Cyclophilin A and TRIM5 α Independently Regulate Human Immunodeficiency Virus Type 1 Infectivity in Human Cells

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Cyclophilin A (CypA), a cytoplasmic, human immunodeficiency virus type 1 (HIV-1) CA-binding protein, acts after virion membrane fusion with human cells to increase HIV-1 infectivity. HIV-1 CA is similarly greeted by CypA soon after entry into rhesus macaque or African green monkey cells, where, paradoxically, the interaction decreases HIV-1 infectivity by facilitating TRIM5 α -mediated restriction. These observations conjure a model in which CA recognition by the human TRIM5 α orthologue is precluded by CypA. Consistent with the model, selection of a human cell line for decreased restriction of the TRIM5 α -sensitive, N-tropic murine leukemia virus (N-MLV) rendered HIV-1 transduction of these cells independent of CypA. Additionally, HIV-1 virus-like particles (VLPs) saturate N-MLV restriction activity, particularly when the CA-CypA interaction is disrupted. Here the effects of CypA and TRIM5 α on HIV-1 restriction were examined directly. RNA interference was used to show that endogenous human TRIM5 α does indeed restrict HIV-1, but the magnitude of this antiviral activity was not altered by disruption of the CA-CypA interaction or by elimination of CypA protein. Conversely, the stimulatory effect of CypA on HIV-1 infectivity was completely independent of human TRIM5 α . Together with previous reports, these data suggest that CypA protects HIV-1 from an unknown antiviral activity in human cells. Additionally, target cell permissivity increased after loading with heterologous VLPs, consistent with a common saturable target that is epistatic to both TRIM5 α and the putative CypA-regulated restriction factor.

Primate cells possess an activity that blocks retroviral replication at a step after viral entry but prior to completion of reverse transcription (29, 30). In rhesus macaques and owl monkeys this activity potently restricts human immunodeficiency virus type 1 (HIV-1) but largely permits infection by simian immunodeficiency virus SIV_{MAC239} (9, 16, 37). Analysis of chimeric viruses generated from HIV-1 and SIV_{MAC239} sequences revealed viral determinants for susceptibility to this restriction activity within CA (15, 18, 41, 50).

Independent screens, one with rhesus macaque cells and the other with owl monkey cells, identified the respective TRIM5 orthologues as necessary for HIV-1 restriction in these species (48, 54). TRIM5 is a member of the tripartite motif (TRIM) protein family, defined by a cluster of RING-finger, B-box, and coiled-coil domains (40, 44). The native function of TRIM5 is unknown, but the ability of this protein to serve as a target for E2 ubiquitylation in a RING-finger-dependent manner (63) suggests that it acts as an E3 ubiquitin ligase *in vivo*.

The alpha isoform of TRIM5 is required for retroviral restriction in most primate species that have been studied (27, 32, 42, 54, 64). Species-specific variation in the carboxy-terminal SPRY domain accounts to a large extent for the variation in retroviral restriction specificity among orthologues (46, 52, 55, 65). For example, TRIM5 α encoded by some African green monkeys (TRIM5 α _{AGM}) and by rhesus macaques (TRIM5 α _{rh}) restricts HIV-1, N-tropic murine leukemia virus (N-MLV), and certain strains of simian immunodeficiency virus (27, 32, 54, 64). Human TRIM5 α (TRIM5 α _{hu}) potently inhibits N-MLV

but, when overexpressed, only moderately restricts HIV-1 and simian immunodeficiency virus (27, 32, 42, 54, 64).

An unusual TRIM5 isoform accounts for the potent HIV-1 restriction activity in owl monkey cells (39, 48). All TRIM5 alleles from this genus that have been examined possess a complete cyclophilin A (CypA) cDNA inserted into intron 7 (39, 45, 48). In the resulting protein, the SPRY domain unique to the alpha isoform has been replaced by CypA. This is particularly intriguing since free CypA binds to the exposed proline-rich loop of HIV-1 CA (21, 22, 35) and promotes HIV-1 infectivity in human cells (12, 13, 21, 58). The CypA-CA interaction is required in the target cell, as opposed to the producer cell (26, 31, 51, 61), but the mechanism by which CypA promotes HIV-1 infection of human cells is unknown. The discovery of the owl monkey TRIM5 gene fusion with CypA raised suspicion that these two proteins might be functionally interdependent.

