Identification of a Cellular Cofactor Required for Infection by Feline Leukemia Virus

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Retroviral infection involves continued genetic variation, leading to phenotypic and immunological selection for more fit virus variants in the host. For retroviruses that cause immunodeficiency, pathogenesis is linked to the emergence of T cell–tropic, cytopathic viruses. Here we show that an immunodeficiency-inducing, T cell–tropic feline leukemia virus (FeLV) has evolved such that it cannot infect cells unless both a classic multiple membrane-spanning receptor molecule (Pit1) and a second coreceptor or entry factor are present. This second receptor component, which we call FeLIX, was identified as an endogenously expressed protein that is similar to a portion of the FeLV envelope protein. This cellular protein can function either as a transmembrane protein or as a soluble component to facilitate infection.

T cell–tropic, cytopathic viruses are generated in cats infected with a cloned, avirulent transmissible form of FeLV (*1*). The cytopathic and pathogenic properties of T cell–tropic FeLV variants (called here FeLV-T) result from the acquisition of mutations in the extracellular surface unit (SU) of the envelope protein (Fig. 1) (*2*). As a result, FeLV-T variants are able to enter cells previously infected with the progenitor transmissible form (*3*). Surprisingly, these variants can also repeatedly reinfect T lymphocytes and replicate to a high copy number in these cells (*2*). This is in contrast to the resistance to infection by homologous virus that typically occurs in retroviral infections as a result of envelope-mediated receptor interference (*4*).

FeLV has at least three distinct interference groups, termed subgroups A, B, and C (*5*). The types of FeLV that are transmitted from cat to cat are members of the A subgroup of FeLV (FeLV-A), and the receptor for this group of viruses has not yet been defined (*6*). The FeLV-B viruses evolve from FeLV-A in the infected cat by recombination with endogenous sequences that are highly related to those of the infectious virus (enFeLV) (*7*), leading to the generation of a chimeric SU that can use a phosphate transporter protein, Pit1, as a receptor (*8*). The FeLV-T variants, which also evolve from FeLV-A, represent something of an anomaly and may form a fourth FeLV receptor interference group (*3*). However, because they do not establish superinfection interference against homologous virus, this has been difficult to ascertain.

To identify a cellular protein required for FeLV-T infection, we used a retroviral gene transfer approach (*9, 10*). D17 canine osteosarcoma cells were used as target cells because they are resistant to FeLV-T infection but can be infected by all other subgroups of FeLV, which suggests that there are no post-entry restrictions to FeLV infection. D17 cells transduced with a feline T cell cDNA library were then infected with FeLV-T viruses carrying two drug-resistance markers, and cell clones were selected in the presence of both drugs (*11*). These resistant cell clones were then challenged with FeLV-T carrying a vector encoding green fluorescent protein, and susceptibility to infection was confirmed by fluorescence microscopy. When D17 cells that had not been transduced with feline cDNA were challenged in parallel throughout the process, no resistant cell clones were obtained; this provided further evidence for the specificity of infection.

The cDNA of interest was cloned after polymerase chain reaction amplification with primers specific to the vector used to construct the library (*9, 12*). Upon analysis of the sequence of this gene, we learned that it was not a membrane receptor as we had expected. Rather, this cDNA encodes a truncated version of an endogenous FeLV envelope glycoprotein. The open reading frame is predicted to encode a 273–amino acid protein that shares 92.3% identity with the NH_2-terminal half of FeLV-B envelope protein, and 95.4% identity within the SU portion (Fig. 1). The COOH-terminus of SU and the transmembrane domain were absent. To determine whether the truncated enFeLV envelope was indeed a factor required for FeLV-T infection, we again introduced the cDNA encoding it into D17 cells. Cells expressing the enFeLV cDNA were rendered susceptible to infection, as judged both by infection with FeLV-T carrying a β-galactosidase gene (Fig. 2A) and by infection with replication-competent FeLV-T (Fig. 2B). In contrast, D17 cells alone or D17 cells expressing enFeLV in the inverse orientation were not permissive to FeLV-T. FeLV-A infected D17 cells but with reduced efficiency (*3*), and infection by the transmissible form of FeLV-A was not affected by the presence of the enFeLV envelope (Fig. 2, A and B). Thus, the enFeLV envelope is required for productive FeLV-T infection, but not for productive infection by the progenitor FeLV-A virus. Given the role that the endogenous FeLV-like envelope protein plays in facilitating FeLV-T infection, we termed this molecule FeLIX (FeLV infectivity X-essory protein).

