virus was used to infect the cells for 2 h at 37 °C. Virus production was measured over time using a micro-RT assay. Cells were then washed and cultured at 37 °C in a 5% CO_2 atmosphere.

Results

FIV isolates entry comparison

FIV-PPRcr is a mutant of FIV-PPR selected for productive growth in CrFK cells.[15] The envelope gene of FIV PPRcr was sequenced, revealing nine amino acid substitutions relative to wild type FIV-PPR (Fig. 1A), including changes D51G and L160V in the region N-terminal to the membrane leader sequence; H247Q in C2; E407K and K412E in V3; R428G and M437T in C3; E656K between the polar domain and the leucine zipper of TM; and V817I in the cytoplasmic tail. Previous work had shown that in part, adaptation of PPRcr for growth in $CD134^-$ adherent cells (either CrFK or G355-5 cells) correlated with the acquisition of ability of its SU to bind to HSPG.[15] Entry assays using beta-galactosidase-expressing pseudovirions coated with either wild type FIV-PPR SU or PPRcr SU were performed to compare the relative ability of these two glycoproteins to facilitate binding and entry as a function of receptor expression (Fig. 1B). The weakest signal level of β-galactosidase activity for each cell line was set at 1, with the signals of other FIV envelopes divided by the weakest signal and calculated as -fold increase. PPRcr SU facilitated entry on CrFK 3-fold better than wild type FIV-PPR SU, whereas wild type PPR SU supported entry into CrFK cells over-expressing CD134 (GFox cells) at a level almost 4800-fold greater than that noted with PPRcr SU. Interestingly, when assayed on

A



B

Virus	Entry efficiency (fold)		
	CrFK	CrFK-CD134 (GFox)	CrFK-CXCR4 (CrFK-fX4)
PPR	1	4776	1
PPRcr	3	1	88
34TF10	107	29	270

Figure 1. (**A**) Schematic representation of the sequence alignment of PPR and PPRcr SU Envelope sequences. A total of nine mutations were noted between field strain FIV-PPR and tissue culture-adapted (TCA) FIV-PPRcr. Four of the above nine amino acid positions were common between the two TCA isolates 34TF10 and PPRcr and are shown as*. (**B**) FIV pseudotyped virions entry into target cells.
Note: The signal of β-galactosidase activity of the lowest FIV envelope entry ability in each cell line was set at 1 and the signals generated as a function of the other FIV envelopes are expressed as—fold differences to the lowest signal.

CrFK over-expressing feline CXCR4, PPRcr-bearing particles bound and entered these cells at levels almost 90-fold over levels obtained with particles pseudotyped with wild type PPR SU (Fig. 1B). Another CD134-independent isolate, FIV-34TF10[39] also demonstrated enhanced entry directly via interactions with CXCR4 compared to the field strain, FIV-PPR. The 34TF10 isolate, however, has not lost the ability to bind CD134 and facilitates entry into GFox cells as well as CrFK (Fig. 1B). The results confirm the loss of CD134 binding by PPRcr SU, but further suggest that both PPRcr and 34TF10 SU glycoproteins have a conformation that more readily associates with CXCR4 directly compared to wild type FIV-PPR SU.

Mutagenesis studies

Point mutants containing single or multiple mutations associated with the PPRcr phenotype were introduced into the wild type FIV-PPR Env background, then assayed for ability to facilitate entry of pseudovirions expressing β-galactosidase as a function of receptor expression (Table 1). The results indicated that E407K was a critical amino acid substitution that totally abrogated CD134-mediated entry on GFox cells and at the same time stimulated CXCR4-only entry by over 12-fold. Similar results were noted with multiple mutations where E407K was present (Table 1). Other single mutants such as D51G, L160V, K412E, M437T, and V817I had a more modest negative effect on CD134-mediated entry into GFox cells and also cause a minor reduction or enhancement on entry into CrFK-fX4 cells. The H247Q mutation was unique in that it had a slight enhancing effect on CD134-mediated entry and an approximate 2-fold enhancement on CXCR4-mediated entry. In contrast, the R428G mutation was associated with severely arrested entry into GFox cells, but had only a weak influence on the entry efficiency in CrFK-fX4 cells. Thus, the R428G mutation contributes to the negative influence on CD134 usage, but makes no apparent contribution to HSPG binding or CXCR4-mediated entry. Mutations in TM, including E656K and its double mutant E656K/V817I contribute strongly to both the loss of entry into GFox cells and enhanced entry into CrFK-fX4 cells. As described above, combined mutants such as E407K/E656K/V817I had apparently enhanced entry capacities on CrFK-fX4 cells, compared with those single mutants. Thus, mutations in TM working alone or in combination with critical residues in SU such as E407K affected the entry process, possibly through interactions associated with the fusion step. Back mutations were also performed in PPRcr SU to verify the fidelity of the above findings (bottom 6 mutants of Table 1). In each instance, the back mutants all restored the expected wild type FIV-PPR SU phenotype.

