Requirements for capsid-binding and an effector function in TRIMCyp-mediated restriction of HIV-1

Felipe Diaz-Grifferoa, Nick Vandegraaffa, Yuan Lia, Kathleen McGee-Estradaa, Matthew Stremlaua, Sohanya Welikalaa, Zhihai Sia, Alan Engelmana, Joseph Sodroskiab,*

a Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute, Department of Pathology; Division of AIDS, Harvard Medical School, Boston, MA 02115, USA
b Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, MA 02115, USA

Received 25 January 2006; returned to author for revision 28 February 2006; accepted 16 March 2006
Available online 2 May 2006

Abstract

In owl monkeys, a retrotransposition event replaced the gene encoding the retroviral restriction factor TRIM5α with one encoding TRIMCyp, a fusion between the RING, B-box 2 and coiled-coil domains of TRIM5 and cyclophilin A. TRIMCyp restricts human immunodeficiency virus (HIV-1) infection by a mechanism dependent on the interaction of the cyclophilin A moiety and the HIV-1 capsid protein. Here, we show that infection by retroviruses other than HIV-1 can be restricted by TRIMCyp, providing an explanation for the evolutionary retention of the TRIMCyp gene in owl monkey lineages. The TRIMCyp-mediated block to HIV-1 infection occurs before the earliest step of reverse transcription. TRIMCyp-mediated restriction involves at least two functions: (1) capsid binding, which occurs most efficiently for trimeric TRIMCyp proteins that retain the coiled-coil and cyclophilin A domains, and (2) an effector function that depends upon the B-box 2 domain.

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Keywords: HIV; Restriction factors; Cyclophilin; Capsid; Uncoating; Retrovirus; Reverse transcription; Tropism

Introduction

Following entry into cells, some retroviruses encounter dominant blocks to infection that act prior to reverse transcription (Munk et al., 2002; Hofmann et al., 1999; Cowan et al., 2002; Besnier et al., 2002; Hatzioannou et al., 2003). Human immunodeficiency virus (HIV-1), for example, is blocked in the cells of most Old World monkeys (Hofmann et al., 1999; Song et al., 2005). Infection by the simian immunodeficiency virus of macaques (SIVmac) encounters similar restrictions in New World monkeys (Hofmann et al., 1999). These restrictions are mediated by TRIM5α, a tripartite motif protein with a RING, B-box 2 and coiled-coil (CC) domain, as well as a carboxy-terminal B30.2(SPRY) domain (Stremlau et al., 2004; Keckesova et al., 2004; Hatzioannou et al., 2004a; Yap et al., 2004; Perron et al., 2004; Nisole et al., 2005). The TRIM5α B30.2 domain is essential for retroviral restriction, and differences in the potency of TRIM5α from different species map to this domain (Sawyer et al., 2005; Stremlau et al., 2005; Nakayama et al., 2005; Yap et al., 2005). The viral determinant of susceptibility to TRIM5α-mediated restriction is the capsid protein, which assembles into the capsid structure that surrounds the viral genomic RNA and enzymes (Hatzioannou et al., 2004b; Owens et al., 2003, 2004; Kootstra et al., 2003). These results suggest that the TRIM5α B30.2 domain may interact with the capsid of the incoming virus, leading to a disruption of the infection process through an unknown mechanism.

Owl monkeys are an unusual New World monkey species that exhibit restrictions to HIV-1 and not SIVmac (Hofmann et al., 1999; Towers et al., 2003). Instead of TRIM5α, owl monkeys express TRIMCyp, a protein that consists of the RING, B-box 2 and CC domains of TRIM5 fused with a carboxy-terminal cyclophilin A (Cyp A) moiety (Sayah et al., 2004; Yap et al., 2004). The TRIMCyp gene was generated by a retrotransposition event involving the TRIM5 and Cyp A genes. Cyp A is known to bind the HIV-1 p24 soluble capsid protein through recognition of
Fig. 1. TRIMCyp deletions and retroviral restriction. (A) The wild-type TRIMCyp protein is depicted in the top figure. The numbers of the amino acid residues at the boundaries of the TRIMCyp domains are shown. The carboxyl terminus of the protein contains either a hemagglutinin (HA) epitope tag or a V5 tag. The regions retained in each of the TRIMCyp mutants are depicted. The position of the C96A change is indicated. (B) HeLa and Cf2Th cells were transduced with pLenti or LPCX vectors, respectively, expressing the indicated TRIMCyp proteins. The TRIMCyp proteins expressed in HeLa cells contain a carboxy-terminal V5 epitope tag; the TRIMCyp proteins expressed in the Cf2Th cells contain a carboxy-terminal HA epitope tag. Cell lysates were Western blotted and probed with antibodies directed against either the appropriate epitope tag (top panels) or β-actin (bottom panels). (C–F) HeLa cells expressing the wild-type and mutant TRIMCyp proteins or control HeLa cells transduced with the empty pLenti vector, were challenged with the indicated amounts of HIV-1-GFP (C, D), SIVagm-GFP (E), or SIVmac-GFP (F). The percentage of GFP-positive cells was determined by FACS. Similar results were obtained in three independent experiments. (G, H) Cf2Th cells expressing TRIMCyp or transduced with the empty LPCX vector were challenged with increasing amounts of FIV-GFP (G) or ELAV-GFP (H). The percentage of GFP-positive cells was determined by FACS. (I) HeLa cells stably expressing the wild-type and mutant TRIMCyp proteins were fixed and stained using a fluorescein isothiocyanate-conjugated anti-HA antibody as described in Materials and methods. Representative confocal microscope images are shown.
a surface-exposed loop (Gamble et al., 1996; Luban et al., 1993). Cyp A binding contributes to the efficiency of HIV-1 infection; the mechanism of this enhancement is unknown, although the prolyl isomerase activity of Cyp A has been suggested to modify the conformation of the HIV-1 capsid protein (Bosco et al., 2002; Bosco and Kern, 2004). Treatment of owl monkey cells with cyclosporine A (CsA), which blocks cyclophilin A-HIV-1 capsid interactions, relieves the restriction to HIV-1 infection in owl monkey cells (Towers et al., 2003). Thus, the interaction of TRIMCyp with the HIV-1 capsid protein is apparently important for the block to infection. TRIMCyp has been shown to bind the monomeric HIV-1 p24 capsid protein (Gamble et al., 1996; Luban et al., 1993). However, there is evidence suggesting that the assembled capsid is the biologically relevant target for TRIM5α and TRIMCyp interaction (Cowan et al., 2002; Forshey et al., 2005; Dodding et al., 2005). The TRIMCyp-mediated block to HIV-1 infection can be abrogated by exposure of owl monkey cells to HIV-1 virus-like particles that can introduce mature, but not unprocessed, capsid structures into the cytoplasm (Forshey et al., 2005). By contrast, expression of monomeric HIV-1 capsid proteins in cells does not relieve restriction (Dodding et al., 2005; Sebastian and Luban, 2005).

