Potent Lentiviral Restriction by a Synthetic Feline TRIM5
Cyclophilin A Fusion

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A synthetic feline TRIM5-cyclophilin A fusion protein (feTRIMCyp) was generated and transduced into
feline cells. feTRIMCyp was highly efficient at preventing infection with human (HIV) and feline (FIV)
immunodeficiency virus pseudotypes, and feTRIMCyp-expressing cells resisted productive infection with either
FIV-Fca or FIV-Pco. The restriction of FIV infection by feTRIMCyp was reversed by the cyclosporine (Cs)
 substitutions (3–5, 11, 31). However, the specificity of the CypA-capsid interaction has been utilized by several species of primates to
target TRIM5 to the lentiviral capsid. Insertion of a CypA cDNA into the untranslated region of exon 8. In the Old World macaques, splicing of the
mRNA transcript fuses the end of exon 6 to the CypA splice acceptor. The potency of the lentiviral restriction by the pri-
mate TRIMCyp proteins offers a novel approach to HIV-1 gene therapy; transduction of either bone marrow stem cells or
peripheral blood CD+ T cells with vectors bearing TRIMCyp fusion proteins should render the cells resistant to HIV infec-
tion and replication. To circumvent a potential immune response by the recipient against Aotus or Macaca TRIMCyps, a
synthetic human TRIM5-CypA fusion protein was generated and shown to confer robust resistance to HIV-1 replication
(18).

Feline immunodeficiency virus (FIV) infection of the domes-
tic cat (Felis catus) offers a well-characterized small-animal model for HIV infection (9, 21, 36). Moreover, with ~0.5
million FIV-infected cats in the United Kingdom alone, there is both a suitable study population and the advanced clinical
facilities that would be required to investigate cutting-edge therapies for immunodeficiency-causing lentiviruses, benefit-
ning both human and veterinary medicine alike. Toward this end, we asked whether a feline-specific TRIMCyp could be
engineered and whether it would display the potent lentiviral restriction activity of the primate proteins. To date, we have
found no evidence of a naturally occurring TRIMCyp fusion protein in either the Felis, Panthera, or Puma lineages (17).
Moreover, the domestic cat lacks a full-length TRIM5 gene due to the presence of a premature stop codon in the feline
TRIM5 exon homologous to human TRIM5 exon 8 (17). However, feline cells express an abundant message for the TRIM5
RBCC (17). As the feline TRIM5 RBCC is encoded by exons 2 to 6, we elected to fuse the start codon of feline CypA to the
last codon of exon 6. The feline TRIM5 RBCC was reamplified from feT5-CXCR (17) using primers feT5a-1 (5′-GC GGAT C
CATGGCTTCTGAACTCCTGAAAT-3′) and feT5a-2 (5′-GCGG

Human immunodeficiency virus type 1 (HIV-1) infection is
inhibited immediately after viral entry by the α isoform of the
tripartite-motif-containing protein TRIM5 (TRIM5α) (8, 23, 29, 32). The C terminus of TRIM5α contains a PRY/SPRY
(B30.2) domain (12, 25), and this domain mediates binding of
TRIM5α to the viral capsid (30, 33). The N-terminal tripartite
motif, or RBCC (RING, B-box, and coiled coil) domain, pos-
sesses an E3 ubiquitin ligase domain (RING) (39), and ubiqu-
itination recruits incoming virions to the proteasome, where
they are degraded. While inhibition of the proteasome pre-
vents degradation of the viral core and enables reverse tran-
scription to proceed, the process of infection does not com-
plete (7, 38), indicating an additional proteasome-independent antiviral function for TRIM5α. Accelerated uncoupling of the
viral capsid from the incoming virion may underlie this pro-
teasome-independent restriction activity (24, 33).

