A soluble envelope protein of endogenous retrovirus (FeLIX) present in serum of domestic cats mediates infection of a pathogenic variant of feline leukemia virus

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INTRODUCTION

Gammaretroviral entry into target cells is mediated by the interaction of viral envelope glycoproteins with virus-specific receptors on the cell surface. Envelope glycoproteins consist of a trimer of heterodimers of a surface subunit (SU) that interacts with a specific receptor and a transmembrane subunit, which mediates the post-binding fusion process (Davey et al., 1997; Eckert & Kim, 2001; Zhao et al., 1998). The PHQ motif that resides at the N terminus of the SU is highly conserved among gammaretroviruses (Bae et al., 1997; Lavillette & Kabat, 2004). Mutations of this motif, especially of a histidine residue (ΔH mutation), result in disruption of the membrane-fusion process, although they do not affect the incorporation of envelope glycoproteins into virions or binding of the envelope proteins to receptors (Barnett et al., 2001; Lavillette et al., 2000). Many studies have revealed that the infectivity abolished by a ΔH mutation can be restored by the addition of a homologous or heterologous soluble SU or receptor-binding domain (RBD) that contains a complete PHQ motif in trans. This transactivation generally occurs more efficiently via a homologous RBD than a heterologous RBD, with the exception that porcine endogenous retroviruses were much more efficiently transactivated by a heterologous RBD derived from Gibbon ape leukemia virus (Lavillette & Kabat, 2004; Lavillette et al., 2000; Farrell et al., 2002; Lavillette et al., 2002).

T-lymphotropic feline leukemia virus (FeLV-T), a highly pathogenic variant of FeLV, induces severe immunosuppression in cats. FeLV-T is fusion defective because in its PHQ motif, a gammaretroviral consensus motif in the N terminus of an envelope protein, histidine is replaced with aspartate. Infection by FeLV-T requires FeLIX, a truncated envelope protein encoded by an endogenous FeLV, for transactivation of infectivity and Pit1 for binding FeLIX. Although Pit1 is present in most tissues in cats, the expression of FeLIX is limited to certain cells in lymphoid organs. Therefore, the host cell range of FeLV-T was thought to be restricted to cells expressing FeLIX. However, because FeLIX is a soluble factor and is expressed constitutively in lymphoid organs, we presumed it to be present in blood and evaluated its activities in sera of various mammalian species using a pseudotype assay. We demonstrated that cat serum has FeLIX activity at a functional level, suggesting that FeLIX is present in the blood and that FeLV-T may be able to infect cells expressing Pit1 regardless of the expression of FeLIX in vivo. In addition, FeLIX activities in sera were detected only in domestic cats and not in other feline species tested. To our knowledge, this is the first report to prove that a large amount of truncated envelope protein of endogenous retrovirus is circulating in the blood to facilitate the infection of a pathogenic exogenous retrovirus.
FeLV-T is fusion defective because its PHQ motif has a substitution of histidine with aspartate. Infection by FeLV-T is transactivated by FeLIX, a truncated envelope glycoprotein released by a defective endogenous FeLV (enFeLV) (Anderson et al., 2000). FeLIX shares high amino acid identity with the SU envelope protein of FeLV subgroup B (FeLV-B) and binds to Pit1, a Na\(^+\)-dependent phosphate symporter, which functions as an FeLV-B receptor (Takeuchi et al., 1992). Although the FeLIX-mediated infection process requires Pit1, FeLV-T RBD seems not to bind to Pit1 directly (Lauring et al., 2002). Furthermore, it was reported that infection by FeLV-T deleted of its RBD could be rescued by soluble cofactors, suggesting that FeLV-T does not require its cognate receptor but can use alternative receptors recognized by the soluble cofactors (Barnett et al., 2003). Because the receptor-independent infection process overcomes the receptor-interference, FeLV-T has a selective advantage in chronically infected cats.

FeLV-T requires two different proteins, FeLIX and Pit1, to infect cells as described above. Despite the fact that Pit1 is expressed in most cells in cats, FeLV-T exhibits T-cell tropism because the expression of FeLIX is restricted to specific cells and tissues (Anderson et al., 2000; McDougall et al., 1994). However, although the expression of FeLIX has been examined in cell lines and tissues, it is not clear whether FeLIX is present in the blood of cats. In this study, we examined the activities of FeLIX in sera of various mammalian species, including feline species, to know the significance of this protein in vivo.

