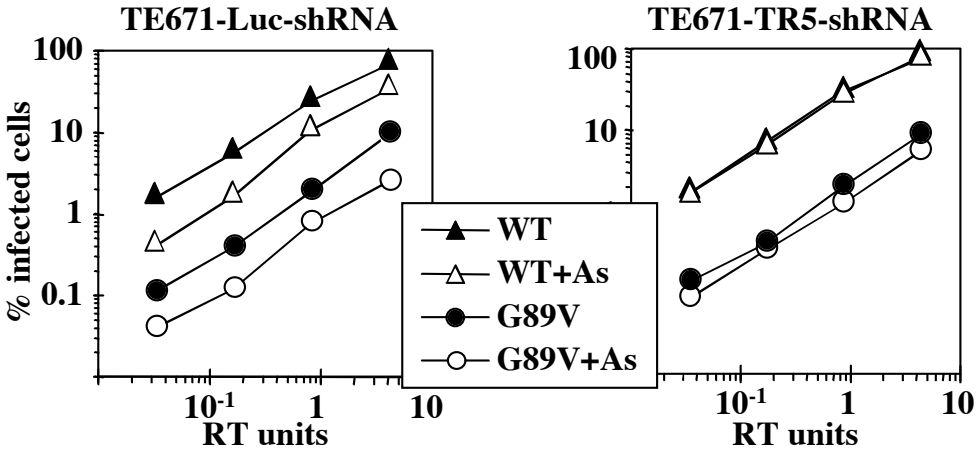

Fig. 5.8. TRIM5α knockdown eliminates the stimulatory effect of As_2O_3 in TE671 cells. TE671-Luc-shRNA and TE671-TR5-shRNA cells were challenged with wild-type and G89V HIV-1_{GFP} in presense and in absence of As_2O_3, as indicated. The percentage of infected cells was determined by flow cytometry.

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

TRIM5α_{hu}-mediated restriction of HIV-1 but not SIV (11). This observation suggests an existence of a restriction factor that specifically targets the A92E CA. Another important observation is the cell-type specificity of this phenotype. Thus, the functional factor seems to be present/active in some cells and lacking in others. The activity of this factor could arise from a gain-of-function change in a protein that is active as a retroviral restriction factor with a specificity to a different retrovirus. In addition, it is possible that a gene silent in the majority of human tissues is active in this cancer cell line. However, it is very unlikely for a cell to develop a unique restriction factor specific for one particular CA mutant. The naturally occurring CypA-independent HIV-1 Group O isolates represent a convincing evidence that such restriction factor may exist.

We next assessed the question whether the A92E phenotype is mediated by an altered TRIM5α_{hu} restriction activity peculiar to HeLa and H9 cell lines. This unusual activity could arise either from alterations in the TRIM5a protein itself, or from differences in putative co-factors involved in TRIM5α_{hu} restriction. It is also possible that the A92E mutation itself renders HIV-1 CA susceptible to TRIM5α_{hu}. We therefore tested the hypothesis that CsA-dependence of CA/A92E results from altered TRIM5α_{hu} activity in HeLa cells.

As mentioned above, TRIM5α_{hu} activity targeting N-tropic MLV or HIV-1 can be overcome by As_2O_3 (13). To test whether the CsA-dependence of HIV-1_{GFP}/A92E reflects sensitivity to TRIM5α_{hu}, HeLa cells were infected with HIV-1_{GFP} vectors in the presence of As_2O_3 over a range of drug concentrations. Infectivity of the HIV-1_{GFP}/G89V control was increased by 4-fold (Fig. 6.1). In contrast, the drug had no effect on HIV-1_{GFP} /A92E (Fig. 6.1), indicating that this CA variant is not TRIM5α_{hu} target.

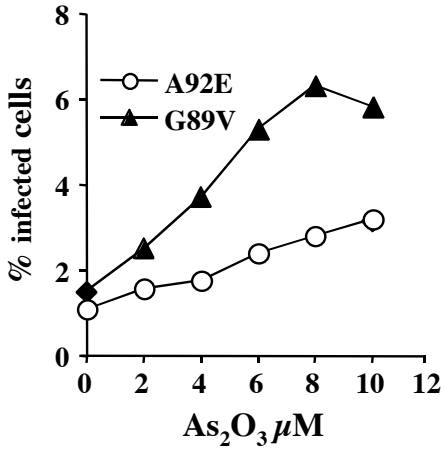

Figure 6.1. Restriction of the CA mutant A92E can not be overcome by As_2O_3. VSV-G pseudotyped HIV-1-GFP vectors bearing either CA mutation A92E or G89V, were used to infect HeLa cells in the presence of As_2O_3. The percentage of GFP positive cells is plotted as a function of As_2O_3 concentration.