Retroviral restriction is overcome by infection at high multiplicity or by flooding target cells with nonreplicating virus-like particles (VLPs) (4, 9, 10, 16, 17, 19, 25, 43, 57, 60). These observations have been explained by postulating the existence of a saturable factor necessary for restriction. Cross-saturation experiments in human cells suggest that, in the absence of CypA, HIV-1 is particularly sensitive to the same factor that restricts N-MLV (61). Since N-MLV restriction in human cells requires TRIM5 α (27, 32, 42, 64), CypA binding to HIV-1 CA might shield incoming HIV-1 cores from restriction by TRIM5 α _{hu}. This hypothesis is supported by the observation that HIV-1 infection is CypA-independent in a human cell clone that was selected for loss of N-MLV restriction activity (47). Additionally, HIV-1 restriction by rhesus macaque or African green monkey TRIM5 α requires CypA (6).

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RNA interference knockdown, single-cycle HIV-1 infectivity assays, and several tools that disrupt the CA-CypA interaction were used here to clarify the relationship between CypA and TRIM5 α_{hu} . We conclude that CypA promotes HIV-1 infectivity via effects on a host factor distinct from TRIM5 α_{hu} .

MATERIALS AND METHODS

Plasmids. pNL4-3 contains an infectious HIV-1 provirus (2). pNL4-3/G89V encodes a mutant CA that disrupts the CypA binding site (51). pNL4-3_{GFP}, or HIV-1_{GFP}, is pNL4-3 with an *env*-inactivating mutation and an enhanced green fluorescent protein (GFP) gene replacing *nef* (28).

p8.9 Δ SB expresses HIV-1 *gag* and *pol* from the cytomegalovirus immediately early (CMVie) promoter and bears inactivating mutations in the RNA packaging region, *env*, and nonessential accessory genes (7). p8.9 Δ SB/A92E is p8.9 Δ SB encoding the CA mutant A92E (7). CSGW Δ N is an HIV-1 vector expressing enhanced green fluorescent protein under the control of the spleen focus-forming virus long terminal repeat (9). It was modified from the parent CSGW vector by blunting a NotI restriction site. pMDG encodes the vesicular stomatitis virus glycoprotein (VSV-G) (8).

pCIG3-N and -B are MLV-derived constructs expressing N- or B-tropic CA, respectively (8). pMIG is a murine stem cell virus (MSCV)-based expression vector containing an internal ribosome entry site-GFP cassette (62). pCL-Eco contains *psi*-minus Moloney MLV expressed from the CMVie promoter (38). pSUPER_{retro.puro} (pSRP; OligoEngine) is an MSCV-derived vector expressing short hairpin RNAs (shRNAs) from the H1 polymerase III promoter (14). pSRP-TRIM5_{hu} expresses an shRNA targeting human TRIM5 mRNA (target sequence, 5'-GCTCAGGGAGGTCAAGTTG-3') (54). pSRP-CypA expresses an shRNA that targets human CypA mRNA. It was made by subcloning the shRNA expression cassette from pMH-CypA147 (48). The control plasmid, pSRP-Luc, encodes an shRNA targeting luciferase (48).

pshRNA.lenti.puro (pshRPL) is an HIV-1-based vector derived from pCSGW Δ N that contains an H1-shRNA cassette as in pSUPER (14) and a puromycin resistance gene driven by the phosphoglycerate kinase (PGK) promoter. To engineer pshRPL-TRIM5_{hu} and pshRPL-Luc, the following oligonucleotides were used for PCR, with pSRP-TRIM5_{hu} or pSRP-Luc as templates: 5'-GCTCTAGAGCGGCCGCTCTCTTCGGTCGGGCGCT-3' and 5'-AGG AATTCTTAATTAAGATCGATCTCTCGAGGTCGAC-3'. The PCR products were subcloned into CSGW Δ N using EcoRI and NotI sites.

Cells and drugs. Adherent cells (293T, TE671, HeLa, and *Mus dunni* tail fibroblasts) were maintained in Dulbecco's modified Eagle medium. A human T-cell line, CEM-SS, was maintained in RPMI medium. All media were supplemented with 10% fetal bovine serum and antibiotics. CsA (Bedford Laboratories) was prepared in dimethyl sulfoxide at 10 mg/ml and diluted in tissue culture medium to 2.5 μ M for each experiment. Dextran sulfate (5 mg/ml; Sigma) was prepared in H₂O and was used at 100 μ g/ml as described previously (8).

