B

Low Multiplicity of Infection **High Multiplicity of Infection**

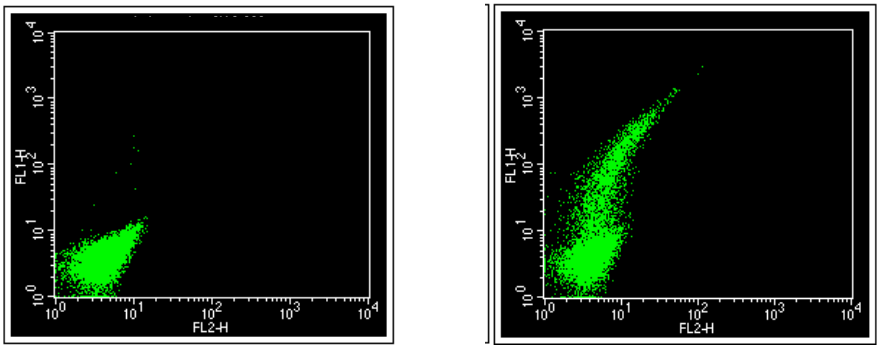
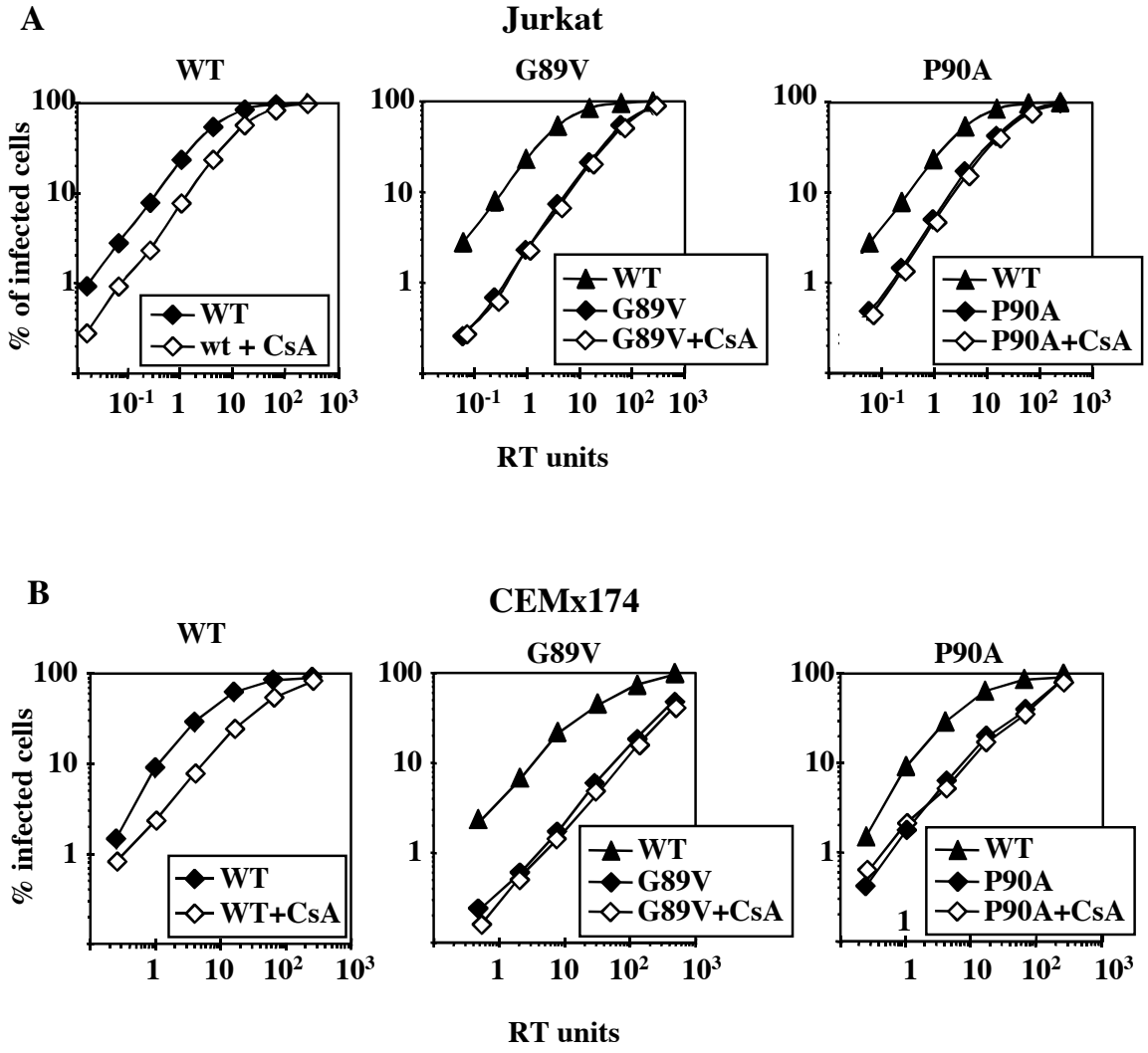