To directly examine the mechanism underlying CsA-dependence of HIV-1/A92E replication, shRNA was used to downregulate endogenous TRIM5α_{hu} in HeLa cells. N-tropic and B-tropic MLV_{GFP} were used to confirm TRIM5α_{hu} knockdown (Fig. 6.2-A 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 (Figs. 6.2-C and D). These results directly demonstrate that the unusual phenotype of this CA mutant is not explained by increased sensitivity to TRIM5α_{hu}-mediated restriction.

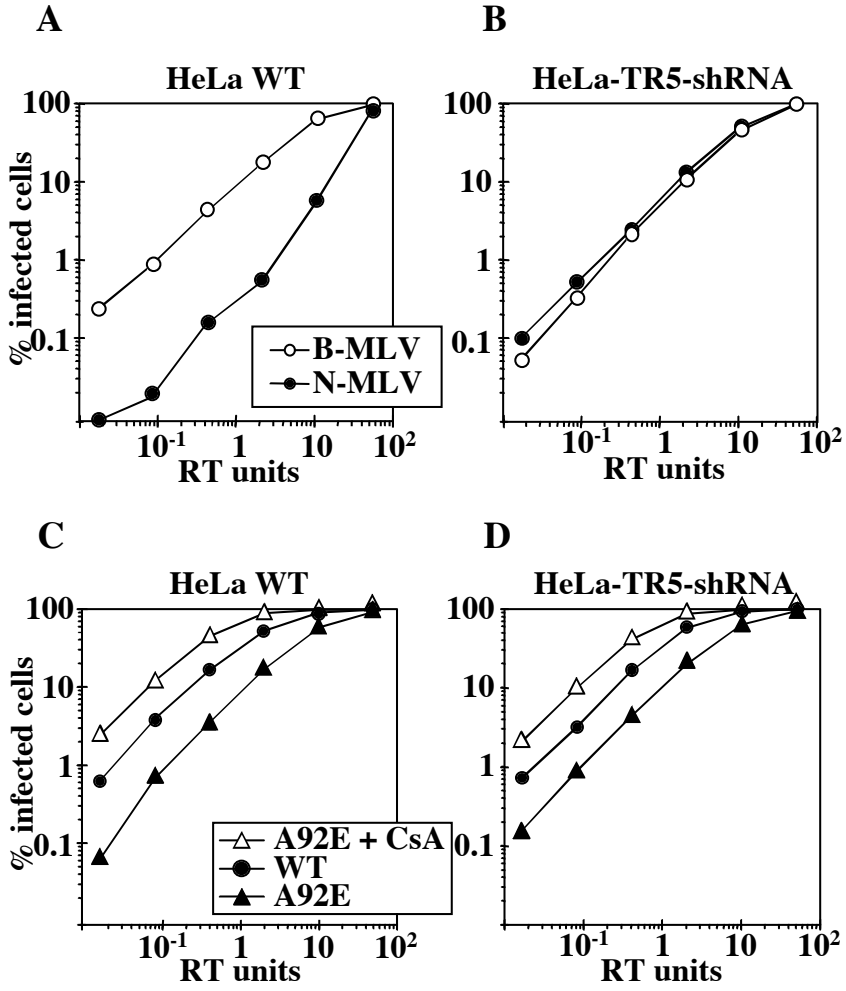

Figure 6.2. CsA-dependence of HIV-1 CA mutant A92E is TRIM5α_{hu}-independent. HeLa cells were transduced with an MLV-based vector delivering an shRNA expression construct specific for human TRIM5. VSV-G pseudotyped, N- and B-tropic MLV_{GFP} were used to infect control HeLa cells (A) or HeLa-TR5-shRNA 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.

