Mechanism of feline immunodeficiency virus envelope glycoprotein-mediated fusion

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Abstract

Feline immunodeficiency virus (FIV) shares remarkable homology to primate lentiviruses, human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV). The process of lentiviral env glycoprotein-mediated fusion of membranes is essential for viral entry and syncytia formation. A detailed understanding of this phenomenon has helped identify new targets for antiviral drug development. Using a model based on syncytia formation between FIV env-expressing cells and a feline CD4+ T cell line we have studied the mechanism of FIV env-mediated fusion. Using this model we show that FIV env-mediated fusion mechanism and kinetics are similar to HIV env. Syncytia formation could be blocked by CXCR4 antagonist AMD3100, establishing the importance of this receptor in FIV gp120 binding. Interestingly, CXCR4 alone was not sufficient to allow fusion by a primary isolate of FIV, as env glycoprotein from FIV-NCSU1 failed to induce syncytia in several feline cell lines expressing CXCR4. Syncytia formation could be inhibited at a post-CXCR4 binding step by synthetic peptide T1971, which inhibits interaction of heptad repeat regions of gp41 and formation of the hairpin structure. Finally, using site-directed mutagenesis, we also show that a conserved tryptophan-rich region in the membrane proximal ectodomain of gp41 is critical for fusion, possibly at steps post hairpin structure formation.

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Introduction

Feline immunodeficiency virus (FIV) (Pedersen et al., 1987) and human immunodeficiency virus (HIV) are lentiviruses that share significant homology in their genomic organization and cause remarkably similar disease in their respective hosts. Both infections are characterized by a progressive depletion of CD4+ T cells leading to immunodeficiency. The mechanism(s) mediating CD4+ T cell loss has yet to be elucidated, but productive infection does not appear to be the only cause (Alimonti et al., 2003). Among other proposed mechanisms, env-mediated cell fusion and death have been implicated in CD4+ T cell depletion (Ferri et al., 2000). Enveloped viruses like HIV and FIV utilize the env glycoprotein on their surface to mediate fusion between viral and cellular membranes. This process is critical for viral entry and infection, and also results in the formation of syncytia that is often seen in vitro (Ferri et al., 2000). Models exploiting the potential of HIV env glycoprotein to mediate syncytia formation have been used to study the interaction between env glycoprotein and cell surface receptors on target cells (Gallo et al., 2003; Jones et al., 1998), kinetics of env-mediated fusion (Reeves et al., 2002), molecular determinants of the fusogenic property of env glycoprotein (Salzwedel et al., 1999), as well as designing and testing various inhibitors of viral entry (Lawless et al., 1996).

The process of env-mediated fusion itself is complex, involving several receptor ligand interactions and conformational changes in the env glycoprotein. HIV env glycoprotein is synthesized as a gp160 precursor and later proteolytically cleaved to the gp120 surface unit and the gp41 transmembrane protein (Chen, 1996; Wyatt and Sodroski, 1998). Gp120 and gp41 are held together by non-covalent interactions and the presence of both proteins is required to mediate fusion (Salzwedel et al., 1993). HIV gp120 binds CD4 and a chemokine receptor, CCR5 or...
CXCR4, on the surface of target cells (Choe et al., 1998), while gp41 mediates fusion (Hart et al., 1991). HIV env-mediated fusion has been studied extensively, and various steps involved in this process have been identified (Gallo et al., 2003). The fusion process is initiated when gp120 binds CD4 resulting in a conformational change in gp120 that allows co-receptor (CCR5/CXCR4) binding. Subsequent conformational changes in gp120 allow insertion of the hydrophobic fusion domain of gp41 into the target cell and exposure of the heptad repeat-1 (HR1) and heptad repeat-2 (HR2) coiled domains of gp41 (Jones et al., 1998). The gp41 molecule on the membrane surface forms highly stable trimeric complexes of HR1 and HR2 running in antiparallel fashion (Lu et al., 1995). An interesting phenomenon first reported for influenza virus (Carr and Kim, 1993) is the interaction of trimeric HR1 and HR2 coiled domains in a zipper-like fashion (Wild et al., 1994b) to form the hairpin structure or the six-helix bundle that brings the viral and cell membrane into close contact resulting in fusion. This phenomenon remains true for a variety of enveloped viruses including HIV (Melikyan et al., 2000) and FIV (Medinas et al., 2002). The importance of this coiling event in the infection process is underscored by the recent demonstration that peptides corresponding to the HR1 and HR2 coiled domains are potent inhibitors of HIV env-mediated fusion (Kilby et al., 1998; Wild et al., 1994a). Similar peptides capable of inhibiting FIV fusion and entry have been identified for FIV (Medinas et al., 2002).

