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Restriction of the felid lentiviruses by a synthetic feline TRIM5-CypA fusion

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Abstract

Gene therapy approaches to the treatment of HIV infection have targeted both viral gene expression and the cellular factors that are essential for virus replication. However, significant concerns have been raised regarding the potential toxic effects of such therapies, the emergence of resistant viral variants and unforeseen biological consequences such as enhanced susceptibility to unrelated pathogens. Novel restriction factors formed by the fusion of the tripartite motif protein (TRIM5) and cyclophilin A (CypA), or “TRIMCyps”, offer an effective antiviral defence strategy with a very low potential for toxicity. In order to investigate the potential therapeutic utility of TRIMCyps in gene therapy for AIDS, a synthetic fusion protein between feline TRIM5 and feline CypA was generated and transduced into cells susceptible to infection with feline immunodeficiency virus (FIV). The synthetic feline TRIMCyp was highly efficient at preventing infection with both HIV and FIV and the cells resisted productive infection with FIV from either the domestic cat or the puma. Feline TRIMCyp and FIV infection of the cat offers a unique opportunity to evaluate TRIMCyp-based approaches to genetic therapy for HIV infection and the treatment of AIDS.

Keywords

FIV; HIV; TRIM5; cyclophilin; TRIMCyp; AIDS

Introduction

In order to efficiently replicate in a host cell, retroviruses must not only be able to make use of available cellular factors, but also overcome dominant intracellular blocks to replication in the host cell known as restriction factors. The study of these factors has uncovered a novel

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Conflict of Interest Statement

All authors declare that there is no conflict of interest.

branch of the innate immune system, targeting specifically various stages of the virus lifecycle (Bieniasz, 2004). One of the major determinants for such virus-host compatibility is the longest (alpha) isoform of the host protein TRIM5, a member of the tri-partite motif family of proteins (Reymond et al., 2001; Stremlau et al., 2004). TRIM5 α from humans specifically inhibits pre-integration stages of murine leukaemia virus N-strain (MLV-N) replication whereas that from rhesus macaques inhibits HIV-1 infectivity (Yap et al., 2004; Hatzioannou et al., 2004; Keckesova et al., 2004). TRIM5 α variants from non-primate species such as cows (*Bos taurus*) (Ylinen et al., 2006; Si et al., 2006) and rabbit (*Oryctolagus cuniculus*) (Schaller et al., 2007a) have also been shown to restrict retroviral infection, suggestive of a common ancestor for mammalian TRIM5 α 's with antiretroviral properties.

The TRIM protein family is large, with at least 68 intact members in the human genome (Nisole et al., 2005). Members of the TRIM protein family typically comprise a RING domain with E3-ubiquitin ligase activity that is capable of auto-ubiquitination, a B-Box-2 domain and a coiled-coil domain, referred to collectively as the RBCC (Reymond et al., 2001). Some TRIM proteins, including TRIM5 α , possess a C-terminal B30.2 (PRY-SPRY) domain that is thought to mediate binding of the TRIM protein to the incoming retroviral capsid (Mische et al., 2005; Sebastian and Luban, 2005; Stremlau et al., 2006). Antiviral activity has been associated with several of these TRIMs, including TRIM19 (Chelbi-Alix et al., 1998), TRIM22 (Tissot and Mechti, 1995) and TRIM32 (Fridell et al., 1995). By far the best understood, however, is TRIM5 α which leads to a block to reverse transcription in most non-permissive cells (Stremlau et al., 2004; Keckesova et al., 2004). Evidence suggests that TRIM5 α homo-dimers bind directly to the retroviral capsid in the cytoplasm (Mische et al., 2005; Sebastian and Luban, 2005; Stremlau et al., 2006) and that the resulting capsid/TRIM5 α complex does not complete reverse transcription due to rapid proteasome-mediated degradation (Diaz-Griffero et al., 2006a; Towers, 2007), as indicated by the observation that under proteasome inhibition restricted virus reverse transcribes but remains uninfected (Anderson et al., 2006; Wu et al., 2006). While inhibition of the proteasome prevents degradation of the viral core and enables reverse transcription to proceed, the process of infection does not complete (Wu et al., 2006; Campbell et al., 2008) indicating an additional proteasome-independent anti-viral function for TRIM5 α . Accelerated uncoating of the viral capsid from the incoming virion may underlie this proteasome-independent restriction activity (Stremlau et al., 2006; Perron et al., 2007). Alternatively-spliced TRIM5 variants (TRIM5 δ and TRIM5 γ lack a B30.2 domain and thus lose their ability to restrict (Stremlau et al., 2004; Passerini et al., 2006). Moreover, these short TRIM5 isoforms have a dominant negative effect, preventing full TRIM5 α restriction by forming heteromers with full-length TRIM5 α (Mische et al., 2005; Perez-Caballero et al., 2005).

