CHAPTER V: TRIM5α AND CYPA INDEPENDENTLY REGULATE HIV-1 INFECTIVITY

Endogenous TRIM5α_{hu} restricts HIV-1. Although TRIMα_{hu} has been shown to exhibit a mild anti-HIV-1 activity when re-introduced into human and feline cells (56, 64, 91, 111, 114), the question whether endogenous TRIM5α_{hu} restricts HIV-1 remained unanswered. Thus, TRIM5α_{hu} activity against HIV-1 could be an overexpression artifact arising from an overwhelming amount of a protein with an unspecific anti-retroviral activity.

To test the importance of TRIM5α_{hu} for HIV-1 infectivity, shRNA was used to downregulate endogenous TRIM5α_{hu}. We chose to work with TE671 cells because of their robust TRIM5α_{hu} phenotype. TE671 cells were transduced with an MSCV-based retroviral vector delivering a pSUPER-shRNA cassette that targets human TRIM5 (25). A construct delivering an shRNA specific for luciferase (103) 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, which is part of the retroviral vector.

A functional assay was utilized to determine if TRIM5α_{hu} knockdown was successful. Since TE671 cells restrict N-MLV more than 100-fold relative to B-MLV in a TRIM5α_{hu}–dependent fashion (56, 64, 91, 129), TE671 cells knocked down for TRIM5 (TE671-TR5-shRNA cells) were challenged with VSV-G pseudotyped B- or N-tropic MLV_{GFP} vectors. The two viral stocks were normalized based on RT activity, as well as on titer in non-restrictive *Mus dunni* tail fibroblasts, as previously described (13, 102, 121). In TE671-Luc-shRNA cells, N-MLV titer was more then 100-fold lower than that

of B-MLV (Fig. 5.1-A). In TE671-TR5-shRNA cells, the N-MLV titer was almost identical to that of B-MLV (Fig. 5.1-B), indicating that TRIM5α_{hu}–dependent restriction activity was eliminated.

The effect of TRIM5α_{hu} knockdown on HIV-1 infectivity was examined using VSV-G pseudotyped HIV-1_{GFP}. The titer of HIV-1_{GFP} was 3-fold higher on TE671-TR5-shRNA cells than on control TE671-Luc-shRNA cells (Fig. 5.1-C), demonstrating that endogenous TRIM5α_{hu} inhibits HIV-1 infectivity. This result is consistent with previous reports that TRIM5α_{hu} overexpression decreases HIV-1 infectivity (56, 64, 91, 111, 114).