Although a detailed understanding of HIV env-mediated fusion has been established, certain aspects of this process such as the events post hairpin structure formation are not clearly defined. In this context, a region of HIV gp41 corresponding to the membrane proximal ectodomain also called the pre-transmembrane region (pre-TM) has gained importance in the fusion process. This region is known to contain highly conserved tryptophans in various lentiviruses including HIV. Mutational analysis shows that tryptophans in the pre-TM region are critical for HIV env-mediated fusion (Salzwedel et al., 1999), suggesting that it may be a second fusion domain in HIV (Suarez et al., 2000) and play a critical role in env-mediated fusion at a post hairpin structure stage (Saez-Cirion et al., 2002). In support of this, Gianneckicini et al. (2003) recently identified an octapeptide spanning the pre-TM region in FIV that has anti-viral activity, most likely by inhibiting fusion.

Although it has been shown that FIV env does not bind CD4 (Hosie et al., 1993; Willett et al., 1997), an interesting finding is that FIV and HIV share the chemokine receptor CXCR4 for viral entry and syncytia formation (Richardson et al., 1999). The laboratory-adapted Petaluma strain of FIV (FIV-pet) can utilize either feline or human CXCR4 for mediating fusion (Willett et al., 1997). Based on this, several previous studies (Medinas et al., 2002; Willett et al., 1998) have utilized Crandell feline kidney (CrFK) cells, chronically infected FIV-pet (CrFKpet), and CXCR4 expressing HeLa cells as a model for studying FIV env-mediated fusion. As most primary isolates of FIV do not infect CrFK cells, the ability of FIV env from various isolates to use CXCR4 as a receptor for viral entry or syncytia formation remains controversial. Although it is widely accepted that the laboratory adapted FIV-pet utilizes CXCR4 to induce fusion in a variety of cells, including the human cell line HeLa, the results with other primary isolates have been varied (De Parseval and Elder, 2001; Willett et al., 2002), suggesting the requirement of yet to be identified receptor–co-receptor.

The objective of the present study was to develop a suitable model to study FIV env-mediated fusion by a primary isolate of FIV in lymphocytic target cell lines. For this purpose we have cloned and expressed the env glycoprotein from a primary isolate of FIV (FIV-NCSU1) in CrFK cells. Cells expressing env glycoprotein from FIV-NCSU1 (CrFKenv/rev) showed fusion with the IL-2-dependent feline T cell line FCD4E cells. Using a quantitative assay based on syncytia formation between CrFKenv/rev cells and FCD4E cells, we were able to block env-mediated fusion at...
various receptor-ligand interactions and conformational changes, and demonstrated the role of CXCR4, gp41-coiled domains, and conserved tryptophan-rich regions of gp41 in FIV env-mediated fusion. This simple model can be used to address numerous questions concerning FIV env interactions with cell surface receptors on T cells and their role in FIV cell entry and pathogenesis.

Results

FIV env gene expression is rev dependent

To study the role of env glycoprotein in FIV pathogenesis, we cloned and expressed the env gene from the pathogenic NCSU1 primary isolate of FIV (Yang et al., 1996). The expression of retroviral genes is complex and involves multiple splicing of viral RNA. Rev, a regulatory protein, reported in both HIV and FIV has been shown to function as a transporter of full-length and partially spliced HIV RNA out of the nucleus (Hadzopoulou-Cladaras et al., 1989; Hammerskold et al., 1989; Phillips et al., 1992). To determine a similar function of rev in FIV we developed two clones, one containing the open reading frame (orf) of the env gene alone (pFIVenv) and other included the env gene along with the 3' exon of the rev gene (pFIVenv/rev) (Fig. 1). Transfection of CrFK cells with these constructs revealed that expression of FIV env as determined by Western blotting and immunocytochemistry could be achieved with the pFIVenv/rev construct but not with

Fig. 2. Expression of FIV env glycoprotein in transfected cells. (A) Western blot showing FIV env expression. Total cellular extracts from transfected CrFK cells were run on SDS page gel, blotted onto PVDF membrane, and probed with SU1–30 mAb against FIV gp120, which also reacts with unprocessed gp160 protein. Lane 1: CrFK infected with Petaluma strain of FIV; lane 2: CrFK transfected with pFIVenv/rev; lane 3: CrFK transfected with pFIVenv; lane 4: CrFK non-transfected. (B) Immunocytochemistry of transfected CrFK cells using serum from an FIV+ cat followed by HRP-conjugated anti-cat antibody and developed with AEC substrate.

