Virus Research 204 (2015) 88–94

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

# Overexpression of feline tripartite motif-containing 25 interferes with the late stage of feline leukemia virus replication

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ARTICLE INFO

*Article history:*
Received 4 November 2014
Received in revised form 27 March 2015
Accepted 19 April 2015
Available online 24 April 2015

*Keywords:*
Cat
Tripartite motif-containing 25 (TRIM25)
Feline leukemia virus
Antiviral activity

ABSTRACT

Tripartite motif-containing 25 (TRIM25) regulates various cellular processes through E3 ubiquitin ligase activity. Previous studies have revealed that the expression of TRIM25 is induced by type I interferon and that TRIM25 is involved in the host cellular innate immune response against retroviral infection. Although retroviral infection is prevalent in domestic cats, the roles of feline TRIM25 in the immune response against these viral infections are poorly understood. Because feline TRIM25 is expected to modulate the infection of feline leukemia virus (FeLV), we investigated its effects on early- and late-stage FeLV replication. This study revealed that ectopic expression of feline TRIM25 in HEK293T cells reduced viral protein levels leading to the inhibition of FeLV release. Our findings show that feline TRIM25 has a potent antiviral activity and implicate an antiviral mechanism whereby feline TRIM25 interferes with late-stage FeLV replication.



## 1. Introduction

Retroviruses prevalent in mammals are integrated into the genome of host cells and disrupt the immune system. Functionally different host-encoded restriction factors, such as APOBEC3, tetherin, and tripartite motif (TRIM) proteins, directed against retroviruses, have been characterized (Chiu and Greene, 2008; Neil et al., 2008; Ozato et al., 2008).

The TRIM protein superfamily includes more than 60 members identified from the mammalian species (Ozato et al., 2008). The N-terminal structure of TRIM proteins comprises an RBCC motif composed of a RING domain, one or two B-boxes, and predicted coiled-coil regions (Meroni and Diez-Roux, 2005; Nisole et al., 2005). The RBCC motif is usually followed by a highly variable C-terminal structure, which is used to classify the 11 TRIM subgroups (Ozato et al., 2008). Members of the TRIM protein family, which are known as ubiquitin ligase proteins, have various distinct cellular functions, including cell proliferation, differentiation, development, and oncogenesis (Ozato et al., 2008). Several TRIM members are induced by interferon (IFN) signaling (Carthagena et al., 2009; Rajsbaum et al., 2008).

Recent studies revealed that several TRIM members regulate retroviral proliferation at multiple steps of the viral replication cycle (Ozato et al., 2008). TRIM5α prevents the uncoating of the human immunodeficiency virus (HIV) genome through the PRY-SPRY domain (Sebastian and Luban, 2005; Wu et al., 2006; Schaller et al., 2007); TRIM25 inhibits the entry of the murine leukemia virus (MLV) (Uchil et al., 2008); TRIM32 and TRIM62 inhibit the expression of MLV genes (Uchil et al., 2008); TRIM25 and TRIM62 restrict the particle release of HIV and MLV (Uchil et al., 2008). According to previous research, 16 TRIM proteins activate NF-κB and/or AP-1 signaling, which contribute to the antiviral response (Uchil et al., 2013). The antiviral effects of the TRIM proteins against HIV-1 are not stronger than those against MLV because NF-κB binding sites that are found in the long terminal repeat (LTR) U3 region of the HIV genome confer resistance to inflammatory TRIM proteins (Uchil et al., 2013).

The retinoic acid-inducible gene I (RIG-I) is a cellular sensor of RNA viral infection that regulates the interferon-beta (IFN-β) response (Gack et al., 2007). It was reported that RIG-I undergoes TRIM25-mediated K63-linked ubiquitination, which promotes IFN-β production (Gack et al., 2007). The RING domain of TRIM25 is essential for this response, while TRIM25 carring a RING-domain mutation suppresses late-stage MLV infection (Uchil et al., 2008). This finding suggests that the suppression of MLV is RIG-I pathway-independent. In a recent study, a RIG-I-independent antiviral response to MLV was reported; however, the involvement of TRIM25 was not shown (Lee et al., 2013).

