Cyclophilin A Renders Human Immunodeficiency Virus Type 1 Sensitive to Old World Monkey but Not Human TRIM5 α Antiviral Activity

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TRIM5 α is an important mediator of antiretroviral innate immunity influencing species-specific retroviral replication. Here we investigate the role of the peptidyl prolyl isomerase enzyme cyclophilin A in TRIM5 α antiviral activity. Cyclophilin A is recruited into nascent human immunodeficiency virus type 1 (HIV-1) virions as well as incoming HIV-1 capsids, where it isomerizes an exposed proline residue. Here we show that cyclophilin A renders HIV-1 sensitive to restriction by TRIM5 α in cells from Old World monkeys, African green monkey and rhesus macaque. Inhibition of cyclophilin A activity with cyclosporine A, or reducing cyclophilin A expression with small interfering RNA, rescues TRIM5 α -restricted HIV-1 infectivity. The effect of cyclosporine A on HIV-1 infectivity is dependent on TRIM5 α expression, and expression of simian TRIM5 α in permissive feline cells renders them able to restrict HIV-1 in a cyclosporine A-sensitive way. We use an HIV-1 cyclophilin A binding mutant (CA G89V) to show that cyclophilin A has different roles in restriction by Old World monkey TRIM5 α and owl monkey TRIM-Cyp. TRIM-Cyp, but not TRIM5 α , recruits its tripartite motif to HIV-1 capsid via cyclophilin A and, therefore, HIV-1 G89V is insensitive to TRIM-Cyp but sensitive to TRIM5 α . We propose that cyclophilin A isomerization of a proline residue in the TRIM5 α sensitivity determinant of the HIV-1 capsid sensitizes it to restriction by Old World monkey TRIM5 α . In humans, where HIV-1 has adapted to bypass TRIM5 α activity, the effects of cyclosporine A are independent of TRIM5 α . We speculate that cyclophilin A alters HIV-1 sensitivity to a TRIM5 α -independent innate immune pathway in human cells.

Viral sequence analysis from infected patients has revealed three subgroups of human immunodeficiency virus type 1 (HIV-1), named M (main), O (other), and N (non-M, non-O), representing three independent zoonotic transfers of simian immunodeficiency virus from chimpanzees (SIVcpz) (16, 38). Remarkably, only that which led to the HIV-1 M group of sequences is responsible for the AIDS pandemic. The narrow host range of primate lentiviruses is further illustrated by the fact that HIV-1 is only able to replicate in chimpanzees and humans and only reliably causes disease in humans. These observations indicate the protective power of species barriers that prevent zoonotic infection. However, their molecular mechanisms are poorly defined and are likely to be diverse and complex.

The antiretroviral innate immune mediator TRIM5 α is an important factor influencing species-specific retroviral replication (21, 25, 33, 41, 48). TRIM5 α targets incoming viral capsids and causes a strong block to infectivity of sensitive retroviruses. The tripartite motif (TRIM) comprises a RING domain, one or two B boxes, and a coiled-coil domain (35). The RING domain is a zinc binding sequence found in E3 ubiquitin or SUMO ligases, and the B boxes and coiled-coils are likely to serve as protein-protein interaction interfaces. Some TRIM splice variants, including TRIM5 α , additionally encode a SPRY

(B30.2; RFP-like) domain at their C terminus. In the case of TRIM5 α , the SPRY domain interacts with the viral capsid and determines antiviral specificity (29, 32, 37, 40, 42, 49). The splice variant TRIM5 γ lacks a SPRY domain and acts as a dominant negative against TRIM5 α , rescuing restricted viral infectivity (32, 41). This observation suggests that more than one SPRY domain may be required in the antiviral complex for effective restriction. The TRIM family is large, comprising around 60 members, and their biochemical function is currently unclear. A number of TRIM proteins have been shown to be up-regulated by influenza virus infection, suggesting a general role in immunity (17), but the fact that polymorphism in TRIM proteins is often associated with developmental abnormalities, such as Opitz G/BBB syndrome (TRIM18) and mulibrey nanism (TRIM37) (1, 34), suggests that at least some TRIM proteins may have roles unrelated to immune function.

The molecular details of the mechanism of TRIM5 α restriction remain unclear, but the simplest model is that TRIM5 α -containing protein complexes interact directly with incoming viral capsids and perturb the ordered activities of the viral core. This leads to a strong block to viral infectivity that prevents viral DNA synthesis in some, but not all, cases (21, 25, 33, 41, 48, 51). Old World monkey TRIM5 α alleles, such as those from rhesus macaque and African green monkey (Agm), are particularly effective against HIV-1, blocking viral DNA synthesis and reducing HIV-1 infectivity by 1 to 2 orders of magnitude.

