AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Virology

Feline Immunodeficiency Virus Vif N-Terminal Residues Selectively Counteract Feline APOBEC3s

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ABSTRACT

Feline immunodeficiency virus (FIV) Vif protein counteracts feline APOBEC3s (FcaA3s) restriction factors by inducing their proteasomal degradation. The functional domains in FIV Vif for interaction with FcaA3s are poorly understood. Here, we have identified several motifs in FIV Vif that are important for selective degradation of different FcaA3s. Cats (*Felis catus*) express three types of A3s: single-domain A3Z2, single-domain A3Z3, and double-domain A3Z2Z3. We proposed that FIV Vif would selectively interact with the Z2 and the Z3 A3s. Indeed, we identified two N-terminal Vif motifs (12LF13 and 18GG19) that specifically interacted with the FcaA3Z2 protein but not with A3Z3. In contrast, the exclusive degradation of FcaA3Z3 was regulated by a region of three residues (M24, L25, and I27). Only a FIV Vif carrying a combination of mutations from both interaction sites lost the capacity to degrade and counteract FcaA3Z2Z3. However, alterations in the specific A3s interaction sites did not affect the cellular localization of the FIV Vif protein and binding to feline A3s. Pulldown experiments demonstrated that the A3 binding region localized to FIV Vif residues 50 to 80, outside the specific A3 interaction domain. Finally, we found that the Vif sites specific to individual A3s are conserved in several FIV lineages of domestic cat and nondomestic cats, while being absent in the FIV Vif of pumas. Our data support a complex model of multiple Vif-A3 interactions in which the specific region for selective A3 counteraction is discrete from a general A3 binding domain.

IMPORTANCE

Both human immunodeficiency virus (HIV) and feline immunodeficiency virus (FIV) Vif proteins counteract their host's APOBEC3 restriction factors. However, these two Vif proteins have limited sequence homology. The molecular interaction between FIV Vif and feline APOBEC3s are not well understood. Here, we identified N-terminal FIV Vif sites that regulate the selective interaction of Vif with either feline APOBEC3Z2 or APOBEC3Z3. These specific Vif sites are conserved in several FIV lineages of domestic cat and nondomestic cats, while being absent in FIV Vif from puma. Our findings provide important insights for future experiments describing the FIV Vif interaction with feline APOBEC3s and also indicate that the conserved feline APOBEC3s interaction sites of FIV Vif allow FIV transmissions in *Felidae*.

The apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC3, A3) family of DNA cytidine deaminases are found in placental mammals with different clade-specific gene copies and arrangements, which plays a vital role in innate immune defenses against retroviruses (see recent reviews [1, 2]). Primates have seven genes (A3A to A3D, A3F to A3H), whereas cats encode four genes (A3Z2a to A3Z2c, A3Z3) (3–5). A3 proteins contain either one or two zinc (Z)-binding domains with the conserved motif of $HxE(x)_{23-28}CxxC$ (where "x" can be any residue) (4). These proteins can target retroviruses and inhibit viral replication by deamination of cytidines in viral single-strand DNA that forms during reverse transcription, introducing G-to-A hypermutations in the coding strand (6–10). In addition, some A3s inhibit virus replication by decreasing reverse transcription and integration via deaminase-independent mechanisms (11–16). To counteract the restriction from A3s, some retroviruses evolved A3-counteracting proteins, such as Vif from lentiviruses, which prevents A3s incorporation into nascent viral particles. Vifs directly bind A3s often in a species-specific manner and recruit them to an E3 ubiquitin ligase complex containing Cullin5 (Cul5), elongin B/C (EloB/C), and RING-box protein RBX2 to induce polyubiquitination and degradation of the A3s by the proteasome (17, 18). Other retroviral proteins that counteract A3s are Bet of foamy viruses, the nucleocapsid of human T cell leukemia virus type 1 and the glycosylated (glyco)-Gag of murine leukemia virus (19–24).

Feline immunodeficiency virus (FIV) is a lentivirus distantly related to human immunodeficiency virus (HIV), which can be isolated from several *Felidae* (25). In most naturally infected domestic cats (*Felis catus*, Fca), FIV causes a severe immune deficiency only in a subpopulation of animals; however, highly pathogenic feline AIDS-inducing FIV isolates have been described (26–29). Thus, FIV infections in cats are a relevant animal model for studying lentiviral AIDS induction and immune control leading to nonprogression (30–33). It is known that the pandemic of HIV originated from cross-species transmission events of SIVs to hu-

Received 11 August 2016 **Accepted** 10 September 2016

Accepted manuscript posted online 14 September 2016

Citation Gu Q, Zhang Z, Cano Ortiz L, Franco AC, Häussinger D, Münk C. 2016. Feline immunodeficiency virus Vif N-terminal residues selectively counteract feline APOBEC3s. J Virol 90:10545–10557. doi:10.1128/JVI.01593-16.

Editor: S. R. Ross, University of Illinois at Chicago

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mans (34). As described in relation to interspecies infections of primate lentiviruses, cross-species transmission of FIV between several *Felidae* were observed (35). For example, pumas are described as being occasionally infected by FIVs of domestic cats and bobcats, and the lion FIV can be transmitted to tigers and leopards (36–40). However, phylogenetic evidence indicates that these FIV transmissions are exceedingly rare events between wildlife cat species, and restriction factors of the host may act as a barrier preventing the spread of FIV (35, 41, 42).

Similar to human A3-mediated restriction of HIV-1Δ*vif*, feline A3s are shown to inhibit FIVΔ*vif* (3, 43–47). Moreover, natural polymorphisms of feline APOBEC3s correlate with FIV and FeLV infection in domestic cats (48). The domestic cat expresses three single-domain A3Z2s (A3Z2a to A3Z2c) and one single-domain A3Z3 protein, as well as double-domain A3Z2Z3 proteins, by readthrough transcription and mRNA alternative splicing (3, 43). Previous studies demonstrated that feline A3Z3 and A3Z2Z3, but not A3Z2s, inhibit FIVΔ*vif* (3, 43), whereas feline A3Z2s strongly restrict the feline foamy virus Δ*bet* (FFVΔ*bet*), and feline A3Z3 and A3Z2Z3 only slightly decrease FFVΔ*bet* infectivity (3, 22). In addition to feline retroviruses, feline A3s also show antiviral activity against HIV-1 (43, 46, 47, 49).

FIV Vif, similar to HIV-1 Vif, forms an E3 ubiquitin ligase complex, to induce feline A3 degradation (50). However, HIV-1 and SIV Vifs need the cofactor CBF-β to stabilize and form this complex (51, 52), whereas FIV and other nonprimate lentiviruses (e.g., maedi-visna virus [MVV], caprine arthritis encephalitis virus, and bovine immunodeficiency virus [BIV]) Vifs do not require CBF-β to induce A3 degradation (53–56). A recent study demonstrated that BIV Vif appears to operate independently of any cofactors, whereas MVV Vif hijacks cellular cyclophilin A as a cofactor in reconstituting the E3 ligase complex (53). Whether FIV Vif recruits any additional protein is unclear. The HIV-1 Vif cannot counteract the strong anti-HIV activity of feline A3Z2Z3; however, binding of HIV-1 Vif and feline A3Z2Z3 was detectable by coimmunoprecipitation assays (44, 47). In contrast to HIV-1 Vif, Vifs from the HIV-2/SIV lineage counteract and induce degradation of feline A3Z2Z3 (44, 47). Residues in feline A3s that are functionally involved in the interaction with FIV Vif were identified by recent studies (44, 48, 57). In contrast, the determinants in FIV Vif that are important for inhibition of the antiviral activity of feline A3s are poorly understood (58).

In the present study, we identified N-terminal Vif sites that regulate the selective interaction of FIV Vif with either feline A3Z2 or A3Z3. These specific Vif sites are conserved in several FIV lineages of domestic and nondomestic cats but absent in FIV Vif from pumas.

MATERIALS AND METHODS

Cells and transfections. HEK293T (293T, ATCC CRL-3216) and HOS (ATCC CRL-1543) cells were maintained in Dulbecco high-glucose modified Eagle medium (Biochrom, Berlin, Germany) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). A3 degradation experiments were performed in 24-well plates; 10^5 293T cells transfected with 50 ng of feline A3Z2 or 250 ng of feline A3Z3 or A3Z2Z3 expression plasmids, together with 30 ng of codon-optimized FIV Vif expression plasmid, pcDNA3.1(+) (Thermo Fisher Scientific, Schwerte, Germany), were used as a control. To produce FIV-luciferase viruses, 293T cells were cotransfected with 0.6 μg of FIV packaging construct, 0.6 μg of FIV-luciferase vector, 0.6 μg of A3 expression plasmid, 0.2 μg of vesicular stomatitis virus G (VSV-G) expression plasmid, and 80 ng of FIV Vif expression plasmid. In some experiments, pcDNA3.1(+) was used instead of Vif or A3 expression plasmids. At 48 h posttransfection, the cells and supernatants were collected. All of the transfections were performed using Lipofectamine LTX (Thermo Fisher Scientific) according to the manufacturer's instructions.