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http://dx.doi.org/10.1016/j.virusres.2015.04.017
0168-1702/

Retroviral infections are also common in cats. Feline leukemia virus (FeLV), which belongs to the genus *Gammaretrovirus* of the family *Retroviridae*, induces lymphoma and bone marrow suppression syndromes and leads to secondary infections because of the virus suppressing the bone marrow and immune system (Hartmann, 2006). FeLV infection as well as feline immunodeficiency virus (FIV) is common in cats and induces immune suppression. It was reported that mammalian TRIM5α, which contains the PRY-SPRY capsid-binding domain, suppresses the proliferation of retroviruses (Saenz et al., 2005; Schaller et al., 2007). However, feline TRIM5 lacks the PRY-SPRY domain and displays no restriction activity against FIV (McEwan et al., 2009). Thus, structural differences among mammalian orthologous TRIM genes are suggested to determine antiviral activity against retroviruses. With the exception of TRIM5, the effects of feline TRIM proteins against retroviral replication have not yet been investigated (McEwan et al., 2009). Several feline TRIM proteins may interfere with the replication of FeLV, similar to those in primates. Our previous research showed that feline TRIM25 comprises an RBCC motif and a PRY-SPRY domain but lacks the approximately 30 amino acids between the coiled-coil and PRY-SPRY domains compared with the human and mouse TRIM25 proteins (Koba et al., 2013). The expression of feline TRIM25 is also up-regulated by type I IFN (Koba et al., 2013). In the present study, the effect of feline TRIM25 on early- and late-stage FeLV replication was examined to investigate whether feline TRIM25 affected retroviral production.

## 2. Materials and methods

### 2.1. Plasmids

The infectious FeLV-B/Gardner-Arnstein clone was kindly provided by Dr. Takayuki Miyazawa at Kyoto University. cDNA was synthesized from total RNA extracted from CRFK cells and was used to amplify the CDS region of feline TRIM25 (AB734707). Primers used to amplify the TRIM25 gene are as follows: forward primer for the wild-type (WT) feline TRIM25, 5′-GAA TTC GCG GAG CTG GTC CCC CTG GCC GAG GAG CTG TCG T-3′; forward primer for the RING-domain-deleted TRIM25 (ΔR), 5′-GAA TTC CGC GCC AGC TAT CAG GAG CGG CCG CAG CTC CAC A-3′; reverse primer for both the WT and ΔR TRIM25, 5′-GAA TTC CTA CTT GCA GGA GCA GAT GGA GAG CGT GGC ACC C-3′. The amplified WT- and ΔR-TRIM25 sequences were cloned by inserting the PCR products into the pCR2.0-TOPO vector (Life Technologies, Foster, CA, USA). Subsequently, an *Eco*RI fragment containing TRIM25 was ligated into a *Bam*HI site of a pCMV-Tag2 protein expression vector (Agilent Technology, Santa Clara, CA, USA). The Schematic structures of WT- and ΔR-TRIM25 are shown in Fig. 1A. FeLV-LTR firefly luciferase reporter vector was constructed by inserting a PCR-amplified LTR sequence into a *Hind*III site of the pGL3-Basic vector (Promega, Madison, WI, USA). NF-κB and AP-1 luciferase reporter vectors were purchased from Promega.

### 2.2. Cell culture

CRFK cells and HEK293T cells were maintained in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin at 37 °C in a humidified 5% $CO_2$ atmosphere.