Figure 1.5. Single cycle viral infectivity assay. (A) Virions are produced by co-transfection of three constructs encoding viral packaging genes, the envelope glycoprotein and the GFP cDNAs into 293T producer cells. Virus is harvested from the supernatant, purified, and used to infect target cells. (B) Typical flow cytometry (FACS) readout measuring the percentage of infected (GFP-positive) cells. GFP fluorescence is detected on the FL1 channel.

CHAPTER II – TARGET CELL CYPA MODULATES HIV-1 INFECTIVITY

CsA disrupts HIV-1 infectivity in a single round infection assays in a variety of human cell lines. To asses the role of CypA during viral entry into the cell, we utilized a system that allowed to study requirements for the early steps of infection in the target cell, separated from replication requirements during viral assembly in the producer cell. The decision to investigate the time point of entry first came from the observation that in primate cells, CypA has been shown to only be relevant during viral entry (123). For these experiments, we utilized replication deficient recombinant HIV-1 reporter vectors that contain a GFP reporter cDNA replacing the virus genome and are pseudotyped with

the vesicular stomatitis virus G (VSVG) envelope protein to enable infection in a receptor independent manner (HIV_{GFP}). HIV_{GFP} vectors were produced by transfection of 293T cells in CsA-free medium, a cell line generally used for virus production because of its high transfection efficiency. Individual vector stocks were normalized by RT activity (13). The infectivity of HIV_{GFP} was assayed in a variety of human cell lines. These included: human T-cell leukemia cell line Jurkat and the lymphoblast T-cell/B-cell hybrid cell line CEMx174; human rhabdomyosarcoma cell line TE671, and human embryonic kidney cell line 293T. CypA binding to CA was disrupted by adding 2.5 μM CsA to the culture medium during the time of infection, or by introducing the G89V or the P90A mutations into CA. Both mutations were shown previously to disrupt the CypA-binding site in CA (46, 77). The percentage of infected (GPF-positive) cells was determined by flow cytometry 48 hours post infection. As depicted in Fig. 2.1-A, B, C and D, HIV-1 infectivity was decreased by about 3- to 5-fold if CsA was present during infection. If CypA binding site to CA was disrupted by the G89V or the P90A mutation, the infectivity of the virus was reduced by 5- to 10-fold, depending on the cell line. Addition of CsA did not decreased the infectivity of CypA-binding mutants any further, confirming that the infectivity decrease was indeed due to the disruption of the CA-CypA interaction. These results clearly demonstrate that the interaction between CA and CypA is necessary for the full infectivity of the virus during the early stages of infection.