Viruses. Vectors and viruses were produced by transfecting 10⁶ 293T cells/well in 6-well plates using Lipofectamine 2000 (Invitrogen). NL4-3_{GFP} vectors were produced by cotransfecting cells with 0.3 μ g of pMDG and 3.7 μ g of either pNL4-3_{GFP} or pNL4-3_{GFP}G89V. HIV-1_{GFP} wild-type and HIV-1_{GFP} A92E vectors were produced using 2 μ g of CSGW Δ N, 0.3 μ g of pMDG, and 1.7 μ g of p8.9 Δ SB, either wild type or A92E mutant. Full-length infectious HIV-1 viruses were produced by transfecting 293T cells with 4 μ g of pNL4-3 or pNL4-3/G89V. To produce MSCV-based shRNA vectors, 293T cells were cotransfected with 1.7 μ g of pCL-Eco, 0.3 μ g of pMDG, and 2 μ g of either pSRP-CypA, pSRP-TRIM5, or pSRP-Luc. To produce HIV-1-based shRNA vectors, 293T cells were cotransfected with 1.7 μ g of p8.9 Δ SB, 0.3 μ g of pMDG, and 2 μ g of either pshRPL-TRIM5 or pshRPL-Luc. For production of N- or B-tropic MLV_{GFP} vectors, cells were cotransfected with 2 μ g of pMIG, 0.3 μ g of pMDG, and 1.7 μ g of either pCIG3-N or pCIG3-B. For production of HIV-1 VLPs, 4.5 $\times$ 10⁶ 293T cells/10-cm-diameter plate were cotransfected with 18 μ g of p8.9 Δ SB and 2 μ g of pMDG using Ca₂PO₄.

All virus- and VLP-containing supernatants were harvested 48 h posttransfection, spun at 300 $\times$ g for 5 min to remove cell debris, and filtered through a 0.45- μ m-pore-size filter (Pall Acrodisc). For infectious NL4-3 viruses and VLPs, clarified supernatant was layered onto a 25% sucrose cushion and accelerated at 100,000 $\times$ g for 2 h in an SW41 rotor. The pelleted virions were resuspended in RPMI medium with 10% fetal bovine serum and stored at -80°C. Virions were normalized prior to infection by measuring reverse transcriptase (RT) activity as previously described (24).

Infection. Adherent cells were plated in 48-well plates (2 $\times$ 10⁴ cells/well). Cells in suspension were seeded in 48-well plates at 8 $\times$ 10⁴ cells/well. Virion

stocks were added to the cells in a final volume of 250 μ l/well. GFP expression in infected cells was analyzed 48 h postinfection by flow cytometry (see below). For infections with full-length virus, dextran sulfate was added 16 h postinfection (100 μ g/ml) to prevent spread of the virus (8). Forty-eight hours postinfection, cells were fixed in 4% formaldehyde-phosphate-buffered saline and assayed for intracellular p24 using a monoclonal anti-p24 antibody (183-H12-5C; NIH AIDS Research & Reference Reagent Program).

To generate TE671 and HeLa cells with stable expression from pSRP vectors, cells were seeded into 24-well plates at 3 $\times$ 10⁴ cells/well, covered with 500 μ l of clarified 293T supernatant, and spinoculated for 70 min at 1,200 $\times$ g. Forty-eight hours posttransduction, cells were subjected to puromycin selection with quantum increases in drug concentration (1, 5, 10, 15, 20, 30, 40, and 50 ng/ μ l) over a period of 10 days.

To generate CEM-SS cells with stable expression from pshRPL vectors, cells were seeded into 24-well plates at 10⁵ cells/well and infected with 50 μ l of clarified 293T supernatant in a total volume of 500 μ l. All cells were subjected to puromycin selection 48 h posttransduction as described above.

Flow cytometry. FACSCalibur and Cellquest Pro software (Becton Dickinson) were used to record GFP fluorescence in the FL1 channel. A quantity of 10⁴ live cells was analyzed per sample.

Western blotting. Cells were normalized by number, and lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting with antibodies to CypA (rabbit polyclonal; Biomol) and β -actin (mouse monoclonal; Sigma). Coomassie blue staining of the membranes confirmed that loading of samples had been normalized.

RESULTS

Endogenous TRIM5 α_{hu} restricts HIV-1. To test the importance of TRIM5 α_{hu} for HIV-1 infectivity, shRNA was used to downregulate endogenous TRIM5 α_{hu} . TE671 cells were transduced with an MSCV-based retroviral vector delivering a pSUPER-shRNA cassette that targets human TRIM5 (14). A construct delivering an shRNA specific for luciferase (48) was used as a control. Pools of transduced cells were selected for maintenance of the shRNA-encoding transgenes by exploiting the PGK-puromycin resistance cassette that is part of the retroviral vector.