Here, we investigate retroviral restriction by TRIMCyp. We study the level of the block with respect to the retroviral infection cycle. We examine the range of lentiviruses susceptible to TRIMCyp restriction and demonstrate that infection by the SIV of African green monkeys (SIVagm) and feline immunodeficiency virus (FIV) can be inhibited by TRIMCyp. The role of each TRIMCyp domain in restriction is studied, and mutant TRIMCyp proteins that efficiently bind
HIV-1 capsids but do not restrict virus infection are identified. Thus, TRIMCyp-mediated restriction involves both capsid-binding and effector functions.

**Results**

*Ability of wild-type and mutant TRIMCyp to restrict various retroviruses*

HIV-1 is the only virus that has currently been shown to be restricted by TRIMCyp (Sayah et al., 2004; Nisole et al., 2004). To examine whether TRIMCyp restriction might extend to other retroviruses and to investigate the contribution of TRIMCyp domains to its antiviral function, we constructed TRIMCyp mutants lacking the RING, B-box 2 and coiled coil domains, or the linker region between the coiled coil and cyclophilin A (Fig. 1A). Several of these mutants were expressed stably and efficiently in human HeLa and canine Cf2Th cells (Fig. 1B). Cells were challenged with VSV G-pseudotyped, GFP-expressing vectors based on HIV-1, SIVmac, SIVagm, FIV and EIAV (Figs. 1C–H). The wild-type TRIMCyp potently inhibited infection by HIV-1, SIVagm and FIV, but did not affect the efficiency of SIVmac and EIAV infection. Thus, retroviruses other than HIV-1 are susceptible to TRIMCyp-mediated restriction.

Deletion of the TRIMCyp RING domain diminished, but did not eliminate, the inhibition of HIV-1 and SIVagm infection (Table 1 and Figs. 1C and E). Deletion of the RING and B-box
Effect of expression of TRIMCyp variants on HIV-1 infection

<table>
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<tr>
<th>TRIMCyp variant</th>
<th>%GFP-positive cells&lt;sup&gt;a&lt;/sup&gt;</th>
<th>HeLa</th>
<th>C2Th</th>
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<td>70 (100%)</td>
<td>97 (100%)</td>
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<td>10 (14%)</td>
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<td>TRIMCyp Δ93</td>
<td>34 (48%)</td>
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<tr>
<td>TRIMCyp Δ128</td>
<td>80 (114%)</td>
<td>61 (62%)</td>
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<tr>
<td>TRIMCyp Δ245</td>
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<td>88 (91%)</td>
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<tr>
<td>TRIMCyp Δ310</td>
<td>ND</td>
<td>91 (94%)</td>
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ND = not determined.

* The percentage of GFP-positive cells after incubation with HIV-1-GFP is shown. Numbers in parenthesis are normalized to the value of GFP-positive cells seen in the control cells transduced with the empty vector.

2 domains completely eliminated HIV-1-inhibitory activity. A slight but reproducible inhibitory effect of the TRIMCyp Δ245 protein on HIV-1 infection of HeLa cells was observed; this may be due to overexpression of cyclophilin A activity, which has been suggested to be detrimental to HIV-1 infection in some contexts (Hatzioannou et al., 2005; Sayah and Luban, 2004; Gatanaga et al., 2006). Expression of the TRIMCyp Δ93, TRIMCyp Δ128 and TRIMCyp Δ245 constructs resulted in partial inhibition of SIV<sub>agm</sub> infection, compared with the wild-type TRIMCyp protein. Thus, the RING and B-box 2 domains of TRIMCyp contribute to the efficiency of the antiviral activity.

In the C96A TRIMCyp mutant, a cysteine residue that is predicted to coordinate a zinc atom in the B-box 2 domain is disrupted. TRIMCyp C96A stably expressed in HeLa cells did not restrict HIV-1 when compared to wild-type TRIMCyp (Fig. 1D). Thus, disruption of the B-box domain by deletion (TRIMCyp Δ128) or by an individual amino acid change (TRIMCyp C96A) impaired the ability of TRIMCyp to restrict HIV (Figs. 1C and D), suggesting that an intact B-box domain is required for efficient antiretroviral activity.

We also analyzed the subcellular localization of wild-type and mutant TRIMCyp proteins (Fig. 1I). Similar to TRIM5α<sub>rh</sub>, TRIMCyp was located in the cytoplasm and also formed cytoplasmic bodies. TRIMCyp Δ93 presented a diffuse cytoplasmic staining pattern. Unlike TRIM5α<sub>rh</sub> Δ93 (Javanbakht et al., 2005), TRIMCyp Δ93 did not exhibit nuclear localization or form nuclear bodies. TRIMCyp Δ128 formed very unusual spiral-like structures in the perinuclear region. TRIMCyp C96A exhibited a pattern of intracellular staining similar to that of wild-type TRIMCyp. TRIMCyp Δ245 and Δ310 were localized diffusely throughout the nucleus and cytoplasm.