Because D17 cells are susceptible to infection by FeLV-B and to a limited extent FeLV-A (Fig. 2A) (*3, 13*), we considered the possibility that a second, more traditional FeLV receptor expressed in this cell type was participating in FeLV-T entry. To address this, we introduced FeLIX into a *Mus dunni* tail fibroblast (MDTF) cell line that is not

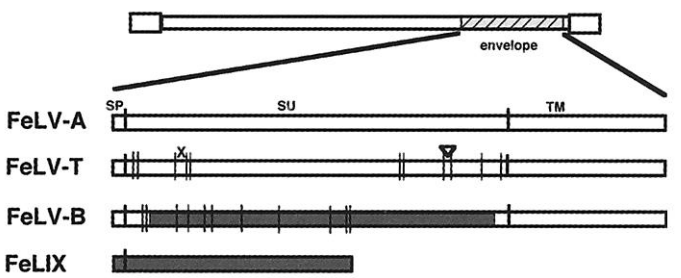

Fig. 1. Schematic representation of the structure of FeLIX in relation to the envelope proteins of infectious FeLV. The locations of the signal peptide (SP), surface unit (SU), and transmembrane (TM) domains are indicated. FeLV-A (61E) is shown as a reference and the 11 amino acids where FeLV-A and FeLV-T (EECC) differ in SU are indicated by vertical lines in FeLV-T. The positions of a 6–amino acid insertion (∇) and deletion (X) in FeLV-T relative to FeLV-A are also shown. The regions of FeLV-B (90Z) that are derived by recombination with enFeLV are shaded. The truncated structure of FeLIX is shown in relation to FeLV-B, and the 11 amino acids where they differ in SU are indicated with vertical lines in FeLV-B; there are also 10 differences in the signal peptide not shown (GenBank accession number AF226623).

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susceptible to infection by FeLV-A or -B (*3*). In these cells, FeLIX did not confer susceptibility to FeLV-T, which confirmed the requirement for a second molecule (Table 1). Because FeLV-B envelope sequences are derived from enFeLV (*7*) and this subgroup is known to use the Pit1 receptor (*8*), we speculated that Pit1 could be acting with FeLIX to facilitate infection. To address this, we examined whether MDTF cells engineered to express both Pit1 and FeLIX were susceptible to FeLV-T infection. Although FeLV-T could not infect MDTF cells expressing either Pit1 or FeLIX alone, the virus was highly infectious for cells expressing both molecules (Table 1). Susceptibility was specific to FeLV-T and was not seen with FeLV-A. Thus, FeLV-T is a simple oncoretrovirus that requires a two-component receptor complex that includes an adapter- or coreceptor-type molecule.

The protein identified as a cofactor for FeLV-T infection does not encode a full-length viral envelope protein. The envelope sequence of FeLIX is >99% identical to the open reading frame encoded by a previously described feline DNA sequence, CFE16, that was identified on the basis of its similarity to exogenous FeLVs (*14*). Because FeLIX encodes a putative signal peptide for extracellular transport but lacks a transmembrane domain, we would predict that this molecule would be shed from the cell surface (*15*). Cell-free supernatant from feline T cells (3201) and from cells engineered to express FeLIX (D17-FeLIX cells) rendered MDTF-Pit1 cells permissive to FeLV-T infection (Table 2). Control experiments with medium from D17-invFeLIX cells did not make MDTF-Pit1 cells permissive to FeLV-T infection. Thus, FeLIX can function as a secreted extracellular protein, in a Pit1-dependent manner, to permit FeLV-T infection.

To investigate whether FeLIX can also function to facilitate entry if it is attached to the cell membrane, we expressed a full-length FeLV-B envelope protein, including both the complete surface and transmembrane domains, in MDTF-Pit1 cells. Unlike cells chronically infected with replication-competent FeLV-B, these cells did not express sufficient SU protein to block homologous virus challenge. We found that MDTF-Pit1 cells expressing FeLV-B envelope were highly infectable by FeLV-T (Table 2); however, the supernatant from MDTF-Pit1–FeLV-B did not confer susceptibility to FeLV-T infection when transferred to MDTF-Pit1 cells, which indicates that the FeLV-B SU protein was not being shed into the medium from these cells (Table 2). These data indicate that the receptor component can also function when tethered to the membrane, which may be occurring either through interactions with the envelope transmembrane domain or through binding to Pit1. Together, these data suggest that FeLIX is likely to be acting at the cell surface, rather than by first binding directly to the virion as a soluble SU protein.