Cell-type specific phenotype of HIV-1 CA variants are not determined by CypA expression levels. The original theory proposed that stoichiometric levels of CypA binding to HIV-1 CA are crucial for the core disassembly after viral entry. Thus, CypA binding to CA was proposed to facilitate uncoating by providing a certain degree of destabilization to the viral core, while in the absence of CypA the core is too stiff to disassemble. If A92E mutation had a destabilizing effect per se, this destabilization of the CA would be suffice to induce disassembly in the absence of CypA (presence of CsA) in Jurkat cells, hence the CsA-insensitivity of HIV-1 in these cells. The CypA expression levels in H9 are much higher. Too much CypA in addition to the destabilizing effect of the A92E mutation causes a rapid decay of the CA shell, impeding essential steps like reverse transcription, so that CypA depletion by addition of CsA is beneficial for viral replication. Thus, differences in CypA expression levels among cell types were thought to be critical for the early events of viral replication.

HeLa, 293T, Jurkat and H9 cell lysates were prepared, normalized by protein concentration using Bradford protein assay, and subjected to Western blotting using an anti-CypA antibody (Biomol). To ensure equal sample loading, a Coomassie Brilliant Blue (Sigma) stain of the blot membrane was performed. Since all cells contained comparable amount of CypA protein (Fig. 6.3), we conclude that CypA expression levels do not contribute to the cell-type specific phenotypes of HIV-1 CA variants.

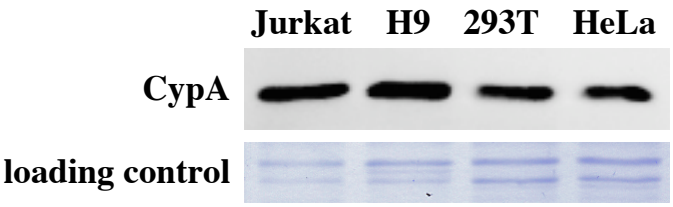

Figure 6.3. CypA expression levels among different cell types. Cells were lyzed, normalized by protein concentration and subjected to Western blotting using the anti-CypA antibody. To verify equal sample loading, the PVDF membrane was stained with Coomassie Blue.

The factor mediating restriction to A92E is not saturable. TRIM5α_{hu} mediated restriction can be saturated by loading cells with VLPs, as long as the VLPs bear CAs from susceptible viruses (123). Although we have shown that the A92E phenotype is not mediated by TRIM5α_{hu}, this phenotype could arise from a similar type of restriction mediated by an unknown factor. Existence of such factor is suggested by the CA-specificity, the modulation by CypA and the cell-type specificity. We next assessed the mechanism by which this factor exerts its restrictive activity. Here, from all CA variants exhibiting the cell-specific CsA resistance/dependence, the A92E mutant was chosen for further studies because of the strongest phenotype with respect to CypA in HeLa cells.

HeLa cells were infected with constant amounts of HIV-1_{GFP}/A92E in the presence of HIV-1 wild-type or mutant VLPs. Although wild-type HIV-1 VLPs were able to increase the titer of wild-type HIV-1 vector by about 4-fold (Fig. 6.4-A), they failed to abrogate restriction to HIV-1_{GFP}/A92E (Fig. 6.4-B). When A92E HIV-1 VLPs were used, they were able to elevate the titer of the wild-type vector by about 3.5-fold. However, this mutant VLP failed to have any effect on the CA with the same A92E

mutation. These results demonstrates that the restriction factor targeting A92E is not saturable by neither homologous nor heterologous HIV-1 VLPs.