A functional assay was utilized to determine whether TRIM5 α_{hu} knockdown was successful. Since TE671 cells restrict N-MLV more than 100-fold relative to B-MLV, in a TRIM5 α_{hu} -dependent fashion (27, 32, 42, 64), TE671 cells knocked down for TRIM5 (TE671-TR5-shRNA cells) were challenged with VSV-G-pseudotyped B- or N-tropic MLV_{GFP}. The two viral stocks were normalized based on RT activity as well as the titer on nonrestrictive *Mus dunni* tail fibroblasts, as previously described (8, 47, 59). In TE671-Luc-shRNA cells, the N-MLV titer was more than 100-fold lower than that of B-MLV (Fig. 1A). In TE671-TR5-shRNA cells, the N-MLV titer was almost identical to that of B-MLV (Fig. 1B), indicating that TRIM5 α_{hu} -dependent restriction activity was eliminated by the shRNA.

The effect of TRIM5 α_{hu} knockdown on HIV-1 infectivity was examined using VSV-G-pseudotyped NL4-3_{GFP} (HIV-1_{GFP}). The titer of HIV-1_{GFP} was threefold higher on TE671-TR5-shRNA cells than on control TE671-Luc-shRNA cells (Fig. 1C), demonstrating that endogenous TRIM5 α_{hu} inhibits HIV-1 infectivity. This result is consistent with previous reports that TRIM5 α_{hu} overexpression decreases HIV-1 infectivity (27, 42, 52, 55).

Inhibition of HIV-1 infectivity by factors that disrupt the CA-CypA interaction is TRIM5 α_{hu} independent. To investigate whether HIV-1 infectivity is more susceptible to TRIM5 α_{hu} -mediated restriction when the CypA-CA interaction is disrupted,

FIG. 1. Knockdown of endogenous TRIM5 α_{hu} enhances HIV-1 infectivity. TE671 cells were transduced with an MLV-based vector delivering an shRNA expression construct specific for human TRIM5 (TR5-shRNA) or luciferase (Luc-shRNA). VSV-G-pseudotyped, N- and B-tropic MLV_{GFP} vectors were normalized by RT and by infectivity on nonrestrictive *Mus dunni* tail fibroblasts and used to infect (A) TE671-Luc-shRNA cells or (B) TE671-TR5-shRNA cells. (C) VSV-G-pseudotyped HIV-1_{GFP} virions were used to infect TE671-Luc-shRNA and TE671-TR5-shRNA cells. The percentage of GFP-positive (infected) cells was determined by flow cytometry. Shown are representative results of a single experiment. Identical results were obtained on three separate occasions using independently produced viral stocks.

TE671-TR5-shRNA cells and TE671-Luc-shRNA cells were infected with HIV-1_{GFP} in the presence of CsA, a competitive inhibitor of the CA-CypA interaction (35). The magnitude of reduction of HIV-1 infectivity by CsA in TE671-TR5-shRNA cells was identical to the magnitude of reduction by the drug in the control TE671-Luc-shRNA cells (Fig. 2A and B). Similarly, the titer of HIV-1_{GFP} G89V, a CA mutant that disrupts interaction with CypA (67), was equally decreased in TE671-TR5-shRNA and TE671-Luc-shRNA cells (Fig. 2C and D). These results show that the reduction in HIV-1 infectivity that results from CsA or the G89V mutation does not result from increased susceptibility to TRIM5 α_{hu} -mediated restriction.

Reduction of HIV-1 infectivity by CypA knockdown is independent of TRIM5 α_{hu} . To verify that the CsA-mediated decrease in HIV-1 titer resulted from the inhibitory effect of CsA on CypA, TE671 cells were transduced with a construct delivering an shRNA specific for CypA, as previously described (48, 51). After selection of a pool of transduced cells with puromycin, CypA was undetectable by Western blotting (Fig. 3A). CypA downregulation resulted in a three- to fivefold decrease in HIV-1 infectivity compared to control cells (Fig. 3B). The magnitude of reduction of HIV-1 infectivity in the context of the CypA knockdown was almost identical to the magnitude of

FIG. 2. Reduction of HIV-1 infectivity by CsA or CA mutant G89V is not mediated by TRIM5 α_{hu} . VSV-G-pseudotyped, RT-normalized, HIV-1_{GFP} wild-type and G89V mutant virions were used to infect TE671-Luc-shRNA (A and C) and TE671-TR5-shRNA (B and D) cells in the presence or absence of CsA, as indicated. The percentage of GFP-positive (infected) cells was determined by flow cytometry. Shown are representative results of a single experiment. Identical results were obtained on three separate occasions using independently produced viral stocks.

reduction of HIV-1 by CsA in control TE671-Luc-shRNA cells (Fig. 3B), indicating that CsA reduces HIV-1 infectivity via effects on CypA. As expected, since the G89V mutation disrupts the CypA binding site in CA, HIV-1 G89V infectivity was decreased no further by CypA knockdown (Fig. 3C).