To evaluate the ability of the TRIMCyp mutants to interfere with the antiretroviral activity of an endogenously expressed TRIMCyp protein, the mutants were stably expressed in owl monkey kidney (OMK) cells (Fig. 2A). The cells were incubated with different amounts of HIV-1-GFP (Fig. 2B). Expression of wild-type and Δ93 TRIMCyp proteins increased the degree of HIV-1 restriction in these cells, whereas the TRIMCyp Δ128 and Δ245 variants slightly decreased the level of HIV-1 restriction. Expression of the coiled-coil domain of TRIMCyp (TRIMCyp CC(128–245)) in OMK cells more potently relieved the restriction of HIV-1 infection mediated by endogenous TRIMCyp (Fig. 2B). To confirm these results, we stably coexpressed the coiled-coil domain of TRIMCyp and wild-type TRIMCyp in C2Th cells (Fig. 2C).

Expression of the coiled-coil domain alone diminished the antiretroviral activity of TRIMCyp (Fig. 2D). One possible explanation for this dominant-negative activity is that the interaction of the coiled-coil domain of TRIMCyp with wild-type TRIMCyp disrupts the antiretroviral activity. To evaluate the ability of these molecules to interact, we attempted to coprecipitate the TRIMCyp coiled coil with wild-type TRIMCyp (Fig. 2E). As expected, TRIMCyp interacted with TRIMCyp CC(128–245), consistent with the observed dominant negative activity.

**Requirement for TRIMCyp interaction with the viral capsid for restriction**

We wished to confirm previous observations suggesting that TRIMCyp interaction with the HIV-1 capsid is necessary for restriction (Towers et al., 2003; Sayah et al., 2004; Nisole et al., 2004; Chatterji et al., 2005) and to examine whether this is true for TRIMCyp-mediated restriction of other viruses such as SIV<sub>agm</sub> and FIV. HeLa cells expressing TRIMCyp or transduced with the control pLenti vector were challenged with either HIV-1-GFP or SIV<sub>agm</sub>-GFP in the presence or absence of cyclosporine A (Figs. 3A and B). Similarly, C2Th cells expressing TRIMCyp or transduced with the control LPCX vector were challenged with FIV-GFP in the presence or absence of cyclosporine A (Fig. 3C). Cyclosporine A, which inhibits the binding of cyclophilin A to its substrates (Luban et al., 1993), completely eliminated the TRIMCyp-mediated restriction of HIV-1, SIV<sub>agm</sub> and FIV. Cyclosporine A treatment also resulted in an increase in FIV infection in the C2Th canine cells transduced with the control vector (Fig. 3C). These results support a model in which TRIMCyp must bind to components of the targeted virus to achieve restriction of infection.

To demonstrate the role of the viral capsid in susceptibility to TRIMCyp-mediated restriction, OMK cells or control C2Th cells were challenged with recombinant GFP-expressing viruses with either wild-type HIV-1 or variant capsids (Figs. 3D and E). As previously reported (Towers et al., 2003), HIV-1 capsids with changes in glycine 89 or proline 90, which are important for cyclophilin A binding (Gamble et al., 1996; Vajdos et al., 1997; Yoo et al., 1997), were less susceptible than the wild-type HIV-1 capsid to the restriction in OMK cells. HIV-1 viruses with SIV<sub>mac</sub> capsid proteins (HIV(SCA)) were also restricted less than the wild-type HIV-1 in these cells. Infection by SIV<sub>mac</sub> with an HIV-1 capsid (SIV (HCA-p2)) was inefficient in the control C2Th cells; in the OMK cells, infection by this virus was undetectable. These results support the importance of the interaction of the cyclophilin A moiety on TRIMCyp with the HIV-1 capsid for restriction of infection.
Fig. 2. Dominant-negative effects of expression of TRIMCyp variants. (A) Owl monkey kidney (OMK) cells were transduced with pLenti vectors expressing the indicated TRIMCyp variants with carboxy-terminal V5 epitope tags. Cell lysates were Western blotted with an antibody against the V5 epitope (top panel) or against β-actin (bottom panel). (B) OMK cells expressing the indicated TRIMCyp variants or transduced with the empty pLenti vector were challenged with HIV-1-GFP and GFP-positive cells were measured by FACS. The results of a typical experiment are shown; the experiment was repeated twice with similar results. (C, D) Cf2Th cells were transduced with a pLenti vector expressing the coiled-coil domain of TRIMCyp with a V5 epitope tag. Afterwards cells were transduced with an LPCX vector expressing HA-tagged wild-type TRIMCyp or with an empty LPCX vector. (C) Cells lysates were analyzed by Western blot using antibodies against HA (upper panel), V5 (middle panel) and β-actin (lower panel). In panel D, the Cf2Th cells were incubated with HIV-1-GFP, and GFP-positive cells were measured by FACS. Results of two experiments were similar, and the results of one experiment are shown. (E) 293T cells were cotransfected with the control pLPCX plasmid or a plasmid encoding wild-type TRIMCyp-HA protein and a plasmid encoding TRIMCyp CC(128–245), which consists of the coiled-coil domain of TRIMCyp tagged with a V5 epitope. The 293T cells were lysed 36 h after transfection. Cell extracts were analyzed by Western blotting using antibodies against the V5 tag, HA tag or β-actin. Cell lysates were used for immunoprecipitation (IP) by the anti-HA antibody. The precipitates were Western blotted (WB) with an anti-V5 antibody (bottom panel). Similar results were obtained in three independent experiments.
Timing and level of TRIMCyp restriction of HIV-1