Consistent with previous reports, we found that FeLIX is expressed at very high levels in feline T cells relative to other feline cell types, whereas the Pit1 receptor appears to be more ubiquitously expressed (Fig. 3A) (*15, 16*). Feline fibroblast cells that are very poorly infectable by FeLV-T were found to express Pit1 RNA, but not a detectable level of FeLIX RNA (Fig. 3A). These cells could be rendered permissive to FeLV-T infection

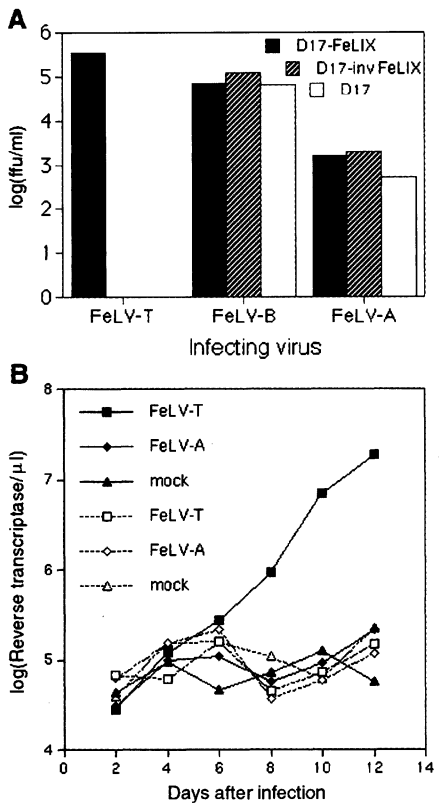

Fig. 2. Infection studies in D17 cells expressing the FeLIX cDNA (D17-FeLIX) or a cDNA clone of FeLIX in the inverse orientation (D17-invFeLIX). To generate these target cells, we transfected the FeLIX cDNA clone (in pCR3.1) into D17 cells and then selected pools of stable cell clones in the presence of G418. (**A**) Single-cycle infection studies using FeLV constructs lacking the packaging signal ($\Delta\Psi$) and carrying a retroviral vector encoding the β-galactosidase gene under the control of the MuLV long terminal repeat. Infected cells were identified as blue foci after incubation with a suitable enzyme substrate (X-gal). FeLV-B (90Z), which efficiently infects D17 cells, was used as a positive control for infection. (**B**) Viral replication kinetics after infection of D17-FeLIX and D17-invFeLIX cells with replication-competent FeLV-T (EECC) and FeLV-A (61E) viruses. Cells were infected with an equal amount of each virus, as judged by FeLV gag antigen levels. The graph presents the average total reverse transcriptase activity in duplicate culture supernatants, as described (*1*). Infections in D17-invFeLIX cells are in open symbols; infections in D17-FeLIX cells are in solid symbols.

Table 1. Infection of murine cells expressing Pit1 and FeLIX. The MDTF cells that express the human allele of Pit1 (MDTF-Pit1) have been described (*20*). Stable pools of MDTF and MDTF-Pit1 cells expressing FeLIX were generated by retroviral gene transfer of FeLIX cDNA and subsequent selection for the drug-resistance marker encoded in the viral genome. Proviral clones EECC (*18*), 61E (*18*), and 90Z (*13*) were used as representative FeLV-T, FeLV-A, and FeLV-B viruses, respectively. The data represent the number of focus-forming units per milliliter (ffu/ml) after infection with the indicated viruses packaging a genome expressing β-galactosidase; <10 ffu/ml indicates that no blue foci were observed in cells infected with up to 100 μl of cell-free viral supernatant.

Virus	Titer (ffu/ml)			
	MDTF cells	MDTF-Pit1 cells	MDTF-FeLIX cells	MDTF-Pit1-FeLIX cells
FeLV-B	<10	2.6×10^5	<10	2.5×10^5
FeLV-T	<10	<10	<10	9.2×10^5
FeLV-A	<10	<10	<10	<10

Table 2. FeLV-T infections in the presence of conditioned media. Media were conditioned on subconfluent cells for 24 hours and diluted as indicated with appropriate media (n.a., not applicable) before application to target cells at the time of infection.

Target cell	Conditioned media	Dilution	Titer (ffu/ml)*
MDTF-Pit1	D17-FeLIX	1:2	1.0×10^6
MDTF-Pit1	D17-FeLIX	1:25	3.6×10^5
MDTF-Pit1	3201	1:2	7.1×10^5
MDTF-Pit1	3201	1:25	1.2×10^5
MDTF-Pit1	D17-invFeLIX	1:2	<10
MDTF-Pit1	None	n.a.	<10
MDTF-Pit1-90Z†	None	n.a.	7.5×10^5
MDTF-Pit1	MDTF-Pit1-90Z	1:2	<10

*See Table 1 for methods. †MDTF-Pit1 cells that stably express the entire FeLV-B-90Z envelope protein under the control of the CMV promoter (in pCDNA3.1 zeo).

by supplying secreted FeLIX (Fig. 3B). Thus, FeLIX may play the more important role in restricting cell specificity of FeLV-T in the infected cat. In this regard, this molecule resembles CD4, which is expressed at highest levels in T lymphocytes and determines the tropism of human immunodeficiency virus–type 1. CD4 may also facilitate infection both as a cell surface molecule and as a soluble protein (*17*). Because FeLIX is shed from the cell and can function as a soluble protein, there may be greater breadth in the cell specificity of T cell–tropic FeLVs in the host than would be predicted by in vitro culture. Indeed, this property of FeLIX may act to augment the severe lymphoid depletion observed in cats infected with FeLV-T (*18*).