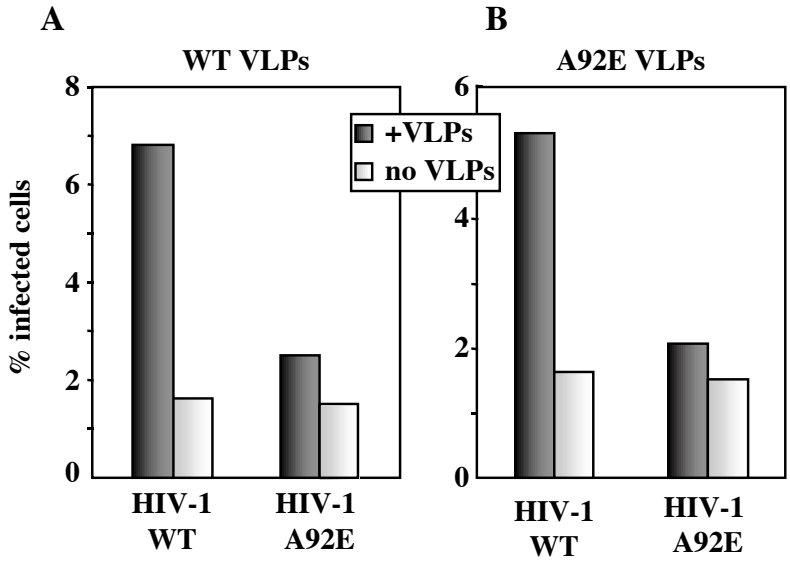

Figure 6.4. Restriction to A92E is not saturable by VLPs. HeLa cells were infected with either wild type or A92E mutant HIV-1 vectors in the presence of the wild-type (A) or A92E mutant (B) VLPs. The percentage of GFP-positive cells was determined by flow cytometry. Identical results were obtained on three separate occasions using independent VLP stocks.

To investigate whether N-MLV can saturate the factor, HeLa cells were infected with fixed amounts of the HIV-1_{GFP}/A92E vector in the presence of increasing amounts of N-MLV VLPs. N-MLV_{GFP} was used as control. As shown before, the VLPs were very effective at saturating restriction to G89V (123), (Fig. 6.5-B). However, no elevating of A92E titer was observed (Fig. 6.5-A). This result demonstrates again that the factor restricting A92E is not saturable by high amounts of incoming CAs.

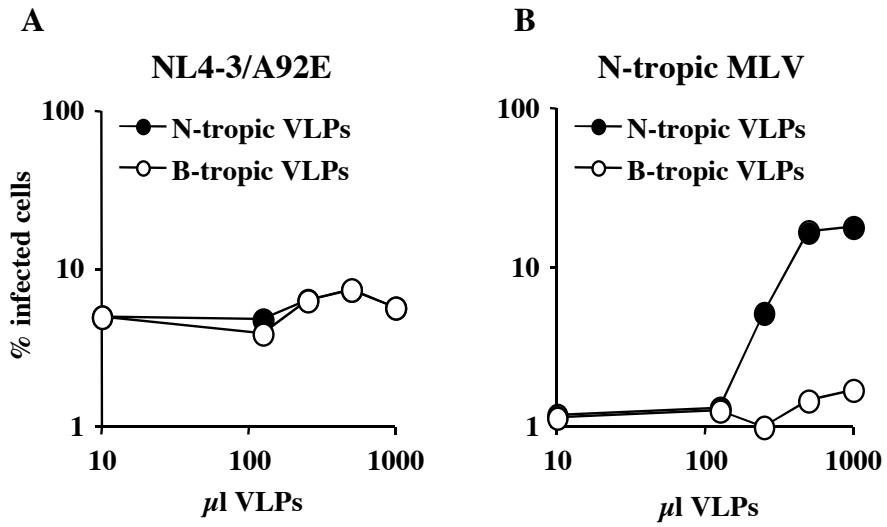

Figure 6.5. Restriction of A92E is not saturable by TRIM5α-susceptible CAs. HeLa cells were infected by VSV-G pseudotyped HIV-1-GFP vector bearing the CA-A92E mutation (A), or by VSV-G pseudotyped N-tropic MLV-GFP vector (B), in the presence of the indicated amounts of N-tropic or B-tropic MLV VLPs. The percentage of GFP-positive (infected) cells, as determined by flow cytometry, is plotted as a function of the VLP dose. Shown are results from a representative experiment. Identical results were obtained on three occasions using a wide range of MOIs.

DISCUSSION

Endogenous TRIM5α_{hu} inhibits HIV-1. The importance of TRIM5α as a factor regulating retroviral infectivity has been demonstrated in numerous studies since the discovery of the protein, as it has been shown to exhibit anti-retroviral activity in cells of several primates. Unfortunately, its strong anti-HIV-1 activity is limited to non-human primates species, whereas the human orthologue is only active against N-MLV.

However, studies had shown that TRIM5α_{hu} can inhibit the titer of wild-type HIV-1 when overexpressed at supranormal levels (56, 64, 82, 91, 114). In this study, we present evidence that endogenous TRIM5α_{hu} targets HIV-1, even though the degree of inhibition is rather modest when compared to the strong HIV-1 restriction in some monkey species (Fig. 24). This innate anti-HIV-1 activity of TRIM5α_{hu} holds significant potential for understanding the epidemiology, clinical course, and pathogenesis of AIDS. Polymorphisms in the gene encoding TRIM5α_{hu} might determine individual susceptibility to acquisition of infection, as well as contribute to duration of clinical latency. In addition, it is possible that differences in TRIM5α_{hu} expression levels influence rates of disease progression.