Because TRIMCyp restriction of HIV-1 can be relieved by cyclosporine A treatment (Owens et al., 2004; Towers et al., 2003), the timing of TRIMCyp action could be investigated. HIV-1-GFP viruses were incubated with control Cf2Th cells transduced with the LPCX vector or Cf2Th cells expressing TRIMCyp, and cyclosporine A was added at various time points. In some arms of the experiment, the lysosomotropic agent ammonium chloride was added along with cyclosporine A to prevent further virus entry into the cells (Seglen, 1983; Matlin et al., 1982; Aiken, 1997). The resulting level of infection was determined by quantitating GFP-positive cells (Fig. 4A). The results indicate that 62 and 78 percent of viruses that enter the cells within the first or second hour, respectively, of incubation are inhibited by TRIMCyp in a manner that is not subsequently reversible by cyclosporine A. The half-life of infectious viruses inside TRIMCyp-expressing cells is less than 1 h.

We also measured the production of HIV-1 reverse transcripts in Cf2Th cells expressing TRIMCyp or TRIM5αrh. The infection of these cells was very inefficient compared with
that of control cells transduced with the LPCX vector (Fig. 4B). Measurement of HIV-1 reverse transcripts in these cells by real-time PCR revealed that even the earliest product of reverse transcription, strong-stop cDNA, was not synthesized efficiently in cells expressing TRIMCyp or TRIM5αrh (Figs. 4C and D). Consistent with these results, strong-stop cDNA was not observed in OMK cells incubated with HIV-1 virus preparations (data not shown). Thus, TRIMCyp blocks HIV-1 infection at an early, post-entry step, before reverse transcription is initiated.

**TRIMCyp oligomerization**

Old World monkey TRIM5α proteins have been shown to form trimers (Mische et al., 2005). To study the oligomerization of wild-type TRIMCyp and TRIMCyp mutants, these proteins were expressed transiently in 293T cells. Cell lysates were cross-linked with glutaraldehyde and then Western blotted. Fig. 5 shows that wild-type and RING- and B-box 2-deleted TRIMCyp proteins formed trimers and higher-order oligomers. By contrast, TRIMCyp Δ245, which is missing the coiled coil, and TRIMCyp CC(128–245), which contains only the coiled-coil, did not form oligomers efficiently. Thus, the TRIMCyp coiled coil is necessary but not sufficient for trimer/oligomer formation.

The HA-tagged wild-type TRIMCyp protein was coexpressed transiently in 293T cells with V5-tagged wild-type and mutant TRIMCyp proteins. Precipitation of the wild-type TRIMCyp protein with an anti-HA antibody coprecipitated TRIMCyp, TRIMCyp Δ93 and TRIMCyp Δ128 proteins, but not the TRIMCyp Δ245 protein (Fig. 6). These results support a model in which the RING and B-box 2 domains are dispensable for TRIMCyp self-association; by contrast, the coiled coil is essential for efficient self-association.

**TRIMCyp interaction with HIV-1 CA–NC complexes**

To investigate the ability of TRIMCyp to interact with the HIV-1 capsid, we incubated 293T cell lysates containing TRIMCyp variants with HIV-1 CA–NC complexes assembled in vitro. These CA–NC complexes have been shown by cryoelectron microscopy to simulate closely the architecture of the bona fide HIV-1 capsid (Ganser-Pornillos et al., 2004). After incubation with TRIMCyp-containing lysates, the CA–NC complexes were pelleted through 70% sucrose cushions, and the pellets were analyzed for CA–NC and TRIMCyp proteins. Fig. 7A shows that TRIMCyp copellets with the HIV-1 CA–NC

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**Fig. 4.** Early post-entry restriction of HIV-1 by TRIMCyp. (A) Cf2Th cells expressing TRIMCyp or transduced with the control vector LPCX were challenged with HIV-1-GFP. Cyclosporine A (CsA), with or without ammonium chloride, was added at the indicated times after virus–cell incubation. The amount of HIV-1-GFP used was sufficient to infect nearly all of the Cf2Th cells transduced with the control LPCX vector. Similar results were obtained in three independent experiments. The percentage of entering viruses that were restricted by TRIMCyp in the period prior to the time at which cells were treated with cyclosporine A and ammonium chloride was calculated using the formula shown. The calculated percentage at each hourly time point is shown. (B–D) Cf2Th cells stably expressing TRIMCyp or TRIM5αrh, or transduced with the empty LPCX vector, were challenged with equal amounts of the HIV-1 luciferase reporter virus or a control virus without envelope glycoproteins (Δenv). Luciferase activity in the cells was measured 48 h later. Infectivity was expressed in relative light units (B). Similar infections were performed and HIV-1 extrachromosomal DNA was extracted at 0 and 6 h post-infection. DNA samples were used to detect strong-stop cDNA (C) and complete LTR copies (D) per 6000 cell equivalents by real-time PCR. Similar results were obtained in three independent experiments.
complexes, and that this association can be completely inhibited by cyclosporine A. TRIMCyp proteins (TRIMCyp Δ93, TRIMCyp Δ128, TRIMCyp Δ95–128, and TRIMCyp C96A) lacking intact RING or B-box 2 domains interacted efficiently with the CA–NC complexes (Figs. 7B and D). By contrast, proteins consisting of the TRIMCyp cyclophilin A domain or the cyclophilin A domain and the linker region did not efficiently bind CA–NC complexes (Fig. 7B). The linker region between the coiled coil and cyclophilin A domain was not necessary for CA–NC association (TRIMCyp Δ232–310) (Fig. 7D). The TRIMCyp coiled coil alone (TRIMCyp CC (128–245)) did not interact with the CA–NC complexes (Fig. 7C). These results suggest that both the coiled coil and cyclophilin A moieties are important for efficient TRIMCyp association with HIV-1 capsid complexes.