To directly test the interdependence of TRIM5 α_{hu} and CypA in mediating HIV-1 restriction, we generated cell lines in which both TRIM5 α_{hu} and CypA were downregulated using shRNA. To accomplish this, the singly transduced TE671-TR5-shRNA and TE671-Luc-shRNA cells (Fig. 1) were subjected to a second round of transduction either with the shRNA specific for CypA or with the control construct targeting luciferase. CypA knockdown was confirmed by Western blotting (Fig. 4A). Both TE671-TR5/CypA-shRNA and TE671-TR5/Luc-shRNA cell lines still failed to restrict N-MLV, confirming that the TRIM5 α_{hu} knockdown was stable (data not shown).

As before (Fig. 1 and 3), cells in which TRIM5 α_{hu} was downregulated were more permissive for HIV-1 infection than control cells, and cells in which CypA was knocked down were less permissive for HIV-1 infection (Fig. 4B). The magnitude of the infectivity decrease due to CypA knockdown was the same in TE671-TR5/CypA-shRNA and TE671-Luc/CypA-shRNA cells, compared to the corresponding control cell lines TE671-TR5/Luc-shRNA and TE671-Luc/Luc-shRNA (Fig. 4B). Similarly, the magnitude of the increase in infectivity due

FIG. 3. Knockdown of CypA reproduces the effect of CsA and G89V on HIV-1 infectivity. TE671 cells were transduced with MLV-based vectors delivering shRNA expression constructs specific for CypA (CypA-shRNA) or luciferase (Luc-shRNA). (A) Cells were normalized by number, and CypA knockdown was demonstrated by Western blotting for CypA and β -actin. VSV-G-pseudotyped, RT-normalized, HIV-1_{GFP} wild-type (B) or G89V mutant (C) vectors were used to infect TE671-CypA-shRNA or TE671-Luc-shRNA cells in drug-free media or in the presence of 2.5 μ M CsA, as indicated. The percentage of GFP-positive (infected) cells was determined by flow cytometry. Shown are representative results of a single experiment. Identical results were obtained on three separate occasions.

to disruption of TRIM5 α_{hu} was the same whether CypA was expressed or not, or whether HIV-1 wild type or G89V was used for infection (Fig. 4B and C). As expected, infectivity of HIV-1 G89V remained unaltered by CypA knockdown (Fig. 4C). These results demonstrate that TRIM5 α_{hu} and CypA independently modulate HIV-1 infectivity. In other words, disruption of TRIM5 α_{hu} does not rescue the reduction in HIV-1 infectivity caused by CypA knockdown.

CypA and TRIM5 α_{hu} independently modulate HIV-1 infectivity in human CD4⁺ T cells. To determine whether the results described above were peculiar to the TE671 rhabdomyosarcoma cell line, the CD4⁺ T-cell line CEM-SS was transduced with an HIV-1-based vector delivering a pSUPER-shRNA cassette (14) specific for human TRIM5 (54). A construct delivering an shRNA specific for luciferase (48) was used as a control. Pools of transduced cells were then selected in puromycin.

As described above for the TE671 cells, TRIM5 α_{hu} knockdown in CEM-SS was confirmed by challenging transduced cells with N- and B-tropic MLV_{GFP} (Fig. 5A and B). HIV-1_{GFP} infectivity was reduced to the same extent by CsA in CEM-SS-TR5-shRNA as it was in CEM-SS-Luc-shRNA (Fig. 5C and D). Similarly, the titer of HIV-1_{GFP} G89V was decreased in both TRIM5 α_{hu} knockdown and control cells (Fig. 5C and D).

FIG. 4. Reduction of HIV-1 infectivity by CypA knockdown is independent of TRIM5 α_{hu} . TE671 cells were transduced with an MLV-based vector delivering an shRNA expression construct specific for human TRIM5 (TR5-shRNA) or luciferase (Luc-shRNA). Each pool of cells was then transduced a second time with shRNA vectors specific for CypA or luciferase. (A) CypA knockdown in the indicated pools of cells was demonstrated by Western blotting for CypA and β -actin. VSV-G-pseudotyped HIV-1 wild-type (WT) (B) or G89V mutant (C) virions were used to infect the indicated pools of cells. The percentage of GFP-positive (infected) cells was determined by flow cytometry. Shown are representative results of a single experiment. Identical results were obtained on three separate occasions.