A
Jurkat
WT
G89V
P90A
% of infected cells
100
10
1
WT
wt + CsA
G89V
G89V+CsA
P90A
P90A+CsA
10^{-1} 1 10 10^2 10^3
RT units
B
CEMx174
WT
G89V
P90A
% infected cells
WT
WT+CsA
G89V
G89V+CsA
P90A
P90A+CsA
1 10 10^2 10^3
RT units

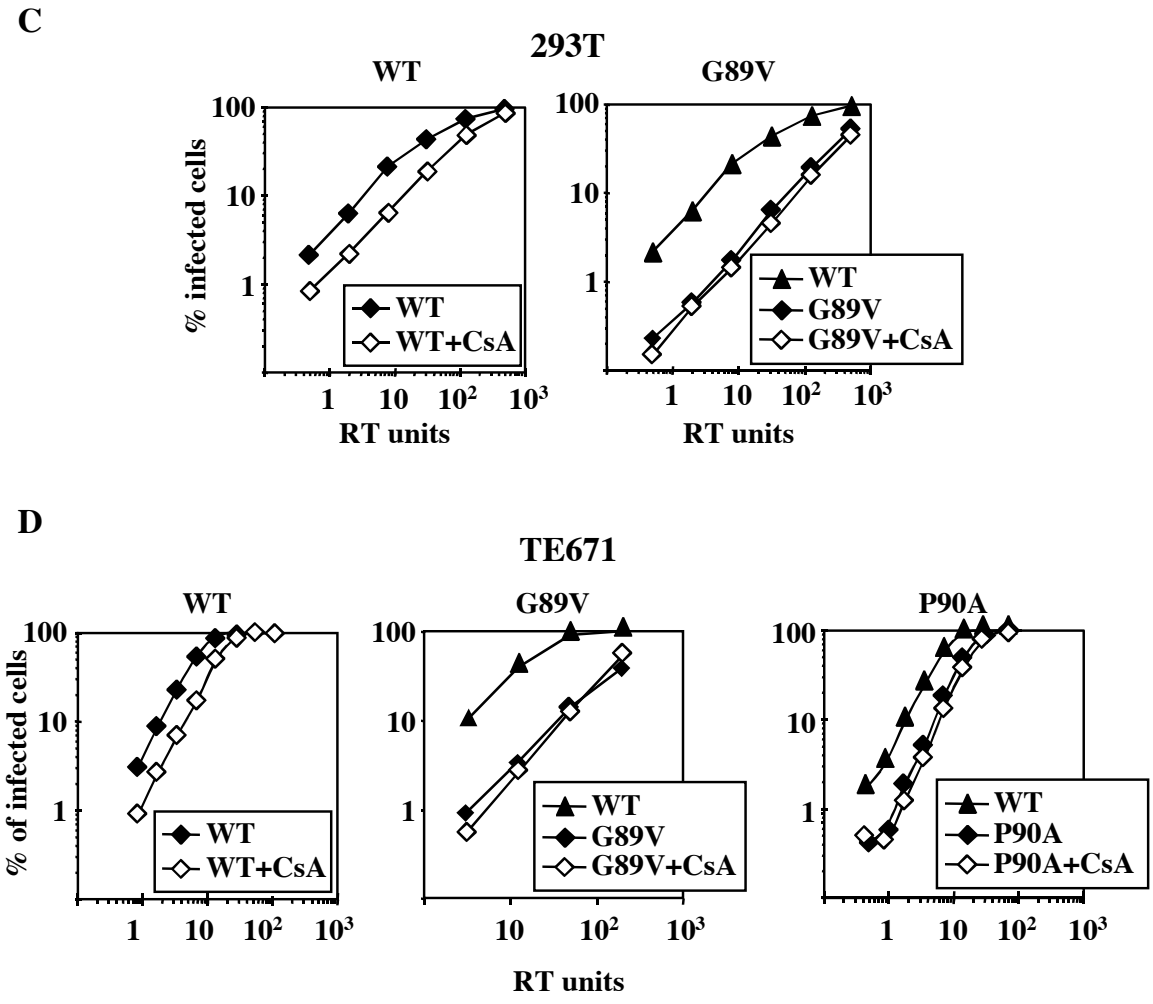


Figure 2.1. HIV-1 infectivity is disrupted by CsA, the G89V and the P90A mutations in CA. VSV-G pseudotyped, RT-normalized HIV-1_{GFP} wild-type (WT), G89V or P90A mutant vectors were produced in 293T cells, purified by filtration and centrifugation through 25% succrose, normalized by reverse transcriptase (RT) activity and used to infect Jurkat (A), CEMx174 (B), 293T (C) and TE671 (D) cells in drug free media or in the presence of 2.5 μM CsA, as indicated. The G89V or P90A mutations abolish CA interaction with CypA. 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.