Vif and A3 plasmids. Domestic cat A3s with a carboxy-terminal hemagglutinin (HA) tag were described previously (3, 43, 49). The codon-optimized Vif gene of FIV-34TF10 and Vif-TLQ-AAA were inserted into pcWPRE containing a C-terminal V5 tag (3, 44). All of the FIV Vif mutants were produced by fusion PCR. FIV Vif-GST (glutathione *S*-transferase) constructs were generated by inserting the full-length FIV Vif or C-terminal truncated FIV Vif into pkGST (59) using HindIII and BamHI. The primers for all FIV Vif constructs are shown in Table 1.

Viruses and infection. To produce FIV single-cycle luciferase viruses (FIV-Luc), 293T cells were cotransfected with the replication-deficient packaging construct pFP93 (60), a gift from Eric M. Poeschla, which only expresses *gag*, *pol*, and *rev*; the FIV luciferase vector pLinSin (3); a VSV-G expression plasmid pMD.G; FcaA3s expression plasmids; FIV Vif expression plasmid; or empty vector pcDNA3.1(+). The reverse transcriptase (RT) activity of FIV was quantified by using a Cavidi HS lenti RT kit (Cavidi Tech, Uppsala, Sweden). For reporter virus infection, 293T cells were seeded in 96-well plate 1 day before transduction. After normalizing for RT activity, the same amounts of viruses based on RT values were used for infection. At 2 days postransduction, the firefly luciferase activity was measured with a Steadylite HTS reporter gene assay system (Perkin-Elmer, Cologne, Germany) according to the manufacturer's instructions on a MicroLumat Plus luminometer (Berthold Detection Systems, Pforzheim, Germany). Each transduction was done in triplicates; the error bar for each triplicate is shown.

Immunoblot analysis. Transfected 293T cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], protease inhibitor cocktail set III [Calbiochem, Darmstadt, Germany]). The expression of A3s and Vif were detected by mouse anti-hemagglutinin (anti-HA) antibody (1:7,500 dilution, MMS-101P; Covance, Münster, Germany) and mouse anti-V5 antibody (1:4,500 dilution, MCA1360; ABDserotec, Düsseldorf, Germany) separately. Tubulin was detected by using mouse anti-α-tubulin antibody (1:4,000, dilution, clone B5-1-2; Sigma-Aldrich, Taufkirchen, Germany), followed by horseradish peroxidase-conjugated rabbit anti-mouse antibody (α-mouse-IgG-HRP; GE Healthcare, Munich, Germany), and developed with enhanced chemiluminescence (ECL) reagents (GE Healthcare). To test the encapsidation of FcaA3 proteins into FIV particles, HEK293T cells were transfected with 600 ng of pFP93, 600 ng of pLinSin, 200 ng of pMD.G, 600 ng of A3 constructs, and 80 ng of FIV Vif or empty vector pcDNA3.1(+). Viral supernatants were collected 48 h later, overlaid on 20% sucrose, and centrifuged for 4 h at 14,800 rpm in a tabletop centrifuge. The viral pellet was resuspended in RIPA buffer, boiled at 95°C for 5 min with Roti load reducing loading buffer (Carl Roth, Karlsruhe, Germany), and resolved on an SDS-PAGE gel. The A3s and tubulin proteins were detected as described above. VSV-G and FIV p24 proteins were detected using mouse anti-VSV-G antibody (1:10,000 dilution, clone P5D4; Sigma-Aldrich) and mouse anti-FIV p24 antibody (1:2,000 dilution, clone PAK3-2C1; NIH AIDS Repository) separately, followed by horseradish peroxidase-conjugated rabbit anti-mouse antibody (α-mouse-IgG-HRP; GE Healthcare), and developed with ECL reagents (GE Healthcare).

Immunofluorescence. HOS cells grown on polystyrene coverslips (Thermo Fisher Scientific) were transfected with expression plasmids for wild-type FIV Vif or mutants using Lipofectamine LTX (Thermo Fisher Scientific). At day 2 posttransfection, the cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min, permeabilized in 0.1% Triton X-100 in PBS for 15 min, and incubated in blocking buffer (10% FBS in PBS) for 1 h, and then the cells were stained by mouse anti-V5 antibody in a 1:1,000 dilution in blocking solution for 1 h. Donkey anti-mouse Alexa Fluor 488 (Thermo Fisher Scientific) was used as a

TABLE 1 PCR primers used in this study

Primer	Sequence (5′–3′)
FIVVif-EcoRI-F	ATGAATTCGCCACCATGAGCGAAGAGGACTG
FIVVIF-V5NotI-R	ATGCGGCCGCTCAGGTGCTGTCCAGGCC
FIVVifEcoRI24F	ATGAATTCGCCACCATGCTGTACATCAGCCGG
FIVVifEcoRI49F	ATGAATTCGCCACCATGGAGACCGGCTTCATC
FIVVif-EcoRI73F	ATGAATTCGCCACCATGATCGGCTACGTGCGG
FIVVifEcoRI103F	ATGAATTCGCCACCATGCAGTACAGACCCGGC
FIVVifEcoRI144F	ATGAATTCGCCACCATGCCAGGCTGGGGCCCTG
FIVVifEEAAEcorI-F	ATGAATTCGCCACCATGAGCGCAGCGGACTGGCAG
FIVVifQVAAEcorI-F	ATGAATTCGCCACCATGAGCGAAGAGGACTGGGCGGCGTCCAG
FIVVifSRAAEcorI-F	ATGAATTCGCCACCATGAGCGAAGAGGACTGGCAGGTGGCCGCGCGGC
FIVVifLFAAEcorI-F	ATGAATTCGCCACCATGAGCGAAGAGGACTGGCAGGTGTCCAGGCGGGCGGCCGCCGTGC
FIVVifGGAA-F	GTGCTGCAGGCCGCCGTGAACAGCGCC
FIVVifGGAA-R	GGCGCTGTTCACGGCGGCCTGCAGCAC
FIVVifYIAA-F	GCGCCATGCTGGCCGCCAGCCGGCTGCCC
FIVVifYIAA-R	GGGCAGCCGGCTGGCGGCCAGCATGGCGC
FIVVifERAA-F	CCCCCGACGCGGCGGAGAAGTACAAGAAGG
FIVVifERAA-R	CCTTCTTGTACTTCTCCGCCGCGTCGGGGG
FIVVifKDAA-F	GAAGTACAAGGCGGCCTTCAAGAAGAGGCTG
FIVVifKDAA-R	CAGCCTCTTCTTGAAGGCCGCCTTGTACTTC
FIVVifKKAA-F	CAAGAAGGACTTCGCGGCGAGGCTGTTCGAC
FIVVifKKAA-R	GTCGAACAGCCTCGCCGCGAAGTCCTTCTTG
FIVVifRLAA-F	GACTTCAAGAAGGCGGCGTTCGACACCGAG
FIVVifRLAA-R	CTCGGTGTCGAACGCCGCCTTCTTGAAGTC
FIVVif53FIAA-F	CCGAGACCGGCGCCGCCAAGCGGCTGCGG
FIVVif53FIAA-R	CCGCAGCCGCTTGGCGGCGCCGGTCTCGG
FIVVif57LRAA-F	CTTCATCAAGCGGGCGGCGAAGGCCGAGGG
FIVVif57LRAA-R	CCCTCGGCCTTCGCCGCCCGCTTGATGAAG
FIVVif61EGIAAA-F	GGCTGCGGAAGGCCGCGGCCGCCAAGTGGAGCTTCCACAC
FIVVif61EGIAAA-R	GTGTGGAAGCTCCACTTGGCGGCCGCGGCCTTCCGCAGCC
FIVVif65WSFAAA-F	GCCGAGGGCATCAAGGCGGCCGCCCACACCCGGGACTAC
FIVVif65WSFAAA-R	GTAGTCCCGGGTGTGGGCGGCCGCCTTGATGCCCTCGGC
FIVVif81VAGAAA-F	GCGGGAGATGGCGGCCGCCAGCACCACC
FIVVif81VAGAAA-R	GGTGGTGCTGGCGGCCGCCATCTCCCGC
FIVVif95YIAA-F	GCGGATGTACATCGCCGCCAGCAACCCCCTGTGG
FIVVif95YIAA-R	CCACAGGGGGTTGCTGGCGGCGATGTACATCCGC
FIVVif119VNAA-F	GAATGGCCCTTCGCGGCCATGTGGATCAAG
FIVVif119VNAA-R	CTTGATCCACATGGCCGCGAAGGGCCATTC
FIVVif126GFMAAA-F	GTGGATCAAGACCGCCGCTGCGTGGGACGACATCGAG
FIVVif126GFMAAA-R	CTCGATGTCGTCCCACGCAGCGGCGGTCTTGATCCAC
FIVVif184CCSS-F	CCAAGAAGTGGTCCGGCGACTCCTGGAACC
FIVVif184CCSS-R	GGTTCCAGGAGTCGCCGGACCACTTCTTGG
Vif25L-A-F	GAACAGCGCCATGGCGTACATCAGCC
Vif25L-A-R	GGCTGATGTACGCCATGGCGCTGTTC
Vif28SR-AA-F	CCATGCTGTACATCGCCGCGCTGCCCCCCG
Vif28SR-AA-R	CGGGGGGCAGCGCGGCGATGTACAGCATGG
Vif30L-A-F	GTACATCAGCCGGGCGCCCCCCGACG
Vif30L-A-R	CGTCGGGGGGCGCCCGGCTGATGTAC
Vif47F-A-F	CAAGAAGAGGCTGGCCGACACCGAGAC
Vif47F-A-R	GTCTCGGTGTCGGCCAGCCTCTTCTTG
Vif24M-A-F	GAACAGCGCCGCGCTGTACATCAGCC
Vif24M-A-R	GGCTGATGTACAGCGCGGCGCTGTTC
Vif26Y-A-F	GAACAGCGCCATGCTGGCCATCAGCCGGC
Vif26Y-A-R	GCCGGCTGATGGCCAGCATGGCGCTGTTC
Vif27I-A-F	GCGCCATGCTGTACGCCAGCCGGCTG
Vif27I-A-R	CAGCCGGCTGGCGTACAGCATGGCGC
FVif25L-S-F	GAACAGCGCCATGTCGTACATCAGCC
FVif25L-S-R	GGCTGATGTACGACATGGCGCTGTTC
FVif25L-G-F	GAACAGCGCCATGGGGTACATCAGCC
FVif25L-G-R	GGCTGATGTACCCCATGGCGCTGTTC
FVif25L-V-F	GAACAGCGCCATGGTGTACATCAGCC
FVif25L-V-R	GGCTGATGTACACCATGGCGCTGTTC
FVif25L-I-F	GAACAGCGCCATGATTTACATCAGCC