Intriguingly, the infectivity of HIV-1 is changed in a species-specific way by inhibition of peptidyl prolyl isomerase activity with cyclosporine A (CSA) (3, 45). Although CSA is active

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against a series of immunophilins, attention has focused on cyclophilin A (CypA) because it is uniquely incorporated into HIV-1 virions, via an interaction with the viral capsid (CA) (15, 43). HIV-1 can also recruit CypA after target cell entry (20, 26, 39, 45). The natural function of CypA within cells is unclear, as is its role in HIV-1 infectivity.

Species-specific effects of CSA on viral infectivity are exemplified by the fact that the HIV-1 titer is decreased in human cells but increased in simian cells by CSA treatment (43, 45). CA-CypA interactions have been shown to be important by preventing CypA-CA interactions with drugs that bind CypA (CSA and analogues) or by mutating the binding site in CA (G89V or P90A) (8, 9, 15, 20, 43, 50). In simian cells CSA treatment generally increases HIV-1 titer. This is particularly true in cells from owl monkey, which restrict HIV-1 and where HIV-1 titer is 2 to 3 orders of magnitude lower than in human cells (23, 45). These observations have recently been explained by the identification of the owl monkey restriction factor as a TRIM5-CypA fusion named TRIM-Cyp. This fusion was generated by transposition of a CypA pseudogene into the TRIM5 gene (30, 36). CSA prevents TRIM-Cyp interacting with HIV-1 CA by competing with CA for TRIM-Cyp binding. Mutation of the CypA binding site in the HIV-1 CA has the same effect, preventing TRIM-Cyp binding and rescuing HIV-1 infectivity in owl monkey cells. Here we investigate the relationship between CypA and TRIM5 α in cells from Old World monkeys, where HIV-1 infectivity is increased by CSA treatment but TRIM5 α is not fused to CypA (41).

MATERIALS AND METHODS

Cell lines and viral vector titrations. Cell lines and growth conditions have been described elsewhere (4, 18, 44). Vesicular stomatitis virus G protein-pseudotyped, green fluorescent protein (GFP)-encoding HIV-1, SIVmac, MLV-N, and MLV-B retroviral vectors were made by transfection of 293T cells as described elsewhere (4, 18, 24). HIV-1 packaging plasmid 8.91 encoding NL4.3 *gag-pol*, *tat*, and *rev* has been described previously (52). Titrations of viral vectors were made on 24-well plates containing 2.5×10^4 cells/well, and infected cells were enumerated 48 h later by fluorescence-activated cell sorting (BD Bioscience). Lentiviral doses were measured by a reverse transcriptase activity enzyme-linked immunosorbent assay (CavidiTech, Uppsala, Sweden) according to the manufacturer instructions. MLV-N and MLV-B titers were measured and equalized on feline CRFK cells, which do not restrict either virus (44), and are expressed as CRFK infectious units per milliliter.

Reduction of CypA or TRIM5 expression using siRNA. Cell lines stably expressing TRIM5 small interfering RNA (siRNA) were made as previously described (51). Cells lines stably expressing CypA siRNA were made using pSuper encoding CypA siRNA as described elsewhere (39). Feline CRFK cells expressing TRIM5 α from African green monkey and rhesus macaque have been described elsewhere (25, 51). CRFK cells expressing TRIM-Cyp were made using a retroviral expression vector encoding TRIM-Cyp, a kind gift of J. P. Stoye, as described in reference 30. Cyclosporine A (Sandoz, Frimley, United Kingdom) was diluted to 1 mM in dimethyl sulfoxide and used at a final concentration of 2.5 or 5 μ M at the time of infection as stated.

Western blot assays. Adherent cells were scraped from plates and pelleted by high-speed microcentrifugation for 1 min. The pellet was resuspended in 250 μ l lysis buffer (0.1 M NaCl, 50 mM Tris HCl, 1% Triton X-100, 2 mM EDTA, 2 mM dithiothreitol, 5 μ M 4-(2-aminoethyl)benzenesulfonyl fluoride, 1.5 mM aprotinin, 10 mM E-64, 10 mM leupeptin) and lysed by sonication for 10 seconds on ice with a Kinematica PT1200E polytron. Equal volumes of total lysate were loaded onto precast 12% polyacrylamide gels (Invitrogen) and run according to the manufacturer's instructions. Electrophoresed proteins were transferred to polyvinylidene difluoride membrane using a Hoefer Semiphor transfer apparatus as recommended by the manufacturer and Western blotted to detect CypA using a rabbit anti-CypA polyclonal antibody (SA296; BioMol) at 1:2,500 and anti-rabbit-horseradish peroxidase conjugate (Amersham) at 1:5,000. Blotting for actin was performed as above but using a rabbit polyclonal antiactin antibody

(Sigma) at 1:2,500. Proteins were detected by enhanced chemiluminescence (Amersham) and exposure to hyperfilm (Amersham).