To determine whether TRIMCyp directly interacts with the HIV-1 capsid, we expressed TRIMCyp as a thioredoxin fusion protein in bacteria and incubated the purified protein with HIV-1 CA–NC complexes assembled in vitro. Pure thioredoxin-TRIMCyp bound the CA–NC complexes, and treatment with cyclosporine A abolished this interaction (Fig. 7E). Only minimal thioredoxin-TRIMCyp was found in the pellet in the absence of added HIV-1 CA–NC complexes. Thus, TRIMCyp directly associates with the HIV-1 capsid in a manner independent of any eukaryotic proteins.

Discussion

New World monkeys of the genus *Aotus*, the owl or night monkeys, are the only known primates to express TRIMCyp (Ribeiro et al., 2005). The TRIMCyp gene apparently arose as a result of a retrotransposition event involving the cyclophilin A and TRIM5 genes (Sayah et al., 2004; Nisole et al., 2004). This retrotransposition occurred after the divergence of owl monkeys from other New World monkeys. TRIMCyp is maintained in all
Fig. 7. TRIMCyp binding to assembled HIV-1 capsids. 293T cells were transfected with wild-type and mutant TRIMCyp constructs tagged with V5 epitopes. Thirty-six hours after transfection, cells were lysed and the lysates mixed with in vitro assembled HIV-1 CA–NC complexes. The mixtures were applied to a 70% sucrose cushion. INPUT represents the mixtures analyzed by Western blot before being applied to the 70% cushion. The INPUT mixtures were blotted for the V5 tag, the HIV-1 CA–NC protein and, in some cases, GADPH. The PELLET from the 70% cushion was analyzed by Western blotting using antibodies against the V5 tag and HIV-1 CA–NC protein. The effect of adding cyclosporine A (CsA) to the mixture of TRIMCyp-containing cell lysate and CA–NC complexes is shown in panel A. In panels B, C, and D, the binding of the indicated TRIMCyp mutants to the HIV-1 CA–NC complexes is examined. In panel E, the binding of the pure bacterially expressed thioredoxin-TRIMCyp fusion protein [HP-Thio-TRIMCyp(V5)] to in vitro assembled HIV-1 CA–NC complexes in the presence or absence of CsA is shown.
extant Aotus species (Ribeiro et al., 2005) and has replaced TRIM5, which encodes the presumably beneficial retroviral restriction factor TRIM5α. This observation implies that TRIMCyp is beneficial to owl monkeys, perhaps more so than TRIM5α. A puzzling aspect of this expectation is that the only virus known to be restricted by TRIMCyp is HIV-1, which is unlikely to have imposed selective pressure on Aotus lineages. Here, we show that several other retroviruses, SIVAgm and FIV, are susceptible to TRIMCyp-mediated restriction. Thus, it is probable that, during owl monkey evolution, the expression of TRIMCyp conferred resistance to infection by retroviruses.

The abrogation of TRIMCyp restriction of HIV-1, SIVAgm and FIV restriction by cyclosporine A implies that the cyclophilin A moiety of TRIMCyp must functionally interact with viral components. For HIV-1, escape from TRIMCyp-mediated suppression can be achieved by alteration of capsid residues 89 and 90, a glycine-proline pair that is known to contact cyclophilin A (Gamble et al., 1996; Vajdos et al., 1997). Cyclophilin A binding to the HIV-1 capsid has been suggested to exert positive, but as yet poorly understood, effects on virus infection (Hatzioannou et al., 2005; Sayah and Luban, 2004; Gatanaga et al., 2006). Perhaps other viruses besides HIV-1 naturally interact with cyclophilin A and thus are rendered susceptible to TRIMCyp restriction in owl monkey cells. Indeed, an inspection of the primary sequences of retroviral capsids reveals the existence of glycine-proline motifs in several lentiviral capsids, in regions analogous to the cyclophilin A-binding loop of HIV-1 (Fig. 8). The exact requirements for susceptibility to TRIMCyp-mediated restriction require further investigation; the mere presence of a glycine-proline motif in a surface-exposed capsid loop is not sufficient for TRIMCyp restriction, as evidenced by equine infectious anemia virus (Fig. 8) (Jin et al., 1999).

Cyclophilin A can interact with the monomeric p24 capsid protein of HIV-1 with low affinity (Gamble et al., 1996; Vajdos et al., 1997; Yoo et al., 1997). TRIMCyp has also been reported to exhibit some binding to HIV-1 capsid proteins, although no estimation of the affinity of this interaction was attempted (Nisole et al., 2004). The natural target for TRIMCyp and TRIM5α is the assembled, mature viral capsid (Dodding et al., 2005; Forshey et al., 2005). Recently, we have demonstrated a specific interaction between TRIM5α variants and in vitro assembled HIV-1 CA–NC complexes such as those used in this study (Stremlau et al., in press). The efficient TRIM5α-capsid interaction is dependent on conditions that preserve the integrity and three-dimensional architecture of the HIV-1 capsid. It is likely that TRIM5α trimerization contributes to the avidity of interaction with retroviral capsids, which exhibit trimeric pseudosymmetry (Ganser et al., 1999; Li et al., 2000). Here, we show that TRIMCyp also interacts with HIV-1 CA–NC complexes assembled in vitro. This interaction is inhabitable by cyclosporine A and is dependent on the integrity of the coiled-coil and cyclophilin A domains of TRIMCyp. Notably, the cyclophilin A moiety of TRIMCyp alone did not bind efficiently to the HIV-1 CA–NC complexes, supporting the contribution of TRIMCyp oligomerization to the avidity of capsid interaction.