Fig. 3. Membrane-expressed FIV env induces syncytia formation with FCD4E cells. (A) FIV env-expressing CrFK (CrFKenv/rev) cells were co-cultured with the feline CD4+ T cell line (FCD4E). At 24 h post co-culture, the plates were fixed and stained with Geimsa stain. (B) Two-color fluorescent dye redistribution assay showing FIV env-mediated fusion between CMFDA-labeled CrFKenv/rev cells and CMTMR-labeled FCD4E cells. Labeled cells were co-cultured for 24 h, following which plates were fixed with 3.7% paraformaldehyde and analyzed by fluorescent microscopy. Fused syncytial plaques (indicated by arrows) are positive for both dyes. (C) Fusion mediated by in vitro-expressed FIV env is linearly related to the number of target cells (FCD4E) added. CrFKenv/rev cells at $2 \times 10^4$ cells/well were co-cultured with FCD4E cells at 2-fold serial dilutions starting at $10^4$ cells/well. Fusion was quantified by counting the number of syncytial plaques formed per well 24 h post co-culture. Data are mean ± standard deviation of quadruplicate wells.
pFIVenv (Figs. 2A and 2B). Env gene transcripts were detected in both pFIVenv- and pFIVenv/rev-transfected cells by RT-PCR (data not shown), suggesting that the role of rev in env gene expression is at a posttranscriptional level. The requirement of rev is absolutely critical for FIV env expression, and this simple mechanism of providing rev from the same plasmid can be used for efficient expression of FIV env from eukaryotic expression vectors. Using this construct we established a stable cell line expressing FIV-NCSU1 env termed CrFKenv/rev that was used in all subsequent studies.

Membrane expressed env glycoprotein causes syncytia formation with a feline CD4+ T cell line

To assess the biological activity of the expressed env glycoprotein, a fusion assay was developed using env-expressing CrFKenv/rev cells and the IL-2-dependent feline CD4+ T cell line (FCD4E). Co-culture of CrFKenv/rev cells with FCD4E cells resulted in the formation of syncytia (Fig. 3A). To confirm that the syncytia seen in the coculture were formed due to fusion between CrFKenv/rev (effector) cells and FCD4E (target) cells, a two-color fluorescent dye redistribution assay was performed. Co-culture of differentially labeled effector and target cells resulted in the formation of syncytial plaques that were double positive for the fluorescent dyes, indicating that the fusion was in fact between effector and target cell (Fig. 3B). For most other experiments, the non-fluorescent syncytial plaque-forming assay was used. A two-fold serial dilution of target cells in the assay showed a linear relationship between the number of target cells added and number of syncytia formed (Fig. 3C). Based on this experiment, 5–2.5 × 10^3 target cells/well gave the most reproducible results.

Blocking of FIV env-mediated fusion

To validate the specificity of env-mediated fusion, either pooled serum from FIV-infected cats (FIV+ serum) or gp41-specific fusion inhibitor T1971 was incorporated in the assay as possible blocking agents. FIV+ serum (shown to have antibodies to FIV env by immunocytochemistry in Fig. 2B) blocked env-mediated fusion in a dose-dependent manner (Fig. 4A). The fusion event mediated by HIV and FIV env involves interaction among coiled domains of gp41 at a post gp120 binding stage. Peptides corresponding to the coiled domains of HIV and FIV gp41 have been shown to be potent inhibitors of env-mediated fusion (Kilby et al., 1998; Medinas et al., 2002). One of these inhibitors, T1971, shown to have significant fusion inhibition against FIV in an earlier study (Medinas et al., 2002) blocked syncytia formation in the CrFKenv/rev-FCD4E syncytia forming assay in a dose-dependent manner, while T1566, a peptide derived from a region outside the coiled domains of FIV gp41, had no inhibitory activity (Fig. 4B). This study confirmed the biological activity and specificity of the cloned env glycoprotein.

CXCR4 is involved in FIV env-mediated fusion of FCD4E cells

It has been reported that FIV utilizes CXCR4 as a receptor for viral entry and cell fusion (Hosie et al., 1998; Willett et al., 1997). To determine if FIV-NCSU1 utilizes CXCR4, antihuman CXCR4 antibodies were assessed for their ability to block fusion. Antihuman CXCR4 monoclonal antibodies (mAb) 12G5 and 44717 failed to block CrFKenv/rev- or CrFKpet-induced fusion with FCD4E (feline) cells up to a concentration of 30 μg/ml, but interestingly, both antibodies completely blocked fusion of CrFKpet with HeLa (human) cells at 3 μg/ml (data not shown). Previous studies (Eggerink et al., 1999; Hosie et al., 1998) and our own observation demonstrated that mAb 44717 cross-reacts with feline CXCR4, whereas 12G5 fails to bind feline cells. We confirmed these findings by flow cytometric analysis of FCD4E cells (data not shown). Failure of cross-reacting mAb 44717 to block fusion medi-
ated by FIV env from both primary FIV-NCSU₁ and lab-adapted FIV-pet strain in feline cells may be due to weak binding affinity or more likely due to failure to mask the FIV env-binding site on the feline CXCR4 receptor. This is supported by our observation that fusion mediated via feline CXCR4 (by either NCSU₁ or Petaluma) could not be inhibited up to a concentration of 30 μg/ml of mAb 44717 while the cell surface staining of feline cells was saturated at 5 μg/ml. Also, fusion via human CXCR4 (CrFKpet-HeLa model) was completely inhibited at 10-fold lower concentration of 3 μg/ml (data not shown).