Cyclophilin A and lentiviral infection

The cyclophilins comprise a large family of proteins found in both prokaryotes and eukaryotes. Cyclophilins are members of the "peptidyl proline isomerases" superfamily (or "immunophilins"), a name which reflects their ability to catalyse *cis-trans* isomerisation around peptidyl-proline bonds (Gothel and Marahiel, 1999). There are at least eight cyclophilin genes expressed in humans, with many other mammalian forms also found,

sharing sequence identity of >50% (Gothel and Marahiel, 1999), but with variable subcellular localisation. The 18 kDa human protein cyclophilin A (CypA) was discovered as the intracellular receptor of immunosuppressive drug cyclosporine A (CsA) (Takahashi et al., 1989). CsA binds CypA and creates a novel binding surface that is able to bind and inhibit the serine/threonine kinase calcineurin. As a primary activity of calcineurin is the activation of NFAT (nuclear factor of activated T cells), CsA thus inhibits T-cell receptor-mediated activation and subsequent expansion of antigen-specific T cells (reviewed in (Clardy, 1995)). CypA was first associated with lentiviral replication when it was found to be packaged into HIV-1 virions via an association with unprocessed Gag polyprotein (Luban et al., 1993; Thali et al., 1994; Franke et al., 1994). These studies found that disruption of the capsid-CypA interaction with CsA prevented CypA incorporation into virions and impacted negatively on HIV-1 replication. However it is during the early stages of viral infection, not incorporation, that CypA impacts on the viral lifecycle (Towers et al., 2003). CypA binds to incoming viral cores via an interaction between the catalytic hydrophobic pocket of the enzyme and an exposed proline-rich loop between helices 4 and 5 on the external surface of capsid N-terminal domain (Gamble et al., 1996). This loop appears to be confined to the lentiviruses as it is absent in other groups of retroviruses. However, whilst the capsid-CypA interaction has been demonstrated for HIV-1, SIVagm and FIV (Zhang et al., 2006; Lin and Emerman, 2006; Diaz-Griffero et al., 2006b) the interaction is not a feature of all lentiviruses: SIVmac, HIV-2 and EIAV do not bind CypA (Braaten et al., 1996b; Yoo et al., 1997; Lin and Emerman, 2006).

The specific association of target cell CypA with the incoming HIV-1 capsid is required for viral infectivity (Braaten et al., 1996a; Braaten et al., 1996b; Braaten and Luban, 2001; Sokolskaja et al., 2004; Hatzioannou et al., 2005). The HIV-1 capsid exists as a mixture of *cis* and *trans* isomers around the G89-P90 peptide bond with 14% of molecules in the *cis* and the remainder in the *trans* conformation (Gitti et al., 1996). Following CypA binding to the HIV-1 capsid, the peptidyl-prolyl bond linking residues G89 and P90 is isomerized (Bosco et al., 2002); CypA catalyses the inter-conversion of the capsid G89-P90 *cis* and *trans* isomers, increasing the rate of reaction by about 100-fold but maintaining the same *cis:trans* ratio (Bosco et al., 2002). These data suggest that CypA performs a catalytic role in promoting the dissociation of the capsid core upon infection of a target cell. The timing of uncoating is critical, if uncoating is delayed or prevented then the pre-integration complex will fail to enter the nucleus of the target cell. In contrast, if the uncoating is accelerated then reverse transcription will fail and the viral capsid will be targeted for proteasomal degradation following ubiquitinylation by TRIM5 α . Indeed, premature uncoating of the viral capsid core has been postulated to be amongst the primary antiviral mechanisms of TRIM5 α (Perron et al., 2007).