(Continued on following page)

TABLE 1 (Continued)

Primer	Sequence (5′–3′)
FVif25L-I-R	GGCTGATGTAAATCATGGCGCTGTTC
FVif25L-F-F	GAACAGCGCCATGTTTTACATCAGCC
FVif25L-F-R	GGCTGATGTAAAACATGGCGCTGTTC
FVif25L-Y-F	GAACAGCGCCATGTATTACATCAGCC
FVif25L-Y-R	GGCTGATGTAATACATGGCGCTGTTC
FVifHindIII-F	ATAAGCTTGCCACCATGAGCGAAGAGGACTGG
FVifFullBamHI-R	ATGGATCCCAGCTCGCCGCTCCACAG
FVif160BamHI-R	ATGGATCCGCTGAAGGCCTTGATGGC
FVif110BamHI-R	ATGGATCCCTTCAGGCCGGGTCTGTAC
FVif80BamHI-R	ATGGATCCCATCTCCCGCACGTAGCC
FVif50BamHI-R	ATGGATCCCTCGGTGTCGAACAGCCTC
FIV_vif_PF	CTTCCTGAAGGGGATGAGTG
FIV_vif_PR	ATCTCTTCCATTCATAGYTCTCC
Env_PR	CCTARTTCTTGCATAGCRAAAGC
A3H2F	TCATCCCCAATGGCACCCACAGC
A3H3R	TCAAACTCTGAGACGGAGGAGGAG

secondary antibody in a 1:300 dilution in blocking solution for 1 h. Finally, DAPI (4′,6′-diamidino-2-phenylindole) was used to stain nuclei for 2 min. The images were captured by using a ×60 objective lens on a Zeiss LSM 510 Meta laser scanning confocal microscope (Carl Zeiss, Cologne, Germany). The images were analyzed by using ZEN 2.1 (blue edition) software (Carl Zeiss).

GST pulldown. To determine Vif and A3 binding, 293T cells were cotransfected with 1 μg of FcaA3 and 1 μg of FIV Vif constructs that expressed a C-terminal GST tag or pkGST empty vector. After 48 h, the cells were lysed in immunoprecipitation-lysis buffer (50 mM Tris-HCl [pH 8], 1 mM phenylmethylsulfonyl fluoride, 10% glycerol, 0.8% NP-40, 150 mM NaCl, and protease inhibitor cocktail set III [Calbiochem]). The lysates were cleared by centrifugation. The supernatants were incubated with 50 μl of preequilibrated glutathione Sepharose beads. After 2 h of incubation at 4°C in end-over-end rotation, the samples were washed four times with lysis buffer on ice. The bound proteins were eluted by boiling the beads for 5 min at 95°C in SDS loading buffer. FcaA3 was detected by immunoblotting with anti-HA antibody. The GST or Vif-GST proteins were observed by Coomassie brilliant blue staining.

Vif sequences from naturally infected cats. Fifteen samples of peripheral blood from domestic cats naturally infected with FIV were subjected to DNA extraction. DNA was extracted using buffer saturated phenol and subjected to three PCRs to detect proviral and genomic DNA. In

TABLE 2 Haplotypes of feline A3Z3 in FIV-infected cats

Vif	A3Z3 haplotype	A3Z3 allele(s)
Vif_FIV_RS09	I	Homozygous
Vif_FIV_RS11	I	Homozygous
Vif_FIV_RS13	I	Homozygous
Vif_FIV_RS02	II	Heterozygous: codon 65 (S or A)
Vif_FIV_RS04	II	Heterozygous: codon 65 (S or A)
Vif_FIV_RS08	II	Heterozygous: codon 65 (S or A)
Vif_FIV_RS14	II	Homozygous: codon 65 (S)
Vif_FIV_RS06	III	Heterozygous: codon 65 (S or A), codon 68 (Q or R), codon 96 (I or V)
Vif_FIV_RS12	III	Homozygous: codon 65 (S); heterozygous: codon 68 (R or Q), codon 94 (T or A)
Vif_FIV_RS03	IV	Heterozygous: codon 65 (S or A), codon 96 (I or V)
Vif_FIV_RS05	IV	Heterozygous: codon 65 (S or A), codon 96 (I or V)
Vif_FIV_RS07	IV	Heterozygous: codon 65 (S or A), codon 96 (I or V)
Vif_FIV_RS10	IV	Heterozygous: codon 65 (S or A), codon 96 (I or V)
Vif_FIV_RS01	V	Heterozygous: codon 65 (I or V)
Vif_FIV_RS15	NC[a]	

[a] NC, not characterized.

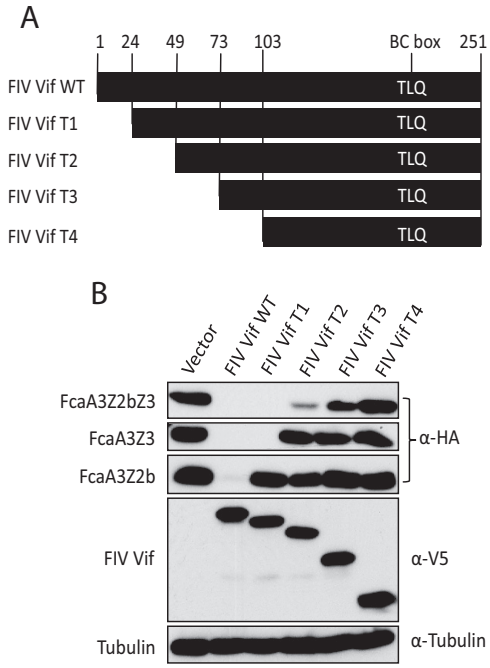

FIG 1 The N-terminal region of FIV Vif determines specific A3 degradation. (A) Schematic structure of FIV Vif (clone-34TF10) N-terminal deletion constructs (T1, T2, T3, and T4). The C-terminal amino acids TLQ that interact with elongin B and elongin C (BC box) of the E3 complex are shown. The numbers represent the amino acids position in FIV Vif. (B) FIV Vif wild type, FIV Vif T1, FIV Vif T2, FIV Vif T3, or FIV Vif T4 were coexpressed with FcaA3Z2bZ3, FcaA3Z3, and FcaA3Z2b. A3s, FIV Vifs, and tubulin proteins were detected by using anti-HA, anti-V5, and anti-tubulin antibodies, respectively.