To further explore a role for CXCR4 in FIV env-mediated fusion, the CXCR4 antagonist AMD3100 that has been shown to block infection of feline cells by various isolates of FIV (Egberink et al., 1999) was used in the fusion assay. Interestingly, AMD3100 efficiently blocked syncytia formation between FIV-NSCU₁ env-expressing CrFKenv/rev cells and FCD4E cells in a dose-dependent manner (Fig. 5A). To determine that AMD3100 was in fact binding to feline CXCR4, a competitive assay was done to block binding of cross-reacting anti-CXCR4 mAb 44717 to FCD4E cells by excess of AMD 3100 and analyzed by flow cytometry. AMD3100 at 1 μM completely blocked binding of mAb 44717 to FCD4E cells (Fig. 5B). These results are in accordance with previous reports of FIV env binding to CXCR4 and confirm that feline CXCR4 is involved in binding and fusion mediated by FIV-NSCU₁ env.

**CXCR4 is required but not sufficient for fusion by a primary isolate (NSCU₁) of FIV**

Having established that CXCR4 was involved in binding and fusion between CrFKenv/rev cells and FCD4E cells, we assessed the ability of FIV-NSCU₁ env to induce syncytia formation with other CXCR4 expressing feline or human cell lines. We tested FCD4E (CD4+ feline T cell line), Fet J cells (CD8+ feline T cell line), 3201 cells (CD4+CD8+ feline lymphoma cell line), and HeLa cells (human epithelial cell line), all of which express CXCR4 on their surface, for their ability to fuse with CrFK cells expressing either the FIV-NSCU₁ env (CrFKenv/rev) or chronically infected with FIV-pet (CrFKpet). Interestingly, only FCD4E cells fused with CrFKenv/rev cells, while all four cell lines fused with CrFKpet cells (Figs. 6A and 6B). These results are consistent with the observation that of all the cell lines tested, FIV-NSCU₁ is capable of infecting only FCD4E cells, whereas the cell culture-adapted FIV-pet isolate has a wider tropism (Verschoor et al., 1995). FACS analysis of CXCR4 expression revealed that FCD4E cells, the only cell line capable of fusion with FIV-NSCU₁...
Fig. 6. CXCR4 is required but not sufficient to mediate fusion by primary isolate of FIV. Cells either expressing FIV-NCSU env (CrFKenv/rev) or infected with FIV-pet (CrFKpet) were tested for their potential to fuse with various cell lines in a co-culture assay as described in Materials and methods. CrFKenv/rev (A) or CrFKpet (B) cells were seeded at $5 \times 10^3$ cells/well; FCD4E, 3201, and Fet-J cells were added at 2-fold serial dilution starting at $10^3$ cells/well and incubated for 24 h following which the plates were fixed, stained, and photographed. For fusion with HeLa cells, HeLa cells were seeded at $10^4$ cells followed by $5 \times 10^2$ CrFKenv/rev or CrFKpet. Either $10^3$ or $5 \times 10^3$ dilution wells shown for Fet-J, 3201, and FCD4E. (C) Flow cytometric analysis of CXCR4 expression on various cell lines. Cells were incubated with antihuman CXCR4 Ab 44717 or isotype control followed by FITC-conjugated secondary Ab and analyzed by flow cytometry.
 env, had the lowest CXCR4 expression (Fig. 6C), suggesting that the level of CXCR4 expression was not a limiting factor in FIV-NCSU1 env-mediated fusion. These findings suggest that in contrast to the cell culture-adapted FIV-pet isolate, the primary FIV isolate NCSU1 may utilize a receptor–co-receptor in addition to CXCR4 for cell entry and fusion, as has been suggested for other primary isolates (De Parseval and Elder, 2001).

**FIV env-mediated fusion is rapid and follows kinetics similar to HIV env glycoprotein**

To determine if the fusion process mediated by FIV env follows similar kinetics as HIV env-mediated fusion, either T1971 or AMD3100 was added at different time points post co-culture. As shown in Fig. 7, the fusion process could be completely blocked when T1971 or AMD3100 was added up to 45 min post co-culture. At time points 1.5 and 2 h, AMD3100 was significantly less effective at blocking syncytia formation than T1971 ($P < 0.01$), suggesting that T1971 acts at time points post CXCR4 binding. Our data indicate that while some of the cells have completed fusion as early as 1 h, it takes up to 4 h for all cells to complete fusion under our assay conditions. The kinetics of fusion in the presence of AMD3100 or T1971 were similar, suggesting that the limiting factor in FIV env-mediated fusion was gp120 binding to receptor–co-receptor and not gp41 hairpin structure formation, which was rapid after CXCR4 engage-
Conserved tryptophans in the pre-transmembrane region of FIV gp41 are essential for fusion