One notable difference between TRIMCyp and TRIM5α binding to the HIV-1 CA–NC complexes is that the linker between the coiled coil and the C-terminal domain is dispensable for TRIMCyp, but not for TRIM5α, binding (Stremlau et al., in press). This linker may influence the spatial relationships between the TRIMCyp and TRIM5α carboxy-terminal domains and the trimer axis. Such spacing may be less critical for the cyclophilin A moiety of TRIMCyp, which interacts with potentially flexible surface-exposed loops on the capsid, than for the TRIM5α B30.2(SPRY) domain, which is hypothesized to interact with rigid, recessed holes on the capsid surface (Mische et al., 2005). The linker region might allow each of the three B30.2(SPRY) domains of TRIM5α to engage their respective pockets on the capsid surface.

Importantly, we demonstrate that the amino-terminal TRIMCyp domains, although contributing to the antiretroviral function of TRIMCyp, are not required for efficient interaction with the HIV-1 CA–NC complex. Consistent with the possible contribution of TRIMCyp oligomerization to capsid-binding avidity, neither the RING domain nor the B-box 2 domain is required for the trimerization of TRIMCyp. Apparently, TRIMCyp mutants with defective or missing RING and B-box 2 domains can bind capsids but do not negotiate an effector function critical for interference with the infection pathway. TRIMCyp mutants with altered RING or B-box 2 domains were at least partially located in the cytoplasm. Therefore, if the effector function dictates proper location in the cell, it must determine a more specific cytoplasmic compartment. Other possibilities for “effector” functions include particular types of self-association or association with cofactors.

Both TRIMCyp and TRIM5α restrict retrovirus infection soon after the entry of the viral capsid into the cytosol (Perez-Caballero et al., 2005). Even the earliest step in reverse transcription, the formation of strong-stop cDNA, does not occur efficiently in cells expressing the restricting protein. The mechanism of TRIMCyp restriction clearly involves some action directed towards the viral capsid. Of interest, it has been observed that the binding of excess cyclophilin A can be detrimental to HIV-1 infection (Gatanaga et al., 2006). Indeed, we observed a mild negative effect on HIV-1 infection of expression of TRIMCyp Δ245, which consists only of the linker region and the cyclophilin A moiety. The increase in capsid-binding avidity resulting from TRIMCyp oligomerization could result in unfavorable levels of the cyclophilin A moiety bound to the HIV-1 capsid. However, only when RING and B-box 2 domains are intact does the full level of restriction ensue. Defining the effector function should advance our understanding of TRIMCyp-mediated restriction.

Of interest, cyclosporine A treatment of canine cells transduced with the empty control vector significantly increased the ability of FIV to infect these cells. This observation raises
the possibility that canine cyclophilin A or a cyclophilin A-dependent factor exerts a negative effect on FIV infection. Our understanding of such host factors may suggest strategies for intervention in retroviral infections.

**Materials and methods**

**Plasmid construction**

The plasmids expressing the wild-type and mutant TRIMCyp proteins tagged with a carboxy-terminal hemagglutinin (HA) epitope were constructed using PCR. The TRIMCyp cDNA was PCR-amplified and digested with EcoRI and ClaI, whose sites were introduced by inclusion in each of the PCR primers. These fragments were cloned into the EcoRI and ClaI site of pLPCX (Stratagene). The plasmids expressing wild-type and mutant TRIMCyp tagged with a carboxy-terminal V5 epitope were constructed by inserting a PCR-amplified TRIMCyp cDNA into the Viral Power plasmid using directional TOPO cloning (Invitrogen).

**Cells and drugs**

HeLa epithelial cells and Cf2Th canine thymocytes were obtained from ATCC. A 10 mM stock solution of cyclosporine A (Sigma) in ethanol was used to achieve a final concentration of 5 μM in cell media.

**Creation of cells stably expressing TRIMCyp variants**

Retroviral vectors encoding wild-type or mutant TRIMCyp proteins were created using the pLPCX vector. Recombinant viruses were produced in 293T cells by cotransfecting the pLPCX plasmids with the pVPack-GP and pVPack-VSV-G packaging plasmids (Stratagene). The pVPack-VSV-G plasmid encodes the vesicular stomatitis virus (VSV) G envelope glycoprotein, which allows efficient entry into a wide range of vertebrate cells. Transduced cells were selected in 1 μg/ml puromycin (Sigma).

Recombinant lentiviruses expressing wild-type and mutant TRIMCyp-V5 proteins were made using the Viral Power system (Invitrogen) according to the manufacturer’s protocol. The resulting virus particles were used to transduce approximately 1×10^6 HeLa cells in the presence of 5 μg/ml polybrene. Transduced cells were selected in 2 μg/ml blasticidin (Invitrogen). Cell lines expressing both wild-type and mutant TRIMCyp proteins were obtained by transduction with both LPCX and Viral Power vectors and dual selection with puromycin and blasticidin.

**Infection with viruses expressing green fluorescent protein (GFP)**

Recombinant HIV-1, SIVmac and SIVagm expressing GFP were prepared as described (Stremlau et al., 2004). Viral stocks
were quantified by measuring reverse transcriptase (RT) activity. For infections, \(3 \times 10^4\) HeLa or Cf2Th cells seeded in 24-well plates were incubated with virus for 24 h. Cells were washed and returned to culture for 48 h and then subjected to FACS analysis with a FACScan (Becton Dickinson). The feline immunodeficiency virus (FIV) vector was obtained from System Biosciences (Mountain View, CA) and was prepared following the manufacturer’s instructions. The equine infectious anemia virus (EIAV) vector was a gift from Dr. John Olsen (1998). FIV and EIAV viral stocks were titrated by serial dilution on Cf2Th cells.