RESULTS

(i) Cyclophilin A has anti-HIV-1 activity in Old World monkey cell lines. In order to examine the effect of CypA on HIV-1 infectivity in Old World monkey cells, we inhibited CypA activity, or reduced CypA levels, and measured HIV-1 infectivity on Agm CV1 cells or rhesus FRhK4 cells. We used vesicular stomatitis virus G protein-pseudotyped HIV-1 vectors encoding GFP as previously described (24). MVP5180 is an O group virus reported to be CypA independent (9, 47). NL4.3(Ba-L) is NL4.3 encoding the CypA binding loop from HIV-1 Ba-L (CA mutations H87Q, A88P, and I91V) and has been reported to be CypA independent and higher titer in simian cells (24, 26). We titrated each virus onto Agm and rhesus cells in the presence or absence of CSA or after stable expression of CypA-specific siRNA (Fig. 1).

The effects of CSA are species and HIV-1 sequence specific. NL4.3 infectivity is increased by CSA treatment in both Agm and rhesus cells (Fig. 1A and D), whereas MVP5180 infectivity is increased in Agm cells but not significantly in rhesus cells (Fig. 1B and E). NL4.3(Ba-L) infectivity is not affected by inhibition of CypA activity (Fig. 1C and F). Reduction of CypA expression has the same effect as treatment with CSA in each case, confirming CypA as the relevant target for CSA (Fig. 1A to F). Importantly, combining CypA siRNA with CSA treatment did not significantly increase NL4.3 infectivity further, in either Agm or rhesus cells, indicating that the reduction of CypA expression by siRNA is significant (Fig. 1G and H). Western blotting for CypA also confirmed that CypA levels are reduced by CypA-specific siRNA (Fig. 1I). In summary, these data indicate that abrogation of CypA activity, either by reduction of CypA expression or by inhibition of CypA enzymatic activity, increases infectivity of wild-type HIV-1 on Old World monkey Agm and rhesus cell lines. Furthermore, mutation of the HIV-1 CA can render HIV-1 insensitive to CypA activity.

(ii) TRIM5 α is required for anti-HIV-1 activity of CypA in simian cells. Previous studies have suggested a link between CypA and the antiretroviral restriction factor TRIM5 α (2, 3, 45). We therefore tested whether TRIM5 is required for the antiviral activity of CypA in simian cells. TRIM5 levels are reduced by stable expression of TRIM5-specific siRNA, and the effect of CSA on HIV-1 infectivity was tested (Fig. 2). In fact, HIV-1 infectivity is not increased by CSA treatment in Agm or rhesus cells that express reduced levels of TRIM5 α . We titrated NL4.3, MVP5180, and NL4.3(Ba-L) onto Agm (Fig. 2A to C) or rhesus (Fig. 2D to F) cells expressing reduced levels of TRIM5 α in the presence and absence of CSA. HIV-1 infectivity on unmodified cells was plotted as a control. The titers of all three viruses were increased by reduction of TRIM5 α expression. Furthermore, inhibition of CypA with CSA had no significant effect in the absence of TRIM5 α expression, with the possible exception of MVP5180 on rhesus cells. These data indicate that in most cases, the negative effect of CypA on HIV-1 infectivity is dependent on TRIM5 α expression and suggest that CypA sensitizes HIV-1 to TRIM5 α . Furthermore, if a virus is insensitive to CypA activity, for example NL4.3(Ba-L), this does not indicate insensitivity to TRIM5 α . Thus, wild-type HIV-1 is maximally sensitive to

FIG. 1. Abrogation of cyclophilin A activity rescues HIV-1 infectivity in Old World monkey cell lines. HIV-1 GFP-encoding vectors were made using Gag from NL4.3, MVP5180, and NL4.3(Ba-L). Threefold serial dilutions were titrated onto Agm CV1 (A to C) and rhesus FRhK4 (D to F) cells. The cells were unmodified and untreated as a control (●), stably expressed siRNA to CypA (▲), or were treated with 5 μ M CSA (○). NL4.3 GFP was also titrated onto Agm CV1 cells (G) or rhesus FRhK4 cells (H) stably expressing siRNA to CypA in the presence (▲) or absence (◆) of 5 μ M CSA. Virus input doses were measured in nanograms of reverse transcriptase as measured by ELISA. Results are representative of two independent experiments performed with two independent preparations of virus. (I) Western blot of extracts of CV1 and FRhK4 cells expressing reduced levels of CypA. Extracts from wild-type cells (wt) and cells expressing siRNA to CypA (KD) are shown. Actin blots are shown as a control for equal loading.