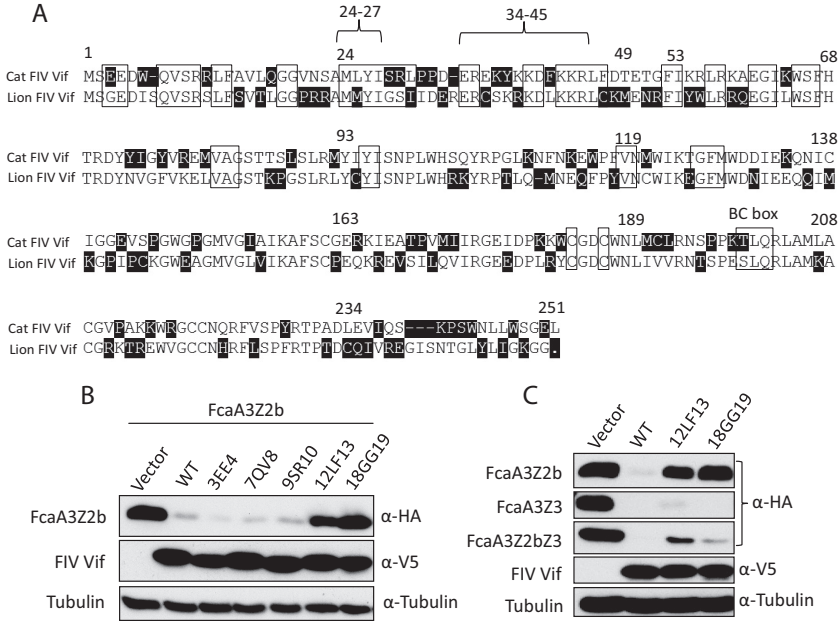

FIG 2 Identification of determinants in FIV Vif important for the degradation of feline A3Z2b. (A) Sequence alignment of domestic cat FIV (clone 34TF10) and lion FIV (subtype B) Vif. The numbers represent amino acids position in domestic cat FIV Vif; the boxes are the relative conserved regions between domestic FIV Vif and lion FIV Vif. The distinct amino acids between two Vif proteins were shown in black. Two extra regions (24-27 and 34-45) are also indicated. (B) FcaA3Z2b was coexpressed with FIV Vif wild type or Vif alanine mutants of the indicated residues. A3, FIV Vif, and tubulin proteins were detected using anti-HA, anti-V5, and anti-tubulin antibodies, respectively. (C) FcaA3Z2b, FcaA3Z3, and FcaA3Z2bZ3 were coexpressed with FIV Vif wild type and alanine Vif mutants of residues 12LF13 or 18GG19. A3, FIV Vif, and tubulin proteins were detected by using anti-HA, anti-V5, and anti-tubulin antibodies, respectively.

order to amplify the *vif* genes, a seminested PCR was developed. In the first round of amplification, the primers FIV_vif_PF and Env_PR (Table 1) were used to obtain a 3-kb amplicon using the Phusion high-fidelity DNA polymerase (New England BioLabs, Ipswich, MA). In the second round of amplification, the primers FIV_vif_PF and FIV_vif_PR (Table 1) were used, and the PCR product was obtained with *Taq* DNA polymerase (Thermo Fisher Scientific). The product was cloned into pCR2.1 vector using a TOPO TA cloning kit (Thermo Fisher Scientific) and submitted to sequencing. The A3Z3 haplotype of each sample was determined according to a previously described protocol (48) using the primers A3H2F and A3H3R and named (see the recent report [57]). Sequences were analyzed with the Geneious software (Biomatters, Auckland, New Zealand). More information about the A3Z3 haplotype and the corresponding FIV Vif sequence is presented in Table 2.

Statistical analysis. Data are represented as means with the standard deviations in all bar diagrams. Statistically significant differences between two groups were analyzed using an unpaired Student *t* test with GraphPad Prism version 5 (GraphPad software, San Diego, CA). A difference was considered statistically significant when the *P* value was <0.05.

Accession number(s). The GenBank accession numbers for FIV Vif are FIV C36 (AY600517.1), FIV 34TF10 (M25381.1), FIV PRR (M36968.1), FIV TM-2 (M59418.1), FIV Shizuoka (LC079040.1), FIV Oma (AY713445), FIV Lion B (EU117991), FIV Lion E (EU117992), FIV puma A (U03982), FIV bobcat A (KF906143), FIV puma B.1 (DQ192583), FIV puma B.2 (KF906194), Vif_FIV_RS09 (KX668638), Vif_FIV_RS11 (KX668640), Vif_FIV_RS13 (KX668642), Vif_FIV_RS02 (KX668631), Vif_FIV_RS04 (KX668633), Vif_FIV_RS08 (KX668637), Vif_FIV_RS14 (KX668643), Vif_FIV_RS06 (KX668635), Vif_FIV_RS12 (KX668641), Vif_FIV_RS03 (KX668632), Vif_FIV_RS05 (KX668634), Vif_FIV_RS07 (KX668636), Vif_FIV_RS10 (KX668639), Vif_FIV_RS01 (KX668630), and Vif_FIV_RS15 (KX668644).

RESULTS

Identification of FIV Vif determinants specific for feline A3Z2 degradation. Previous studies have shown that feline A3 cytidine deaminases can act as restriction factors for FIV, which are counteracted by the FIV Vif protein (3, 43, 45, 47). However, the molecular interaction between FIV Vif and feline A3s is poorly understood. In order to identify determinants in the FIV Vif protein that are specific to the degradation of different feline A3 proteins, we used a FIV (clone 34TF10) from domestic cats (*Felis catus*, Fca), here referred to as FIV. First, we generated several FIV Vif constructs that had N-terminal deletions. We deleted amino acids 1 to 24, 1 to 49, 1 to 73, or 1 to 103 of FIV Vif, respectively, termed FIV Vif T1, FIV Vif T2, FIV Vif T3, and FIV Vif T4 (Fig. 1A). Cotransfection experiments of cat-derived A3s and FIV Vif expression plasmids were performed in 293T cells. All A3 constructs expressed the corresponding A3 protein with a C-terminal HA tag, whereas Vif was expressed as a C-terminal V5 tag fusion protein. Immunoblots of protein extracts from cells coexpressing both A3 and Vif were used as a readout for the degradation of the respective A3 proteins. The results showed that wild-type FIV Vif induced degradation of single-domain feline A3Z2b and A3Z3 and double-domain A3Z2bZ3 in agreement with previous reports (43, 44). FIV Vif T1 induced degradation of single-domain feline A3Z3 and double-domain A3Z2bZ3 but could not mediate the degradation of feline A3Z2b, which suggested the possibility that amino acids 1 to 24 of FIV Vif are specific for interaction with feline A3Z2b (Fig. 1B). FIV Vif T2 failed to deplete feline A3Z3 and A3Z2b but moderately induced the degradation of feline A3Z2bZ3 (Fig. 1B). All three feline A3 proteins showed resistance to FIV Vif T3 and T4 (Fig. 1B). Taken together, these results implied that amino acids 1 to 24 of FIV Vif are specific to feline A3Z2b degradation, whereas the feline A3Z3 interaction site may localize to residues 24 to 49 of FIV Vif.