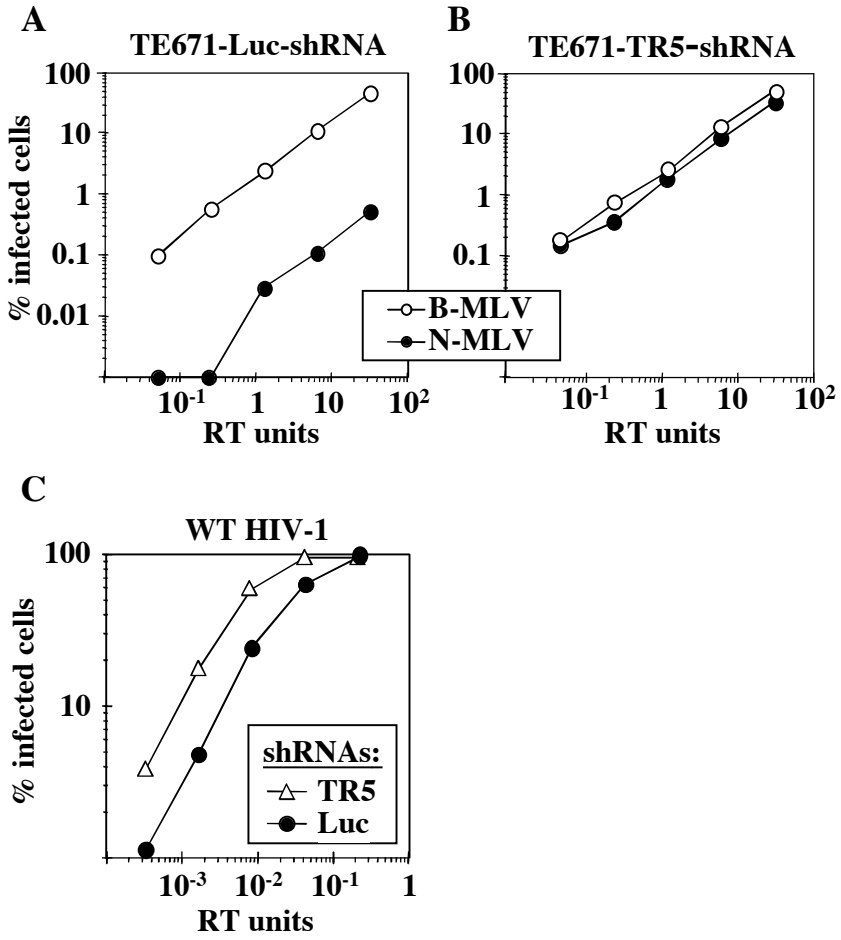

Figure 5.1. Knockdown of endogenous TRIM5α_{hu} enhances HIV-1 infectivity. TE671 cells were transduced with an MLV-based vector delivering an shRNA expression construct specific for human TRIM5 (TR5-shRNA), or luciferase (Luc-shRNA). VSV-G pseudotyped, N- and B-tropic MLV vectors were normalized by RT and by infectivity on non-restrictive *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.

Inhibition of HIV-1 infectivity by factors that disrupt the CA-CypA interaction is TRIM5α_{hu}-independent. To investigate whether HIV-1 is more susceptible to TRIM5α_{hu}–mediated restriction when the CypA-CA interaction is disrupted, 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 (77). The magnitude reduction of HIV-1 infectivity by CsA in TE671-TR5-shRNA cells was identical to the magnitude reduction by the drug in the control TE671-Luc-shRNA cells (Figs. 5.2-A and B). Similarly, the titer of HIV-1_{GFP}-G89V, a CA mutant that disrupts interaction with CypA (134), was equally decreased in TE671-TR5-shRNA and TE671-Luc-shRNA cells (Fig. 5.2-C 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 TRIM5α_{hu}-mediated restriction.