Table 1. FIV pseudotyped virions entry into target cells.

Mutants	Entry efficiency (fold)	
	CrFK-CD134 (GFox)	CrFK-CXCR4 (CrFK-fX4)
PPR	1.0 ± 0.0	1.0 ± 0.0
D51G	0.4 ± 0.1	0.8 ± 0.1
L160V	0.8 ± 0.0	1.2 ± 0.2
H247Q	1.2 ± 0.1	2.4 ± 0.4
E407K	**–**	**11.9 ± 1.1**
K412E	0.9 ± 0.1	0.7 ± 0.0
R428G	0.1 ± 0.0	0.8 ± 0.2
M437T	0.4 ± 0.0	3.2 ± 0.9
E656K	**0.1 ± 0.0**	**6.3 ± 0.9**
V817I	0.4 ± 0.0	1.0 ± 0.0
D51G/L160V	0.3 ± 0.1	1.0 ± 0.0
E407K/K412E	**–**	**5.8 ± 0.4**
E407K/M437T	**–**	**37.6 ± 4.7**
H247Q/E407K	**–**	**30.5 ± 3.8**
H247Q/M437T	0.4 ± 0.0	4.8 ± 0.4
H247Q/E407K/M437T	**–**	**46.4 ± 5.2**
E656K/V817I	**0.2 ± 0.1**	**11.8 ± 1.9**
E407K/E656K/V817I	**–**	**88.0 ± 8.2**
PPRcr	1.0 ± 0.0	1.0 ± 0.0
K407E	**13.9 ± 0.1**	**–**
E412K	0.9 ± 0.0	0.7 ± 0.08
K656E	1.9 ± 0.0	0.0 ± 0.0
I817V	0.7 ± 0.0	0.7 ± 0.0
K407E/E412K	**40.2 ± 3.0**	**0.1 ± 0.0**
K656E/I817V	5.5 ± 0.2	0.6 ± 0.0

Notes: Entry facilitated by wild-type PPR or PPRcr Env was arbitrarily set at 1 and the other entry data were normalized to this value. An arbitrary value of 0.2× or less relative to entry facilitated by PPR Env in GFox cells was defined as significant entry reduction. The arbitrary value of 5× or more relative to entry facilitated by PPR Env in CrFK-fX4 cells was defined as significant entry enhancement (bolded numbers). By contrast, an arbitrary value of 0.2 or less relative to entry facilitated by PPRcr Env in CrFK-fX4 cells was set as significant entry reduction and the arbitrary value of 5 or more relative to entry facilitated by PPRcr Env in GFox cells was set as significant entry enhancement (bolded numbers). An arbitrary value of 0.1 or less relative to entry facilitated by PPR or PPRcr Env in both GFox and CrFK-fX4 cells is shown as bolded, italic numbers. "-" no entry detected.

Binding assay of FIV SU to CD134- or CXCR4-expressing CrFK cells

To determine whether the binding properties of FIV-PPR and FIV-PPRcr SU correspond directly

to the entry data above, an assessment of the relative binding of PPR and PPRcr SU to CrFK, GFox cells and CrFK-fX4 cells (Fig. 2) was performed. As expected, binding of PPR SU-Fc to control CrFK cells was low (Fig. 2, top panel). Binding was not inhibited by AMD3100, indicating that the binding detected here was not via CXCR4. Binding was, however, reduced by competition with heparin or by pre-treatment of the cells with heparinase, indicating low-level binding to HSPG. In contrast, the binding of PPRcr SU (Fig. 2, second panel from top) to CrFK cells was relatively high, again