TRIM5 – cyclophilin A fusion proteins

The specificity of the CypA-capsid interaction has been utilised by several species of primate to target TRIM5 α to the lentiviral capsid. Insertion of a CypA cDNA between exons 7 and 8 of TRIM5 α in the New World monkey *Aotus trivirgatus* (owl monkey) generated a TRIM5-CypA fusion (TRIMCyp, or TRIM5CypA1 (Stoye and Yap, 2008)) with potent lentiviral restriction activity (Sayah et al., 2004; Nisole et al., 2004). Moreover, gene fusions

have been detected in three species of Old World macaques, *Macaca mulatta* (rhesus macaque) (Wilson et al., 2008; Newman et al., 2008), *Macaca nemestrina* (pig-tailed macaque) (Liao et al., 2007; Newman et al., 2008; Virgen et al., 2008; Brennan et al., 2008) and *Macaca fascicularis* (crab-eating macaque) (Brennan et al., 2008), 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 (TRIM5CypA2 (Stoye and Yap, 2008)). The two TRIMCyps display distinct antiviral specificities. *Aotus* TRIMCyp displays potent restriction activity against HIV-1 and FIV but fails to restrict SIV_{MAC} or EIAV (Sayah et al., 2004; Nisole et al., 2004; Diaz-Griffero et al., 2006b). In contrast, *Macaca* TRIMCyp fails to restrict HIV-1, SIV_{MAC}, EIAV or MLV, but does restrict HIV-2, SIV_{AGM}Tan and FIV (Wilson et al., 2008; Virgen et al., 2008). The molecular basis for the specificity of the antiviral function resides in a point mutation in the CypA domain of the two TRIMCyps, H69R (Virgen et al., 2008) (H69 in *Macaca* while R69 in *Aotus*).

The potency of the lentiviral restriction by the primate TRIMCyp proteins has raised the possibility of novel approaches to HIV-1 gene therapy; the transduction of either bone marrow stem cells or peripheral blood CD4⁺ T cells *in vitro* with vectors bearing TRIMCyp fusion proteins should render the cells resistant to HIV infection. Upon re-infusion, the transduced cells should repopulate the host immune system with cells that are able to resist viral replication and cytopathicity. Given that TRIMCyp fusion proteins have now been identified in several primate species, TRIMCyps would appear to be well-tolerated by the host with no obvious detrimental effects to immune function. However, given the amino acid sequence divergence between non-human primate TRIM5 and human TRIM5, introduction of *Aotus* or *Macaca* TRIMCyps into humans may induce a specific immune response to the transgene product. To circumvent a potential immune response by the recipient against the primate TRIMCyp, a synthetic human TRIM5-CypA fusion protein has been generated (Neagu et al., 2009) by fusing human TRIM5 PRY/SPRY domain at serine 322 with a CypA cDNA. The resulting synthetic TRIMCyp, hT5-S322-Cyp was found to confer robust resistance to HIV-1 replication (Neagu et al., 2009). However, the possibility remains that the novel splice junction created by fusion of the TRIM5 and CypA proteins will in itself create an epitope that will be recognised by the human immune system.

TRIM, CypA and feline immunodeficiency virus

FIV infection of the domestic cat (*F. catus*) offers a well-characterized small animal model for HIV infection (Pedersen et al., 1987; Willett et al., 1997; Elder et al., 1998). Infection with FIV results in the development of an immunodeficiency similar to AIDS in humans, characterised by a progressive depletion of CD4⁺ helper T lymphocytes and clinical signs including wasting/cachexia, recurrent gingivitis/stomatitis, neurological disorders and an increased likelihood of malignancy. With approximately 0.5 million FIV-infected cats in the United Kingdom alone, the development of a gene therapy approach to the treatment of FIV infection would represent not only a significant advance in animal welfare but also proof of concept for the gene therapy of HIV infection in humans. As previous studies have demonstrated that FIV-Fca is highly sensitive to restriction by TRIM5 proteins (Saenz et al., 2005; Diaz-Griffero et al., 2006b; Munk et al., 2007; Schaller et al., 2007b; Wilson et al.,