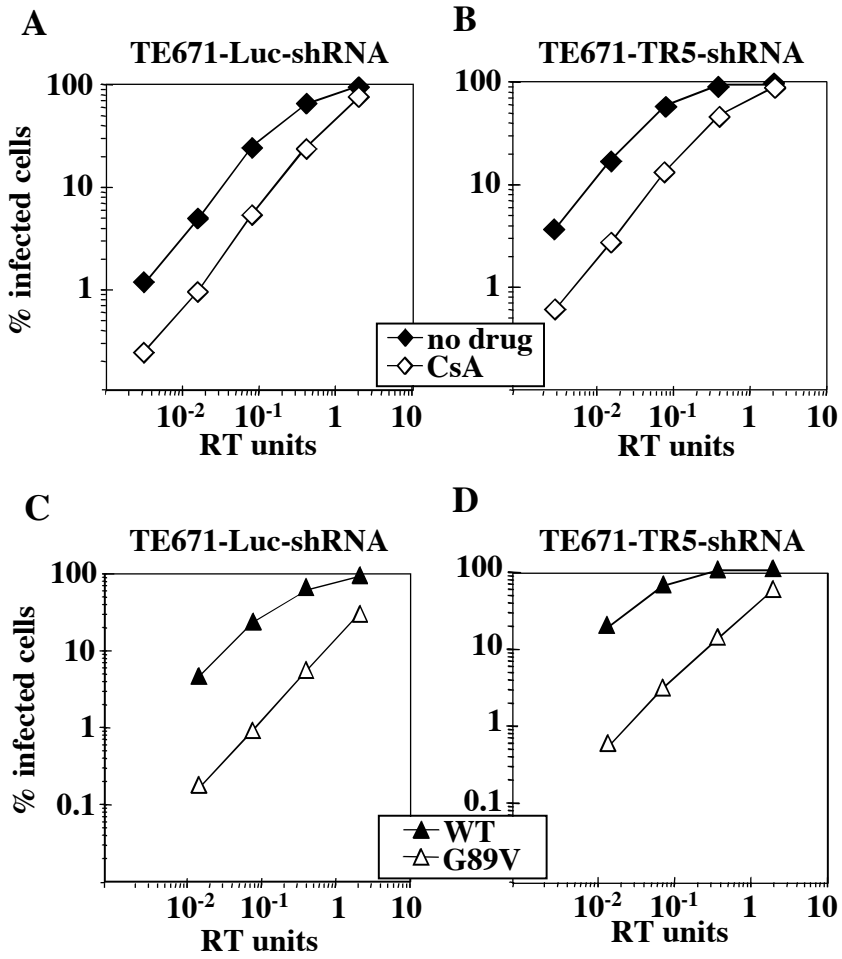

Figure 5.2. Reduction of HIV-1 infectivity by CsA or CA mutant G89V is not mediated by TRIM5α_{hu}. VSV-G pseudotyped, RT-normalized, HIV-1_{GFP} wild-type and G89V mutant virions were used to infect TE671 Luc-shRNA (A and C) and TE671 TR5-shRNA (B and D) cells in the presence or absence of CsA, as indicated. 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 infectivity by CypA knockdown is independent of TRIM5α_{hu}. To directly test the interdependence of TRIM5α_{hu} and CypA in mediating HIV-1 restriction, we generated cell lines in which both TRIM5α_{hu} and CypA were downregulated using shRNA. To accomplish this, the singly-transduced TE671-TR5-shRNA and TE671-Luc-shRNA cells (Fig. 5.2) were subjected to a second round of transduction with either the shRNA specific for CypA or with the control construct targeting luciferase. CypA knockdown was confirmed by western blotting (Fig. 5.3-A). Both TE671-TR5/CypA-shRNA and TE671-TR5/Luc-shRNA cell lines still failed to restrict N-MLV, confirming that the TRIM5α_{hu} knockdown was stable (data not shown). As before, cells in which TRIM5α_{hu} was downregulated were more permissive for HIV-1 infection than control cells (Fig. 5.3-B), and cells in which CypA was knocked down were less permissive for HIV-1 infection (Fig. 2.2). The magnitude of the infectivity decrease due to CypA knockdown was the same in TE671-TR5/CypA-shRNA and TE671-Luc/CypA-shRNA cells, as compared to the corresponding control cell lines TE671-TR5/Luc-shRNA and TE671-Luc/Luc-shRNA (Fig. 5.3-B). Similarly, the magnitude increase in infectivity due to disruption of TRIM5α_{hu} was the same whether CypA was expressed or not, or whether HIV-1 wild-type or G89V was used for infection (Fig. 5.3-B and C). As expected, infectivity of HIV-1 G89V remained unaltered by CypA knockdown (Fig. 5.3-C). These results demonstrate that TRIM5α_{hu} and CypA independently modulate HIV-1 infectivity. In other words, disruption of TRIM5α_{hu} does not rescue the reduction in HIV-1 infectivity caused by CypA knockdown.

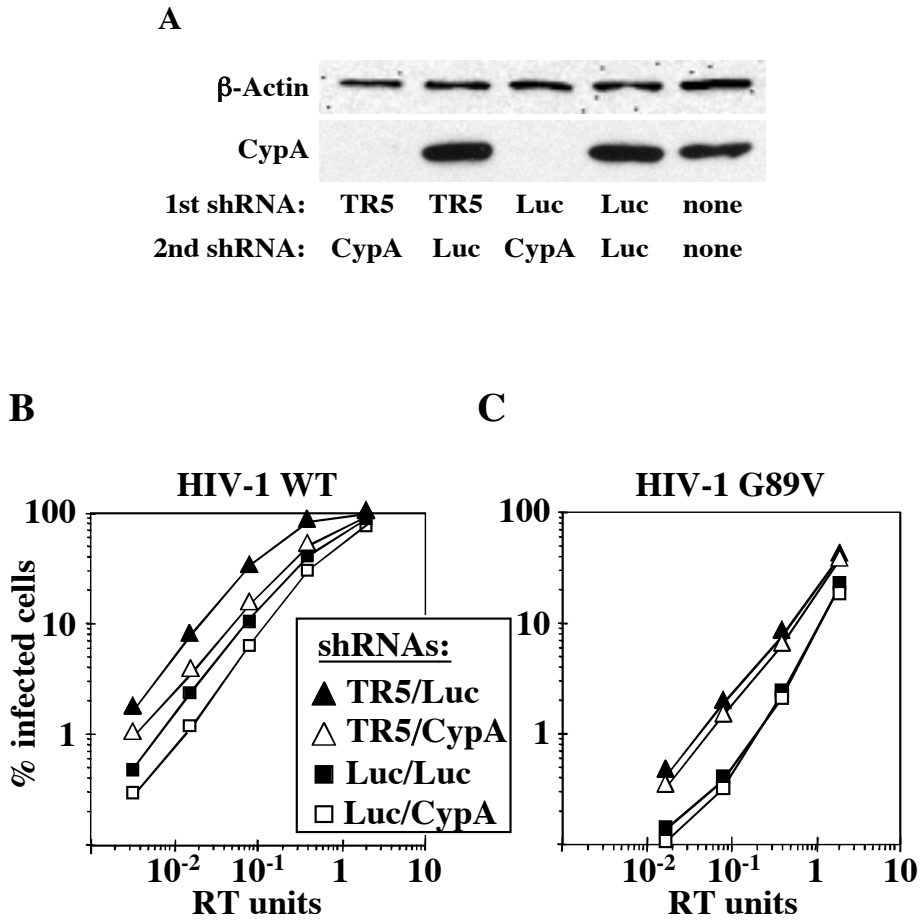

Figure 5.3. Reduction of HIV-1 infectivity by CypA knockdown is independent of TRIM5α_{hu}. CypA knockdown in TE671 TR5/CypA-shRNA and TE671 Luc/CypA-shRNA cells was demonstrated by Western blot for CypA and β-actin (A) . VSV-G pseudotyped HIV-1 WT (B) or HIV-1 G89V virions (C) were used to infect TE671 TR5/CypA-shRNA, TE671 TR5/Luc-shRNA, TE671 Luc/CypA-shRNA and TE671 Luc/Luc-shRNA cells. The percentage of GFP-positive cells was determined by flow cytometry. Shown are representative results of a single experiment. Identical results were obtained on three separate occasions.