TRIM5 α in the presence of active CypA, whereas certain mutant HIV-1 sequences are sensitive to TRIM5 α irrespective of CypA activity [NL4.3(Ba-L)].

In the absence of an antibody to measure TRIM5 α expression levels, we functionally tested the reduction in TRIM5 α expression. In Agm cells expressing TRIM5 siRNA, HIV-1 infectivity is increased by 10-fold (Fig. 2A), and the titer of Agm TRIM5 α -sensitive MLV-N GFP is the same as TRIM5 α -insensitive MLV-B GFP (Fig. 2G). Untreated Agm cells infected with MLV-N and MLV-B are shown as a control (Fig. 2H). In rhesus cells, a significant reduction in TRIM5 α expression is indicated by a 10-fold increase in HIV-1 infectivity (Fig.

FIG. 2. Reduction of TRIM5 α protein levels renders HIV-1 infectivity insensitive to cyclosporine A. HIV-1 GFP-encoding vectors were made using Gag from NL4.3, MVP5180, or NL4.3(Ba-L). Threefold serial dilutions were titrated onto Agm CV1 cells (A to C) and rhesus FRhK4 cells (D to F). The cells were unmodified and untreated as a control (●), stably expressed siRNA to TRIM5 (▲), or stably expressed siRNA to TRIM5 and were treated with 5 μ M CSA (◇). To control for the reduction of TRIM5 expression, we show that MLV-N (■) and MLV-B (□) GFP-encoding vectors have similar titers on Agm CV1 (G) or FRhK4 rhesus (I) cells expressing reduced levels of TRIM5 α . MLV-N and MLV-B infectivities on untreated cells are shown as control Agm (H) and rhesus (J). Lentiviral input doses are measured in nanograms of reverse transcriptase as measured by ELISA. MLV input doses are shown as infectious units per milliliter on permissive feline CRFK cells. Results are representative of two independent experiments performed with two independent preparations of virus.

2D) and a slight increase in MLV-N infectivity (Fig. 2I). These data are concordant with previous data showing weak restriction of MLV-N but not MLV-B by rhesus TRIM5 α (21). Untreated rhesus cells infected with MLV-N and MLV-B are shown as a control (Fig. 2J).

(iii) Expression of simian TRIM5 α enables permissive feline CRFK cells to restrict HIV-1 in a cyclosporine-sensitive way. If CypA sensitized HIV-1 to restriction by Agm and rhesus TRIM5 α , we would expect that HIV-1 infection of feline

FIG. 3. Expression of simian TRIM5 α enables permissive feline CRFK cells to restrict HIV-1 in a cyclosporine-sensitive way. Serial dilutions of HIV-1 encoding GFP were titrated onto feline CRFK cells expressing Agm TRIM5 α (A), rhesus TRIM5 α (B), or unmodified CRFK cells as a control (C) in the presence of 5 μ M CSA ($\diamond$) or untreated as a control ($\blacklozenge$). Viral dose was measured by assay of reverse transcriptase (RT) by ELISA. (D) A fixed dose of HIV-1, chosen to infect around 1% of the target cells, was used to infect unmodified CRFK cells ($\bullet$), CRFK cells expressing Agm TRIM5 α ($\blacktriangle$), or rhesus TRIM5 α ($\circ$) in the presence of a serial dilution of CSA from 0 to 10 μ M. The fold increase in infectivity after CSA treatment was measured and is plotted. Results are representative of two independent experiments performed with two independent virus preparations.