TRIM5α_{hu} and CypA independently regulate HIV-1 infectivity. Since the discovery of the HIV-1 CA/CypA interaction (77), 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 TRIM5α_{hu}-dependent restriction of N-MLV when CypA is removed (123) set the stage for the most recent model, namely, that CypA protects HIV-1 from restriction by TRIM5α_{hu}. Discovery of the owl monkey fusion protein TRIMCyp supported this model, as its existence hinted that CypA and TRIM5α are functionally interrelated (103). Recent studies of the CypA role in TRIM5α mediated restriction in cells from rhesus macaques and African green monkeys showed that these TRIM5 orthologues are indeed CypA dependent in these species (11).

Though all the results described above indicate that CypA protects HIV-1 from TRIM5α_{hu}, it was disappointing to find that elimination of TRIM5α expression does not

alter HIV-1 CypA-dependency. Experiments presented here show that both endogenous TRIM5α_{hu} and CypA influence HIV-1 infectivity, but, in contrast to findings in Old world primates (11), is was clearly demonstrated here that endogenous TRIM5α and CypA regulate HIV-1 infectivity independently of each other (Figs. 5.2 and 5.3). These findings held for HIV-1 infection of both adherent cells and in $CD4^+$ T lymphocytes (Fig. 5.4). In addition, the effects were the same regardless of whether HIV-1 entered target cells via direct fusion with the cytoplasm or via an endocytic pathway (Figs. 5.5 and 5.6). This finding stands in apparent contrast with the earlier report stating that pseudotyping HIV-1 with VSVG frees the virus from CypA dependence (4). This discrepancy can be explained by the fact that the earlier study examined the effect of CsA when the drug was administered during virus assembly, while the phenomena studied here concern CypA's role in post entry effects.

Evidence that CypA modulates restriction. Though much progress has been made over the past 13 years, the exact role of CypA in HIV-1 replication remains a mystery. We have now demonstrated that TRIM5α is not directly involved in restriction of CypA-free HIV-1. However, our experiments suggest that CypA provides protection of the virus core from a restriction activity in the target cell.

What is the evidence that CypA-free HIV-1 CA is targeted by a restriction factor rather then just being a case of a replication defect? One clue has been obtained from experiments with HeLa and H9 cells, which have shown that CypA-dependent phenotype of wild-type HIV-1 is cell-type specific (1, 55, 109, 133), (Figs. 13 and 14). This observation hints that there is a factor responsible for restriction that is present in some

cell types and missing in others.

Most importantly, experiments with a human cell line TE671 provided the strongest support for this hypothesis. This cell line, which is known for its potent TRIM5α_{hu} phenotype, was subjected to selection for loss of N-MLV restriction, until a clone that had no restriction activity against N-MLV could be isolated. In this clone, dubbed 17H1, HIV-1 replication has become CypA independent (102), indicating that CypA-free HIV-1 is inhibited by the same activity that restricts N-MLV. However, consistently with findings presented here, neither TRIM5α_{hu} sequence nor TRIM5α_{hu} expression levels were altered in 17H1 (102). These very important observations suggest that (i) CypA-free HIV-1 is subject to retroviral restriction and (ii) TRIM5α_{hu} – mediated restriction and the restriction of CypA-free HIV-1 are parts of one pathway. To accommodate these reports and the data presented here, we propose a modification of the previous model: CypA does not protect HIV-1 from TRIM5α_{hu} but from an unknown restriction factor in the target cell.

Cross-saturation and retroviral restriction pathways. Given that restriction of N-tropic MLV requires TRIM5α_{hu} (56, 64, 91, 129), and given that CypA and TRIM5α_{hu} act independently to regulate HIV-1 infectivity (Fig. 5.2), how does one explain the heightened ability of N-MLV and HIV-1 to cross-saturate when CypA is removed (123) (Fig. 5.7)? Cross-saturation is another evidence for the existence of a common factor required for restriction of both viruses. Biochemical experiments (104), as well as extensive genetic experiments (56, 64, 91, 129), indicate that TRIM5α_{hu} is the recognition element for N-MLV CA. 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 (102), which belies the presence of yet another unknown factor that is epistatic to the CA recognition elements and required for restriction of both N-MLV and CypA-free HIV-1 (Fig.). It also explains the requirement of TRIM5α_{hu} for saturation of restriction by N-MLV VLPs (Fig. 7.1): TRIM5α_{hu} is serving as a necessary adapter between the N-MLV CA and the epistatic common factor.