CypA and TRIM5α_{hu} independently modulate HIV-1 infectivity in human CD4$^+$ T cells. TE671 is a rhabdomyosarcoma cell line that is useful for studying innate antiviral resistance mechanisms because of it's robust TRIM5α_{hu}-phenotype. However, HIV-1

does not naturally infect this type of tissue. To extend our findings to cells more relevant for HIV-1 infection, shRNAs were used to downregulate TRIM5 expression in human T cell lines. After several failed attempts to knock down TRIM5α_{hu} using the retroviral-based pSRP vector, we generated a lentiviral vector expressing the pSUPER-cassette specific for TRIM5α_{hu}. This vector allowed a far more efficient transduction of T cells. An isogenic construct delivering an shRNA specific for luciferase (103) was used as a control. Pools of transduced cells were selected in puromycin. As described above for the TE671 cells, TRIM5α_{hu} knockdown in CEM-SS was confirmed by challenging transduced cells with N- and B-tropic MLV_{GFP} (Fig. 5.4-A and B). Infectivity of VSV-G pseudotyped HIV-1_{GFP} was reduced to the same extent by CsA in CEM-SS-TR5-shRNA as it was in CEM-SS-Luc-shRNA (Fig. 5.4-C and D). Similarly, the titer of HIV-1_{GFP} G89V was decreased in both TRIM5α_{hu} knockdown and control cells (Figs. 5.4-C and D). This shows that the finding that CypA acts independently of TRIM5α_{hu} to modulate HIV-1 infectivity holds in human T lymphocytes.