CsA affects HIV-1 infectivity via effects on target cell CypA. Human genome encodes about 15 known cyclophilins, all of which are potential targets for CsA. To investigate whether reduced HIV-1 infectivity resulted from the inhibitory effect of CsA on CypA and not any other of the PPIase family member, RNA interference was used to downregulate endogenous CypA expression. TE671 cells were transduced with a retroviral vector delivering an shRNA specific for human CypA and a puromycin resistance gene, generating the TE671-CypA-shRNA cell line. Alignment of the shRNA CypA targeting sequence against the human genome (http://www.ncbi.nlm.nih.gov/genome/seq/HsBlast.html) revealed no homologous sequences other than CypA. Any other human cyclophilin family member (22) possesses at least five nucleotide mismatches with respect to the shRNA. A vector delivering an shRNA sequence targeting firefly luciferase was utilized as a control, generating the TE671-Luc-shRNA cell line. Pools of transduced cells were selected in puromycin, after which CypA was undetectable by Western Blotting (Fig. 2.2-A).

As shown in Fig. 2.2-B, CypA downregulation resulted in a three- to fivefold decrease of HIV-1 infectivity as compared to control TE671-CypA-shRNA cells. The magnitude of reduction of HIV-1 infectivity in the context of the CypA knockdown was almost identical to the magnitude of reduction of HIV-1 by CsA in control TE671-Luc-shRNA. CypA knockdown had no effect on the infectivity of the control vector bearing the G89V mutation (Fig. 2.2-C). These results clearly demonstrate that reduction of HIV-1 infectivity during viral entry by CsA occurs solely via effects on CypA.