These results confirm that CypA acts independently of TRIM5 α_{hu} to modulate HIV-1 infectivity in human CD4⁺ T cells.

Effects of CypA and TRIM5 α_{hu} on HIV-1 infectivity are independent of the route by which virions enter target cells. Previous studies indicated that pseudotyping with VSV-G renders HIV-1 insensitive to CsA (3). VSV-G and the HIV-1 *env*-encoded glycoproteins (gp120/gp41) are believed to promote virion entry into target cells via two distinct mechanisms. VSV-G enters cells via an endocytic pathway, while the HIV-1 *env* glycoproteins mediate direct fusion into the target cell cytoplasm (5, 36, 53). To determine whether the functional interdependence of CypA and TRIM5 α_{hu} is altered by the entry mechanism, CEM-SS-TR5-shRNA and CEM-SS-Luc-shRNA cells were challenged with full-length, infectious HIV-1_{NL4-3}. In the presence of CsA, or the G89V mutation, HIV-1 infectivity was decreased to the same extent in CEM-SS-TR5-shRNA cells as it was in control CEM-SS-Luc-shRNA cells (Fig. 6). These findings were confirmed in TE671 cells transfected with CD4 (data not shown). Thus, CypA and TRIM5 α_{hu} act independently to regulate HIV-1 infectivity, irrespective of the route of entry utilized by the virus.

FIG. 5. Reduction of HIV-1 infectivity by CsA or the G89V mutant is independent of TRIM5 α_{hu} in human T cells. CEM-SS cells were transduced with an HIV-based vector delivering an shRNA expression construct specific for human TRIM5 or luciferase as a control. VSV-G-pseudotyped, N- and B-tropic MLV_{GFP} virions were used to infect CEM-SS-Luc-shRNA cells (A) or CEM-SS-TR5-shRNA cells (B). VSV-G-pseudotyped, HIV-1_{GFP} wild-type, or G89V mutant vectors were used to infect CEM-SS-Luc-shRNA cells (C) and CEM-SS-TR5-shRNA cells (D) in drug-free medium or in the presence of 2.5 μ M CsA, as indicated. The percentage of GFP-positive (infected) cells was determined by flow cytometry. Shown are representative results of a single experiment. Identical results were obtained on three separate occasions using independently produced viral stocks.

Disruption of the CypA-CA interaction enhances the efficiency by which HIV-1 VLPs saturate restriction. Prior to the discovery that TRIM5 restricts retroviruses in primate cells (48, 54), and prior to the widespread use of RNA interference in mammalian systems, HIV-1 VLPs were shown to enhance N-MLV infectivity, if the CA-CypA interaction was disrupted using CsA or the CA G89V mutation (61). Given that TRIM5 α_{hu} exhibits a mild inhibitory effect on wild-type HIV-1 (Fig. 1), one would expect wild-type HIV-1 VLPs to also increase the N-MLV titer, though small phenotypes in such technically difficult saturation experiments might be difficult to detect. The effect of HIV-1 VLPs on N-MLV titer was reexamined here in the context of CypA and TRIM5 knockdown cells.

TE671-CypA-shRNA cells or control TE671-Luc-shRNA cells were infected with a constant amount of N-MLV_{GFP} in the presence of increasing amounts of HIV-1 VLPs, either wild type or G89V. Consistent with previous observations (61), HIV-1 G89V VLPs enhanced N-MLV titer in both TE671-CypA-shRNA and control TE671-Luc-shRNA cells (Fig. 7A). In TE671-Luc-shRNA cells, wild-type HIV-1 VLPs increased N-MLV titer approximately threefold, though these effects were only evident at the highest quantities of VLPs adminis-

FIG. 6. Promotion of HIV-1 infectivity by CypA is independent of TRIM5 α_{hu} , regardless of the route of viral entry. HIV-1_{NL4-3} virions were used to infect CEM-SS-Luc-shRNA (A) and CEM-SS-TR5-shRNA cells (B), in the presence or in absence of CsA, as indicated. HIV-1_{NL4-3} virions, either wild type or G89V mutant, as indicated, were used to infect CEM-SS-Luc-shRNA (C) and CEM-SS-TR5-shRNA cells (D). The percentage of infected cells was determined by flow cytometry after immunostaining for CA. Shown are representative results of a single experiment. Identical results were obtained on three separate occasions using independently produced viral stocks.

tered (Fig. 7B). The same VLPs were much more efficient at abrogating restriction to N-MLV in TE671-CypA-shRNA cells, indicating the importance for the saturation mechanism of a factor that selectively recognizes HIV-1 CA in the absence of CypA (Fig. 7B).