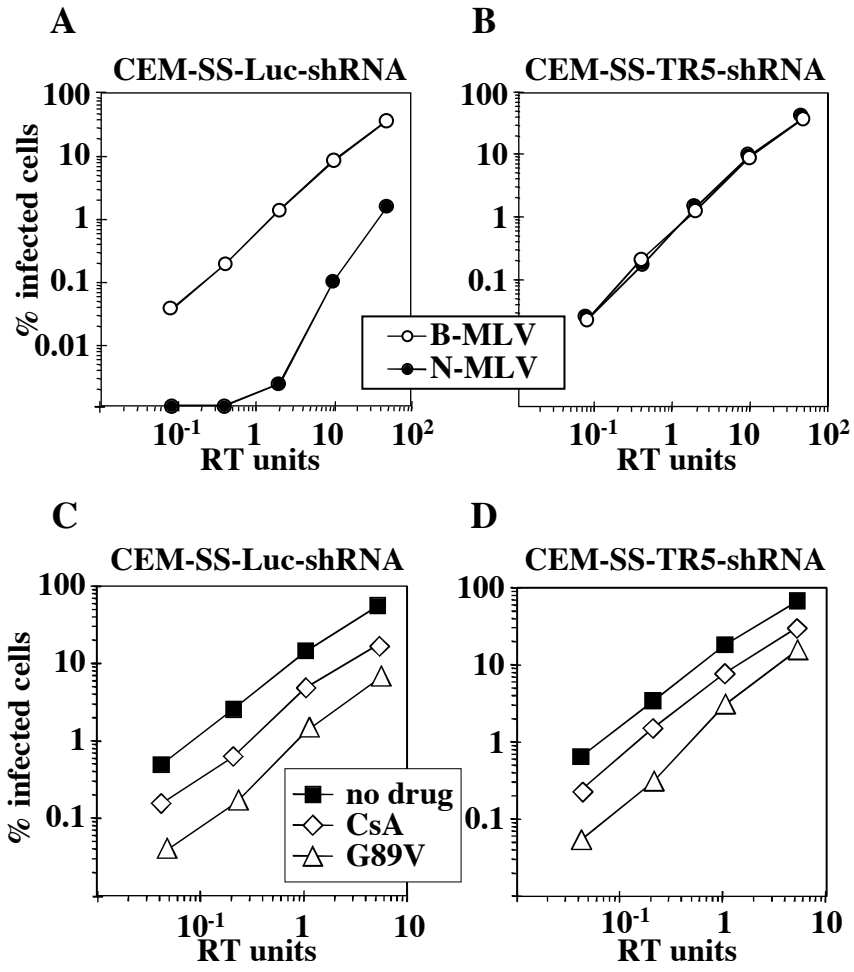

Figure 5.4. Reduction of HIV-1 infectivity by CsA or G89V mutation is independent on TRIM5α_{hu} in human T-cell lines. CEM-SS cells were transduced with an HIV-based vector delivering an shRNA expression construct specific for human TRIM5, or luciferase as a control. VSV-G pseudotyped, N- and B-tropic MLV vectors were normalized by RT and used to infect Luc-shRNA cells (A) or TR5-shRNA cells (B). VSV-G pseudotyped NL4-3_{GFP} virions were used to infect CEM-SS Luc-shRNA cells (C) and CEM-SS TR5-shRNA cells (D), in the presence or absence of CsA, as indicated. The percentage of GFP-positive (infected) cells was determined by flow cytometry.

Effects of CypA and TRIM5α_{hu} on HIV-1 infectivity are independent of the route by which virions enter target cells. Our studies (Fig. 3.4) and studies by others (4) indicated that pseudotyping with VSV-G renders HIV-1 relatively insensitive to CsA during virus production. 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 (8, 78, 112).

To determine if the functional independence of CypA and TRIM5α_{hu} is altered by the entry mechanism, TE671-TRIM5-shRNA and TE671-Luc-shRNA control cells were transiently transfected with a pcDNA construct containing human CD4 under the control of the CMV immediate early promoter. 48 hours post transfection, the cells were challenged with fully infectious wild-type and G89V HIV-1_{NL4-3}. The infection was stopped 16 hours later by adding dextrane sulfate to the medium. Infectivity reduction caused by CsA was of the same magnitude (about 4- to 5-fold) in cells lacking TRIM5α_{hu} expression as it was in cells with wild-type expression of TRIM5α_{hu} (Fig. 5.5-A and B). Similarly, titer reduction caused by the G89V mutation was of equal degree in both cell lines (Fig. 5.5-C and D). These results demonstrate that disruption of HIV-1 infectivity by the lack of CypA binding to CA is independent of TRIM5α_{hu}, and this independence is regardless of the route of viral entry. It is noteworthy that in this experiment, unlike in others, the infectivity disruption caused by CsA is greater than the disruption caused by the G89V mutation. This is explained by the fact in the case of a full-length infectious HIV-1, new virions are produced and released from infected cells into the CsA containing medium, from where they spread to new cells. Thus, this observation confirms previous

findings that CsA disrupts envelope-mediated viral entry into the cell when present during production, as shown before (Figs. 2.3 and 2.5).