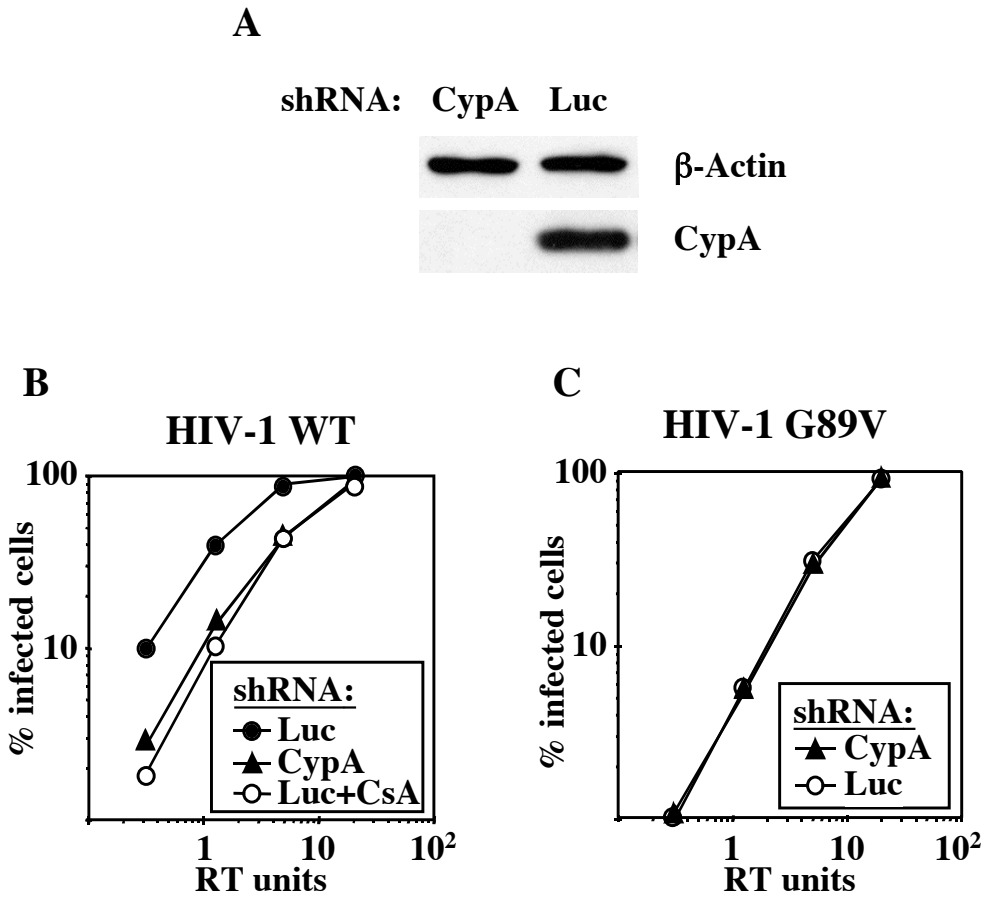


Figure 2.2. Knockdown of CypA by RNAi reproduces the effect of CsA and G89V. TE671 cells were transduced with MLV-based vectors delivering shRNA expression constructs specific for CypA or luciferase. (A) Cells were normalized by number and CypA knockdown was demonstrated by western for CypA and β-actin. VSV-G pseudotyped, RT-normalized, HIV-1_{GFP} wild-type (B) or G89V mutant (C) vectors were used to infect CypA-KD or Luc-KD cells in drug free media or in the presence of 2.5 μM 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.

CsA administration at different times in the virus life cycle has additive inhibitory effects on HIV-1 infectivity. Next, the importance of CypA-CA interaction on viral assembly and viral entry was investigated in a site-by-site experiment. This experiment aimed to determine whether CypA-CA interaction plays role during assembly of the virus as well, and to investigate whether it promotes HIV-1 infectivity via the same mechanism as it does for entry. To this aim, single-cycle replication assays with full-length infectious HIV-1 were performed. Fully infectious HIV-1_{NL4-3} (NL4-3/WT) virions were produced by transfection of 293T cells. Viral stocks were harvested 48h post transfection, purified by pelleting through a 25% sucrose cushion in order to avoid drug carry-over, normalized by RT activity prior to infections, and used to infect human $CD4^+$ T cell line Jurkat. To preclude virus spread and ensure only one round of infection, dextrane-sulfate was added to infected cells 16 hrs post-infection. 2.5 μM CsA was added to tissue culture medium either during production of the virus, during viral entry, or during both production and entry. The percentage of infected cells was determined by flow cytometry after staining with anti-CA antibody (13).

When 2.5 μM CsA was added during virion production, infectivity of wild-type virions was disrupted by approximately 2-fold (Fig. 2.3-A) and the CypA-content of purified virions was severely reduced, as demonstrated by western blotting (Fig. 2.4). When Jurkat target cells were infected in the presence of CsA, a 6-fold decrease in infectivity was observed. If CsA was added both during assembly and entry, there was a 12-fold reduction in titer (Fig. 2.3-A), indicating that the effects of the drug during production and entry were additive and there might be arising from distinct mechanisms.