A VLP titration experiment was also performed in the context of TRIM5 α_{hu} knockdown. Here cells were challenged with a fixed amount of HIV-1_{GFP} G89V in the presence of increasing quantities of either N-MLV or B-MLV VLPs. Compared with B-MLV VLPs, N-MLV VLPs specifically increased HIV-1_{GFP} G89V titer in TE671-Luc-shRNA cells (Fig. 7C). No infectivity increase was observed in TE671-TR5-shRNA cells (Fig. 7D). These results indicate that TRIM5 α_{hu} is required for saturation of restriction machinery by N-MLV VLPs.

HIV-1 CA mutant A92E is not hypersensitive to TRIM5 α_{hu} . HIV-1 CA mutant A92E was selected by serial passage of HIV-1_{NL4-3} in the presence of a nonimmunosuppressive analogue of CsA (1, 26, 51, 66). In some cell lines, A92E renders HIV-1 CsA resistant. In HeLa cells, A92E is CsA dependent and its infectivity is stimulated by removal of target cell CypA (1, 26, 51, 66). The inhibitory effect of CypA on A92E replication in HeLa cells is reminiscent of the situation in Old

FIG. 7. Abrogation of N-MLV restriction in TE671 cells by HIV-1 VLPs is enhanced by CypA knockdown. TE671-CypA-shRNA or TE671-Luc-shRNA cells were infected with constant amounts of N-MLV_{GFP} in the presence of increasing quantities of HIV-1 G89V VLPs (A) or HIV-1 WT VLPs (B). TE671-Luc-shRNA (C) or TE671-TR5-shRNA (D) cells were infected with constant amounts of HIV-1_{GFP} G89V in the presence of increasing quantities of N- or B-MLV VLPs. The percentage of GFP-positive (infected) cells was determined by flow cytometry. Identical results were obtained on three separate occasions using independent VLP stocks.

FIG. 8. CsA dependence of HIV-1 CA mutant A92E is TRIM5 α_{hu} independent. HeLa cells were transduced with an MLV-based vector delivering an shRNA expression construct specific for human TRIM5. VSV-G-pseudotyped, N- and B-tropic MLV_{GFP} virions were used to infect control HeLa cells (A) or HeLa-TR5-shRNA cells (B). VSV-G-pseudotyped, HIV-1_{GFP} WT or A92E mutant virions were used to infect control HeLa cells (C) or HeLa-TR5-shRNA (D) cells, in the presence or absence of CsA, as indicated. The percentage of infected cells was determined by flow cytometry. Shown are representative results of a single experiment. Identical results were obtained on three separate occasions using independently produced viral stocks.

World primate cells, where CypA is required for TRIM5 α_{hu} -mediated restriction of HIV-1 (6).

To directly examine the mechanism underlying the CsA dependence of A92E replication, shRNA was used to downregulate endogenous TRIM5 α_{hu} in HeLa cells. N-tropic and B-tropic MLV_{GFP} virions were used to confirm TRIM5 α_{hu} knockdown (Fig. 8A and B), as described above. Cells were then challenged with HIV-1_{GFP} wild type or A92E mutant, in the presence or absence of CsA. HIV-1_{GFP} A92E showed the same CsA dependence in HeLa-TR5-shRNA cells as in the control HeLa cells (Fig. 8C and D). These results indicate that the unusual phenotype of this CA mutant is not explained by increased sensitivity to TRIM5 α_{hu} -mediated restriction.

DISCUSSION

Endogenous TRIM5 α_{hu} restricts HIV-1. In this report, several issues were addressed concerning CypA and TRIM5 α_{hu} . Previous studies had suggested that HIV-1 is susceptible to restriction by TRIM5 α_{hu} , but only when interaction between CA and CypA is disrupted (61). Other studies had shown that supranormal levels of TRIM5 α_{hu} inhibit the titer of wild-type HIV-1, even in the presence of CypA (27, 42, 52, 55). Here, endogenous levels of TRIM5 α_{hu} were shown to restrict HIV-1

in cells with normal CypA expression (Fig. 1). This innate anti-HIV-1 activity of TRIM5 α_{hu} holds significant potential for understanding the epidemiology, clinical course, and pathogenesis of AIDS. Polymorphisms in the gene encoding TRIM5 α_{hu} , or differences in TRIM5 α_{hu} expression level, might determine an individual's susceptibility to the acquisition of HIV-1 infection. Alternatively, TRIM5 α_{hu} might be added to the long list of HIV-1 replication barriers that contribute to the duration of clinical latency.