Our previous study demonstrated that FIV Vif from lions (sub-

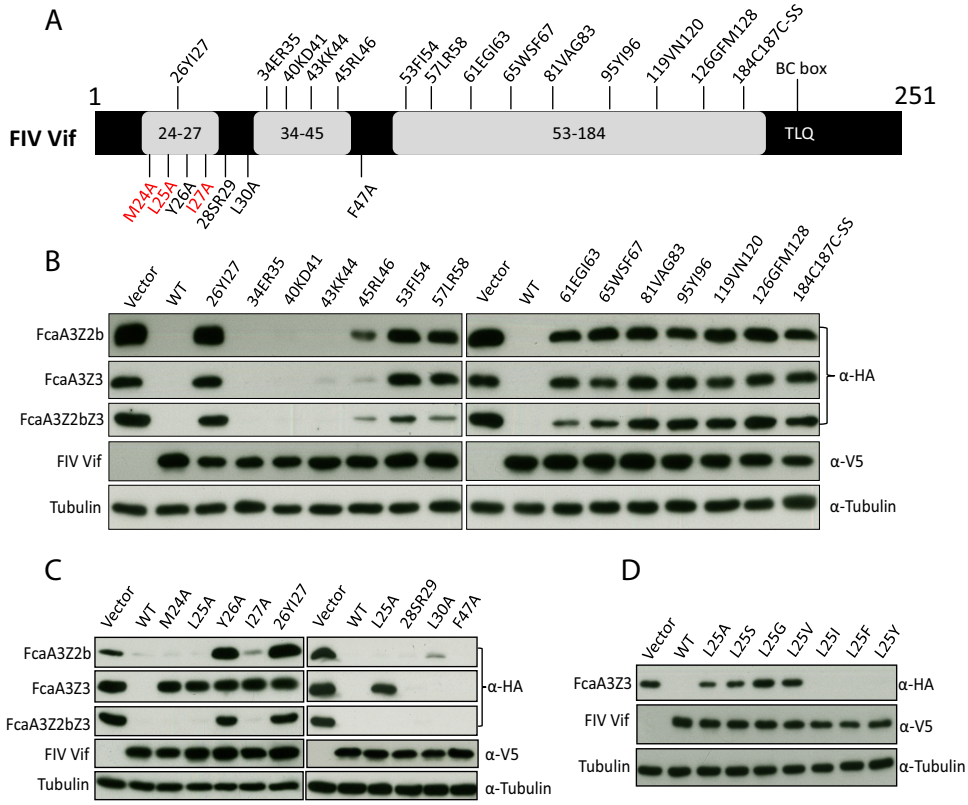

FIG 3 Identification of determinants in FIV Vif that confer degradation of feline A3Z3. (A) FIV Vif schematic structure, locations of tested mutations indicated. The numbers indicate the positions of amino acids; all these amino acids were mutated to alanines, and additionally some residues were mutated to other amino acids. The residues that determine FIV Vif degradation activity against FcaA3Z3 are shown in red. (B and C) FcaA3Z2b, FcaA3Z3, or FcaA3Z2bZ3 were coexpressed with FIV Vif wild type and Vif mutants. A3, FIV Vif, and tubulin proteins were detected by using anti-HA, anti-V5, and anti-tubulin antibodies, respectively. (D) Leucine 25 of FIV Vif was replaced by several amino acids: alanine (A), serine (S), glycine (G), valine (V), isoleucine (I), phenylalanine (F), and tyrosine (Y). FcaA3Z3 was coexpressed with FIV Vif wild type and Vif mutants. A3, FIV Vif, and tubulin proteins were detected by using anti-HA, anti-V5, and anti-tubulin antibodies, respectively.

type B) also counteracted feline A3s from the domestic cat (44). Thus, we analyzed sequences of domestic cat FIV Vif and lion FIV Vif and identified conserved amino acids that localized at residues 1 to 24 of domestic cat FIV Vif as potential feline A3Z2b interaction sites (Fig. 2A). Next, these conserved residues were mutated in cat FIV Vif to alanines and tested for their degradation activity of feline A3Z2b. De Filippis et al. showed that mutations of a big residue into alanine or glycine rarely lead to major rearrangements in the direct three-dimensional environment (61). The results showed that alanine mutations in residues 3EE4, 7QV8, and 9SR10 did not alter the FIV Vif activity to degrade feline A3Z2b, whereas replacing 12LF13 and 18GG19 by alanines abolished FIV Vif-mediated A3Z2b degradation (Fig. 2B). In addition, we found that mutation in 12LF13 and 18GG19 motifs had no influence on FIV Vif-induced degradation of feline A3Z3 and A3Z2bZ3 (Fig. 2C). These results demonstrated that residues of 12LF13 and 18GG19 of FIV Vif selectively determine the degradation of feline A3Z2b.

Identification of feline A3Z3 interaction sites of FIV Vif. Figure 1 suggested that the amino acids from 24 to 49 of FIV Vif interacted with feline A3Z3. To identify the specific feline A3Z3 interaction residues in this region, we analyzed the sequence of cat FIV Vif and lion FIV Vif. We found that residues 24 to 27 and residues 34 to 45 were quite conserved, whereas amino acids 27 to 34 of Vif were more variable (Fig. 2A). Then, we replaced the conserved residues (26YI27, 34ER35, 40KD41, 43KK44, and 45RL46) in cat FIV Vif by alanines (Fig. 3A). In addition, we chose nine conserved motifs in the region between residues 53 and 184 for alanine mutations (53FI54, 57LR58, 61EGI63, 65WSF67, 81VAG83, 95YI96, 119VN120, 126GFM128, and 184C187C) (Fig. 3A). All FIV Vif mutants were coexpressed with either one of the three feline A3 proteins (A3Z2b, A3Z3, and A3Z2bZ3), and immunoblots were used to evaluate the expression of A3 proteins and FIV Vif mutants. The result showed that all FIV Vif mutants displayed similar expression levels (Fig. 3B). The alanine mutant of 26YI27 showed no degradation activity of any feline A3 protein, whereas FIV Vif mutants of 34ER35, 40KD41, and 43KK44 diminished feline A3Z2b, A3Z3, and A3Z2bZ3 levels as efficient as did wild-type FIV Vif (Fig. 3B). Alanine mutations introduced in position 45RL46 of FIV Vif had a minor influence on feline A3 degradation (Fig. 3B). Mutants of residues 53FI54, 57LR58, 61EGI63, and 65WSF67 slightly induced degradation of feline A3Z2b, not much of A3Z3, and triggered quite efficient degradation of feline A3Z2bZ3 (Fig. 3B). All three feline A3 proteins were mostly resistant to alanine mutants of Vif residues 81VAG83, 95YI96, 119VN120, 126GFM128 and 184C187C (Fig. 3B). We next focused on the motif of residues 26YI27 in FIV Vif. Single mutations around this motif in FIV Vif were generated (M24A, L25A, Y26A,

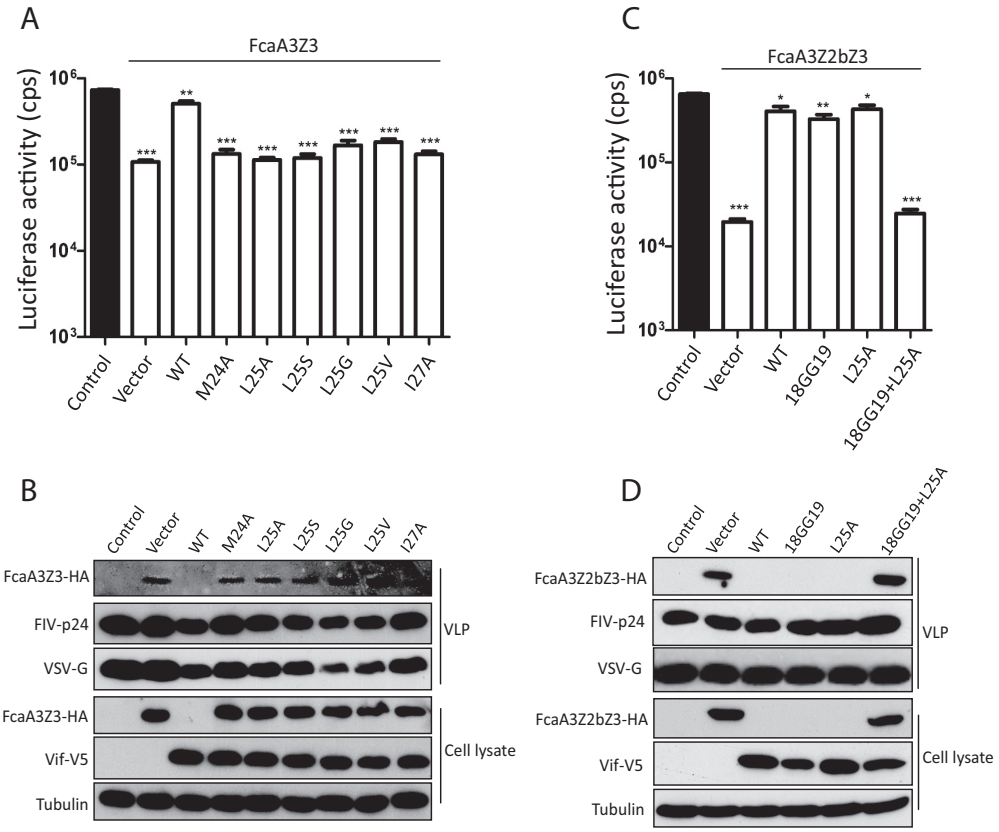

FIG 4 FIV Vif mutants cannot counteract the antiviral activity of feline A3s. (A and C) Single-round FIVΔ*vif* luciferase reporter virions were produced in the presence of feline A3 expression plasmids (FcaA3Z3 or FcaA3Z2bZ3) with FIV Vif wild type or Vif mutants; pcDNA3.1(+) was added as a control (vector). The infectivity of reporter vectors was determined by quantification of the luciferase activity in 293T cells transduced with vector particles. (B and D) Cell lysates of the FIV producer cells examined in panels A and C were used to detect the expression of feline A3s and FIV Vif by anti-HA and anti-V5 antibodies, respectively. Cell lysates were also analyzed for equal amounts of total proteins using anti-tubulin antibody. Feline A3s encapsidated into FIV viruslike particles (VLPs) were detected by anti-HA antibody. VSV-G and FIV p24 proteins in VLPs were also detected by anti-VSV-G and anti-FIV p24 antibodies separately. Asterisks represent statistically significant differences (***, $P < 0.001$; **, $0.001 < P < 0.01$; *, $0.01 < P < 0.05$ [Dunnett's *t* test]).