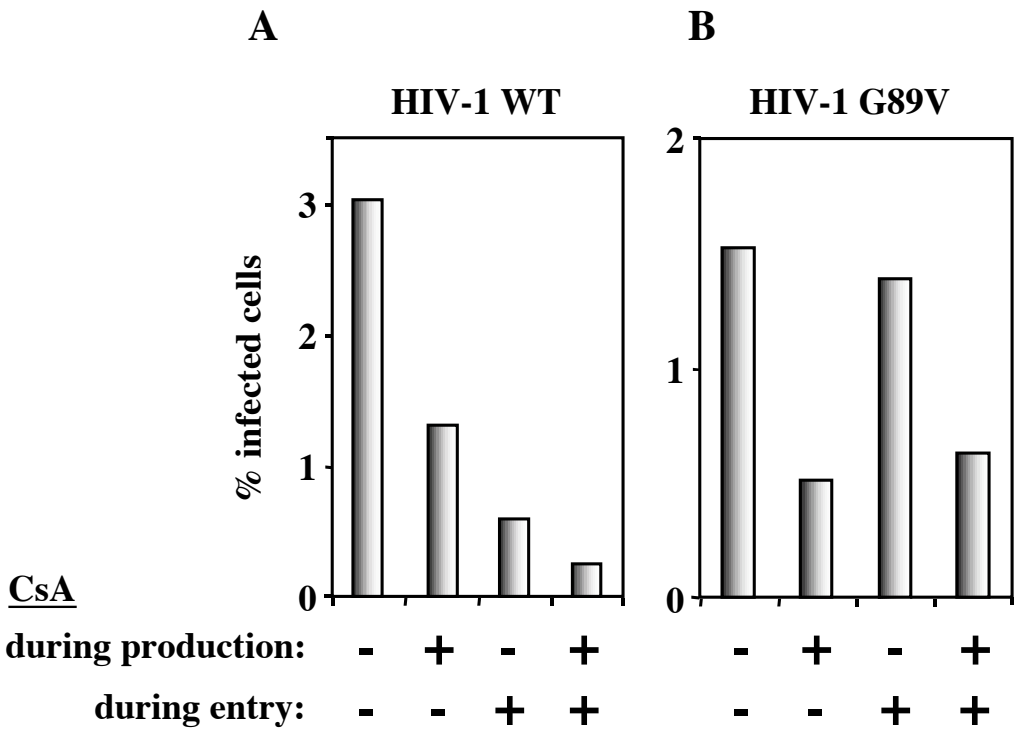


Figure 2.3. Inhibition of HIV-1 infectivity by CsA is CA-dependent if drug is administered to target cells during virus entry but independent of the CA-CypA interaction if drug is present during virion production. Full infectious HIV-1_{NL4-3} virions, either wild-type (A) or CA mutant G89V (B), were produced from 293T cells, pelleted though 25% sucrose, normalized by RT activity, and used to infect Jurkat cells. The percentage of cells infected was determined by flow cytometry after immunostaining for CA. 2.5 μM CsA was added either during virion production or during infection, or both, as indicated. Shown are representative results of a single experiment. Identical results were obtained on three separate occasions using independently produced viral stocks of varying multiplicities of infection.

Inhibition of infectious HIV-1 virion production by CsA is independent of the CA-CypA interaction. Since interaction with CypA is disrupted by mutations in the proline-rich loop connecting HIV-1 CA helices IV and V, in particular by a CA mutation G89V (134), it is expected that treatment of target cells with CsA will not decrease the titer of this virus any further. NL4-3/G89V mutant virions were produced by transfection of 293T cells and used to infect Jurkat cells. Again, CsA was added at the times of production, infection, or both production and infection of the virus. As expected, CypA incorporation into NL4-3/G89V virions was significantly decreased as determined by western blotting (Fig. 2.4). Indeed, CsA addition to target cells during infection with NL4-3/G89V virus did not decrease viral infectivity (Fig. 2.3-B). However, the infectivity of NL4-3/G89V virus produced in the presence of CsA was reduced to the same extent as was the wild-type virus (compare Figs. 2.3-A and 2.3-B). If both producer and target cells were treated with CsA, the reduction in NL4-3/G89V infectivity was identical to drug-free infection with NL4-3/G89V that was produced in the presence of drug (Fig. 2.3-B). These results indicate that CsA inhibition of infectious virion production does not involve the CA-CypA interaction, and is possibly CA-independent.