Effects of CypA on HIV-1 infectivity are independent of endogenous TRIM5 α_{hu} . Endogenous TRIM5 α_{hu} and CypA both influence HIV-1 infectivity, but, in contrast to findings in Old World primates, where HIV-1 restriction by TRIM5 α is CypA dependent (6), it was clearly demonstrated here that endogenous TRIM5 α_{hu} and CypA influence HIV-1 infectivity independently of each other (Fig. 2 and 4). These findings held for HIV-1 infection of adherent cells and of CD4⁺ T cells (Fig. 5). In apparent contrast to a previous study with CsA (3), the effects of the two host factors were the same whether HIV-1 entered target cells via direct fusion with the cytoplasm or via an endocytic pathway (Fig. 6). The discrepancy with the earlier report can be explained by the fact that the phenomena studied here concern CypA's role in postentry events (26, 51, 61) and the previous study examined the effect of CsA when the drug

was administered during virion production (3). The inhibitory mechanism of the drug given during virion production, in fact, does not involve disruption of CypA or the CA-CypA interaction (51).

Evidence that CypA protects HIV-1 from a restriction factor. Since the discovery of the HIV-1 CA/CypA interaction (35), several models have been proposed to explain the role of CypA in HIV-1 replication. The finding that HIV-1 is particularly good at saturating the TRIM5 α_{hu} -dependent restriction of N-MLV when CypA is removed (61) set the stage for the most recent model, namely, that CypA protects HIV-1 from restriction by TRIM5 α_{hu} . This model was supported by the discovery of the owl monkey fusion protein TrimCyp (48), the existence of which hinted that the functions of CypA and TRIM5 were somehow associated. In cells from rhesus macaques and African green monkeys, the TRIM5 α orthologues are indeed CypA dependent (6). Ever-more-convincing evidence was provided when a TE671 clone (17H1) that had been selected for loss of N-MLV restriction was found to be permissive for HIV-1 in a CypA-independent manner (47). Though all the results described above indicate that CypA protects HIV-1 from a host restriction factor, it was disappointing to find that neither TRIM5 α_{hu} expression levels nor the TRIM5 α_{hu} sequence was altered in clone 17H1 (47) (data not shown). To accommodate these reports, and the data presented here, the model requires modification: CypA does not protect HIV-1 from TRIM5 α_{hu} but from an unknown restriction factor. Though much progress has been made over the past 13 years, the exact role of CypA in HIV-1 replication remains a mystery.

Possible mechanisms by which CypA regulates HIV-1 CA recognition by the restriction machinery. CypA catalyzes the *cis-trans* isomerization of peptidyl-prolyl bonds (20, 33, 34, 56), and HIV-1 CA has been shown to be a catalytic substrate by nuclear magnetic resonance (11). In the absence of CypA, nearly 90% of the covalent bonds between glycine 89 and proline 90 are in the *trans* conformation (23). If restriction machinery only recognizes CA in its *trans* conformation, then CypA might protect HIV-1 by assisting in the formation of the restriction-resistant *cis*-isoform. Alternatively, CypA binding might mask CA residues or induce an allosteric change in CA that prevents recognition by the restriction machinery.

Cross-saturation and retroviral restriction pathways. Given that restriction of N-tropic MLV requires TRIM5 α_{hu} (27, 32, 42, 64), and given that CypA and TRIM5 α_{hu} act independently to regulate HIV-1 infectivity (Fig. 4), how does one explain the heightened ability of N-MLV and HIV-1 to cross-saturate when CypA is removed (61) (Fig. 7)? Cross-saturation implies the existence of a common factor required for restriction of both viruses. Biochemical experiments (49), as well as extensive genetic experiments (27, 32, 42, 64), indicate that TRIM5 α_{hu} is the recognition element for N-MLV. Data presented here indicate that an unknown factor must be required for HIV-1 CA recognition in the absence of CypA. How then can these findings be reconciled? The answer is suggested by the phenotype of clone 17H1 (47), which belies the presence of yet another unknown factor that is epistatic to the CA recognition components and required for restriction of both N-MLV and CypA-free HIV-1.

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