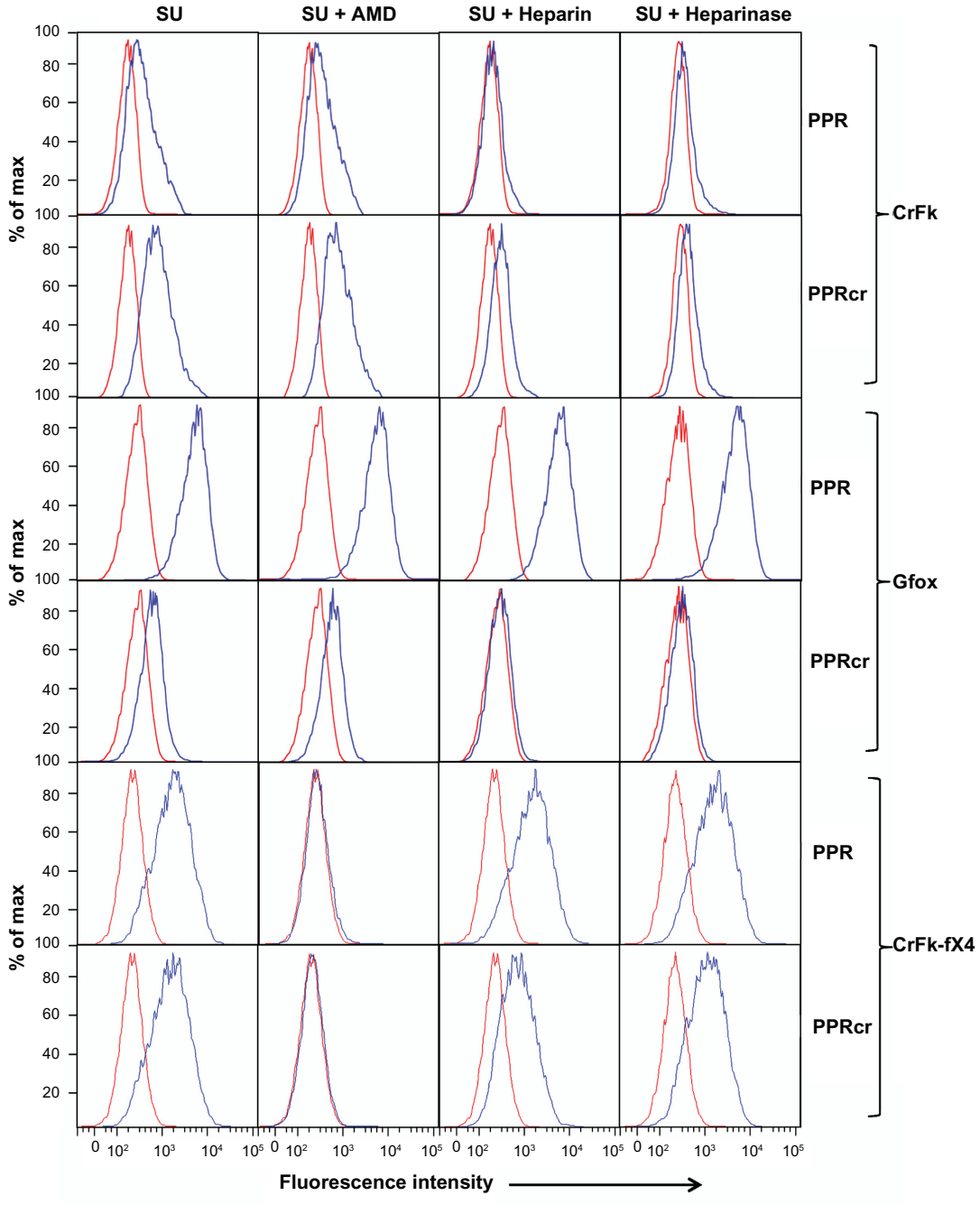


Figure 2. FACS analysis of PPR and PPRcr SU-Fc binding to CrFK, GFox, and CrFK-fX4 cells.
Notes: Binding of SU-Fc to CrFK (top two panels), GFox (CD134-expessing CrFK, middle two panels), CrFK-fX4 (CXCR4-expessing CrFK, bottom two panels) cells, respectively. Binding of SU-Fc to cells was analyzed at 25 °C in the presence or absence of AMD3100 (AMD) or heparin or after heparinase (10 U/mL) treatment of cells. Binding inhibited by AMD3100 (AMD) or heparin indicates that the binding is mediated by CXCR4 or HSPG, respectively. Heparin also interferes with PPRcr SU-Fc binding to CXCR4. Heparinase treatment caused no significant reduction in the binding of PPR SU-Fc but blocked the binding of PPRcr SU-Fc. Red indicates background binding and blue represents detected SU-Fc binding. Results are representative of three independent experiments.

primarily occurring via HSPG binding, as indicated by reduction with heparin competition or heparinase treatment.

