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A Single Amino Acid Substitution in the Transmembrane Envelope Glycoprotein of Feline Immunodeficiency Virus Alters Cellular Tropism

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The cellular tropism of the feline immunodeficiency virus (FIV) is affected by changes in variable region 3 (V3) of the surface (SU) envelope glycoprotein (Verschoor, E. J., et al., J. Virol. 69:4752–4757, 1995). By using high-dose DNA transfection, an FIV molecular clone with a non-CRFK-tropic V3 acquired the ability to replicate in CRFK cells. A single point mutation from a methionine to a threonine in the ectodomain of its transmembrane (TM) envelope glycoprotein was responsible for this change in viral tropism. This substitution is located in the putative SU interactive region, between the fusion peptide and the membrane-spanning region. Our results show that this region of the TM envelope glycoprotein constitutes an additional determinant for cell tropism.

Feline immunodeficiency virus (FIV) infection of cats causes a slow, progressive loss of immune functions, eventually leading to a disease comparable to AIDS in humans (26). FIV replicates both in vivo and in vitro in $CD4^+$ and $CD8^+$ T lymphocytes, B lymphocytes, macrophages, and astrocytes (2, 3, 10, 12). FIV isolates are usually obtained from cultures of peripheral blood mononuclear cells (PBMC) of infected cats. Some FIV isolates also have the ability to replicate in Crandell feline kidney (CRFK) cells (22, 27, 37). Both PBMC and CRFK cells carry the putative fCD9 receptor for FIV (16, 35), indicating that affinity for a second receptor may be involved in the cell tropism of FIV. Second receptors for infection by the human immunodeficiency virus type 1 (HIV-1) have recently been identified and belong to the chemokine receptor family, with CCR-5 and CXCR-4 as the main receptors for macrophage- and T-cell-line-tropic isolates, respectively (8, 11, 13, 38). The human CXCR-4 molecule also appears able to mediate the entry of CRFK-tropic FIV Petaluma (36). A key role for the interaction of HIV-1 with its second receptor is played by the third variable region (V3) of the surface (SU) envelope glycoprotein (5, 13, 38). In line with this observation, the HIV-1 V3 was for long known to be involved in cell tropism and to be an immunodominant region able to elicit neutralizing antibodies (6, 7, 17, 33). An analogous function appears to reside in the V3 region of the SU envelope glycoprotein of FIV which also contains an immunodominant neutralization domain and a determinant of cell tropism (9, 19, 28, 31). In this study, we investigated if regions of the envelope glycoprotein of FIV, other than V3, could influence cell tropism as well.

In a previous study, we generated FIV mutants carrying changes in V3, which were replication competent in feline thymocytes but not in CRFK cells (31). One of these non-CRFK-tropic mutants, the pPET-REK molecular clone (arginine, glutamic acid, and lysine at positions 397, 407, and 409, respectively), which has the same overall charge in the V3 as the CRFK-tropic molecular clone pPET-RKE and shares amino acids at crucial positions with the CRFK-tropic FIV-14 Petaluma molecular clone (23), was chosen for further study. Basically, the pPET-RKE molecular clone is the FIV-14 Petaluma molecular clone in which envelope sequences were replaced by those of a CRFK-tropic variant of FIV-UT113, and pPET-REK is a non-CRFK-tropic derivative in which two amino acids in the V3 crucial for cell tropism were exchanged (Table 1).

To facilitate the generation of mutants, a high dose of DNA (10 μg) from pPET-REK was transfected to CRFK-HO6 cells with pPET-RKE as a positive control. By using the Lipofect AMINE reagent (Life Technologies), each molecular clone was transfected into 1.25×10^5 CRFK-HO6 cells. These were seeded in a well of a 24-well plate and were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum (FCS) and antibiotics. Both pPET-REK and pPET-RKE transiently produced p24 viral antigen as determined by enzyme-linked immunosorbent assay (ELISA), but only the CRFK-tropic virus derived from pPET-RKE continued to produce p24 antigen.

Three days after transfection the cells were transferred to a 25-cm^2 flask enabling growth to confluency. Cultures were monitored at regular intervals for the production of p24 antigen. While clone pPET-RKE continuously produced viral antigen, p24 production in the pPET-REK-transfected cells reappeared only after 17 days, indicating that a mutant with a CRFK-tropic phenotype had arisen (Fig. 1).