CRFK cells expressing simian TRIM5 α proteins might be sensitive to CSA treatment. To test this we titrated HIV-1 onto CRFK cells expressing Agm TRIM5 α (Fig. 3A), rhesus TRIM5 α (Fig. 3B), or unmodified CRFK cells (Fig. 3C) as a control, in the presence or absence of 5 μ M CSA. As predicted by data in Fig. 2, expressing Agm or rhesus TRIM5 α in feline cells renders them able to restrict HIV-1 infectivity in a CSA-sensitive way. HIV-1 infection of unmodified feline cells is high titer and insensitive to CSA treatment (Fig. 3C). However, CSA treatment rescues HIV-1 infectivity by around sixfold after expression of rhesus TRIM5 α and by around threefold after Agm TRIM5 α expression. The fact that the restoration of HIV-1 infectivity by CSA is less effective in the feline cells overexpressing TRIM5 α (Fig. 3) than it is in cells making endogenous levels of TRIM5 α (Fig. 2) probably reflects higher protein levels in the feline overexpressing cells. To test whether

FIG. 4. Cyclophilin A is not required for restriction of MLV or SIVmac by TRIM5 α . (A) Threefold serial dilutions of MLV-N ($\circ$) or MLV-B ($\bullet$) encoding GFP were titrated onto unmodified Agm CV1 cells as a control. (B) MLV-N (open symbols) and MLV-B (solid symbols) encoding GFP were titrated onto unmodified Agm CV1 cells in the presence of 5 μ M CSA ($\triangle$) or onto Agm CV1 cells stably expressing siRNA to CypA ($\diamond$). (C) Threefold serial dilutions of SIVmac encoding GFP were titrated onto unmodified, untreated Agm CV1 cells as a control ($\bullet$), Agm CV1 cells in the presence of 5 μ M CSA ($\blacktriangle$), or Agm CV1 cells expressing siRNA to CypA ($\blacklozenge$). MLV input doses are shown as infectious units on permissive feline CRFK cells, and SIVmac input doses are shown as nanograms of reverse transcriptase (RT) as measured by ELISA. Results are representative of two independent experiments performed with two independent preparations of virus.

higher CSA concentrations will rescue HIV-1 infectivity on these cells further, we measured infectivity of a fixed dose of HIV-1 over a range of CSA concentrations. These data show that 8 μ M CSA is optimal. We also found that SIVmac infectivity was not rescued in CRFK cells expressing Agm TRIM5 α by CSA treatment (not shown).

(iv) **Cyclophilin A is not required for restriction of MLV or SIVmac by TRIM5 α .** We then tested whether CypA activity is uniquely required for TRIM5 α antiviral activity against HIV-1 or whether it is also required for Agm TRIM5 α restriction of other viruses. Abrogation of CypA activity with CSA, or by using CypA-specific siRNA, had no significant effect on the infectivity of Agm restriction-sensitive MLV-N, or insensitive MLV-B, in Agm CV1 cells (Fig. 4). Untreated MLV infectivities are plotted as a control (Fig. 4A). There are small increases in MLV-N titer after reduction of CypA expression, but this virus is still strongly restricted in the absence of CypA activity. We also examined Agm-TRIM5 α -sensitive SIVmac (Fig. 4C), and neither CypA reduction nor CypA inhibition significantly affected its infectivity. Similar experiments performed on FRhK4 cells gave similar results (data not shown). The effects of CSA and CypA knockdown are therefore specific to HIV-1, suggesting that CypA has a direct effect on HIV-1 rather than an effect on TRIM5 α . This notion is consistent with the observation that HIV-1 is the only retrovirus known to package CypA into virions during viral assembly and to have an exposed proline residue on its CA subject to CypA-mediated peptidyl prolyl isomerization (6, 9).

(v) **TRIM5 α restricts HIV-1 independently of CypA binding to HIV-1 CA.** Figures 1 and 2 indicate that CypA activity is required for maximal restriction of HIV-1 NL4.3 and MVP5180 by Agm and rhesus TRIM5 α . This is reminiscent of the restriction in owl monkey cells, where TRIM5 is actually fused to CypA to form the protein named TRIM-Cyp. The CypA domain recruits the tripartite motif to the HIV-1 capsid, leading to strong restriction of infectivity (30, 36). TRIM-Cyp restriction of HIV-1 is blocked by

FIG. 5. TRIM5 α restricts HIV-1 independently of CypA binding to HIV-1 CA. Wild-type HIV-1 NL4.3 or MVP5180 (◆) and their mutant CA G89V counterparts (◇) were titrated onto Agm CV1 cells (A and B) and rhesus FRhK4 cells (C and D). The HIV-1 G89V mutant was also titrated onto cells expressing reduced levels of CypA protein (○). Wild-type HIV-1 (E and F) or their G89V mutant counterparts (G and H) were also titrated onto unmodified, permissive feline CRFK cells (▲), CRFK cells expressing rhesus TRIM5 α (○), Agm TRIM5 α (◇), or owl monkey TRIM-Cyp (●). Virus input doses are measured in nanograms of reverse transcriptase, as measured by ELISA. Results are representative of two independent experiments performed with two independent preparations of virus.