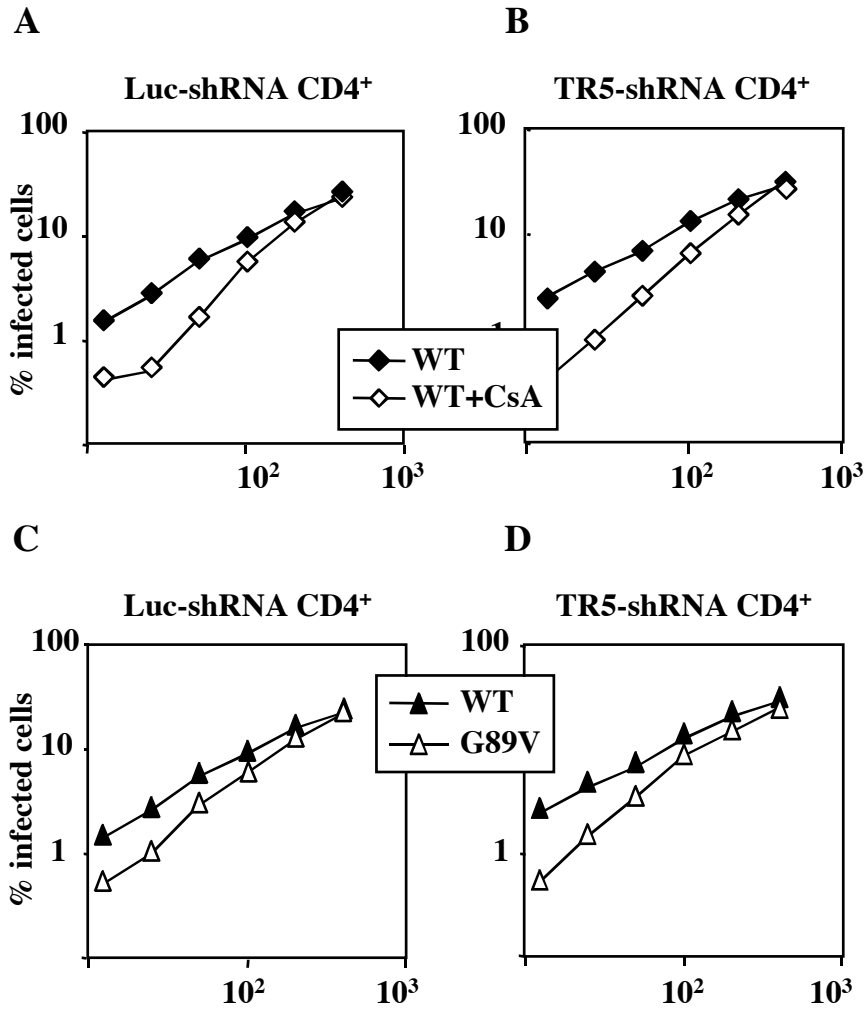

FIG. 5.5. Promotion of HIV-1 infectivity by CypA is independent of TRIM5a$_{hu}$ regardless of the route of viral entry. TE671-Luc-shRNA (A,C) and TE671-TR5-shRNA (B,D) cells were transiently transfected with CD4, and challenged with wild-type (A, B) and G89V (C, D) HIV-1$_{NL4-3}$ virions in presence or in absence of CsA, as indicated. 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.

To further investigate the functional interdependence of CypA and TRIM5α_{hu} with respect to 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. 5.6). Thus, CypA and TRIM5α_{hu} act independently to regulate HIV-1 infectivity, irrespective of the route of entry utilized by the virus.

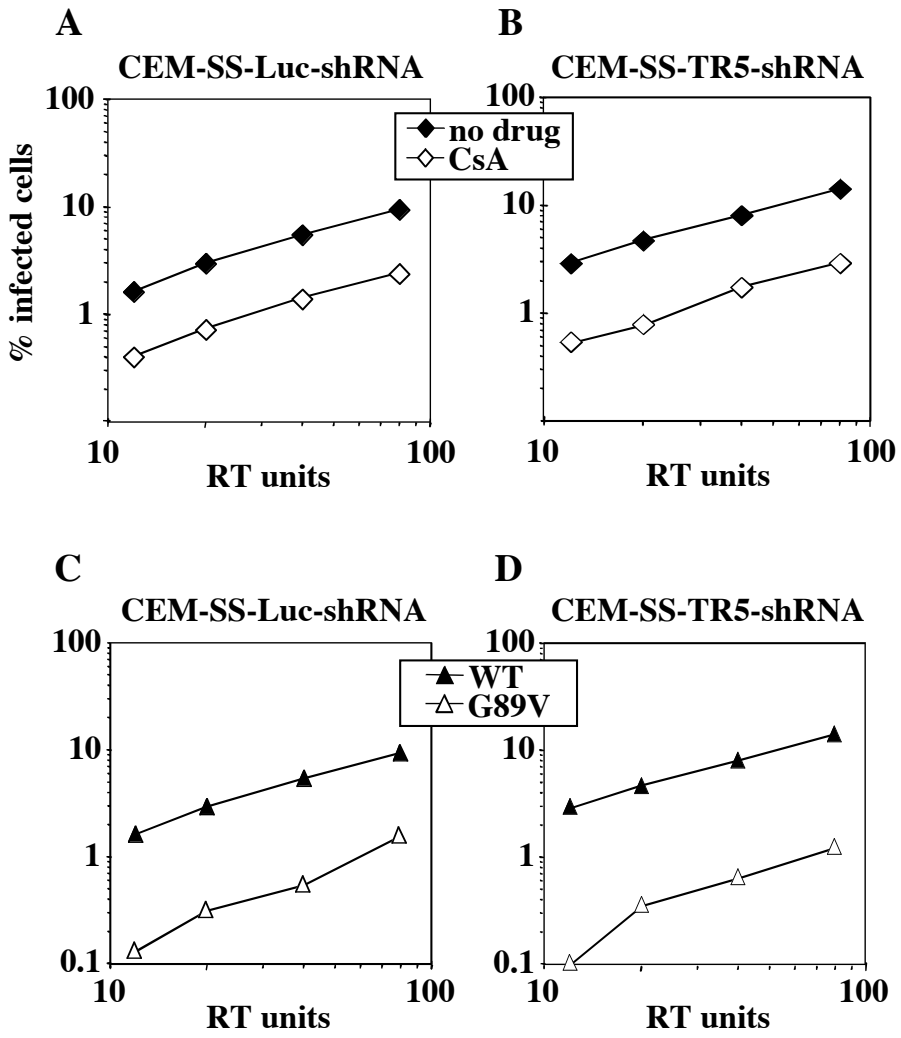

Figure 5.6. Promotion of HIV-1 infectivity by CypA is independent of TRIM5a$_{hu}$ regardless of the route of viral entry. HIV-1_{NL4-3} virions were used to infect CEM-SS Luc-shRNA (A), and CEM-SS TR5-shRNA cells (B), in presence or in absence of CsA, as indicated. The percentage of infected cells was 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.