### 2.3. Analysis for subcellular localization of feline TRIM25

CRFK cells were seeded at $3 \times 10^5$ cells in 2 ml of growth medium on a 35-mm plastic dish on the day before transfection. The cells were transfected with 1 μg of plasmid-expressing FLAG-tagged TRIM25 using Lipofectamine LTX (Life Technologies). At 24 h post-transfection, the cells were fixed with 4% paraformaldehyde in PBS for 10 min and were permeabilized on ice for 30 min with 0.1% Triton X-100. The cells were blocked for 1 h at room temperature with 10% goat serum in 0.1% Tween 20 in PBS and were then reacted with an anti-FLAG M2 antibody (Sigma, St. Louis, MO, USA) in a blocking buffer at 4 °C overnight. The cells were then stained with Alexa Fluor 488 anti-mouse secondary antibody and TOPRO3 (Life Technologies) in the blocking buffer for 2 h. TOPRO3 was used as a nuclear stain. The cells were examined with an LSM510 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany) using 400× magnification.

### 2.4. Effects of feline TRIM25 against intracellular viral RNA and proteins

HEK293 T cells were seeded at $3 \times 10^5$ cells per well in a 12-well plate and were transfected with 0, 20, 50, and 100 ng of plasmid expressing TRIM25 and 100 ng of plasmid carrying FeLV-B. The total DNA per transfection was adjusted to 200 ng by adding an empty pCMV-Tag2 vector. Total RNA was extracted using the ISOGEN RNA extraction reagent (Nippon Gene, Tokyo, Japan). cDNA was synthesized after DNase I treatment using the PrimeScript first-strand cDNA Synthesis Kit (Takara Bio, Otsu, Japan) according to the manufacturer's instructions. DNase I treatment was performed using RQ1 RNase-free DNase (Promega). To quantitate the FeLV transcript levels, quantitative real-time PCR (qRT-PCR) was performed using the 7500 Fast Real-Time PCR System (Life Technologies). Primers designed to amplify a 119- bp fragment containing the FeLV *gag* region are as follows: forward primer FeLV-GA-F, 5′-ACA GGC GAA GAA AGG CAA AGG G-3′ and reverse primer FeLV-GA-R, 5′-TTG GGA CGG GTC AAG GGG AAA G-3′. The initial qRT-PCR reaction was performed at 95 °C for 20 s to activate the polymerase in the Fast SYBR Green Master Mix (Life Technologies), followed by 40 cycles of denaturation at 95 °C for 3 s, and annealing and extension at 60 °C for 30 s. A melting curve was generated to verify the specificity of each reaction. All reactions were performed in triplicate to ensure the accuracy of quantitation. These data were analyzed by the absolute quantification method using 7500 Software v2.0.5 (Life Technologies). To quantitate the FeLV transcript levels, serial dilutions of plasmids containing the FeLV *gag* region were run to prepare a standard curve (Fig. 2A), and the human *GAPDH* gene was amplified to normalize FeLV products. Human *GAPDH* primer sequences were obtained from a previous study (Yao and Duh, 2004) and are as follows: forward primer f114, 5′-GAG TCA ACG GAT TTG GTC GT-3′ and reverse primer r260, 5′-GAC AAG CTT CCC GTT CTC AG-3′. To generate a standard curve for cycle thresholds versus copy numbers, a vector containing a targeting fragment was constructed by inserting the PCR fragment into the pCR2.0-TOPO vector using a TOPO-TA cloning Kit (Life Technologies) according to the manufacturer's instructions. At 48 h post-transfection, the amounts of intracellular viral proteins were determined by Western blotting. The cells were lysed in CytoBuster protein reagent (Novagen, WI, USA) containing a protease inhibitor cocktail (Roche, Basel, Switzerland). Cellular and viral proteins were detected using anti-FLAG M2 antibody (Sigma), anti-β-actin antibody (Sigma), and mouse anti-FeLV p27 IgG antibody PF12J-10A (Abcam, Cambridge, UK).

### 2.5. Effects of feline TRIM25 against viral release

HEK293T cells seeded in a 12-well plate were co-transfected with 100 ng of plasmid carrying FeLV-B and from 0 to 100 ng of plasmid-expressing TRIM25 as described above. The total DNA per transfection was adjusted by adding an empty pCMV-Tag2 vector. Twenty-four hours after the transfection, the medium was replaced