I27A, and L30A), and, in addition, residues 28SR29 and F47 were replaced by alanines (Fig. 3A and C). The immunoblotting results of the A3 degradation test revealed that mutations M24A, L25A, and I27A of FIV Vif specifically blocked the capacity to induce the degradation of feline A3Z3 but had no influence on the degradation of feline A3Z2b and A3Z2bZ3 (Fig. 3C). Interestingly, the mutation Y26A impaired FIV Vif degradation for all three feline A3 proteins. In contrast, mutations in residues 28SR29, L30, and F47 of FIV Vif had no effect on feline A3 degradation (Fig. 3C).

Next, we constructed several derivatives of FIV Vif in which residue 25 was replaced by different amino acids (Fig. 3D). However, L25A, L25S, L25G, and L25V mutants could not degrade feline A3Z3, whereas L25I and L25F mutants, as well as the L25Y mutant, degraded feline A3Z3 as efficiently as did wild-type FIV Vif (Fig. 3D). Compared to the amino acids alanine, serine, glycine, and valine, the residues isoleucine, phenylalanine, and tyrosine have a more complex side chain. These results suggest that the specific spatial distance of the FIV Vif-A3Z3 interaction area determines the FIV Vif-induced degradation of feline A3Z3.

FIV Vif mutants fail to counteract the antiviral activity of feline A3s. Previous studies reported that FIV Vif inhibited feline A3s by E3-complex-induced degradation and thus prevented A3 incorporation into FIV particles (43, 50). Thus, we analyzed the anti-FIV activity of feline A3Z3 in the presence of wild-type or mutant FIV Vifs by using a single-round FIV-luciferase reporter virus. As previously reported (44), feline A3Z3 inhibited FIVΔ*vif* 5- to 7-fold, which could be counteracted by wild-type FIV Vif (Fig. 4A). However, the presence of defined FIV Vif mutants (M24A, L25A, L25S, L25G, L25V, and I27A) destroyed this FIV Vif activity (Fig. 4A). The immunoblots of virus-producing cells indicated that FIV wild-type Vif decreased the protein level of feline A3Z3 and prevented feline A3Z3 incorporation into FIV viral particles (Fig. 4B). However, the tested FIV Vif mutants had no effect on the protein level of feline A3Z3 in cells and failed to generate A3-free virions (Fig. 4B).

FIV Vif counteracts feline A3s by interacting with both single Z2 and Z3 domains (43, 44). Hence, we generated a FIV Vif mutant in which the A3Z2 interaction sites 18GG19 and the A3Z3 interaction site L25 were replaced by alanines, termed FIV Vif.18GG19+L25A. In testing this Vif mutant, we found that it did not neutralize the antiviral activity of feline A3Z2bZ3. The Vif mutants of residues 18GG19 and L25 rescued most of the infectivity of FIVΔ*vif* compared to infections without Vif (vector) (Fig. 4C). The corresponding immunoblots from virus-producing cells and viral particles demonstrated that FIV Vif.18GG19+L25A did

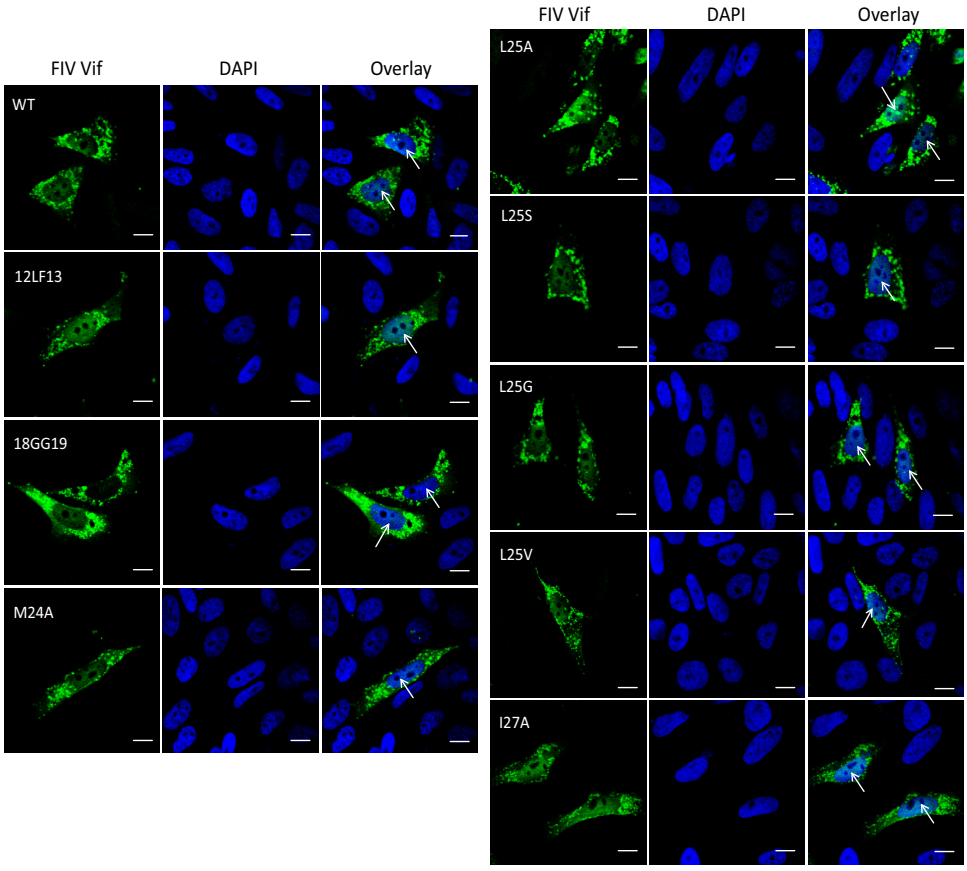

FIG 5 Cellular localization of FIV Vif and Vif mutants. HOS cells were transfected with FIV Vif wild type and Vif mutants. All Vif mutants were generated by replacing the indicated residues by alanines. The numbers represent the amino acid positions. To detect FIV Vif and Vif mutant (green), immunofluorescence staining was performed with an anti-V5 antibody. Nuclei (blue) were visualized by DAPI staining. The white bar indicates 10 μm. The white arrows indicate Vif protein in the nucleus.

not influence feline A3Z2bZ3 protein levels and its viral incorporation (Fig. 4D).

FIV Vif mutants failing to degrade A3s bind to A3. The results presented above demonstrate that FIV Vif residues 12LF13, 18GG19, M24, L25, and I27 determine the specific degradation of either feline A3Z2 or A3Z3. To determine whether mutations in these Vif sites alter the cellular localization of Vif, wild-type and mutant FIV Vifs were expressed in HOS cells. The cellular localization was determined by confocal microscopy. The results showed that wild-type FIV Vif was mainly localized to the cytoplasm. We also observed some small levels of wild-type FIV Vif localizing to the nucleus (Fig. 5), which was consistent with a previous study (62). The cellular localizations of the FIV Vif mutants were found to be identical to wild-type FIV Vif (Fig. 5). These results suggest that mutations in 12LF13, 18GG19, M24, L25, and I27 of FIV Vif impair the degradation of selective feline A3s but do not alter the subcellular localization of FIV Vif.