**RESULTS**

**FeLIX activity in 3201 cell culture supernatant**

FeLIX is a soluble cofactor that transactivates FeLV-T infection, identified in feline thymic lymphoma 3201 cells. To confirm that 3201 cells release FeLIX into the culture supernatant, we performed an immunoprecipitation assay with rabbit anti-FeLIX serum (termed 573) (Fig. 1a). Simian COS cells which do not express FeLIX were transfected with pSG5-FeLIX, expressing FeLIX, and the control plasmid pSG5, and named COS(FeLIX) and COS(SG5) cells, respectively. As shown in Fig. 1(a), the COS(FeLIX) cells expressed a 35 kDa protein, namely FeLIX, in intracellular and extracellular fractions, but FeLIX was not detected in COS(SG5) cells. Similarly, FeLIX was also detected in both cell lysates and supernatants of 3201 cells. Although the identity of the other bands detected by the anti-FeLIX antibody in COS cells was unknown at present, these bands might be related to endogenous retroviral envelope proteins containing similar epitope(s) of the FeLIX protein. After a short metabolic labelling (30 min) and chase period, cell extracts and culture supernatants were immunoprecipitated (Fig. 1b). The FeLIX protein was rapidly detected as a 35 kDa intracellular protein, which was secreted into the culture supernatant during the 6 h chase period, suggesting that FeLIX was efficiently released from the cells and accumulated in the culture supernatants with time. Therefore, we used the 3201 cell culture supernatant (termed 3201 sup) as a source of FeLIX in the subsequent experiments.

To examine FeLIX activity (transactivation of FeLV-T infection) in the 3201 sup, a LacZ pseudotype assay was performed in the presence of serially diluted 3201 sup (2\(^9\) to 2\(^{-11}\)) (Fig. 2). Feline fibroblastic AH927 cells were used as targets because they are highly susceptible to LacZ pseudotype viruses of both FeLV-A and FeLV-B, named as LacZ(FeLV-A) and LacZ(FeLV-B), respectively, but scarcely susceptible to the FeLV-T pseudotype virus [LacZ(FeLV-T)] in the absence of FeLIX (Nakaya et al., 2010).

![Fig. 1](image)

**Fig. 1.** (a) Metabolic labelling of 3201 cells, and COS cells transfected with pSG-MC1 and pSG5. Cells were labelled with \([^{35}S]\)methionine-cysteine for 3 h. Cells were disrupted and cell extracts (C) and culture supernatant (S) were immunoprecipitated with a rabbit anti-FeLIX antibody (termed 573) and preimmune normal rabbit serum (NRS). (b) Pulse–chase experiments. 3201 cells (lanes 1 and 2) or COS cells transfected with pSG-MC-1 (lanes 3–8) or pSG5 (lanes 9 and 10) were labelled for 30 min (lane 3) and then chased with excess unlabelled amino acids for 30 min (lanes 1, 4 and 9), 1 h (lane 5), 2 h (lane 6), 4 h (lane 7) and 6 h (lanes 2, 8 and 10). Cell lysates (upper) and culture supernatants (lower) were immunoprecipitated with 573 serum.
FeLIX in cat's serum mediates infection of FeLV-T