preventing the CypA domain from interacting with CA by using CSA or by HIV-1 CA mutation (30, 36, 45). In order to consider the role of CA-CypA interactions in the antiviral activity of CypA, we asked whether HIV-1 mutants (CA G89V) that are unable to bind CypA are restricted in Agm or rhesus cells. We compared infectivity of NL4.3 and MVP5180 G89V mutants to their wild-type counterparts (Fig. 5A to D). Consistent with previous reports, the NL4.3 G89V titer is slightly higher than wild-type virus

on rhesus but not Agm cells (20, 45) (Fig. 5A and C). MVP5180 G89V is more strongly restricted in simian cells (Fig. 5B and D). Thus, despite being unable to interact with CypA, both G89V HIV-1 mutants are strongly restricted on Agm and rhesus cells. Neither G89V mutant is sensitive to CSA treatment (data not shown). Importantly the infectivity of the G89V mutants is not affected by reduction of CypA expression. Titration of both HIV-1 NL4.3 and MVP5180 G89V mutants on either Agm (Fig. 5A and B) or rhesus (Fig. 5C and D) cells expressing reduced levels of CypA does not increase the titer of the mutant viruses. However, infectivity of wild-type viruses is significantly increased by reduction of CypA expression (Fig. 1).

To further test their sensitivity to restriction, we titrated NL4.3, MVP5180, and their G89V mutants on permissive CRFK cells or CRFK cells expressing Agm or rhesus TRIM5 α or owl monkey TRIM-Cyp. In concordance with the results shown in Fig. 5A to D, both wild-type and G89V mutants are strongly restricted by Agm and rhesus TRIM5 α proteins (Fig. 5E to H). Furthermore, and as previously reported (30, 36, 45), wild-type HIV-1 viruses, but not their G89V mutants, are strongly restricted by TRIM-Cyp. HIV-1 G89V titers are therefore similar in cells expressing TRIM-Cyp and in unmodified CRFK cells. These data clearly show that HIV-1 mutants unable to bind CypA can be restricted by TRIM5 α , but not TRIM-Cyp. They indicate a key mechanistic difference between the role of CypA in restriction by TRIM5 α and TRIM-Cyp.

(vi) Cyclosporine A reduces HIV-1 infectivity in human cells independently of TRIM5 α . In human cells CSA has been shown to reduce HIV-1 infectivity (15, 20, 24, 43, 50). It has been assumed that CypA is required for maximal efficiency of a poorly defined capsid-dependent step, early after target cell entry. A role for restriction factors in the effects of CSA was suggested by the fact that HIV-1 treated with CSA, but not untreated virus, was able to titrate restriction of MLV-N in human cells (45). We therefore examined the effect of knocking down TRIM5 α expression on HIV-1 sensitivity to CSA in human cells. We titrated HIV-1 onto human cells expressing TRIM5-specific siRNA, or unmodified TE671 cells, in the presence and absence of CSA (Fig. 6). Reducing TRIM5 α expression had no effect on the reduction of HIV-1 infectivity by CSA (compare Fig. 6A and B). We also noted that reduction of TRIM5 α expression had only a small effect on the infectivity of HIV-1, consistent with the notion that HIV-1 is largely insensitive to restriction by human TRIM5 α . Preventing CypA binding to HIV-1 CA by mutation has also been shown to reduce HIV-1 infectivity in human cells. We therefore tested whether the infectivity of the HIV-1 CA mutant G89V, which cannot bind CypA, was influenced by reduction of TRIM5 expression. In fact, the infectivity of HIV-1 CA G89V is not increased by reduction of TRIM5 expression (Fig. 6C and D). These data are therefore consistent with the notion that restriction by TRIM5 α cannot account for the reduction of HIV-1 infectivity observed after inhibition of CypA activity or prevention of the CypA-CA interaction.

In order to show that we had effectively knocked down TRIM5 α expression, we demonstrated equal infectivity of TRIM5 α -sensitive MLV-N and TRIM5 α -insensitive MLV-B on TE671 expressing TRIM5 siRNA (compare Fig. 6E and F). Together these data show that the ability of CSA to reduce HIV-1 infectivity in human cells is independent of TRIM5 α .