FIV-PPR SU bound strongly to the CD134$^+$ GFox cells (Fig. 2, third panel from top) and was neither inhibited by AMD3100 nor heparin, consistent with binding via CD134.[30,10] PPRcr SU (Fig. 2, fourth panel from top) bound much more weakly to GFox and, as with control CrFK cells, the low level binding detected was inhibited by heparin competition or heparinase treatment, indicative of HSPG interaction.

Both PPR SU and PPRcr SU bound strongly to CrFK-fX4 cells over-expressing CXCR4 (Fig. 2, fifth and sixth panels from top, resp.) and the binding could be inhibited completely by the CXCR4 antagonist, AMD3100. No inhibition was noted with heparin competition or heparinase treatment on PPR SU binding on these cells (fifth panel from top), but the latter treatments reduced PPRcr SU binding by approximately 60% (bottom panel), consistent with previous observations.[14,15] The additional interaction with HSPG in combination with CXCR4 may explain the enhanced entry (Fig. 1B) afforded by PPRcr SU in CrFK-fX4 cells relative to that facilitated by FIV-PPR SU.

Neutralization sensitivity of pseudovirions for entry into CrFK-fX4 cells

The above findings are consistent with the notion that FIV-PPRcr, in gaining the ability to infect CrFK and other CD134$^-$, CXCR4$^+$ cells, also gained the capacity to bind HSPG and to bind CXCR4 directly without need of interactions with the primary binding receptor. In essence, it appeared that PPRcr SU had taken on a conformation similar to that of wild type SU when associated with CD134; ie, an open conformation suitable for direct high affinity interaction with CXCR4. We utilized SU2-5, a CD134-dependent anti-V3 monoclonal antibody[32] to assess the neutralization sensitivity of pseudovirions either psuedotyped with FIV-PPR or FIV-PPRcr Env for entry into CrFK-fX4 cells (Fig. 3). Antibody SU2-5 blocked FIV-PPR entry in the absence of CD134 interaction by approximately 20% relative to the entry level in the absence of antibody. However, under the same conditions, FIV-PPRcr-facilitated entry was blocked by greater than 95%, consistent with increase availability of the epitope recognized by the neutralizing monoclonal antibody; ie, a more open conformation for the host range mutant in the absence of CD134.

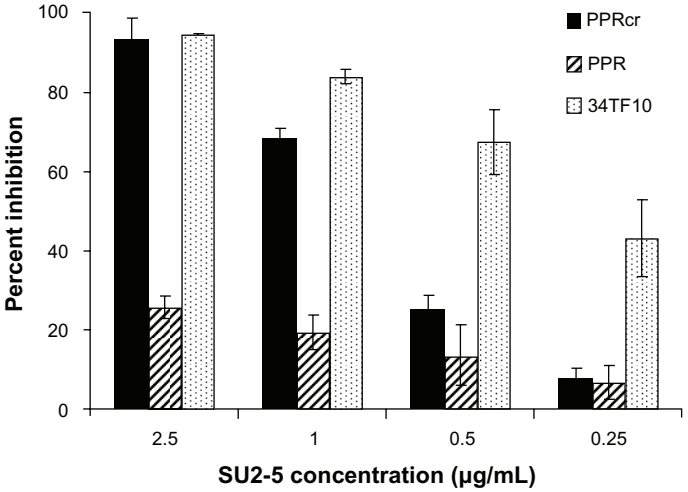


Figure 3. Neutralization sensitivity of pseudovirions for entry into CXCR4$^+$, CD134$^-$ cells.
Notes: β-gal-expressing pseudovirions with PPRcr, PPR or 34TF10 envelope were produced as described in "Materials and Methods". SU2-5, a CD134-dependent neutralizing anti-V3 antibody (32), was preincubated with pseudovirions at 37 °C for 60 min at indicated concentrations and the virus was then used for single round infection of CrFK-fX4 cells (PPRcr and PPR) or G355-5 cells (34TF10). β-galactosidase expression was then assessed 48 h after infection. Percent inhibition was calculated as described in "Materials and Methods". Results are means ± standard deviations (SD) for three independent experiments.