To characterize the determinants of binding of FIV Vif to feline A3, GST-pulldown assays were performed. We tested FIV Vif constructs with a GST tag, which had a C-terminal deletion, expressing the first 160 (Vif.1-160), 110 (Vif.1-110), 80 (Vif.1-80), or 50 (Vif.1-50) amino acids of Vif (Fig. 6A). For the full length of the FIV Vif, we inactivated the BC box (TLQ-AAA) to prevent A3 degradation activity. These five Vif constructs were cotransfected with a feline A3Z2bZ3 expression plasmid into 293T cells. After 48 h, the cells were harvested, and Vif was pulled down by using glutathione-Sepharose beads. The binding between FIV Vif and feline A3Z2bZ3 was evaluated by detecting the A3Z2bZ3 protein in the pulldown complex. We found that GST alone could not pull down feline A3Z2bZ3, whereas it was detected in the pulldown complex of Vif.TLQ-AAA, Vif.1-160, Vif.1-110, and Vif.1-80 (Fig. 6B). Vif.1-50 displayed very weak binding to feline A3Z2bZ3 compared to the GST control (Fig. 6B). These results suggest that residues 50 to 80 of FIV Vif confer binding to feline A3Z2bZ3. However, the specific A3Z2 and A3Z3 degradation determinants locate at residues 1 to 50 of FIV Vif (Fig. 2 and 3). To test whether these determinants were involved in the binding of A3Z2 and A3Z3, we generated several derivatives of Vif.1-110 in which residues 12LF13, 18GG19, M24, L25, or I27 were replaced by alanines separately (Fig. 6A). The binding to A3Z2 or A3Z3 of these mutants was detected by GST-pulldown assays. We found that Vif.1-110, Vif.1-110-12LF13, and Vif.1-110-18GG19 had an identical protein level in the pulldown complex and immunoprecipitated similar amounts of feline A3Z2b protein (Fig. 6C). Vif.1-110 could also bind to feline A3Z3, and introducing M24A and L25A into Vif.1-110 did not alter its binding affinity to feline A3Z3 (Fig. 6D). Compared to Vif.1-110, we detected fewer Vif.1-110-I27A and feline A3Z3 proteins in immunoprecipitated complexes

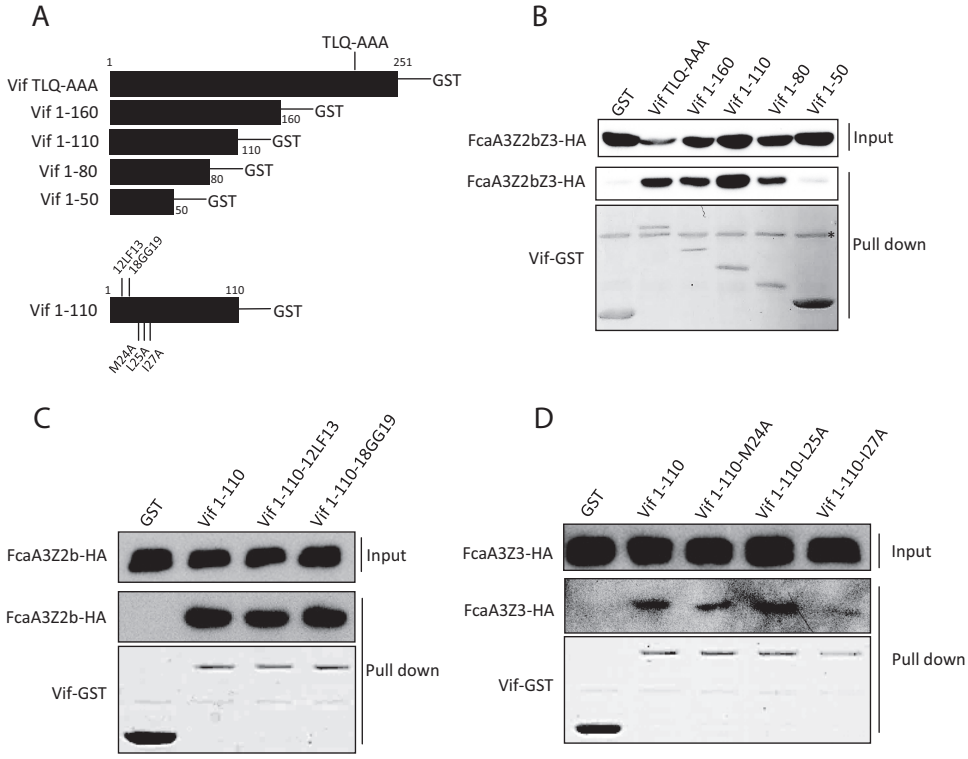

FIG 6 Binding of FIV Vif to feline A3s. (A) Schematic structure of C-terminal truncations of FIV Vifs. TLQ-AAA represents the inactive BC box of FIV Vif. FIV Vif TLQ-AAA, FIV Vif.1-50, FIV Vif.1-80, FIV Vif.1-110, and FIV Vif.1-160 constructs were fused with a C-terminal GST tag. (B, C, and D) 293T cells were cotransfected with an expression plasmid encoding GST or different FIV Vif constructs fused with a C-terminal GST, as indicated. At 48 h, the transfected cells were lysed, followed by incubation with glutathione-Sepharose beads. The feline A3s of input and bound fractions were detected by immunoblots using anti-HA antibody. Pulldown fractions were also used for Coomassie blue staining to show the GST or Vif-GST. Asterisks (*) indicate unspecific bands.

(Fig. 6D). Taken together, these results suggest that the specific A3Z2 and A3Z3 degradation determinants in FIV Vif (12LF13 and 18GG19 for A3Z2; M24, L25, and I27 for A3Z3) do not determine to any great extent the Vif-A3 binding, which, however, is regulated by the region from residues 50 to 80 of the FIV Vif.

The specific A3Z2 and A3Z3 interaction sites are conserved in FIV Vif variants except for puma FIV_{Pco} Vif. After the identification of the selective A3Z2 and A3Z3 interaction sites of domestic cat FIV Vif (clone FIV-34TF10), 15 Vif sequences that belong to naturally FIV-infected cats and their respective A3Z3 haplotypes were analyzed. We identified three animals with haplotype I, four with haplotype II, two with haplotype III, four with haplotype IV, and one animal displayed haplotype V (48). A3Z3 haplotype of one sample was not determined. All 15 Vifs isolated showed that the A3Z2 and A3Z3 interaction sites are highly conserved (Fig. 7A). It was described that FIVs from several felid species showed genetic divergence, which suggests virus-host adaptations and rare cross-species transmissions in the wild (35, 63). Thus, we analyzed the Vif sequences of additional domestic cat FIV strains and Vifs from several nondomestic cat FIVs. We found that the feline A3Z2 and A3Z3 interaction sites are conserved in domestic cat FIV Vif from subtypes A, B, and D (Fig. 7B). FIV Vif from Pallas's cats (*Otocolobus manul*) had one substitution (F13Y) at the feline A3Z2 interaction sites (Fig. 7B). The A3Z2 and A3Z3 interaction sites in lion FIV Vif from subtypes B and E were identical to domestic cat FIV Vif except for one substitution (L25M) in FIV lion (*Panthera leo*) subtype E Vif (Fig. 7B). Interestingly, Vif from three FIV_{Pco} strains of puma (*Puma concolor*) and one FIV_{Pco} strain of bobcat (*Lynx rufus*) had an evident difference compared to the other FIV Vifs, especially the Vif from the FIV subtype B of puma had two discontinuous deletions at the N-terminal region (Fig. 7B) (64). Taken together, the sequence analysis suggests that the A3Z2 and A3Z3 interaction sites of the FIV Vif protein are highly conserved in different domestic cat FIV Vifs in agreement with its importance in counteracting A3 restriction of the host and interestingly conserved, as well, in some FIVs from nondomestic cats.

DISCUSSION

Previous studies have identified several determinants in HIV-1 Vif, which confer selective interactions with A3F or A3G (e.g., 39YRHHY44 for interaction with A3G and 13DRMR17 for interaction with A3F) (65–68). In addition, some motifs of HIV-1 Vif regulate its binding to both A3G and A3F (e.g., $55VxIPx_4L64$, 69YxxL72, and $96TQx_5ADx_2I107$, where "x" can represent any amino acid) (66, 69, 70). In this study, we identified two motifs (12LF13 and 18GG19) in the FIV Vif N-terminal region that specifically determine its interaction with feline A3Z2 (Fig. 2). We also found that the residues M24, L25, and I27 of FIV Vif mediate the selective interaction with feline A3Z3 (Fig. 4). Only by impairing the feline A3Z2 and A3Z3 interaction sites together was it possible to generate a FIV Vif that lost its counteractivity against the feline dou-