**Protein analysis**

Cellular proteins were extracted with radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris, pH 7.4; 100 mM NaCl; 1% sodium deoxycholate; 0.1% sodium dodecyl sulfate [SDS]; 1% NP-40; 2 mg/ml aprotinin; 2 mg/ml leupeptin; 1 mg/ml pepstatin A; 100 mg/ml phenylmethylsulfonyl fluoride). The cell lysates were analyzed by SDS-PAGE (10% acrylamide), followed by blotting onto nitrocellulose membranes (Amer- sham Pharmacia Biotech). Detection of protein by Western blotting utilized monoclonal antibodies directed against the HA (Roche) or V5 (Invitrogen) epitope tags and monoclonal antibodies to β-actin (Sigma). Detection of proteins was performed by enhanced chemiluminescence (NEN Life Sciences Products), using antimouse (for V5 and β-actin) and antirat (for HA) secondary antibodies (Amersham Pharmacia Biotech).

**TRIMCyp localization experiments**

Localization of TRIMCyp variants in expressing cells was investigated as previously described (Diaz-Griffero et al., 2002). Briefly, cells were grown overnight on 12-mm-diameter coverslips and fixed in 3.9% paraformaldehyde (Sigma) in phosphate-buffered saline (PBS; Cellgro) for 30 min. Cells were washed in PBS, incubated in 0.1 M glycine (Sigma) for 10 min, washed in PBS, and permeabilized with 0.05% saponin (Sigma) for 30 min. Samples were blocked with 10% donkey serum (Dako, Carpinteria, CA) for 30 min, and incubated for 1 h with antibodies. The anti-HA FITC-conjugated 3F10 antibody (Roche) was used to stain HA-tagged TRIM5Cyp proteins, respectively. Subsequently, samples were mounted for fluorescence microscopy using the ProLong Antifade Kit (Molecular Probes, Eugene, OR). Images were obtained with a BioRad Radiance 2000 laser scanning confocal microscope with Nikon 60× N.A.1.4 optics.

**Immunoprecipitations**

At 48 h after transfection, 293T cells were removed from the plate and washed with PBS. 293T cells from nearly confluent 100-mm plates were lysed in 500 µl IP buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM DTT, 0.5% NP-40, Protease Inhibitor Cocktail). Insoluble material was pelleted at 22,000 \(\times\) g for 2 hours and the supernatant was used for immunoprecipitation. Equal amounts of protein (approximately 200–500 µg, as determined by the BioRad assay) were incubated with 30 µl protein A-Sepharose (50%) and 5 µl of anti-HA antibody (Roche) for 2 hours at 4 °C. The precipitates were then washed three times with IP buffer and twice with PBS. After the final supernatant was removed, 30 µl of 2× sample buffer (120 mM Tris–HCl, pH 6.8, 20% glycerol, 4% SDS, and 0.02% bromphenol blue) was added, and the samples were boiled for 5 min. Supernatants were loaded on SDS-polyacrylamide gels and analyzed by Western blotting using anti-HA or anti-V5 antibodies.

**HIV-1 luciferase reporter virus**

The plasmid pNLX.Luc(R-) and the expression vector encoding vesicular stomatitis virus G (VSV-G) glycoprotein (pSVS-G) have been previously described (Lu et al., 2004). Single-round viruses carrying the VSV-G envelope glycoprotein were derived by co-transfecting 293T cells with pNLX.Luc(R-) and pVSV-G at the ratio of 10:1 in the presence of calcium phosphate. Culture supernatants were collected 36 h after transfection, filtered through 0.45-µm-diameter pores, and treated for 1 h at 37 °C with 40 U/ml Turbo DNase (Ambion) prior to use. Cell-free virus titers were determined with an exogenous \(^{32}\)P-based reverse transcriptase assay. Twenty-four hours prior to infection, cells (2 × 10⁵/well) were plated in 6-well trays. For infections with recombinant viruses, cells were incubated with 1 × 10⁶ (Luciferase assays) or 5 × 10⁶ (DNA assays) cpm of RT activity in a final volume of 500 µl for 2 h. Medium was then added to a final volume of 2 ml, and the cells were cultured until harvesting. At 48 h after infection, cells were washed once in PBS and then lysed in 150 µl of Passive Lysis Buffer (Promega Corp.). Cell lysates were centrifuged at 20,630 \(\times\) g for 15 min at 4 °C to pellet debris, diluted 1:100 in Passive Lysis Buffer, and then 20 µl was analyzed using the Luciferase Assay Reporter System (Promega Corp.) as recommended by the manufacturer. Luciferase values were normalized to the total protein content of extracts as determined by the Bio-Rad Protein Assay (Bio-Rad Laboratories).

**Real-time PCR**

Extrachromosomal HIV-1 DNA was extracted from cells following infection as previously described (Vandegraaff et al., 2001) and DNA resuspended at approximately 6000 cell equivalents/µl (c.e./µl). Relative levels of total DNA in extracts were determined by amplification of mitochondrial DNA sequences using a semi-quantitative real-time PCR (qPCR). Primers for amplification of mitochondrial DNA (designed to amplify both canine and human sequences) were 5′-GGGATAACAGCGCAATCCTATTC and 5′-ACGTAGGACTTTAATCGTTGAAC.

PCR reactions (30 µl) were performed using the QuantiTect Sybr Green PCR Kit (Qiagen) under conditions recommended by the manufacturer. Reactions were cycled as follows: 95 °C
for 15 min, followed by 40 cycles of 94 °C for 15 s, 58 °C for 30 s, and 72 °C for 30 s. Melting curve analyses (from 55 °C to 90 °C, read every 1 °C) were conducted to confirm amplification of predominantly a single DNA species. PCR reactions assessing strong-stop and late reverse transcription products were performed using primers and probes as previously described (Julias et al., 2001; Butler et al., 2001). PCR reactions (30 μl) were performed using the Quantitect Probe PCR Kit (Qigagen) under conditions recommended by the manufacturer. Reactions were cycled as follows: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Serial dilutions of known amounts of plasmid pNLX.Luc(R-) served as a copy number standard to generate standard curves. PCR reactions were performed in a DNA Engine Opticon thermal cycler (MJ Research Inc., Waltham, Mass), and results were analyzed using the OpticonMONITOR Analysis Software version 2.01 supplied by the manufacturer.