FeLIX is expressed in lymphoid organs (e.g. thymus, spleen, and lymph node) but not detected in other tissues (e.g. muscle, brain, small intestine, kidney and liver) (McDougall et al., 1994; Lauring et al., 2001). Because FeLIX is a soluble factor and expressed constitutively in these lymphoid organs, we presumed that it is present in blood in cats. To test this assumption, the LacZ pseudotype assay was performed in the presence of serially diluted cat serum (2⁰ to 2⁻²) as described above. Similar to 3201 sup, cat serum did not affect the infectivity of LacZ(FeLV-A) or LacZ(FeLV-B), although a slight reduction in titre was observed at higher concentrations of serum (Fig. 3a, b). The FeLIX activity of cat serum was also blocked by the anti-FeLIX antibody (Fig. 4b). It has been reported that pseudotyped viruses produced in human cells are inactivated by certain temperature-sensitive factor(s) in the serum of cats (Watanabe et al., 2004). Therefore, we speculated that the reduction was caused by factors in the serum. However, heat treatment (56 °C, 30 min) appeared not to be effective, although a slight restoration was observed. The kinetics of LacZ(FeLV-T) infection with the cat serum was similar to that with 3201 sup (Fig. 3c), indicating that the cat serum also had FeLIX activity and rendered AH927 cells susceptible to infection. While the dilution of 3201 sup which gave a titre of LacZ(FeLV-T) in AH927 cells of 1 × 10⁴ f.f.u. ml⁻¹ was 1 : 64, that of cat serum was 1 : 4096, showing that the FeLIX activity of the cat serum was much stronger than that of 3201 sup. As observed in LacZ(FeLV-A) and LacZ(FeLV-B), the titre of LacZ(FeLV-T) declined at higher concentrations of the serum. Interestingly, heat treatment abolished all FeLIX activity. These results indicate that the FeLIX glycoprotein was heat labile and the conformational determinants of its receptor-binding or virus-cofactor interactions were disrupted by the heat treatment.

Immunoprecipitation assay to detect FeLIX in cat plasma

We attempted to confirm the presence of FeLIX in plasma of cats by immunoprecipitation using the anti-FeLIX antibody. As a result, we clearly detected FeLIX in plasma of two cats (Fig. 5) but not in plasma of dogs (data not shown).

Absence of FeLIX activities in sera from animals other than domestic cats

Most mammals have their own endogenous retroviruses, some of which are closely related to enFeLV (Herniou et al., 1994), at the step of receptor-interaction (McDougall et al., 1994). However, our results showed that 3201 sup did not affect FeLV-B infectivity although it had FeLIX activity (Fig. 2b). Similar observations, showing that FeLIX does not block FeLV-B infection, were reported by us and another group (Anderson et al., 2000; Shojima et al., 2006).

Fig. 2. Single-round infection studies of LacZ(FeLV-A) (a), LacZ(FeLV-B) (b) and LacZ(FeLV-T) (c) in the presence of serially diluted 3201 sup. The viral titre was calculated by end point dilution. The data are the means ± standard deviations of three independent experiments.

The infection of LacZ(FeLV-T) was transactivated by addition of 3201 sup in a dose-dependent manner (Fig. 2c), indicating that 3201 sup rendered target cells permissive to FeLV-T. This transactivation property of 3201 sup is ascribed to the FeLIX protein because treatment with the anti-FeLIX antibody specifically blocked LacZ(FeLV-T) infection (Fig. 4a). These findings showed that 3201 sup contains FeLIX protein at a functional level in terms of transactivating FeLV-T infection. FeLV-A is ecotropic and infects mainly feline cells using feline thiamine transport protein 1 (Mendoza et al., 2006). LacZ(FeLV-A) gave constant titres regardless of the concentrations of 3201 sup (Fig. 2a), showing that FeLV-A infectivity was not affected by FeLIX as previously reported (Anderson et al., 2000). On the other hand, it is thought that FeLIX functions as a natural resistance factor against infection by FeLV-B because FeLIX is highly homologous to FeLV-B RBD and interferes with FeLV-B...
If the retroviruses of various mammals besides domestic cats code for a transactivation cofactor similar to FeLIX, the sera of these mammals may confer FeLV-T infectivity. endFeLVs are found in the genome in the genus Felis including domestic cats but not in other species of felids despite their close relation to domestic cats in terms of the evolutionary process. Therefore, it is believed that endFeLVs entered the germ line prior to the evolutionary divergence of the domestic cat lineage species (Benveniste et al., 1975; Roca et al., 2004). When we tested for the presence of the FeLIX activity in various mammalian species using 10-fold-diluted sera, FeLIX activities were detected only in domestic cats (n=3) and not in other feline species tested (Table 1). Because the three domestic cats that exhibited FeLIX activities in their sera were not infected with any exogenous FeLVs, as confirmed using a commercial FeLV detection kit, the effects of exogenous FeLVs could be excluded. Furthermore, other mammalian sera tested had no transactivation activity either, suggesting that the various endogenous retroviruses of these mammals did not affect FeLV-T infectivity.