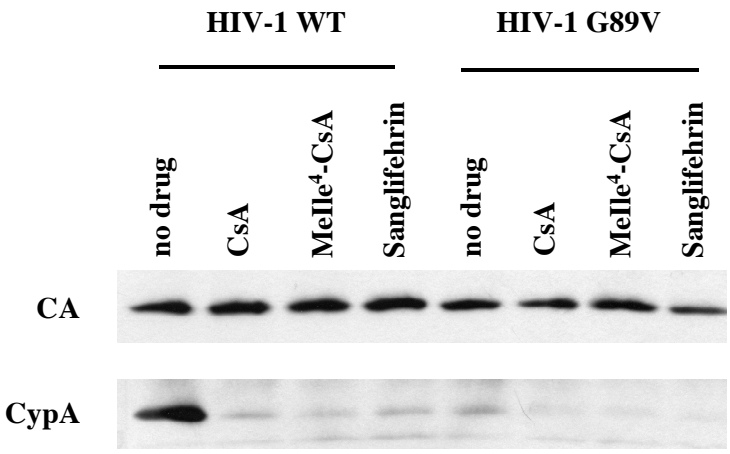


Figure 2.4. Disruption of CypA incorporation into virions by competitive inhibitors of CypA-CA interaction. HIV-1_{NL4-3} virions, either wild-type or G89V mutant were produced and purified as in Fig. 2.1. Where indicated, 2.5 μM CsA, MeIle^4-CsA or Sanglifehrin were added during virion production. Purified virions were subjected to western blotting using antibodies against CypA and CA.

MeIle^4-CsA and Sanglifehrin inhibit infectious HIV-1 virion production via a CA-independent mechanism. The experiments above indicate that the CA-CypA interaction is uniquely important during virion entry, as opposed to virion assembly. To clarify the mechanism by which CsA inhibits the production of infectious virions we examined the effect of two additional drugs. As a complex with CypA, CsA binds and inhibits the calcium-dependent phosphatase calcineurin, which leads to disruption of signaling pathways downstream of calcineurin and results in blocked proliferation of T-cells (47, 75). MeIle^4-CsA is an analogue of CsA that binds CypA as tightly as the parent compound but is unable to form a complex with calcineurin, and is therefore devoid of

the immunosuppressive activity (45, 120). Sanglifehrin is structurally unrelated to CsA, but it also binds to cyclophilins (39, 100) and might be expected to disrupt the CypA-CA interaction. In fact, both MeIle^4-CsA or Sanglifehrin blocked the incorporation of CypA into nascent virion particles (Fig. 2.4).

MeIle^4-CsA or Sanglifehrin treatment of producer cells reduced infectivity of wild-type virions by about 2.5-fold and 1.5-fold, respectively, as compared to virions produced with no drug (Fig. 2.5-A). The effect of the two drugs on the titer of nascent NL4-3/G89V virions was identical to the effect of the two drugs on the wild-type virus (Fig. 2.5-B). This result again confirms that the mechanism of inhibition is independent of the CA-CypA interaction, since the ability of this CA mutant to bind CypA is disrupted. It is however noteworthy that all three drugs that are able to disrupt HIV-1 infectivity if added during production of the virions share the common feature of binding to CypA. The relatively smaller reduction of HIV-1 infectivity by Sanglifehrin is explained by the lower permeability of this drug into cells, as compared to the other drugs CsA and MeIle^4-CsA (39).