TRIMCyp cross-linking assay

Approximately 1 × 10^7 293T cells transfected with 5 μg of plasmids expressing TRIMCyp variants were lysed in IP buffer and cross-linked with different concentrations (0–5 mM) of glutaraldehyde for 5 min as previously described (Mische et al., 2005). After cross-linking, samples were taken up in 2× sample buffer and incubated at 37 °C for 30 min. Samples were analyzed by SDS-PAGE and Western blotting with an anti-V5 antibody (Invitrogen).

HIV CA–NC expression and purification

HIV-1 CA–NC protein was expressed, purified, and assembled as previously described (Ganser et al., 1999). The pET11a expression vector (Novagen) expressing CA–NC of HIV-1 was used to transform BL-21(DE3). CA–NC expression was induced with 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) when cells achieved an optical density at 600 nm of 0.6. After 4 h of induction, cells were harvested and resuspended in 20 mM Tris–HCl (pH 7.5), 1 mM ZnCl2, 10 mM 2-mercaptoethanol and protease inhibitors (Roche). Bacteria were lysed by sonication and debris was pelleted for 30 min at 35,000×g. Nucleic acids were stripped from the solution by using 0.11 equivalents of 2 M (NH4)2SO4 and the same volume of 10% polyethyleneimine. Nucleic acids were removed by stirring and centrifugation at 29,500×g for 15 min. Protein was recovered by addition of 0.35 equivalents of 2 M (NH4)2SO4. Protein was centrifuged at 98,200×g for 15 min and resuspended in 100 mM NaCl, 20 mM Tris–HCl (pH 7.5), 1 mM ZnCl2 and 10 mM 2-mercaptoethanol. Protein was dialyzed against 50 mM NaCl, 20 mM Tris–HCl (pH 7.5), 1 mM ZnCl2, and 10 mM 2-mercaptoethanol.

In vitro assembly of HIV-1 capsid complexes

HIV-1 capsid complexes were assembled in vitro by diluting the HIV-1 CA–NC protein to a concentration of 0.3 mM in 50 mM Tris–HCl (pH 8.0), 0.5 M NaCl and 2 mg/ml DNA oligo (TG)50. The mixture was incubated at 4 °C overnight and centrifuged at 8600×g for 5 min. The pellet was resuspended in assembly buffer (50 mM Tris–HCl (pH 8.0), 0.5 M NaCl) at a final protein concentration of 0.15 mM (Ganser-Pornillos et al., 2004).

TRIMCyp binding to HIV-1 CA–NC complexes

293T cells were transfected with plasmids expressing different TRIMCyp or TRIM5 proteins. Forty-eight hours after transfection, cell lysates were prepared as follows: Washed cells were resuspended in hypotonic lysis buffer (10 mM Tris, pH 7.4, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT). The cell suspension was frozen and thawed and then incubated on ice for 10 min. Afterwards, the lysate was centrifuged at 14,000×g in a refrigerated microcentrifuge for 5 min. The supernatant was supplemented with 1/10 volume of 10× PBS and then used in the binding assay. Five milliliters of in vitro assembled CA particles were incubated with 200 μl of cell lysate at room temperature for 1 h. A fraction of this mixture was stored (INPUT). The remainder of the mixture was spin through a 70% sucrose cushion (70% sucrose, 1× PBS and 0.5 mM DTT) at 100,000×g in an SW55 rotor (Beckman) for 1 h at 4 °C. After centrifugation, the supernatant was carefully removed, and the pellet was resuspended in 1× SDS-PAGE loading buffer (PELLET). The level of TRIMCyp or TRIM5 proteins was determined by Western blot.

TRIMCyp expression in E. coli

Owl monkey TRIMCyp was cloned in the pBAD vector following the manufacturer’s directions (pBAD/TOPO ThioFusion, Invitrogen). This vector fused a Histidine patch thiorodoxin to the amino terminus of TRIMCyp and a V5-6His tag at the carboxyl terminus. DH5α cells were transformed, and expression was induced by using 0.2% l-arabinose. Induction was performed at an optical density (OD600) of 0.6 for 5 h. Cells were harvested and lysed by sonication in Buffer A [200 mM NaCl, 50 mM Tris–HCl (pH 8.0), 1 mM DTT and protease inhibitors (Roche)]. The lysate was centrifuged at 15,000 rpm in a JA-10 rotor (Beckman). The pellet was washed twice in buffer A containing 0.5% Triton X-100 and twice in buffer A containing 2 M urea. The remaining pellet was dissolved in buffer A containing 8 M urea. Supernatant was diluted 1/10 in buffer A and purified by using a Ni-NTA resin (Qiagen). Protein was eluted by using buffer A in 200 mM imidazole. Two dialysis steps against buffer A were performed. The thioredoxin-TRIMCyp protein was snap frozen in liquid nitrogen and stored at −80 °C. The thioredoxin-TRIMCyp protein was estimated to be 90% pure by Coomassie staining of SDS-polyacrylamide gels.

Acknowledgments

We thank Ms. Yvette McLaughlin and Sheri Farnum for manuscript preparation, and the National Institutes of Health (AI063987, HL54785, AI45405, and a Center for AIDS.
Research Award [AI28691]), the International AIDS Vaccine Initiative, the Bristol-Myers Squibb Foundation, the William A. Haseltine Foundation for the Arts and Sciences, and the late William F. McCarty-Cooper for financial support. M.S. was supported by a National Defense Science and Engineering Fellowship and is a Fellow of the Ryan Foundation.

References