2008; Virgen et al., 2008), a feline-specific TRIMCyp would represent an excellent candidate for FIV gene therapy. Prior to the generation of a feline TRIMCyp, it was important to assess whether feline CypA bound the FIV capsid. In order to analyse the interaction between feline CypA and the FIV capsid, the N-terminal domain of FIV capsid and feline CypA were expressed in a prokaryotic expression system and purified by affinity chromatography. Protein-protein interactions were quantified by isothermal titration calorimetry (ITC). The FIV capsid was found to bind to feline CypA with a similar affinity (6.2 μ M) to the interaction between the HIV-1 capsid and human CypA (5.3 μ M) (Dietrich et al., 2010). Thus, using feline CypA to target the TRIM5 RBCC to the viral capsid appeared technically feasible. However whether the feline TRIM5 RBCC would constitute a functional restriction factor when targeted by fusion to CypA required experimental confirmation. Previous studies had indicated that the truncated feline TRIM5 could act as a dominant negative to the antiviral activity of human TRIM5 (McEwan et al., 2009) suggesting that the expressed protein adopted a conformation that allowed it to heteromerise with human TRIM5 however in the absence of a PRY/SPRY domain no antiviral activity could be ascribed to feline TRIM5.

Generation of a synthetic feline TRIMCyp

To assess whether a synthetic TRIMCyp of entirely feline origin could be synthesised that would display the potent lentiviral restriction activity of the primate proteins, feline TRIM5 and feline CypA cDNAs were fused experimentally (Figure 1). 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 (McEwan et al., 2009). However, felid cells express an abundant mRNA for the TRIM5 RBCC (McEwan et al., 2009). As the feline TRIM5 RBCC is encoded by exons 2 to 6, the start codon of feline CypA was fused to the last codon of exon 6 of *TRIM5*. Thus, the synthetic feline TRIMCyp was designed to mimic the naturally occurring TRIMCyp (TRIM5CypA2) of the rhesus macaque. Although the CypA cDNA has inserted into the 3' untranslated region of the rhesus macaque TRIM5 gene, the CypA splice acceptor is used to splice the CypA alongside the splice donor of TRIM5 exon 6 following mRNA processing, yielding a TRIMCyp fusion at TRIM5 exon 6. The selection of exon 6 for the splice junction in feline TRIMCyp to mimic the old world monkey TRIMCyp would appear to have been somewhat fortuitous given that a systematic screening of potential sites for gene fusion in exon 8 (PRY/SPRY domain) of human TRIM5 yielded several non-functional synthetic human TRIMCyp proteins before the identification of serine 322 in hT5-S322-Cyp as a compatible site for gene fusion (Neagu et al., 2009).

The resulting feline *TRIMCyp* transgene was cloned into a retroviral vector, transduced into Crandell feline kidney cells (CrFK) and stably selected. CrFK cells support the replication of CD134-independent feline lentiviruses such as FIV-Fca Petaluma-F14 or FIV-Pco-CoLV. Following stable transduction with a retroviral vector bearing the feline TRIMCyp fusion, cells were rendered refractory to the replication of feline lentiviruses (Figure 2). Given that TRIM5 targets an early stage of viral replication, the process of viral entry, the feline TRIMCyp expressing cells were then infected with FIV (VSV) or HIV(VSV) pseudotypes bearing a marker gene (green fluorescent protein, GFP). The FIV(VSV) and HIV(VSV)-GFP pseudotypes undergo a single cycle of viral entry and gene expression and thus

distinguish entry from productive infection. Feline TRIMCyp expression reduced infection with pseudotypes bearing either FIV or HIV Gag significantly (Figure 3), confirming that viral replication was targeted at an early stage of the cycle, most likely during the process of viral entry. Feline CypA has an arginine at residue 69, similar to human CypA and the CypA domain of *Aotus* TRIMCyp and, consistent with this, the feline TRIMCyp failed to restrict infection with SIV_{MAC}(VSV)-GFP pseudotypes (Dietrich et al., 2010).

While previous data had suggested that feline TRIM may act as a dominant negative when co-expressed with a functional TRIM5 α (McEwan et al., 2009), the inhibition of both viral entry and viral replication by expression of the TRIMCyp fusion protein provide confirmation that the feline TRIM5 RBCC is indeed functional when targeted to the viral capsid by the CypA and that the absence of anti-lentiviral activity in cells expressing either endogenous or exogenous feline TRIM5 likely results from the truncation of the capsid-targeting PRY/SPRY domain.