Cyclophilin A (CypA) associates with the HIV-1 capsid (16)
and is present in viral particles (10, 34). CypA is a ubiquitous
cytoplasmic protein that catalyzes the cis/trans isomerization of
peptidyl-prolyl bonds, and following binding to HIV-1 capsid,
the peptidyl-prolyl bond linking residues G89 and P90 is isomerized (2). The specific association of target cell CypA
with the incoming HIV-1 capsid is required for viral infectivity
(3–5, 11, 31). However, the specificity of the CypA-capsid in-
teraction has been utilized by several species of primates to
target TRIM5 to the lentiviral capsid. Insertion of a CypA
cDNA between exons 7 and 8 of TRIM5 in the New World
monkey Aotus trivirgatus (owl monkey) generated a TRIM5-
CypA fusion (TRIMCyp) with potent lentiviral restriction ac-
tivity (20, 29). Moreover, gene fusions have been detected in
three species of Old World macaques, Macaca mulatta (rhesus
macaque) (19, 37), Macaca nemestrina (pig-tailed macaque) (6,
14, 19, 35), and Macaca fascicularis (crab-eating macaque) (6),
resulting from insertion of a CypA cDNA into the untranslated
region of exon 8. In the Old World macaques, splicing of the
mRNA transcript fuses the end of exon 6 to the CypA splice
acceptor. The potency of the lentiviral restriction by the pri-
mate TRIMCyp proteins offers a novel approach to HIV-1 gene therapy; transduction of either bone marrow stem cells or
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Feline CypA was amplified from cDNA derived from the domestic cat primary T-cell line Mya-1 using primers directed to the predicted feline CypA (GenBank AANG01610851), fCypA R69 5'-H11032 Nde (5'-AACATATGGGTCA ACCCCATCGTG-3') and feCypA 3' Mlu (5'-AAACGCGTT TAGATTTGTCCACAGTC-3'). The amplicon was cloned into the prokaryotic expression vector pOPTH using NdeI and MluI restriction sites and subsequently reamplified using primers feCypA-1 (5'-ATAATACAGCCCTTTAAGAATGTT CAACCCATCGTG-3') and feCypA-2 (5'-GCGTGACTTT AGATTTGTCCACAGTCAGC-3'). Feline CypA binding to FIV capsid (CA) was confirmed by isothermal titration calorimetry (ITC), as previously described (26). FIV capsid N-terminal domain (feCAN), feCypA, and human CypA (huCypA) were overexpressed in Escherichia coli and purified by Ni-nitrilotriacetic acid (NTA), gel filtration, and ion-exchange chromatography. Titrations were carried out at 10°C, and binding isotherms fit to a standard one-state model to give the stoichiometry (N), enthalpy change (ΔH), and equilibrium association constant (Kₐ), from which the change in Gibbs energy (ΔG) and entropy (ΔS) and the equilibrium dissociation constant (Kₐ') were derived. FeCAN bound feCypA and huCypA with Kₐ of 6.2 μM and 7.2 μM, respectively, compared to a value of 5.3 μM for HIV-1 CA binding to huCypA (previous reports indicated a Kₐ of 5 to 15 μM for HIV-1 and huCypA [26, 40]). Mutation of HIV-1 CA proline-rich loop residues G89 and P90 abrogated CypA binding in HIV-1 (40).
To test whether CypA interacted with FIV in a similar manner, the P90A mutant that was previously shown to prevent CypA packaging within FIV virions (15) was tested for feCypA binding. No detectable interaction was found, suggesting that, like HIV-1, the proline-rich loop is critical in feCAN-CypA interactions.

The TRIM5 RBCC and CypA amplification products were annealed and used as templates to generate a TRIMCyp gene fusion by reamplification with feT5a-1 and feCypA-2. The TRIMCyp fusion was cloned into BamHI and SalI sites of the retroviral vector pDON-AI-2neo (Takara Bio Europe S.A.S., Saint-Germain-en-Laye, France), and the nucleic acid sequence of the TRIMCyp fusion was confirmed (GenBank accession number HM246715). Crandell feline kidney cells (CrFK, line ID10) were transduced with murine leukemia virus pseudotypes bearing the TRIMCyp fusion, and stable lines were selected in G418. Stable expression of the feline TRIM-Cyp fusion rendered ID10 cells resistant to infection with either the HIV(VSV) (vesicular stomatitis virus envelope glycoprotein) or FIV(VSV) pseudotype (Fig. 1). The specificity of the inhibitory effect was confirmed by the addition of specific antagonists of the CypA-capsid interaction; either Cs (Fig. 1C and E) or its nonimmunosuppressive derivatives NIM811 (28) (Fig. 1D and F) and Debio-025 (27) (Fig. 1E and F). While 2.0 μM Cs displayed a modest reversal of the inhibition of HIV pseudotype entry by TRIMCyp (Fig. 1A), it was unable to reverse the inhibition of HIV pseudotype infection (Fig. 1B). In contrast, the Cs derivatives NIM811 (Novartis, Basel, Switzerland) and Debio-025 (Debiopharm, Lausanne, Switzerland) at 2.0 μM reversed the inhibition of infection with both HIV (Fig. 1C and E) and FIV (Fig. 1D and F) pseudotypes. Titrating the CypA antagonists confirmed the differential sensitivities of the HIV and FIV pseudotypes to reversal of the TRIMCyp restriction; restriction of HIV was readily reversed by NIM811 (Fig. 1H) and Debio-025 (Fig. 2I), while Debio-025 (Fig. 2J) alone reversed the restriction of FIV to near control levels of infection. The data suggest that the feline TRIMCyp fusion targets the HIV and FIV capsids specifically during viral entry and that inhibition of FIV is extremely potent. Next, we asked whether the TRIMCyp fusion would inhibit productive infection with replication-competent lentiviruses from the domestic cat FIV-Fca (Petaluma-F14 strain) (Fig. 2A and B) or puma FIV-Pco (Fig. 2C and D). Replication of both FIV-Fca and FIV-Pco was blocked completely by expression of the TRIMCyp fusion protein (Fig. 2B and D), while both viruses replicated well in cells transduced with vector only (Fig. 2A and C). FIV-Fca replication was accompanied by the formation of prominent syncytia (Fig. 2E). The CypA antagonists NIM811 and Debio-025 (2.5 μM) blocked replication with FIV-Fca and FIV-Pco in the control cells, indicating an important role for CypA in the replication of these viruses in CrFK cells and consistent with previous studies suggesting a role for CypA in the replication of FIV (15). Debio-025 was more potent than NIM811 and blocked FIV-Fca replication completely. In contrast, both Debio-025 and, to a lesser extent, NIM811 countered the inhibition of viral growth by TRIMCyp. The most marked effect was with FIV-Fca, where Debio-025 restored viral growth to a level at which small syncytia could be visualized (Fig. 2H, arrows) and