To verify this observation, the supernatant from the pPET-REK transfected culture was centrifuged, filtered (0.45 μm), and incubated with uninfected CRFK-HO6 cells. Abundant viral replication was detected within 7 days, and genomic DNA was isolated from the cells by the method of Boom et al. (1). The envelope sequence was amplified using primers with flank-

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TABLE 1. FIV replication[a]

Virus	Replication in	
	Thymocytes	CRFK-HO6 cells
pPET-REK	+	−
pPET-REK/T	+	+
pPET-*CRFK*-REK	+	+
pPET-*CRFK*-REK/M	+	−
pPET-GEK	+	−
pPET-GEK/T	+	+

[a] FIV replication was assessed by p24 antigen capture ELISA. Results shown were consistent with those from three separate experiments.

ing 5′ *Mlu*I (ACGCGT) or 3′ *Sal*I (GTCGAC) restriction sites TAGACGCGTAAGATTTTTAAGATACTCTGATG (nucleotide [nt] 6512 to 6543) and CTTGTCGACTAAGTCTGA GATACTTCATCATTCCTCC (nt 8861 to 8825), respectively.

PCR was performed for 30 cycles (1 min at 94°C, 1 min at 55°C, and 2.5 min at 68°C) in a final volume of 50 μl by using the Expand Long Template PCR System (Boehringer Mannheim). The reaction mixture contained 100 ng of cellular DNA, 50 ng of each primer, 50 mM Tris (pH 9.2), 1.75 mM $MgCl_2$, 14 mM $(NH_4)_2SO_4$, 200 μM of each deoxynucleoside triphosphate, and 0.75 μl of enzyme mix. The PCR product was purified by preparative gel electrophoresis, digested with *Mlu*I and *Sal*I, and cloned into the pPETΔENV vector which lacks the corresponding envelope sequences (31).

Resultant clones, named pPET-*CRFK*-REK, were transfected into CRFK-HO6 cells using the LipofectAMINE procedure as described before except that 0.5 μg instead of 10 μg of DNA per well was used. Cells were either cultured and expanded in 25-cm^2 flasks as before or cocultured with 10^5 feline thymocytes. After 24 h thymocytes were removed, washed once with phosphate-buffered saline, and cultured separately in a 96-well plate in Iscove's medium supplemented with 200 U of recombinant interleukin-2 (Eurocetus) per ml, 2.5 μg of concanavalin A per ml, and 10% FCS. Viral replication was detected in cultures of thymocytes and CRFK-HO6 cells, whereas virus from the parental clone pPET-REK replicated only in cultures of thymocytes.

The envelope genes of two independently isolated clones of pPET-*CRFK*-REK were sequenced, and the amino acid sequences were analyzed. Four amino acid changes were detected when compared with the sequence of pPET-REK: an arginine to glycine mutation (R→G at residue 108) within the leader of the envelope glycoprotein, an aspartic acid to asparagine mutation (D→N at residue 290) and a threonine to alanine mutation (T→A at residue 507) within the SU envelope glycoprotein, and a methionine to threonine mutation (M→T at residue 751) in the TM envelope glycoprotein. Amino acid changes within V3 of the SU envelope glycoprotein were not found.

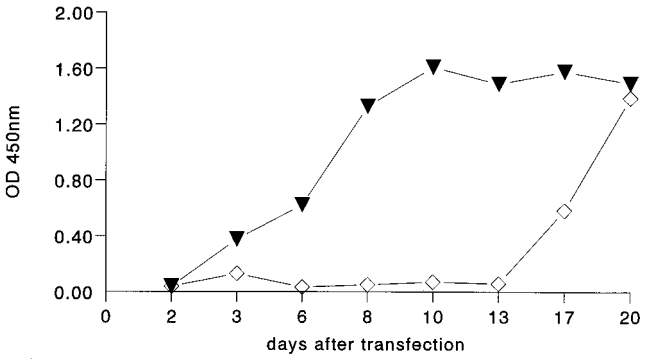

FIG. 1. Replication curves of FIV clones. Virus replication in CRFK-HO6 cells after transfection of a non-CRFK-tropic molecular clone (V3 sequence REK) and a CRFK-tropic clone (V3 sequence RKE). Viral antigen production was measured by using a p24 antigen capture ELISA. ▼, pPET-RKE; ◇, pPET-REK.