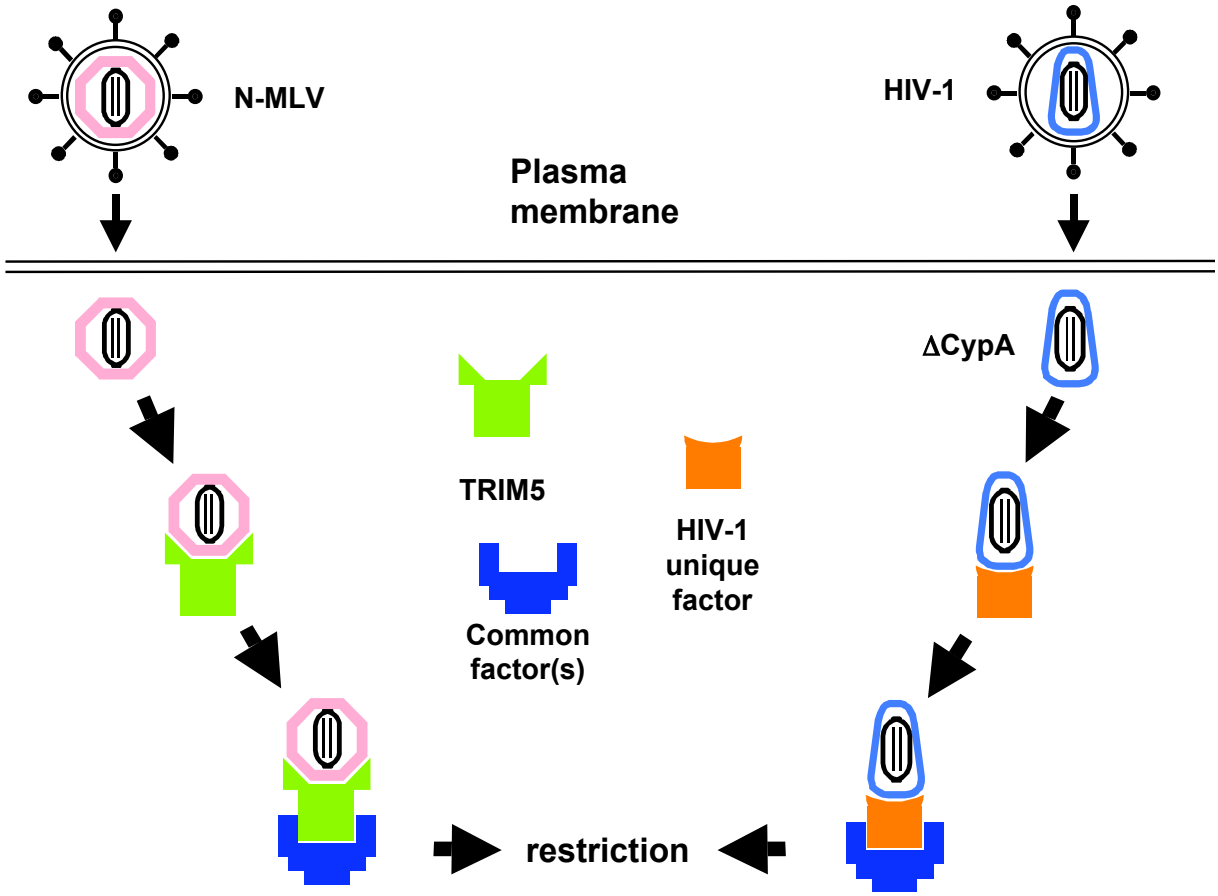

Figure 7.1. Proposed model for restriction of N-MLV and HIV-1. N-MLV CA and CypA-free HIV-1 CA are recognized by unique CA binding factors which then utilize a common factor to target the viruses for restriction through a common, saturable pathway.