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 (103, 114), and prior to the widespread use of RNAi 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 (123). Given that TRIM5α_{hu} exhibits a mild inhibitory effect on wild-type HIV-1 (Fig. 2.2), one would expect wild-type HIV-1 VLPs to also increase N-MLV titer. However, saturation experiments are technically difficult due to VLP toxicity at high concentrations, and such small phenotypes 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 (123), HIV-1 G89V VLPs enhanced N-MLV titer in both TE671-CypA-shRNA and control TE671-Luc-shRNA cells (Fig. 5.7-A). As expected, in TE671-Luc-shRNA cells, wild-type HIV-1 VLPs increased N-MLV titer approximately 3-fold, though these effects were only evident at the highest, almost toxic quantities of VLPs administered (Fig. 5.7-B). 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. 5.7-B).

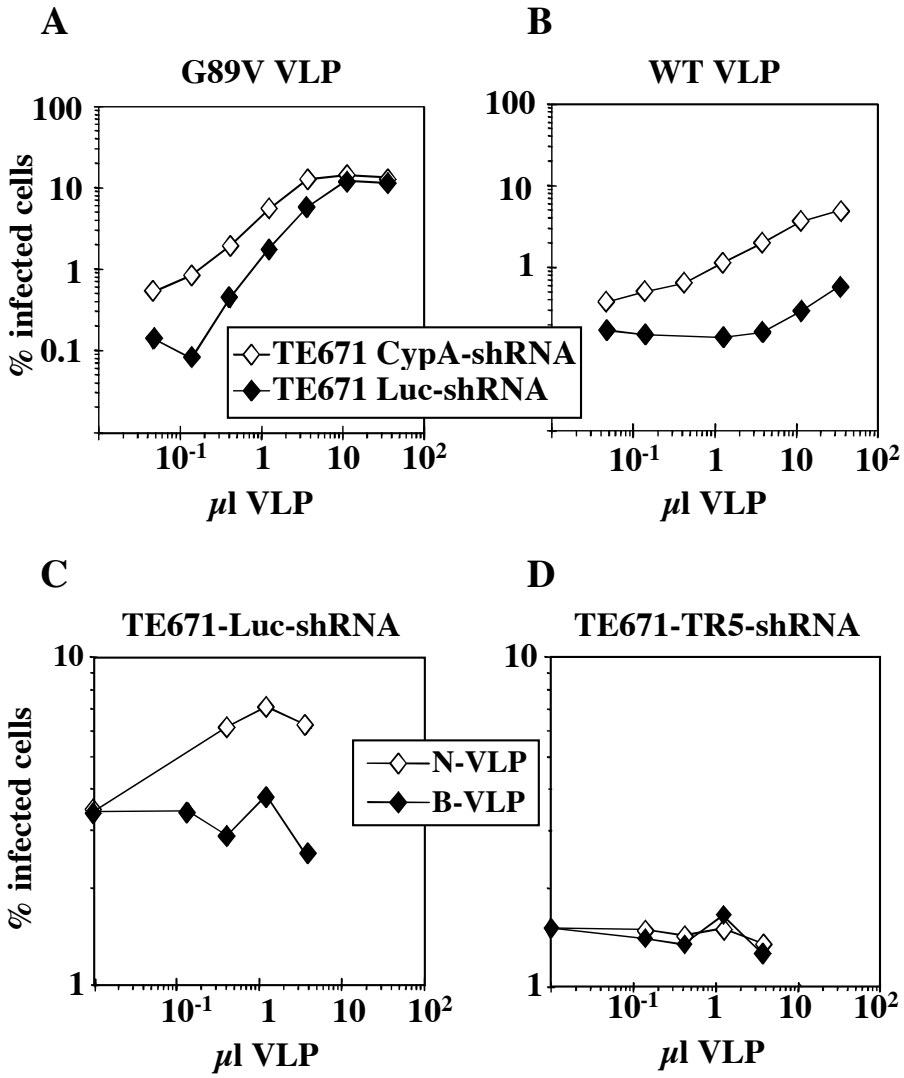

Figure 5.7. HIV-1 VLPs ability to abrogate N-MLV restriction in TE671 cells is enhanced if CypA expression is knocked down. Upper panel: TE671 CypA-shRNA or TE671 Luc-shRNA cells were infected with stable amounts of N-MLV reporter vector in the presence of increasing doses of HIV-1 WT VLPs (A) or HIV-1 G89V VLPs (B). Lower panel: TE671 Luc-shRNA (C) or TE671 TR5-shRNA (D) cells were infected with fixed amounts of HIV-1 G89V reporter vector in the presence of increasing doses 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.