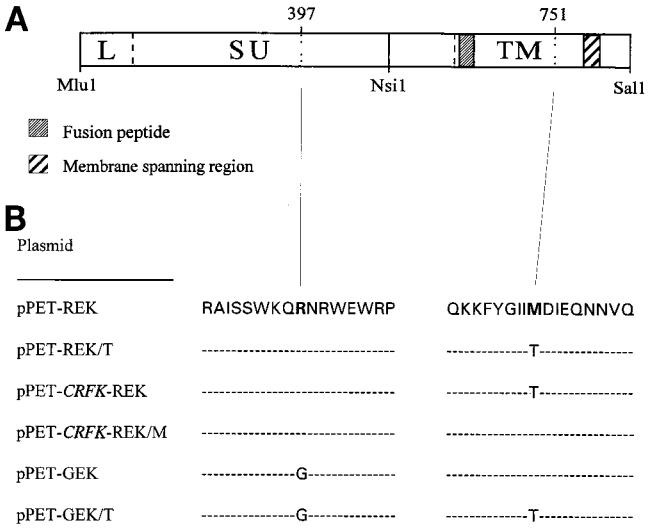

FIG. 2. (A) Schematic representation of the envelope regions and the restriction sites used in this study for the exchange of the DNA fragments. (B) Positions of the amino acids differing between the clones are indicated according to the FIV-UT113 envelope gene sequence (EMBL accession no. X60725). Amino acid sequences of V3 and TM of the parental, adapted, and chimeric FIV clones.

To investigate the role of the individual amino acid changes, envelope chimeras were constructed by exchanging fragments between clone pPET-REK and pPET-*CRFK*-REK. Envelope sequences derived from these clones were first cloned into vector pSH, a derivative of pSP73 containing *Mlu*I and *Sal*I cloning sites (31). The 5′ *Mlu*I-*Nsi*I (1,293 bp) and 3′ *Nsi*I-*Sal*I (1,045 bp) fragments were exchanged between the envelope genes of the two clones (Fig. 2). The resultant chimeric *Mlu*I-*Sal*I fragments were then cloned into pPETΔENV. By using sequence analysis it was confirmed that the fragment exchanges were performed correctly. The results of the transfection experiments with these chimeras are shown in Fig. 2.

Transfection of the chimeric clones led to progeny which replicated in feline thymocytes with similar kinetics (Fig. 3A), with viral replication detectable after 7 days. In CRFK-HO6 cells, however, only virus derived from clone pPET-*CRFK*-REK and from the chimeric clone pPET-REK/T, which is identical to its pPET-REK parent except that it contains the methionine to threonine change (amino acid 751) derived from pPET-*CRFK*-REK, replicated (Fig. 3B). The three substitutions present in the N-terminal *Mlu*I-*Nsi*I fragment of clone pPET-*CRFK*-REK/M (M at position 751) did not confer CRFK cell tropism, showing that the TM envelope glycoprotein of FIV harbors a determinant for cell tropism.

To substantiate these results, we also constructed a chimeric clone pPET-GEK/T containing the methionine to threonine mutation in the TM envelope glycoprotein at position 751. The parent clone pPET-GEK (identical to pPET-REK except for a glycine residue at position 397) was chosen, because the non-CRFK-tropic pPET-GEK (31) and the CRFK-tropic pPET-GKE resemble the pPET-REK–pPET-RKE pair. As shown in