DISCUSSION
In this study, we demonstrated that FeLIX is present in cat serum at a level high enough for it to be functional. Therefore, it is assumed that cats are tolerant of the enFeLV envelope protein, which might partly explain the observation that most cats chronically infected with FeLV do not produce sufficient amounts of neutralizing antibodies against exogenous FeLVs (Hardy, 1993). Furthermore, although the host range of FeLV-T was thought to be restricted to cells expressing FeLIX, our results suggest that FeLV-T can infect cells expressing Pit1 regardless of the expression of FeLIX, and the host cell range should be broad in vivo. However, experimental infections showed that proviral genomes of FeLV-T were detected only in the lymphoid tissues and the host range was generally...
Fig. 5. Immunoprecipitation assay with anti-FeLIX antibody for detection of FeLIX in cat plasma. COS cells transfected with and without pSG-FeLIX were used as positive and negative controls, respectively (lanes 1 and 2). The cell lysates (lanes 3 and 5), supernatants (lanes 4 and 6) and cat plasma (lanes 7 and 8) were immunoprecipitated with anti-FeLIX antibody covalently conjugated with protein G. Proteins were subjected to immunoblot analysis using the anti-FeLIX antibody.

concordant with the tissues expressing FeLIX (Anderson et al., 2000). These results indicated that FeLV-T infection correlated with FeLIX expression despite the presence of FeLIX inside the cats. Some uncharacterized factor(s) other than FeLIX may play a role in the determination of the tissue tropism of FeLV-T.

FeLV infections have only rarely been reported in wild felids, but certain wild felids such as wild cougar, Namibian cheetah, Iberian lynx and Florida panther infected with FeLV have been reported (Jessup et al., 1993; Marker et al., 2003; Meli et al., 2009; O’Brien et al., 2012). The extensive screening of various mammalian sera revealed FeLIX activity only in the sera of domestic cats. Thus, wild felids may be the least at risk of infections from FeLV-T.

Several types of enFeLVs (enFeLV-AGTT, -GGAG, -CTCT, -AGAG and -ATGC) were identified based on their full-length sequences and none were genetically fixed in cats (Roca et al., 2004, 2005). Taking these findings into consideration, there might be cats which do not have enFeLV encoding FeLIX [enFeLV(FeLIX)] in their genome. Therefore, we studied the presence of FeLIX activity in 88 cats from a diverse geographical cohort (5 and 54 from Hokkaido and Kagoshima prefectures in Japan, respectively, and 29 from Vietnam). All of these cats exhibited FeLIX activities, indicating that enFeLV(FeLIX) was common to domestic cats and entered the germ line earlier than other enFeLVs isolated recently, probably before domestication and subdivision by breeding. Although sera of other Felis species (sand cat, jungle cat, black-footed cat, and Chinese desert cat) were not available in this study, the evaluation of FeLIX activities in these animals will help to determine exactly when enFeLV(FeLIX) entered the germ line.

### METHODS

**Cell lines.** Feline fibroblastic AH927 cells (McDougall et al., 1994), African green monkey kidney COS cells and human medulloblastoma TELCeB6 cells, which express murine leukemia virus (MLV) core particles incorporating an MFGnlsLacZ vector (Cosset et al., 1995), were grown in Dulbecco’s modified Eagle’s medium (DMEM) (high glucose) supplemented with 10% heat-inactivated FCS, 100 μg streptomycin ml⁻¹ and 100 IU penicillin ml⁻¹. Feline T-lymphoma 3201 cells were maintained in RPMI 1640 growth medium supplemented with 10% FCS, 2 mM L-glutamine, 10 μM 2-mercaptoethanol, 100 μg streptomycin ml⁻¹ and 100 IU penicillin ml⁻¹.

**Sera.** Sera were collected from various mammalian species: domestic cat (Felis silvestris catus), lion (Panthera leo), Amur tiger (Panthera tigris altaica), Sumatran tiger (Panthera tigris sumatrae), leopard (Panthera pardus), snow leopard (Uncia uncia), clouded leopard (Neofelis nebulosa), snow leopard (Uncia uncia), clouded leopard (Neofelis nebulosa), leopard cat (Prionailurus bengalensis), dog (Canis familiaris), horse (Equus caballus), cow (Bos taurus), goat (Capra hircus) and pig (Sus domesticus). Blood collections from lion, Amur tiger, Sumatran tiger, leopard, snow leopard and clouded leopard in zoos were done when these animals were medically checked periodically. All studies were approved by the Kyoto University Institutional Animal Care and Use Committee.