Sequence analysis of HIV and FIV env glycoproteins revealed significant homology in the gp41 region and not in the gp120 region. A region of HIV gp41 N terminal to the membrane-spanning region called the pre-TM region has been shown to have conserved tryptophans (W) that are critical for membrane fusion (Salzwedel et al., 1999). Based on the observation that the pre-TM region of FIV gp41 also has conserved tryptophans (Fig. 8A), we used site-directed mutagenesis to replace W at positions 766, 769, and 772 with Alanine (A). The mutant env/rev construct thus generated [W(1–3)A] was transfected into CrFK cells and tested for its fusion-inducing ability with FCD4E cells. As

![Fig. 8](image_url)

Fig. 8. The conserved tryptophan-rich region in gp41 is critical for fusion. (A) Schematic diagram of FIV gp41 showing the location of the tryptophan-rich region that is conserved in various lentiviruses and among various isolates of FIV. (B) Expression and fusion potential of wild-type (WT) and mutant env W(1–3)A in which all three tryptophans were replaced by alanine. CrFK cells were transiently transfected with WT or W(1–3)A env constructs and analyzed for env expression by Western blotting and fusion by a co-culture assay as described above.
shown in Fig. 8B, the W(1–3)A env showed similar levels of expression and processing as the wild type (WT), but was completely defective in forming syncytia with FCD4E cells. As with HIV, this region appears to be critical for fusion by FIV env and that the presence of tryptophans is required for this property.

Discussion

The present study was undertaken to develop an in vitro model to study interactions of membrane-expressed FIV env with receptors on T cells that leads to env-mediated fusion. We have used this model to study env receptor interactions as well as kinetic analysis of FIV env-mediated fusion. Several critical questions regarding FIV env expression and function are answered in our study. Firstly, failure to express FIV env from a plasmid lacking 3’ rev-coding region suggests that the expression of FIV env, similar to HIV env, is rev dependent. Rev is involved in the transport of unspliced and partially spliced env mRNA from the nucleus (Malim et al., 1989). Similar constructs have been used to express HIV env glycoprotein in transfected cells (Hammerskjold et al., 1989). In some studies with HIV, rev has been provided in trans from another plasmid to obtain env expression (Moir and Poulin, 1996). In our system, providing rev from the same plasmid is sufficient to get efficient express env. Using this construct we were able to study the various steps involved in FIV env-mediated fusion and correlate it to findings with HIV.

The first step in HIV env-mediated fusion is the binding of gp120 to CD4 and CXCR4 on T cells (Choe et al., 1998). Although several studies have ruled out a role of CD4 as a receptor for FIV (Hosie et al., 1993; Willett et al., 1997), CXCR4 has been well established as a necessary receptor or co-receptor for the laboratory-adapted Petaluma isolate of FIV (Hosie et al., 1993; Richardson et al., 1999), while results with primary isolates have been varied. In support of this, a recent report by Willett et al. (2002) shows that expression of feline CXCR4 alone was sufficient for viral entry and fusion by laboratory-adapted FIV-pet, but not the primary isolate FIV-Glasgow. Similarly, studies by De Parseval and Elder (2001) have shown a yet unidentified 40-kDa protein on T cells that bind recombinant FIV gp120 from the primary isolate FIV-PPR. In the same study, CXCR4 agonist SDF-1 failed to inhibit recombinant FIV-PPR gp120 binding to T cells. In both these studies, the authors fail to show direct evidence that the env glycoprotein from primary isolates bind to or utilize feline CXCR4. Using CXCR4 antagonist AMD3100 we show that CXCR4 is in fact a receptor (co-receptor) for FIV-NCSU1 and plays a necessary role in FIV env-mediated cell fusion by both primary- and laboratory-adapted strains of FIV. Cross-reacting antihuman CXCR4 antibody failed to block fusion in our model even though excess AMD3100 blocked binding of this antibody to FCD4E cells. We believe that the failure to inhibit fusion via cross reacting antibody is likely due to failure to mask the FIV env-binding site on feline cells. This is supported by the report of Willett et al. (1998) showing that the second extracellular loop of CXCR4 that is required for FIV env binding contains the majority of differences between feline and human CXCR4.