![FIG. 2. Inhibition of viral replication by feline TRIMCyp and its reversal by CypA antagonists. CrFK (ID10) cells stably transduced with a retroviral vector bearing feline TRIMCyp (B and D) or vector only (A and C) were infected with FIV-Fca (Petaluma-F14 strain) (A and B) or FIV-Pco (CoLV strain) (C and D). Infections were performed in the presence or absence of medium supplemented with 2.5 μM CypA antagonist NIM811 or Debio-025 or their respective solvents, DMSO and ethanol (EtOH). Supernatants were collected and assayed for RT activity by nonisotopic RT assay (LentiRT; CavidTech, Sweden). CON, control. (E to H) Syncytium formation in CrFK cells infected with FIV-Fca. Cells expressing vector only (E and G) or TRIMCyp (F and H) and infected with FIV-Fca in the presence of Debio-025 (G and H) or ethanol solvent control (E and F) were fixed and stained at 10 days postinfection with 1.0% methylene blue-0.2% basic fuchsin in methanol. The arrows indicate small syncytia, magnified in the inset (H).](http://jvi.asm.org/)

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Thus, where endogenous CypA and ectopically expressed TRIMCyp are coexpressed, the CypA antagonists appear to tip the balance in favor of viral replication. In support of this hypothesis, we repeated the viral replication assay in the presence of a reduced antagonist concentration (2.0 μM) (Fig. 3). While growth of FIV-Pco in control cells was suppressed efficiently by both NIM811 and Debio-025 at 2.0 μM (Fig. 3C), growth of FIV-Fca was reduced modestly (Fig. 3A). However, in the presence of suboptimal antagonist, replication of FIV-Fca in the TRIMCyp-expressing cells was restored completely by Debio-025 and partially by NIM811 (Fig. 3B), with viral replication accompanied by prominent syncytium formation.

As FIV-Fca (Petaluma F14) and FIV-Pco (cougar lentivirus [CoLV]) are cell culture-adapted viral strains, we generated ID10 and ID10-TRIMCyp cells stably expressing CD134 to examine the effect of the feline TRIMCyp fusion on primary FIVs. Replication of the GL8 (Fig. 4A) and PPR (Fig. 4B) strains of FIV-Fca was blocked completely by the feline TRIMCyp fusion protein. Finally, we confirmed the specificity of TRIMCyp by comparing infection of feline TRIMCyp-expressing cells with FIV, HIV-1, and simian immunodeficiency virus (SIVmac). SIVmac CA does not possess CypA-binding activity, and accordingly, SIVmac pseudotypes infected feline TRIMCyp-expressing ID10 cells with efficiency similar to that with control cells (Fig. 4D), while parallel infections with either FIV (Fig. 4C) or HIV-1 (Fig. 4E) pseudotypes were blocked completely.

By designing a feline TRIM5-CypA gene fusion based on the naturally occurring TRIMCyp of *A. trivirgatus*, we postulated that a feline TRIMCyp with robust antilentiviral activity would result. In doing so we confirmed that the feline TRIM5 retains full ability to restrict both FIV and HIV-1; it simply lacks a capsid-targeting SPRY domain. The feline CypA targets the feline TRIM5 RBCC to the lentiviral capsid efficiently, generating a potent restriction factor of entirely feline origin. Indeed, feTRIMCyp ablated completely the ability of feline lentiviruses to grow in vitro. Accordingly, feTRIMCyp should facilitate in vivo analyses of the viability of gene therapy.
TRIMCyp without the potential pitfall of the host generating an immune response against xenoantigens. The TRIMCyp-based approach to lentiviral gene therapy offers advantages over other potential approaches to therapy; for example, by targeting viral entry, it denies the virus the opportunity to replicate, and thus the virus cannot generate escape mutants. As TRIMCyp does not target the function of an endogenously expressed molecule (recent targets for HIV gene therapy have included the coreceptor CCR5 [1, 13, 22]), it is unlikely to have side effects that are detrimental to the host. Treatment of the lentivirus-infected host using TRIMCyp fusions may be achieved by transduction of bone marrow-derived hematopoietic progenitor stem cells and repopulation of the host immune system following bone marrow ablation. However, transduction of peripheral-blood-derived CD4+ T cells and ex vivo expansion of the transduced cells prior to repopulation of the host immune system may be sufficient to overcome the immunodeficiency associated with AIDS. Accordingly, the successful in vitro studies described here offer strong support for clinical trials of feline TRIMCyp both as a therapy for FIV infection and as a model for the gene therapy of HIV infections in humans.

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