**Expression plasmid of FeLIX.** To construct the plasmid expressing the FeLIX gene, an EcoRI–BamHI fragment of pEN-MC-1 (McDougall et al., 1994) containing the coding region of the gene was cloned into the EcoRI and BamHI sites of the pSG5 expression plasmid (Strategene) and designated pSG-FeLIX. In this plasmid, the expression of the FeLIX gene was driven by the simian virus 40 early gene promoter.
Radioimmunoprecipitation assay. COS cells were grown to 90% confluency in a six-well plate and transfected with 9 µg plasmid DNA by the calcium phosphate coprecipitation method. Four hours later, the cells were washed with FCS-free medium and glycerol shocked for 2 min before receiving fresh medium. Following a further overnight incubation, the transfected cells were removed with trypsin and reseded in DMEM growth medium. Cells were incubated in methionine-free medium for 1 h at 37 °C, after which 37 kBq of [35S]methionine-cysteine label (Translabel; NEN) was added and cells were incubated for 30 min or 3 h. The labelled cells were pelleted, rinsed in PBS, and disrupted in lysis buffer (10 mM Tris/HC1, pH 8.0, 0.14 M NaCl, 1% Triton X-100, 1% deoxycholate, 1 mM PMSE, and 0.1 U aprotinin ml−1). After the removal of debris by centrifugation, lysates or culture supernatants were incubated with the complex of protein G-Sepharose beads and rabbit anti-FeLIX serum (termed 573) (McDougall et al., 1994) overnight at 4 °C. After successive washes with 0.5 mM LiCl and lysis buffer (three times), the adsorbed proteins were eluted by incubation in sample buffer at 100 °C for 3 min. Proteins were resolved by SDS-10% PAGE and the gels were fixed in 5% methanol/7.5% acetic acid for 15 min, treated with autoradiography enhancer solution for 15 min, dried under vacuum and exposed to Kodak X-OMAT films.

Preparation of pseudotype viruses and FeLIX. TELCeB6 cells were transfected with pFBFeLV-A(61E), pFBFeLV-B(GA) and pFBFeLV-T(ECC) (Nakata et al., 2003) using FuGENE6 (Roche). After selection of the transfectants with 50 µg ml−1 virus supernatants were harvested from the pooled plasmid-resistant cell population and filtered through a 450 nm Millipore filter. The 3201 sup was harvested at the point when the density of cells was 1 × 106 ml−1 and filtered through a 450 nm Millipore filter.

JacZ pseudotype assay. Titration of the lacZ pseudotype virus was performed as described by Takeuchi et al. (1994). Briefly, AH927 cells (a feline embryonic fibroblast cell line) were seeded in 48-well plates 1 day before infection. The lacZ pseudotype virus was inoculated into the cells with cat serum or 3201 sup. After 4 h of infection in the presence of 8 µg polybrene ml−1 for viral adsorption, the virus was removed and cells were cultured in the growth medium. After infection, the cells were stained with 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside in situ, and lacZ-positive foci were counted. The assays were performed in the presence or absence of serially diluted 3201 sup or mammalian sera.

Inactivation of FeLIX activity. 3201 sup and serum samples were incubated with the anti-FeLIX antibody or preimmune normal rabbit serum (NRS) at 37 °C for 1 h.

Immunoprecipitation assay. Whole-cell lysates were prepared by resuspending the cells in radioimmunoprecipitation assay lysis buffer. COS cells transfected with and without FeLIX expression plasmid (pSG-FeLIX) were used as positive and negative controls, respectively. The cell lysates, supernatants and cat plasma were immunoprecipitated with the rabbit anti-FeLIX antibody (McDougall et al., 1994) covalently conjugated with protein G. Proteins were subjected to SDS-PAGE on a 10% gel and then transferred electrophoretically onto a PVDF membrane, and the blots were probed with the anti-FeLIX antibody. The secondary antibody was an anti-rabbit IgG antibody conjugated with horseradish peroxidase, followed by visualization using a Super Signal West Femto system (Thermo Fisher Scientific), and images were obtained and processed by using a luminescent image analyser (LAS4000 Mini; Fujifilm).

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