Comparison of FIV-PPRcr and FIV-34TF10

FIV-34TF10, a molecular clone recovered from passage of the original FIV-Petaluma isolate[5] on CrFK cells,[39] grows to a much higher titer on CrFK or G355-5 cells than the more recently derived FIV-PPRcr isolate and its SU facilitates entry into wild type CrFK, GFox, and CrFK-fX4 cells to a greater degree than FIV-PPRcr SU (Fig. 1B). However, the 34TF10 isolate lacks a functional OrfA gene due to a stop codon in the OrfA coding sequence[38] and does not productively infect CD4$^+$ T cells, the normal in vivo target for FIV unless the stop codon in the OrfA gene is repaired.[42] Even though the 34TF10 SU facilitates entry into GFox cells (Fig. 1B) and binds to CD134 (Fig. 4A), it does not productively infect CD134$^+$ GFox cells;[43] (Fig. 4C). At least one important role of OrfA is to down-regulate CD134 expression on virus-infected cells[43] and we surmise that the lack of OrfA expression by FIV-34TF10 results in the viral envelope

interacting with CD134 on the cell surface or on internal membranes, preventing virus egress.[43] If this hypothesis is correct, then FIV-PPRcr, which does not bind CD134, should productively infect GFox cells. Overall virus expression in FIV-PPRcr infection is much lower than for FIV-34TF10 on CD134$^-$ G355-5 cells (Fig. 4B). However, the former virus grows productively on CD134$^+$ GFox cells whereas FIV-34TF10 does not (Fig. 4C), consistent with the above hypothesis.