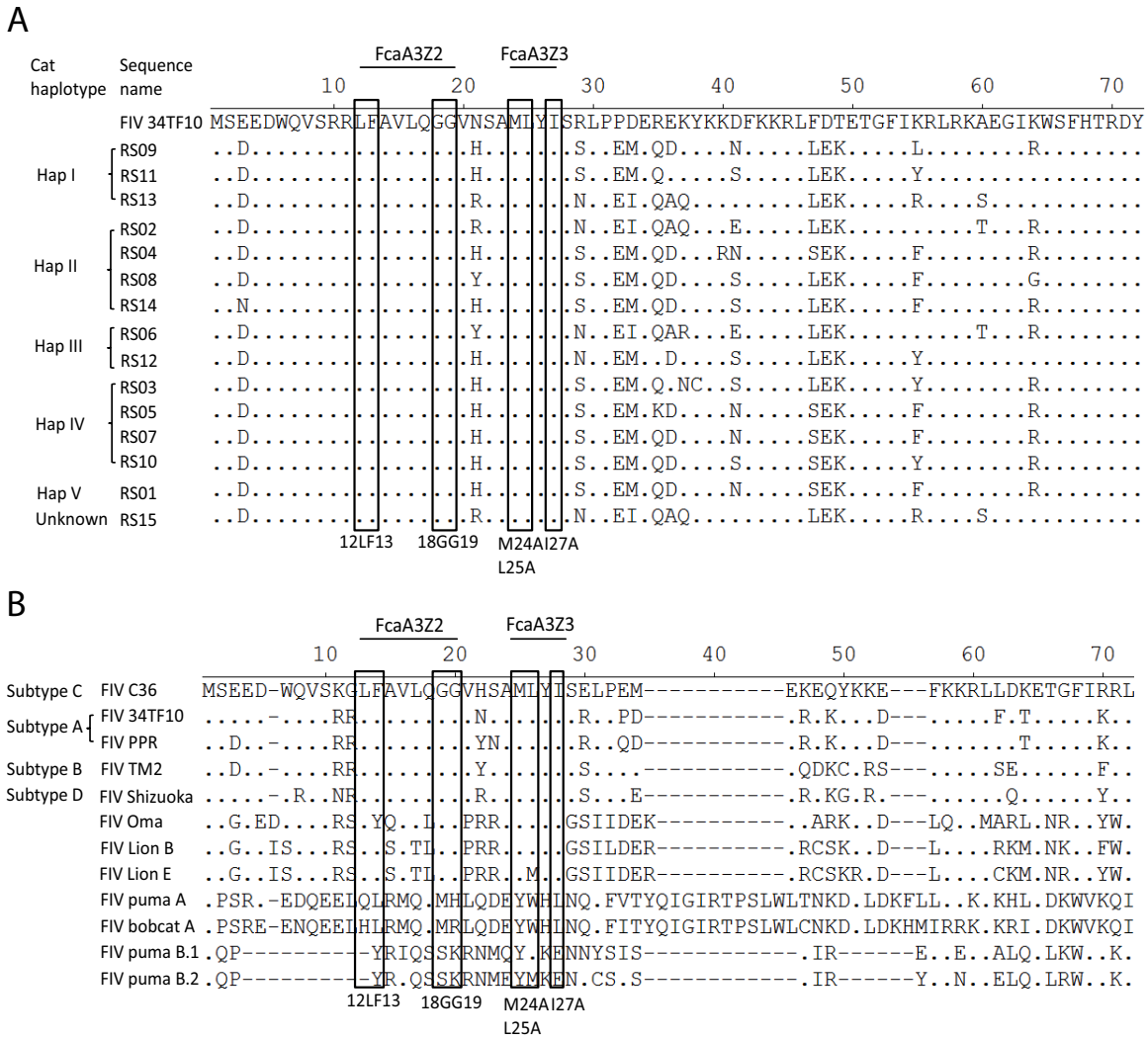

FIG 7 Sequence alignment of Vif from different FIV stains. (A) Sequence alignment of Vif (N terminus) naturally isolated from cats infected with FIV in Brazil with known A3Z3 haplotypes. (B) Subtype A of FIV includes FIV C36, FIV 34TF10, and FIV PRR. Subtype B includes FIV TM2. FIV Shizuoka belongs to subtype D. Other FIV Vifs sequence are from nondomestic cats of FIV Oma (Pallas's cats), FIV lion B, FIV lion E, FIV puma A, FIV bobcat A, and FIV puma B. The different residues between these FIV Vifs are shown. Dots represent identical residues with Vif of FIV C36. Hyphens indicate sequence deletion. The feline A3Z2 and A3Z3 interaction sites are boxed.

ble-domain A3Z2Z3 (Fig. 4C and D). In addition, nine discontinuous, conserved Vif motifs (Y26, 53FI54, 57LR58, 61EGI63, 65WSF67, 81VAG83, 95YI96, 119VN120, and 126GFM128) were identified that were shown to be necessary for inducing the degradation of all three feline A3s (Fig. 3B and C). Why mutations in these residues blocked the degradation of feline A3s was not investigated here. We can speculate that amino acid changes in some of these motifs affect the integrity of the Vif protein. However, some conserved residues of Vif likely form part of the Vif-A3 interface. In addition, some of these residues may interact with cellular proteins important for A3 degradation. Recently, the HIV-1 Vif structure was reported, and it shows that the region forming the β1 and β6 strands and the α2 helix of HIV-1 Vif are involved in CBF-β binding (71). Impairing the α2 helix [T(Q/D/E)x_5ADx_2(I/L)] of HIV-1 Vif disrupted the neutralizing activity of HIV-1 Vif toward both A3G and A3F (69). Whether the nine discontinuous motifs of FIV Vif are involved in the interaction with the E3 ligase or an unknown cofactor needs more investigation.

A previous study demonstrated that A3F.E289 and HIV-1 Vif.R15 display a strong interaction by applying molecular docking (72). In addition, it was reported that several electrostatic interfaces of HIV-1 Vif are involved in binding to A3F and A3C (68, 72). In this study, the specific feline A3Z3 interaction sites (M24, L25, and I27) of FIV Vif belong to hydrophobic residues, and FIV Vif counteraction against feline A3Z3 was also determined by the size of the side chain of FIV Vif residue 25 (Fig. 3C and D). These results may indicate that the interactions of FIV Vif with feline A3Z3 and HIV-1 Vif with human A3s are quite different. Yoshikawa et al. demonstrated that the exposed surface area of feline A3Z3 residue 65 determines its interaction with FIV Vif (57). Whether M24, L25, or I27 of FIV Vif directly interact with I65 of feline A3Z3 by the specific spatial distance needs further investigation.

In this study, we found that FIV Vif protein localized to both cytoplasm and nucleus, but it was mainly found be cytoplasmic (Fig. 5), which is consistent with a previous observation (62). We also observed that the FIV Vif protein formed several puncta in the cytoplasm that may be caused by Vif oligomerization (Fig. 5). Previous reports showed that HIV-1 Vif also localized to both the cytoplasm and nucleus compartments (73, 74). Mutations of feline A3Z2 and A3Z3 interaction sites of FIV Vif did not alter the cellular localization of Vif (Fig. 5).

Unexpectedly, these specific Vif mutations did not disrupt the binding of FIV Vif to feline A3Z2 and A3Z3 in pulldown assays. When we tested different N-terminal Vif fragments, the region from amino acids 50 to 110 of FIV Vif was found to be important for binding to feline A3s; this may explain why mutations in conserved residues of FIV Vif regions from amino acids 50 to 110 disrupted the degradation activity of FIV Vif against all three feline A3s (Fig. 3B). Several previous observations describe that Vif binding to A3s is important but insufficient to induce degradation (44, 75, 76). Based on a recent wobble model (72), we speculate that the 50-110 region of FIV Vif contains the main Vif-A3 interaction interface, whereas the specific feline A3Z2 and A3Z3 interaction sites at the N-terminal 1-50 region provide additional stabilizing contacts.

The A3G and A3F interaction sites of Vif from different HIV-1 strains are relatively conserved. The feline A3Z2 and A3Z3 interaction sites identified here are conserved in Vifs from different FIV subtype stains of domestic cat, one FIV of Pallas's cats, and two FIV subtype strains of lion (Fig. 7). Vifs of puma and bobcat FIVs (FIV_{Pco}) are quite different from the other FIV Vifs (Fig. 7B). It was reported that puma FIV has a high divergence, and multiple puma FIV strains circulate in pumas (36, 39, 77). One recent study demonstrated that puma FIV Vif is inactive against A3Z3s derived from pumas and the domestic cat (58). However, it is important to point out that the described Vif was derived from puma FIV subtype B, which has two deletions at the N-terminal region (Fig. 7B). Puma FIV can cause infections in domestic cats, but these infections often are abortive (78–80), which indicates an immune defense from the domestic cat. Specifically, increased A3-related G-to-A mutations were detected in the viral genomes of puma FIV subtype B during infections in domestic cats (81). These observations indicate that the two N-terminal Vif deletions in puma FIV subtype B might impair viral cross-species transmission. However, it is also important to clarify how this virus evades the restriction from its host A3s.

In summary, we identified here specific interaction sites in FIV Vif for the degradation of feline A3Z2 and A3Z3. Several motifs of FIV Vif were also identified that were important for the degradation of all feline A3s. These results provide important insights for future experiments describing the FIV Vif interaction with A3s and other cellular proteins.

ACKNOWLEDGMENTS

We thank Wioletta Hörschken for excellent technical assistance and Ananda Ayyappan Jaguva Vasudevan for the RT assays. We thank Eric Poeschla for plasmid pFP93.

The reagent anti-FIV p24 monoclonal (PAK3-2C1) was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.

FUNDING INFORMATION

This work, including the efforts of Carsten Münk, was funded by Heinz Ansmann Foundation. This work, including the efforts of Qinyong Gu and Zeli Zhang, was funded by China Scholarship Council (CSC).

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