Primary isolates of FIV, including NCSU1, neither infect nor mediate fusion in CXCR4-expressing CrFK cells (Hod-datsu et al., 1996), which suggests that a second receptor may be required for entry by these viruses. The differences between FIV-pet and primary isolates have been largely attributed to variation in the env gene sequence, more specifically changes in the V3 loop of FIV-pet resulting in a net positive charge in this region have been implicated in the broader tropism and fusogenicity of FIV-Pet (Verschoor et al., 1995). We show that while CrFK cells infected with FIV-pet (CrFKpet) would fuse with a variety of cell lines expressing CXCR4, such as Jef J, 3201, HeLa, and FCD4E cells, cells expressing FIV-NCSU1 env (CrFKenv/rev) fused only with FCD4E cells. This is again in agreement with the tropism of NCSU1, which productively infects only FCD4E cells or primary lymphocytes (English et al., 1993). This suggests that though CXCR4 is required, it is not sufficient for fusion by primary isolates of FIV and that another receptor or co-receptor expressed on FCD4E cells may be involved. Although FCD4E cells express CD4, it is not a receptor for FIV as has been shown previously (Hosie et al., 1993). Our findings confirm that FIV-NCSU1 binds feline CXCR4, but expression of CXCR4 alone was not sufficient to induce fusion and that another receptor–co-receptor may be involved.

Following HIV gp120 binding to CD4/CXCR4, a conformational change in the env glycoprotein occurs that results in insertion of the N terminal fusion domain of gp41 into the target cell membrane and exposure of the coiled domains HR-1 and HR-2 of gp41 (Wild et al., 1994b). Interaction among the coiled domains of gp41 in a zipper-like fashion (Wild et al., 1994b) brings the membranes close together facilitating fusion. Lentiviral env glycoprotein is present as a trimer on the membrane surface, and hence interaction of the coiled domains results in formation of a six-helix bundle structure. Peptides corresponding to either the HR-1- or HR-2-coiled domains of HIV or FIV gp41 have the unique property of inhibiting six-helix bundle formation and thus viral entry (Wild et al., 1994a). These peptides have evolved into a new generation of anti-HIV drugs premiered by T-20 (Enfuvirtide) (Moyle, 2003). Gp41-specific peptide inhibitors are also powerful tools to study env interactions post receptor–co-receptor binding. We utilized an FIV-specific fusion inhibitor T1971, previously shown to block CrFKpet fusion with HeLa cells (Medinas et al., 2002), to block fusion in our model in a dose-dependent manner. Inhibition by gp41-specific fusion inhibitors is unique because it inhibits env response at a post-CXCR4 binding stage (Gallo et al., 2001).
Utilizing CXCR4 antagonist AMD3100 and gp41-specific fusion inhibitor T1971, we studied the kinetics of FIV-NCSU-1 env-mediated fusion by inhibiting this process at different time points post co-culture. As shown in Fig. 7, we see a lag phase of around 45 min in fusion mediated by FIV-NCSU1, which is not too different from 20 to 30 min reported for HIV (Melikyan et al., 2000). T1971 was more efficient than AMD3100 at inhibiting fusion at time points 1.5 and 2 h. This correlates with the proposed sequence of events involved in env-mediated fusion in that CXCR4 binding precedes and induces gp41 hairpin formation. Nevertheless, the overall kinetics of fusion in the presence of T1971 or AMD 3100 were similar, suggesting that the limiting factor in FIV env-mediated fusion is not gp41 hairpin structure formation, which probably occurs rapidly after receptor–co-receptor binding.

The events after hairpin formation that may be important for fusion in HIV have not been well characterized. However, a tryptophan-rich region of gp41 proximal to the membrane-spanning domain termed pre-transmembrane region has gained importance following a report by Salzwedel et al. (1999) that conserved tryptophans in this region are critical for fusion. Further, others have shown that this region is a novel fusogenic domain (Suarez et al., 2000) and that it has lectin-like properties and binds to sphingomyelin- and cholesterol-rich structures in cell membranes (Saez-Cirion et al., 2002). Sequence analysis showed that tryptophans in this region were conserved among various lentiviruses and also within various strains of FIV (Fig. 8A). We used site-directed mutagenesis to replace tryptophans (W) in this region with alanine (A) resulting in a fusion-defective mutant. We hypothesize that the hydrophobic nature of W in this region may facilitate fusion at a step post six-helix bundle formation by mediating mixing of lipid components of effector and target membranes. Interestingly, Giannecchini et al. (2003) have recently reported that an octapeptide spanning this region has anti-FIV activity by inhibiting virus entry. The authors have suggested that the peptide inhibits viral entry by binding to components on the cell surface rather than any region in the viral gp41 protein. They also found that tryptophans were critical for the inhibitory activity of the peptides.

Using the CrFKenv/rev-FCD4E syncytia model, we have delineated various steps that are involved in cell fusion and syncytia formation by the env glycoprotein from FIV-NCSU1 primary isolate (Fig. 9). The first step involves gp120 binding to CXCR4 and possibly another receptor–co-receptor on the cell surface. This is followed by conformational changes in the env protein that result in exposure of gp41 heptad repeat domains and six-helix bundle formation. Finally, our data indicate that the tryptophan-rich pre-TM region has a critical role in FIV env-mediated fusion probably at a step post six-helix bundle formation.