Developing TRIMCyp as a gene therapy for AIDS

The next stage in developing feline TRIMCyp for use in gene therapy in FIV infected cats will require a close examination of the effects of TRIMCyp expression on the function and development of feline T cells. The presence of TRIMCyps in the *Aotus* and *Macaca* primate lineages would suggest that TRIMCyp expression may be accommodated by the host when expressed from birth, however it is possible that a detrimental effect may manifest following ectopic expression of TRIMCyp in transduced primary cells. An insight into the likely therapeutic utility of TRIMCyp fusions in human has been provided by Neagu et al (2009) in their study of the effect of stable expression of a synthetic human TRIMCyp on human T cell function. HT5-S322-Cyp (hT5Cyp) was cloned into the bicistronic lentiviral vector scALPS in which TRIMCyp expression is driven from the SFFV promoter and a GFP marker gene is controlled by the human CypA promoter (Neagu et al., 2009). Human CD4⁺ T cells stably transduced with hT5Cyp proliferated at the same rate, produced similar levels of interleukin-2 (IL-2) and expressed similar levels of cell-surface CD4, CXCR4 and MHC class I as cells transduced with either vector only or a hT5Cyp mutant lacking biological activity (hT5CypH126Q) (Neagu et al., 2009). Human CD4⁺ T cells stably transduced with hT5Cyp were then transplanted into *Rag2*^{-/-}/ γ c⁻ mice, a mouse strain that lacks B, T and NK cells and which does not reject xenografts (Mazurier et al., 1999). Following engraftment of Clodronate-primed and irradiated *Rag2*^{-/-}/ γ c⁻ mice with hT5Cyp-transduced human T cells, stable expression of a GFP marker gene (incorporated into the hT5Cyp-expression vector) was detected in a substantial proportion of thymic T cells (~50%) (Neagu et al., 2009). Following challenge of the engrafted mice with HIV-1, those expressing hT5Cyp resisted infection as evidenced by reduced plasma viral load and maintenance of CD4⁺ T cell number in peripheral blood and in lymphoid tissues (Neagu et al., 2009). In contrast, when *Rag2*^{-/-}/ γ c⁻ mice were transplanted with hT5Cyp transduced CD34⁺ haematopoietic progenitor cells, although T and B cell compartments were successfully reconstituted by 8 weeks post-transplantation, the CD4⁺ T cells that emerged did not express the GFP marker gene (Neagu et al., 2009). Irrespective of these findings, the mice reconstituted with hT5Cyp-expressing CD34⁺ progenitor cells achieved a mean viraemia following HIV-1 challenge that was less than 30% of that observed in mice

reconstituted with a non-functional TRIMCyp construct (hT5CypH126Q) (Neagu et al., 2009). Thus initial studies with a synthetic human TRIMCyp offer great hope for the use of TRIMCyp as a relatively benign approach to gene therapy for lentiviral infections. Translating the findings with *Rag2*^{-/-}/ γ c- mice and engrafted TRIMCyp-expressing human T cells to animals models for AIDS such as FIV infection of the domestic cat would validate the two approaches to therapy; adoptive transfer of transduced T cells and reconstitution with CD34+ stem cells.