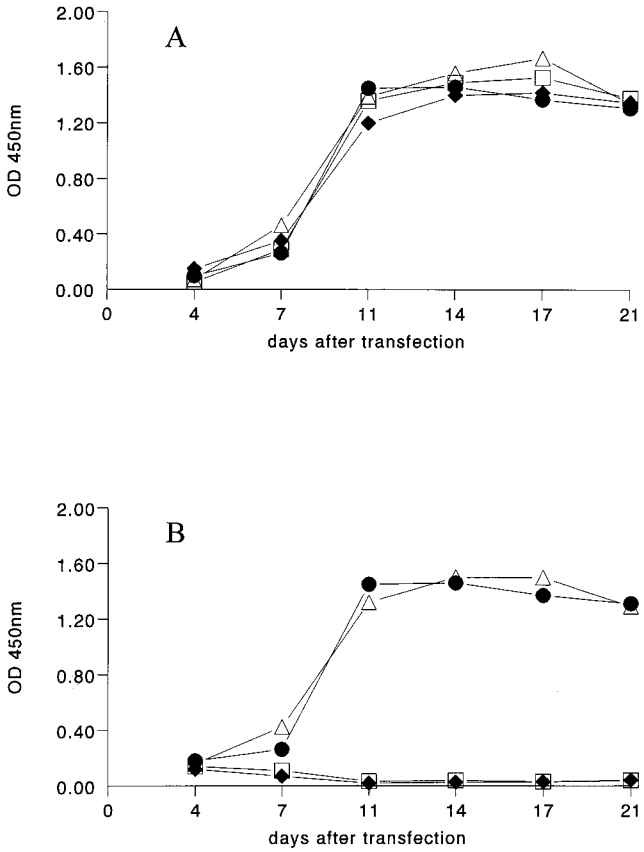

FIG. 3. Replication kinetics of viral progeny derived from molecular clone pPET-REK, pPET-*CRFK*-REK, and clones chimeric in their envelope sequences. Viral replication in feline thymocytes (A) and CRFK-HO6 cells (B) was monitored by measuring p24 production in the culture supernatants. ◆, pPET-REK; △, pPET-*CRFK*-REK; ●, pPET-REK/T; □, pPET-*CRFK*-REK/M.

Table 1, virus derived from the chimeric clone pPET-GEK/T also replicated in CRFK cells, supporting the role of the threonine residue at position 751 in determining cell tropism.

The mapping of the determinant of cell tropism in the envelope glycoprotein is in agreement with findings that the 3′ end of the FIV genome is decisive for cell tropism and the viral entry-related capacity to induce syncytia (18). The capacity to induce syncytia has been shown to be located in the envelope gene and to concur with CRFK cell tropism (25). Our data and those of others (28, 31) have indicated that the V3 region can determine cell tropism. Here, we showed that a single amino acid change in the TM envelope glycoprotein can alter cell tropism.

The methionine to threonine change at amino acid position 751 is located between the V7 and V8 region of the TM envelope gene and does not include a potential N-glycosylation site (24, 30). Both CRFK-tropic clones derived from the isolate Petaluma p34TF10 (29) and FIV-14 (22) contain a methionine residue at position 751. This indicates that a threonine at amino acid 751 is not directly correlated with CRFK cell tropism but that the context of the envelope protein plays a role as well. Constraints in the pPET-REK envelope protein and its V3 apparently gave way to a mutational pathway in the TM with one transition that altered a methionine (ATG) to a threonine (ACG) rather than for example multiple changes in the V3 region which would also have resulted in the CRFK-tropic phenotype.

The 751 position of the M→T mutation is located in the ectodomain of the TM envelope glycoprotein between the fusion peptide (amino acids 616 to 640) and the membrane-spanning region (amino acids 786 to 812). Lombardi et al. (20) showed that four peptides located in this region (just downstream of the described mutation), spanning amino acids 757 to 806, exhibit a specific antiviral effect in CRFK cells. They exert their antiviral activity after addition of the virus, suggesting that the peptides interfere with virus replication at a step subsequent to binding to the primary receptor.

According to the predicted structure of retroviral TM envelope glycoproteins, the structure of the TM glycoprotein of FIV parallels that of HIV-1 (14, 24). The corresponding region of the M→T mutation in HIV-1 is involved in the interaction with SU and in oligomerization, plays a role in viral entry, and is a target for neutralizing antibodies (4, 15, 21, 34). The involvement of this region of the TM envelope glycoprotein in entry would fit in a model (32) in which the viral envelope glycoproteins bind their receptor, after which conformational changes occur that mediate binding to the second receptor. Binding to the second receptor involves the V3 region and induces secondary conformational changes that free the fusion peptide of the TM envelope glycoprotein from interaction with SU envelope glycoprotein and exposes it to the cellular membrane, allowing the entry process to occur.

In accord with the latter model and that of Gallaher et al. (14), the M→T mutation in the ectodomain of the TM envelope glycoprotein would alter the cell tropism of FIV by allowing alternative conformational changes during the entry process. We, however, cannot as yet exclude the possibility that postentry processes are influenced by the M→T mutation. Our findings that determinants of cell tropism of FIV are located in the V3 region of the SU and in the ectodomain of the TM envelope glycoprotein, suggest that despite the obvious differences between the lentiviruses, the basic processes of entry are similar; a notion that could help to elucidate the pathogenesis of the individual viruses.

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