Materials and methods

Antibodies and reagents

Antihuman CXCR4 antibodies 12G5 and 44717 were obtained from BD Biosciences (San Jose, CA). Anti-FIV gp120 antibody SU1–30 was obtained from Custom Monoclonals (West Sacramento, CA). Gp41 fusion inhibitor T1971 and control peptide T1566 were a kind gift from Robyn Medinas (Trimeris Inc., Durham, NC). CXCR4 antagonist AMD3100 was a kind gift from Dr Edward Hoover, (Colorado State University).

Fig. 9. Proposed sequence of events leading to FIV env-mediated fusion. Step1: FIV gp120 binds to CXCR4 (inhibited by AMD3100) and an unknown receptor–co-receptor on the surface of target cells. Step 2: a conformational change in the env glycoprotein allows insertion of gp41 hydrophobic domain in the target membrane and exposure of the coiled domain. Step 3: the coiled domains interact with each other (inhibited by T1971) to form the hairpin structure. Step 4: the pre-TM region of gp41 may mediate mixing of the lipid components of the effector and target membranes resulting in fusion [the W(1–3)A mutant is unable to mediate fusion].

Infected cell
Cell lines and cell culture

The CrFK or CrFKpet cell line (Crandell feline kidney cells persistently infected with a CrFK-adapted strain of FIV-Petaluma; stock number ATCC-CCL-94; American Type Culture Collection [ATCC]) was maintained in Dulbecco’s modification of Eagle’s medium (high glucose; Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), streptomycin (100 μg/ml), penicillin (100 IU/ml), L-glutamine (2 mM), HEPES (15 mM), and sodium pyruvate (2 mM) at 37 °C in 5% CO₂. The HeLa cell line (stock number ATCC-CCL-2; ATCC) was maintained in DMEM (high glucose) supplemented as described above. The feline T cell line (FCD4E) (an interleukin 2 [IL-2]-dependent feline CD4⁺ lymphocyte cell line described by English et al., 1993) was maintained in RPMI 1640 medium (Mediatech) supplemented with 10% FBS, streptomycin (100 μg/ml), penicillin (100 IU/ml), L-glutamine (2 mM), HEPES (15 mM), sodium pyruvate (2 mM), β-mercaptoethanol (2.5 × 10⁻⁵ M), and recombinant human IL-2 (rhIL-2) (100 U/ml; AIDS Reference and Reagent Program) at 37 °C in 7% CO₂. Other feline T cell lines Fet-J and 3201 (Hohdatsu et al., 1996) were maintained as described for FCD4E cells in the absence of IL-2.

Cloning of FIV env

Plasmid pFIVenv was generated by PCR amplification of the env gene from a plasmid containing the molecular clone JSY3 of FIV-NCSU1 (Yang et al., 1996) using primer BamH1FIVenv> (ATTGGATCCCGCACAATAATTATGGCAGA) and <FIVenvEcoR1 (GCGGGAATTCAGTCTGAGATCTTCCATCAT). For plasmid pFIVenv/rev, which contained the 3’ exon of rev, the forward primer was the same as above (BamH1FIVenv>) and the reverse primer was located at the end of the LTR region <FIVrevEcoR1 (ATAAAGTTCAGTTCTGGCCGGATTC) (underlined sequences show restriction enzyme sites). PCR-amplified products were digested with BamH1 and EcoR1 and cloned into BamH1- and EcoR1-digested pcDNA3 vector (Invitrogen). The cloned products were sequenced at the automated DNA sequencing facility at University of North Carolina (Chapel Hill, NC).

Transfection of cells

Subconfluent monolayers of CrFK cells were transfected with 1 μg of plasmid DNA of either pFIVenv or pFIVenv/rev using Effectene transfection system (Qiagen Inc., Valencia, CA) as per manufacturer’s instructions. Forty-eight hours after transfection, the cells were seeded at low density in selection medium containing G418 at 700 μg/ml ( GibcoBRL, Gaithersberg, MD). The cells were selected by limiting dilution for at least 3 weeks before analysis and use in experiments. Selected cells were maintained in 400 μg/ml G418 throughout the study.

Detection of env expression

Immunocytochemistry and Western blotting were used to detect expression of FIV env in transfected cells. For immunocytochemistry, transfected and selected CrFK cells were seeded in 96-well plates and grown till subconfluent. Cells were fixed with methanol 0.03% H₂O₂ for 30 min followed by blocking with 0.1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 h. Cells were then stained with serum from an FIV-infected cat diluted 1:1000 in blocking buffer (0.1% BSA in PBS) for 2 h. Subsequently, cells were stained with horseradish peroxidase (HRP) conjugated goat anti-cat antibody (Cappel Research products, Durham, NC) for 1 h. Finally, the plates were developed with 3-amino-9-ethylcarbazole (AEC) substrate kit (Biogenex, San Ramon, CA) and analyzed by microscopy. For Western blotting, total cellular lysates were run on a 4–12% polyacrylamide gel, transferred to PVDF membrane, and blocked with Tris-buffered saline (TBS) containing 0.05% Tween 20 with 1% gelatin, overnight. Blocked membranes were blotted with monoclonal antibody to FIV env, SU1–30, at 1 μg/ml followed by HRP-conjugated goat anti-mouse antibody (Pierce Biotechnology Inc., Rockford, IL) at 0.1 μg/ml in blocking buffer. The membranes were developed with enhanced chemiluminescence (ECL) system using ECL Western blotting kit (Pierce Biotechnology Inc.). The developed membrane was analyzed by Lumimagger (Boheringer, Germany).