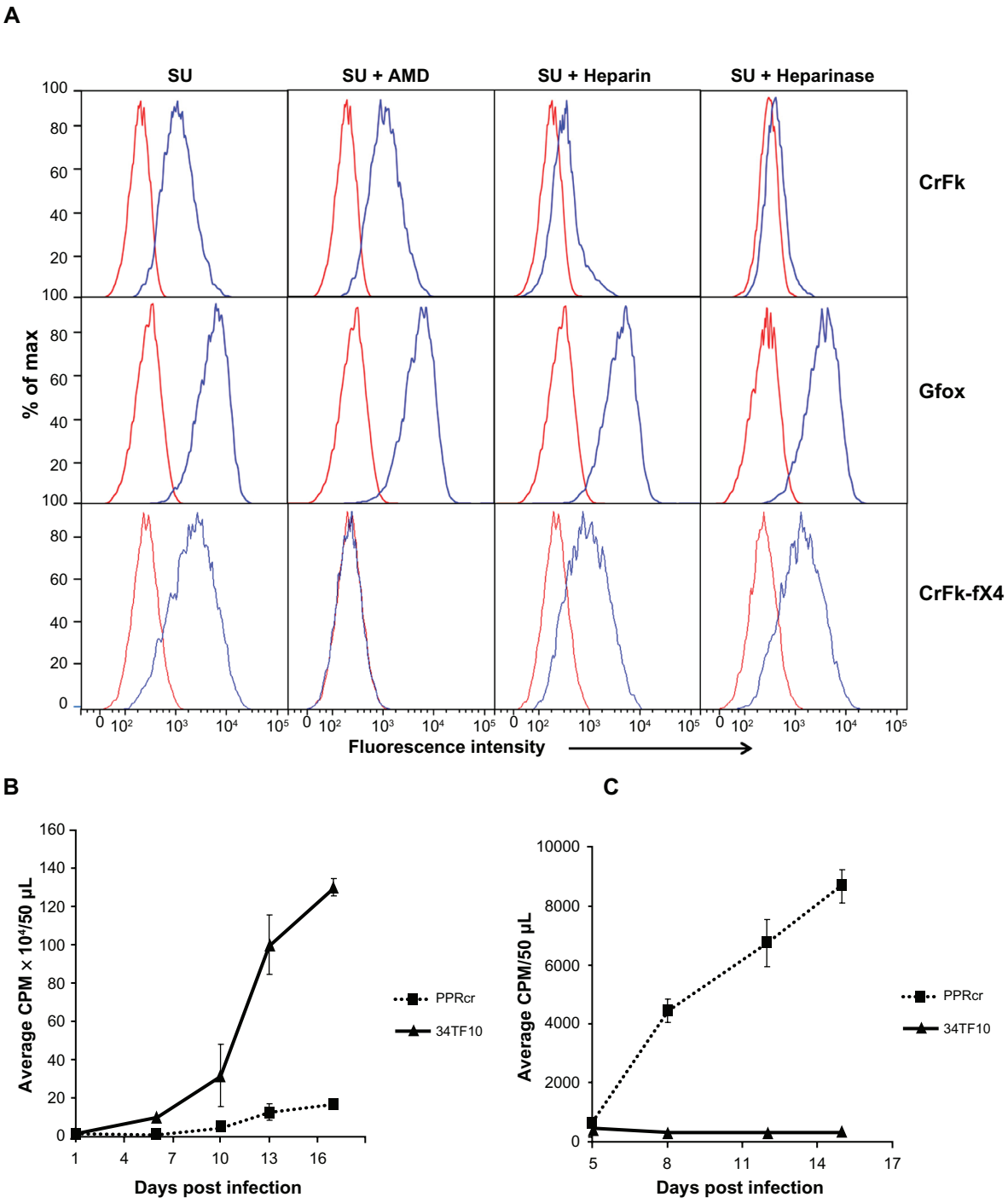


Figure 4. Binding and infection assay of 34TF10. (**A**) Binding of 34TF10 SU-Fc to CrFK, GFox, CrFK-fX4 cells, respectively. The binding assay was performed as described in Figure 2. (**B**) Comparison of the infectivity of FIV-PPRcr and FIV-34TF10 in G355-5 (**B**) and GFox (**C**) cells. Both 34TF10 and PPRcr can productively infect G355-5 cells but the infectivity of PPRcr is much lower.
Notes: GFox cells can be productively infected with PPRcr strain but not with 34TF10. Virus production was monitored by reverse transcriptase assay over a period of two weeks. Results are the means ± SD for triplicate determinations.

Discussion

The purpose of the present study was to more precisely define the key amino acid residues that are required for CD134 and HSPG interactions in order to gain insight into the nature of the two-receptor mechanism for virus entry. Our previous studies showed that FIV PPRcr adapted to propagate in CrFK cells acquired HSPG binding capacity at the cost of losing the ability (or need) to bind CD134.[15] These observations offer an opportunity to define key amino acid residues responsible for CD134 interaction. Comparison of the sequences of wild type FIV-PPR to PPRcr Env revealed 9 amino acid substitutions in Env (SU and TM) associated with changes in cell tropism, as assessed on a panel of target cells ex vivo. *Immunoadhesins comprised of PPR and PPRcr SU with Fc tags were prepared and used to assess* the distinct receptor binding phenotypes of each Env. In addition, site-directed mutagenesis was performed to place individual mutations and various combinations into the wild type background to assess the relative contribution of each mutation on entry into target cells.

In general, there was concordance between the three assays as to infection phenotype based on receptor utilization; ie, PPRcr SU bound better to CrFK cells than did PPR SU (Fig. 2) and PPRcr Env (SU plus TM) facilitated entry 3-fold better on CrFK than did PPR Env (Fig. 1B). In contrast, wild type PPR SU bound to a much greater extent to $CD134^+$ GFox cells than did PPRcr SU (Fig. 2) and PPR Env facilitated entry into GFox to a much greater degree than PPRcr Env (Fig. 1B). However, there was a telling discrepancy between SU binding and facilitation of entry into CrFK-fX4 cells that over-express CXCR4. Both PPR and PPRcr SU bound to these cells to approximately the same degree and binding of both was blocked by AMD3100 (Fig. 2). However, PPRcr Env facilitated entry of pseudovirions into these cells 88-fold better than did PPR Env (Fig. 1B). The results imply that PPRcr SU binding occurs in a more efficient/productive manner than PPR SU. As reported previously,[44] E407K near the V3 loop in SU was primarily responsible for the loss of interaction with CD134. Results in the present study show that E407K alone or in combination with other mutations in Env including mutations in TM (E656K and V817I) contributed to enhance entry via CXCR4 without requirement for primary interaction with CD134 (Table 1). In addition, the mutations in TM alone enhanced CXCR4-directed entry (Table 1), which may partially explain the greater degree of entry facilitated by PPRcr Env than PPR Env on CrFK-fX4 cells. Furthermore, results of neutralization assays (Fig. 3) using a monoclonal antibody that is CD134-dependent when used against wild type FIV-PPR[32] revealed that neutralization of PPRcr was CD134-independent. Neutralization of entry into G355-5 cells facilitated by 34TF10 Env was also blocked by the CD134-dependent SU2-5 antibody (Fig. 3), Together, the findings are consistent with an "open" conformation for both PPRcr SU and 34TF10 SU that allows efficient binding to CXCR4 without involvement of CD134.