Possible mechanisms by which CypA regulates HIV-1 CA recognition by the restriction machinery. As a member of the cyclophilin family, CypA catalyzes the *cis-trans* isomerization of peptidyl-prolyl bonds (41, 65, 71, 117). In fact, HIV-1 CA is so far the only protein that has been found to be a CypA catalytic substrate in vitro, as shown by nuclear magnetic resonance (18). In the absence of CypA, nearly 90% of the covalent bonds between glycine 89 and proline 90 are in the *trans* conformation (50). If restriction machinery only recognizes the CA *trans*-isomer, then CypA might protect HIV-1 by assisting in the formation of the restriction-resistant *cis*-isoform. Alternatively, CypA binding might simply mask CA residues that are important for the interaction between CA and the restriction factor. Finally, CypA binding might induce an allosteric change in CA that prevents recognition by the restriction machinery.

CA variants with unusual phenotypes. Previous experiments suggested that the CsA-dependence of the A92E mutant in H9 cells results from elevated levels of CypA in H9 cells, when compared to Jurkat cells (2, 133). This hypothesis seems not to hold up in that CsA-dependence of A92E is more pronounced in HeLa than in H9 cells (data not shown) yet the levels of CypA expression in HeLa are not significantly elevated as compared to 293T cells, a cell line which exhibits the same phenotype as Jurkat cells (Fig. 6.3).

The phenotype of the A92E mutant in HeLa cells resembles TRIM5α_{hu}-mediated restriction in non-human primate cells, where HIV-1 replication is inhibited at an early post-entry step of infection and can be rescued by CsA (64, 103, 123, 129). We used two different approaches, suppression of TRIM5α_{hu} activity by As_2O_3 and downregulation of

TRIM5 protein by RNAi, to address the question of whether the CsA-dependent phenotype of HIV-1 CA variants in HeLa cells results from altered TRIM5α_{hu} activity in these cells. Both experiments indicated that TRIM5α_{hu} is not restricting NL4-3/A92E (Figs. 6.1 and 6.2). Additionally. we attempted to saturate the putative restriction factor using HIV-1 VLPs bearing the A92E mutation. Evidence of a saturable restriction factor was not obtained (Figs. 6.4 and 6.5).

The group O viruses HIV-1_{CA9} and HIV-$1_{MVP5180}$ are resistant to cyclosporine in Jurkat or PBMCs (22, 127). Based on this phenotype similarity with the A92E mutant we hypothesized that the Group O viruses would behave similarly in HeLa cells in single-cycle assays. Vectors bearing the CypA-binding loop from these viruses (Fig. 2.6) indeed exhibited the same cell-type specific response to CsA (Figs 2.8, 2.7 and 2.9). Thus, viruses bearing CAs that confer this phenotype occur in infected patients. Also, the dramatically different phenotypes of different human cell lines raises the possibility that such variations might occur among people and might render individuals differentially susceptible to HIV-1 infection.

Conclusions. Very little is known about processes that occur shortly after a retrovirus fuses with the cell membrane. The step in the retroviral life cycle that is generally referred to as “uncoating” is a logical, yet still a hypothetical process. Although a lot of progress has been made to understand the details, only a few clues are available about mechanisms of this phase in the retroviral infection, and about cellular factors that orchestrate this process. Fv1 restriction has been described and characterized many years ago, but even after isolation of Fv1, its mechanism of action remains unknown. CypA

was shown to mediate HIV-1 infectivity via interactions with HIV-1 CA, but only recently has it become clear that it is altering recognition of the retrovirion latticework by host restriction factors. The recent discovery of TRIM5α has provided an enormous advance to our knowledge of host factors that regulate post-entry replication steps in the retroviral life cycle.

Retroviral restriction represents an addition of a new chapter into our knowledge about mechanisms and factors of the innate immunity. One of the most devastating diseases of our time, AIDS, is caused by a retrovirus, but despite enormous efforts to develop antiretroviral treatment or preventive measures, the means available today are not sufficient to combat the disease. Antiretroviral drugs represent a powerful tool against HIV-1, but rapidly emerging resistances to available compounds make them inefficient in controlling the HIV-1 epidemic. Efforts to develop anti-HIV-1 vaccines are impeded by the inability of the adaptive immune system to produce an adequate response to HIV-1 infection. The emerging knowledge about natural mechanisms of innate antiretroviral immunity, combined with gene therapy approaches, will open up a possibility to develop a new class of anti-HIV-1 drugs.