FIG. 6. Cyclosporine A reduces HIV-1 infectivity in human cells independently of TRIM5 α . Threefold serial dilutions of wild-type HIV-1 NL4.3 (A and B) or HIV-1 CA G89V (C and D) were titrated onto TE671 cells stably expressing TRIM5 siRNA (A and C) or on unmodified cells as a control (B and D) in the presence (○) or absence (●) of 2.5 μ M CSA. Threefold serial dilutions of MLV-N (◇) and MLV-B (◆) were titrated onto TE671 cells expressing TRIM5 siRNA (E) or unmodified TE671 cells (F). Effective reduction of TRIM5 α expression is illustrated by equal MLV-N and MLV-B titers on cells treated with TRIM5 siRNA. HIV-1 input doses are measured in picograms of reverse transcriptase, as determined by ELISA. MLV input doses are shown as infectious units on permissive feline CRFK cells. Results are representative of two independent experiments performed with two independent preparations of virus.

We conclude that HIV-1 is insensitive to restriction by TRIM5 α and is rendered less infectious by CSA in human cells by a TRIM5 α -independent mechanism.

DISCUSSION

Here we have shown that the peptidyl prolyl isomerase enzyme CypA has an inhibitory effect on the infectivity of wild-type HIV-1 in cells from Old World monkeys (Fig. 1). Inhibition of CypA activity with CSA, or reduction of CypA expression using siRNA, strongly increases HIV-1 infectivity. Critically, the positive effect of CSA on HIV-1 infectivity is dependent on the antiviral factor TRIM5 α , as shown by loss of CSA sensitivity in cells expressing reduced TRIM5 α levels (Fig. 2). Furthermore, expression of Apm or rhesus TRIM5 α

in permissive feline cells renders them able to restrict HIV-1, and this antiviral effect is partially relieved by treatment with CSA (Fig. 3). An incomplete rescue of HIV-1 infectivity by CSA in feline cells overexpressing TRIM5 α (Fig. 3) compared to almost complete rescue in cells making TRIM5 α endogenously (Fig. 2) probably reflects higher protein levels in the feline cells. High expression levels of restriction factors are known to broaden antiviral specificity (5, 31, 51) and may broaden the antiviral specificity of simian TRIM5 α to HIV-1 CA in the absence of CypA. Regardless of expression levels, the data in Fig. 1 to 3 indicate that maximal restriction of wild-type HIV-1 by Apm or rhesus TRIM5 α depends on CypA. Moreover, TRIM5 α 's requirement for CypA is unique to restriction of wild-type HIV-1. Reducing CypA expression or inhibiting CypA activity has no effect on the ability of Apm TRIM5 α to strongly restrict MLV-N or SIVmac, suggesting that CypA acts on HIV-1 CA rather than on TRIM5 α (Fig. 4).

We have also investigated whether CypA binding to HIV-1 CA is important for restriction by TRIM5 α , as it is for restriction by owl monkey TRIM-Cyp. TRIM-Cyp recruits its tripartite motif to HIV-1 CA via its CypA domain and strongly restricts HIV-1 infectivity (30, 36). Using CypA for effector domain recruitment leads to absolute sensitivity to either drugs or HIV-1 CA mutations that prevent CypA-CA interactions (Fig. 5). In the case of Apm, or rhesus, TRIM5 α restriction of HIV-1, prevention of CypA-CA interactions does not rescue restricted infection. This is shown by strong restriction of HIV-1 G89V in Apm or rhesus cells as well as in feline cells expressing Apm or rhesus TRIM5 α proteins (Fig. 5). These data indicate that CypA does not recruit TRIM5 α to the incoming HIV-1 capsid, as it does for TRIM-Cyp.

HIV-1 and the closely related SIVcpz from chimpanzees are unique in incorporating CypA into their cores and, importantly, this recruitment leads to *cis-trans* isomerization of the peptide bond of CA proline residue P90 (6, 7, 9). We propose therefore that an interaction between CypA and incoming HIV-1 core leads to an alteration in the conformation of the capsid target for simian TRIM5 α and that this leads to altered sensitivity to restriction. A TRIM5 α sensitivity determinant in this part of the capsid is supported by mutations in the SIVmac CA, within a few residues of P90, that render SIVmac insensitive to restriction by squirrel monkey TRIM5 α (51). Further, mutations close to P90 have been shown to influence HIV-1 species-specific infectivity (19, 20). These data support a role for this exposed part of the capsid in TRIM5 α sensitivity, and an altered CA conformation might reasonably influence TRIM5 α binding directly or indirectly and subsequent restriction of infectivity. In fact, peptidyl prolyl isomerization has been shown to control protein-protein interactions in other systems, for example, the Itk tyrosine kinase (10, 13, 28). The notion that CypA alters HIV-1 CA sensitivity to TRIM5 α by P90 isomerization is also supported by the observation that altering residues close to the CypA binding site in NL4.3(Ba-L) renders TRIM5 α restriction of this virus independent of CypA activity. These changes could either prevent CypA from isomerizing P90, or the TRIM5 α binding site could assume a conformation in which the isomerization status of P90 does not impact on TRIM5 α binding and restriction. Importantly, the NL4.3(Ba-L) CA has been shown to bind CypA (11, 26).