FIV-34TF10 is the first infectious molecular clone we derived[39] and has a long passage history in $CD134^-$, adherent cells such as CrFK and G355-5. As such, it is much more highly adapted for rapid growth than the recent FIV-PPRcr isolate and grows to much higher titer (Fig. 4B). SU binding studies indicate that primary binding of PPRcr (Fig. 2) and 34TF10 (Fig. 4) occurs via HSPG interactions and is inhibited by heparin or heparinase treatment of the target cells. The two tissue culture adapted (TCA) isolates share 4 mutations that distinguish both isolates from FIV-PPR, including V817I in TM (Fig. 1A, asterisks); 34TF10 has additional amino acid differences, some of which must be compensatory mutations that recover growth potential while maintaining CD134 independence. Interestingly, both 34TF10 and PPRcr share the E407K mutation that eliminated CD134 binding by PPRcr, yet 34TF10 has maintained CD134 binding capacity even though it is not required for infection;[14] (Fig. 4). These observations are consistent with the notion that conformational changes are occurring in Env that influence both CD134 binding as well as availability of the contiguous site in V3 responsible for CXCR4 interaction. Changes in TM presumably impact on virus entry via altering fusion kinetics, since it appears unlikely that these residues would be available for direct interactions with relevant amino acids in SU. Further dissection of this issue awaits three-dimensional structural information.

Previous studies have revealed the interesting observation that FIV-34TF10, which is defective for OrfA expression[38] grows well in CrFK or G355-5 cells, but show no productive infection of $CD134^+$ GFox cells;[43] if the stop codon in OrfA is

repaired (34TF10-OrfArep), the virus grows productively in GFox cells and CD134-expressing T cells.[43] 34TF10 Env facilitates entry into GFox, albeit at a rate much lower than PPR Env, but 29-fold higher than PPRcr Env (Fig. 1B). One major function of OrfA is to down-regulate surface expression of CD134 and given that all retroviruses down-regulate their receptors after infection, this must be an important function. Taken together, the findings are consistent with the notion that interactions between 34TF10 Env and CD134 during egress prevent virus release and block productive infection. The observation that FIV-PPRcr, which does not bind CD134, productively infects GFox cells is consistent with this argument.

The findings emphasize several important points regarding FIV Env/CD134 receptor interactions as well as the two-receptor mechanism for virus infection in general. The results precisely map critical residues in Env interacting with feline CD134. As many parallels exist in both the pathology and molecular structure of FIV and HIV, identification of structures critical for receptor interaction by FIV isolates will contribute to defining potential targets for development of broad-based treatment for FIV and HIV infections.

Author Contributions

Conceived and designed the experiments: Q-YH, JE. Analyzed the data: Q-YH, JE, EF. Wrote the first draft of the manuscript: Q-YH, EF. Agree with manuscript results and conclusions: Q-YH, EF, JE. Jointly developed the structure and arguments for the paper: Q-YH, EF, JE. Made critical revisions and approved final version: Q-YH, JE. All authors reviewed and approved manuscript.

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Competing Interests

Author(s) disclose no potential conflicts of interest.

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Disclosures and Ethics

As a requirement of publication author(s) have provided to the publisher signed confirmation of compliance with legal and ethical obligations including but not limited to the following: authorship and contributorship, conflicts of interest, privacy and confidentiality and (where applicable) protection of human and animal research subjects. The authors have read and confirmed their agreement with the ICMJE authorship and conflict of interest criteria. The authors have also confirmed that this article is unique and not under consideration or published in any other publication, and that they have permission from rights holders to reproduce any copyrighted material. Any disclosures are made in this section. The external blind peer reviewers report no conflicts of interest.

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