A VLP titration experiment was also performed in the context of TRIM5α_{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. As compared with

B-MLV VLPs, N-MLV VLPs specifically increased HIV-1_{GFP} G89V titer in TE671-Luc-shRNA cells (Fig. 5.7-C). No infectivity increase was observed in TE671-TR5-shRNA cells (Fig. 5.7-D). These results indicate that TRIM5α_{hu} is required for saturation of restriction machinery by N-MLV VLPs.

Arsenic Trioxide (As_2O_3) stimulates viral infectivity. As_2O_3 is a drug originally used to treat acute promyolytic leukemia in patients bearing an oncogenic PML / α-retinoic receptor fusion protein, which arises during chromosomal translocation. Curiously, PML itself is a nuclear protein from the TRIM family of proteins, which has been shown to be involved in processes associated with infection by some DNA viruses, and with HIV-1. As_2O_3 has also been found to promote degradation of the PML protein itself, and to counteract TRIM5α-mediated restrictions to MLV and HIV-1 in both human and simian cells. However, very little is known about the mechanism by which arsenic mediates this stimulatory effect on N-MLV and HIV-1 infection. We therefore decided to directly investigate the role of TRIM5α in this stimulation of HIV-1 replication. TE671-TR5-shRNA and TE671-Luc-shRNA cells were challenged with wild-type and G89V HIV-1_{GFP} vectors in presence or absence of 2.5μM As_2O_3. In control cells, addition of arsenic resulted in a 3- to 5-fold increase of infectivity of both wild-type and G89V HIV-1_{GFP} (Fig. 5.8-A). In TE671-TR5-shRNA cells, the stimulatory effect of Arsenic was eliminated (Fig. 5.8-B), demonstrating that TRIM5α is required for this effect to occur.

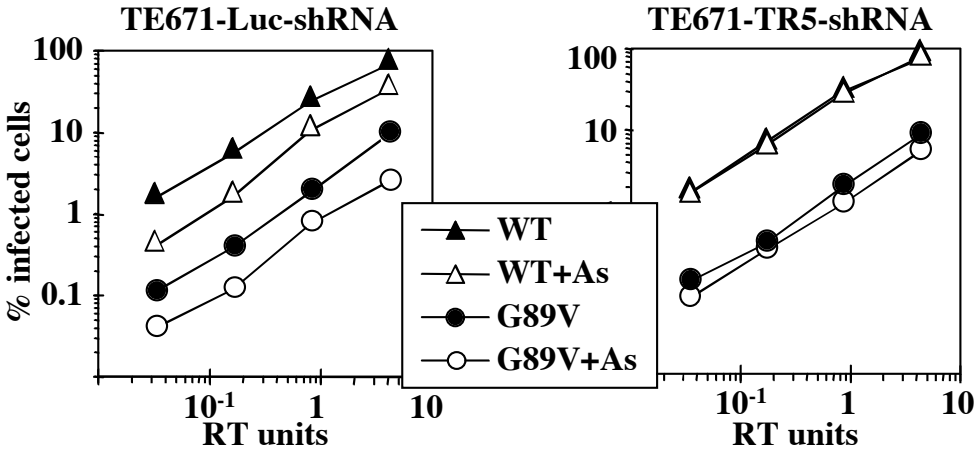

Fig. 5.8. TRIM5α knockdown eliminates the stimulatory effect of As_2O_3 in TE671 cells. TE671-Luc-shRNA and TE671-TR5-shRNA cells were challenged with wild-type and G89V HIV-1_{GFP} in presense and in absence of As_2O_3, as indicated. The percentage of infected cells was determined by flow cytometry.

CHAPTER VI: CELL-TYPE SPECIFIC PHENOTYPES OF HIV-1 CA VARIANTS ARE NOT DETERMINED BY TARGET CELL TRIM5α_{hu}

CA variants with cell-type specific phenotypes are not subject to restriction by TRIM5α_{hu}. The CA mutant A92E and the CA chimera containing CypA regions from HIV-1 Group O isolates exhibit unusual phenotypes with respect to target cell CypA. Recently emerging knowledge about retroviral restriction factors has led is us to speculation that this phenomenon might involve retroviral restriction. The modulation of infectivity by CsA and the fact that this phenotype is peculiar to a specific CA variant is reminiscent of the situation in old world primate cells, where CypA is required for