Several approaches to the gene therapy of HIV infection have been proposed, from targeting directly the expression of viral genes using either RNA-based agents such as antisense RNA, ribozymes, aptamers and RNAi, or protein-based agents including dominant negative proteins, intrabodies, intrakines, fusion inhibitors and zinc-finger nucleases (reviewed in (Rossi et al., 2007)) to inhibiting viral entry by selective knockdown of expression of the viral co-receptors CXCR4 and CCR5 (Anderson and Akkina, 2005a; Anderson and Akkina, 2005b; An et al., 2007; Perez et al., 2008; Kumar et al., 2008). While many of these approaches have demonstrated robust inhibition of HIV replication *in vitro*, significant concerns have been identified in regard to possible toxicity to the host and the likelihood of off-target effects. Moreover, just as HIV mutates rapidly to evade conventional reverse transcriptase-based therapies, viruses may evolve to escape RNAi-based approaches. Perhaps one of the most promising developments to emerge in recent years has been the selective targeting of the HIV co-receptor CCR5, a member of the seven transmembrane domain superfamily of proteins and receptor for the chemokines CCL3 (MIP-1 α), CCL4 (MIP-1 β) and CCL5 (RANTES). Individuals homozygous for a 32-base pair deletion in CCR5 (*CCR5*^{Δ32}) display resistance to infection with HIV-1 (Samson et al., 1996; Dean et al., 1996). Because of the redundancy built-in to the chemokine receptor signalling system (CCL3 and 4 also bind CCR1 and CCR4, while CCL5 also binds CCR1, CCR3 and DARC (CD234)) the loss of CCR5 does not appear to impair immune function. Using zinc-finger nucleases (ZFN) to disrupt endogenous CCR5 expression, human CD4+ T cells were rendered resistant to infection with HIV-1 *in vitro* and using the NOG mouse model for HIV-1 infection (human haematopoietic stem cell transplanted *NOD/SCID/IL2R*^{null} mice (Ito et al., 2002; Watanabe et al., 2007a; Watanabe et al., 2007b)) and engrafting with ZFN-modified CD4+ T cells, mice developed lower viral loads following challenge with HIV-1 (Perez et al., 2008). While this approach to gene therapy for HIV-1 infection offers great promise for the future, a potential drawback is the emergence of CCR5-independent viruses. Further, CCR5 deletion has been implicated in the defective control of parasitic infections in murine model systems for *Trypanosoma cruzi* (Hardison et al., 2006) and *Cryptococcus neoformans* (Huffnagle et al., 1999) and defective control of infection with West Nile virus in humans (Lim et al., 2006; Glass et al., 2006), suggesting that this approach to HIV therapy may not be feasible in countries where these pathogens are endemic. TRIMCyp-based approaches to lentiviral gene therapy offer advantages over other such approaches to therapy. By targeting viral entry the virus is denied the opportunity to replicate and thus escape mutants cannot be generated. Moreover, as TRIMCyp does not target the function of an endogenously expressed molecule, it is unlikely to have side effects that are detrimental to the host. Theoretically, it is possible that fusing a biologically potent CypA domain onto a TRIM5 RBCC in a species that has not evolved to accommodate a TRIMCyp fusion product

(humans or cats) may reveal unforeseen cryptic biological activities that are deleterious to immune function. Future studies should address the selective targeting of the TRIMCyp transgene to CD4+ T cells while minimising the likelihood of off-target effects. FIV infection of the domestic cat offers a unique opportunity to investigate the potential therapeutic utility of this approach to lentiviral gene therapy in a well-studied small animal model system for AIDS.

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Figure 1.

Scheme for the generation of a synthetic feline TRIM5 CypA gene fusion. As feline TRIM5 is truncated by the presence of a STOP codon in the genomic region homologous to human TRIM5 exon 8, the start codon of feline CypA was fused to the feline TRIM5 cDNA at the 3' end of exon 6. The gene fusion site for the synthetic feline TRIMCyp is also shown in comparison with the naturally-occurring macaque and owl monkey TRIMCyps, and the synthetic human TRIMCyp (huT5-S322-Cyp) (Neagu et al., 2009).

Figure 2.

Inhibition of FIV replication by stable expression of a synthetic feline TRIMCyp fusion protein. Duplicate cultures of CrFK cells were transduced with retroviral vectors bearing feline TRIMCyp (TRIMCyp6 and TRIMCyp7) and stably selected. TRIMCyp-expressing or vector-only control (CON) cells were then infected with FIV-Fca (Petaluma) or FIV-Pco (COLV). Viral growth was monitored by non-isotopic reverse transcriptase assay.

Figure 3.

Stable expression of feline TRIMCyp inhibits viral entry. CrFK cells stably transduced with feline TRIMCyp expression vectors (TRIMCyp6&7) or vector only (CON) were infected with HIV(VSV) or FIV(VSV)-GFP pseudotypes and viral entry quantified by flow cytometry (mean percentage, $n=3, +/ -SE$). Infection with either FIV or HIV-based pseudotypes was reduced significantly by feline TRIMCyp.