FIV syncytial assay

The FIV syncytia forming assay utilized FCD4E cells and CrFK cells transfected with pFIVenv/rev (CrFKenv/rev). CrFKenv/rev cells were plated in a 96-well flat-bottom plates at a concentration of 2 × 10⁴ cells/well in 100 μl of DMEM medium and allowed to adhere overnight. The following day, the culture medium was aspirated and FCD4E cells, 5 × 10⁴ in 100 μl of medium, were added to give reproducible numbers of syncytia. After 24 h, the plates were fixed and stained with crystal violet (stock stain: 2.5 g of crystal violet, 1.25 g of Giemsa stain, 500 ml of 80% methanol; working solution: 200 ml of stock and 200 ml of 80% methanol). Stained syncytial plaques (fused cells that are five cell diameters or greater) were counted using an inverted microscope. When the number of syncytia formed within 24 h was compared to the number of FCD4E cells plated, there was a linear correlation between the number of target cells plated and the number of syncytia produced. For studies with CrFK cells chronically infected with Petaluma strain of FIV (CrFKpet) and HeLa cells, the fusion assay used has been described previously (Medinas et al., 2002). Briefly, HeLa cells were plated at 10⁴ cells/well in 96-well plates, and the following day 5 × 10² CrFKpet cells were added. Plates were fixed and stained the next day as described above. Various treatments such as serum, peptides, or AMD3100 were added in a 2-fold serial dilution series in 100 μl of FCD4E cell medium to the
CrFK cells before co-culture. For anti-CXCR4 mAb treatment, the FCD4E or HeLa cells were pre-incubated with serial dilutions of the Ab for 1 h before co-culture with CrFK cells. For fusion assays with different feline cell lines, either CrFKenv/rev or CrFKp66 was seeded in 96-well plates at 2 × 10^4 cells/well. FCD4E, Fet-J, or 3201 was added 24 h later at 2-fold dilutions starting at 2 × 10^4 cells/well. Plates were stained and observed for syncytia formation 24 h later as described above.

**Flow cytometry**

Different cell lines were stained with antihuman CXCR4 antibody 44717 that cross-reacts with feline CXCR4, followed by FITC-conjugated goat anti-mouse IgG antibody (BD Biosciences). Stained cells were analyzed by flow cytometry using FACS Calibur (BD Biosciences). For inhibition of anti-CXCR4 antibody binding to feline cells, feline cells were incubated with either isotype control or 44717 antibody in the presence or absence of AMD3100 (0.5 μg/ml) for 1 h followed by washing and incubation with FITC-conjugated goat anti-mouse antibody and analyzed as above.

**Site-directed mutagenesis**

PCR-mediated site-directed mutagenesis was used to induce a tryptophan (W) to alanine (A) mutation in the pre- TM region of FIV env. The following primer pairs were used to generate two fragments: Fragment 1, BamH1FIVenv> (sequence shown above) and <W(1–3)A-antisense (CGCTCTACGATCCATTTCCGCTTTTGTGTTATCCCC); Fragment 2, W(1–3)A-sense> (GCGGA- AGATGCGGT AGGAGCCATGGAAATATCCACAA- TAC) and <FIVrevEcoR1 (sequence shown above). Fragments 1 and 2 were then used in an overlap PCR to generate a mutant construct that was digested with EcoR1 and BamH1 and cloned into pcDNA3 vector as described above. The presence of mutation in the construct was confirmed by sequencing.

**Two-color fluorescent dye redistribution assay**

For the two-color fluorescent dye redistribution assay, CrFKenv/rev or CrFK control cells were labeled with cytoplasmic dye 5-chloromethylfluorescein diacetate (CMFDA) at a concentration of 0.5 μm in PBS for 30 min at 37 °C followed by washing twice with medium and seeded in 96-well plates at 2 × 10^4 cells/well. The cells were allowed to adhere overnight following which FCD4E cells labeled with cytoplasmic dye 5- and 6-[(4-chloro- methyl) benzoyl] amino] tetramethyl rhodamine (CMTMR) at a concentration of 5 μm were added to the wells at 5 × 10^3/well. The cells were co-cultured for 24 h following which the plates were washed to remove non-adherent cells and fixed with 3.7% paraformaldehyde. Fixed plates were analyzed by fluorescent microscope.

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**References**