Our studies show that FeLV-T has evolved to recognize the same receptor, Pit1, that is used by FeLV-B. This finding may suggest that cells expressing Pit1 are particularly attractive targets during the persistent stages of infection but are not primary cell targets during transmission. Our data also suggest that FeLV-T can infect cells in the cat that have already been infected with FeLV-B, which may allow FeLV-T to maximize its potential cell targets during chronic stages of infection.

Endogenous retrovirus-like sequences are found in many mammals, including mice, cats, and primates (*19*). The origin and function of endogenous proviral sequences have long been a topic of speculation. The studies described here add another layer of intrigue to the mystery of these cellular viral remnants and provide the first example of an infectious, pathogenic retrovirus that has usurped a related endogenous protein as a host cell factor for viral infection. In this manner, T cell–tropic FeLV appears to use a novel strategy to increase its selective advantage in the host.

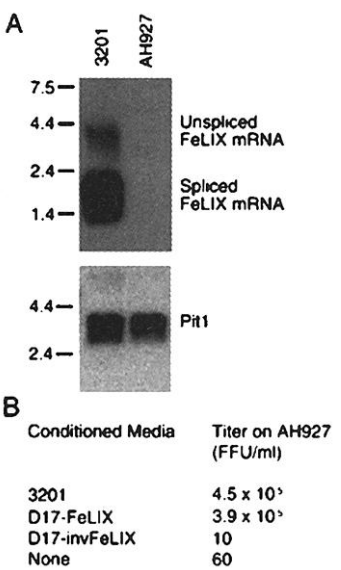

Fig. 3. Expression of FeLIX and Pit1 in feline cells in relation to infectivity. **(A)** Northern blot analyses of feline T (3201) and fibroblast (AH927) cell lines for expression of FeLIX and Pit1. Upper panel: 10 μg of total RNA was loaded in each lane; bands corresponding to the predicted unspliced and spliced FeLIX mRNA are shown. Lower panel: The first blot was stripped and probed with a fragment from feline Pit1. **(B)** Feline AH927 cells were infected in the presence of a 1:2 dilution of the indicated conditioned supernatants, as described in Table 2.

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11. The feline cDNA library was generated using the method and the vector provided by Kitamura (*9*). Viral particles containing the library were generated by cotransfecting 293T cells with the library DNA plus plasmids encoding a packaging-defective FeLV-gag-pol and amphotropic MuLV envelope. Cell-free virus was used to infect D17 cells; after 2 days, cells were challenged with FeLV-ΔΨ EECC virus. FeLV-ΔΨ EECC was engineered to be defective in packaging its own genome but competent to package and transfer a variety of retroviral vectors encoding selectable marker genes (*3*). Two viruses carrying vectors with different drug-selectable markers were applied simultaneously, one encoding neomycin phosphotransferase and the other encoding a histidinol dehydrogenase gene, and infected cells were selected in the presence of G418 and histidinol. When single-cell clones became visible, they were isolated and expanded.
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Candidate Taste Receptors in *Drosophila*

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Little is known about the molecular mechanisms of taste perception in animals, particularly the initial events of taste signaling. A large and diverse family of seven transmembrane domain proteins was identified from the *Drosophila* genome database with a computer algorithm that identifies proteins on the basis of structure. Eighteen of 19 genes examined were expressed in the *Drosophila* labellum, a gustatory organ of the proboscis. Expression was not detected in a variety of other tissues. The genes were not expressed in the labellum of a *Drosophila* mutant, *pox-neuro*^{70}, in which taste neurons are eliminated. Tissue specificity of expression of these genes, along with their structural similarity, supports the possibility that the family encodes a large and divergent family of taste receptors.

Although two putative mammalian taste receptors have recently been described (*1*), remarkably little is understood in general about taste receptors across species. A computer algorithm that seeks proteins with particular structural properties, as opposed to proteins with particular sequences, identified a large family of candidate odorant receptors from the *Drosophila* genomic database (*2*). Here, we report that further analysis of genes identified by this algorithm revealed one gene that defines a distinct large family of membrane proteins (*3*); 43 members of this family have been identified in the first 60% of the *Drosophila* genome that has been sequenced thus far (*3*). If the sequenced part of the genome is

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