Competitors have recently described an involvement of

CypA in TRIM5 α restriction of HIV-1 (2). Our data support this work but with an important difference. Berthoux et al. have proposed that the HIV-1 mutant CA G89V, which is unable to recruit CypA, is unrestricted in Acm and rhesus cells. This study cannot differentiate, therefore, between a model in which CypA recruits TRIM5 α to the HIV-1 CA, as it does for TRIM-Cyp, and the model we propose in which CypA alters the conformation of the TRIM5 α sensitivity determinant and sensitivity to restriction. We discount a model in which the CypA-HIV-1 CA complex is a better target for TRIM5 α restriction than HIV-1 CA alone, because the CypA binding virus NL4.3(Ba-L) is restricted in a CypA-independent way (Fig. 2 and 4) and also because an HIV-1 CypA binding mutant (HIV-1 G89V) is restricted in simian cells (Fig. 5). Formally, it is possible that CypA binding has some other effect on the HIV-1 capsid, but we believe a change in conformation due to peptidyl prolyl isomerase activity is the most likely explanation. The difference in TRIM5 α sensitivity of HIV-1 G89V between Berthoux's work and ours remains unexplained. The high titer of G89V in simian cells appears to be particular to the study by Berthoux et al., as two other studies have shown that preventing CypA binding by the mutation G89V or P90A does not significantly increase HIV-1 infectivity in cells from Old World monkeys (19, 45).

HIV-1 is only very weakly sensitive to restriction by TRIM5 α in human cells, despite being subject to peptidyl prolyl isomerization at CA P90 by CypA (6, 7). Intriguingly, inhibition of CypA in human cells reduces HIV-1 infectivity by a TRIM5 α -independent mechanism (Fig. 6). This implies that HIV-1 CA conformation relies on CypA activity for insensitivity to restriction by as-yet-unidentified factors. The existence of further CypA-sensitive antiviral factors is supported by the observation that certain HIV-1 mutants, for example HIV-1 CA A92E, appear to be restricted in a TRIM5 α -independent way in certain human cell lines (20, 39). Remarkably, CSA treatment, or reduction of CypA expression, rescues their infectivity, as is the case with wild-type HIV-1 in simian cells. Notably, unlike the saturable restriction by TRIM5 α , infectivity of these mutant viruses is not rescued by high doses of mutant VLP. CSA treatment also enables wild-type HIV-1 to saturate restriction of MLV-N by TRIM5 α in human cells (45). This suggests that TRIM5 α might be saturated by CSA-treated HIV-1, although it could also indicate saturation of a TRIM5 α cofactor.

HIV-1's dependence on CypA activity in human cells might not contradict an innate immune role for cyclophilins. If cyclophilins contribute to innate immune function by isomerizing prolines on incoming viral proteins, then in order to replicate in human cells HIV-1 might have to tolerate CypA activity. This could lead to dependence on CypA if the prolines targeted are functionally important to viral replication or targets for mediators of innate immunity. Such speculation is encouraged by recent work showing that hepatitis C virus is dependent on CypB activity for replication (46). Vaccinia virus, too, appears to depend on immunophilin activity for replication, although the details of this interaction remain unclear (14). An innate immune role for CypA is also supported by recent work in plants. *Aridopsis* cyclophilin can isomerize prolines on an incoming *Pseudomonas* protease AvrRpt2, leading to activation of the specific innate immune mediator RIN4 (12). This model is strikingly similar to that proposed here, in which

CypA sensitizes HIV-1 to Old World monkey TRIM5 α by isomerizing proline residues on the incoming HIV-1 capsid.

A definitive role for cyclophilins in cell biology remains elusive. A nonessential function is indicated by the fact that yeast knocked out for all eight cyclophilin genes remain viable (12, 22). A role for CypA in immune function is supported by the use of CSA as an immunosuppressive agent. Current models propose that the interaction between calcineurin and the CypA-CSA complex that leads to immunosuppression only occurs in the presence of CSA. In the absence of CSA, CypA does not appear to regulate calcineurin. However, it is remarkable that calcineurin is also inhibited by a complex between another immunophilin, FK506 binding protein (FKBP12), and the immunosuppressant FK506 (27). Together, these examples of cyclophilin activity suggest a central role for peptidyl prolyl isomerization in immunity, and the continued study of immunophilins and their role in infectious disease promises to reveal important details of immune activity and host virus interactions.

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