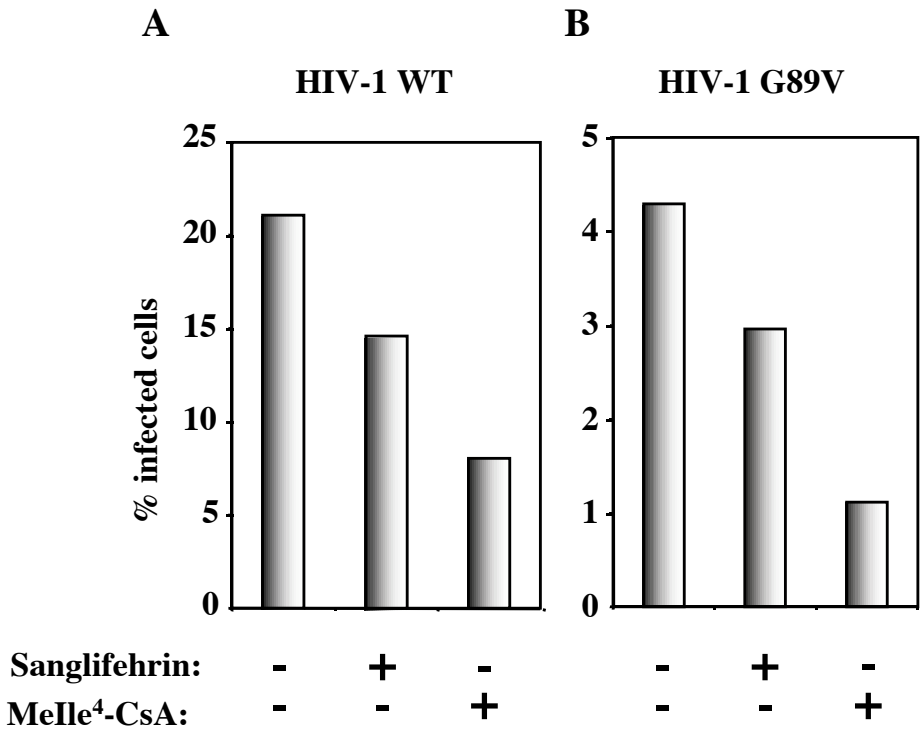


Figure 2.5. MeIle^4-CsA or Sanglifehrin inhibit infectious HIV-1 virion production independently of the CA-CypA interaction. Wild-type (A) or G89V (B) virions were prepared as in Fig. 2.1, normalized by RT activity, and used to infect Jurkat cells. Where indicated, 2.5 μM MeIle^4-CsA or Sanglifehrin was added during virion production.

Producer cell CypA is not required for production of fully infectious HIV-1 virions.

At this point, it cannot be excluded that CypA is required for a CA-unrelated process during production of viral proteins or virion assembly, and that the CypA-binding drugs inhibit this process, thus disrupting viral infectivity. Alternatively, CypA-binding drugs could be affecting one of the other 15 known mammalian cyclophilins. Thus, the effect observed here could arise from inhibition of a different cyclophilin, the role of which in

HIV-1 replication is unknown.

To determine if producer cell CypA is necessary to render HIV-1 virions fully infectious, siRNA was used to downregulate CypA expression. HeLa cells were transduced with a retrovirus expressing a short-hairpin RNA (shRNA) (25) specific for CypA cDNA. To avoid loss of shRNA construct expression in a pool of transduced cells, single cell clones of the transduced HeLa cells were screened by western blotting for CypA expression (Fig. 2.6-A), and clones with wild-type levels of expression (control) or undetectable expression (CypA KD) were selected for further analysis.

The effect of producer cell CypA on HIV-1 virion infectivity was assessed by transfecting CypA KD and control HeLa cells with pNL4-3 to produce HIV-1 virions. Both transfected CypA KD and control virion producer cells were treated with CsA to determine if the inhibitory effect of the drug was dependent upon the presence of CypA. Virions were purified from the culture supernatant and Western blotting confirmed the absence of CypA in association with virions produced by the CypA KD cells or from the control cells treated with CsA (Fig. 2.6-B). After normalization by RT activity, infectivity of all viral stocks was assayed in Jurkat target cells. Virions produced by CypA KD cells were as infectious in Jurkat cells as were virions produced by control HeLa cells (Fig. 2.6-C). Additionally, reduction in virion infectivity due to CsA was of equal magnitude when the drug was added to CypA KD or control cells during virus production (Fig. 2.6-C). These results indicate that producer cell CypA plays no role in HIV-1 virion infectivity and that the inhibitory effects of CsA during virion production are CypA-independent.

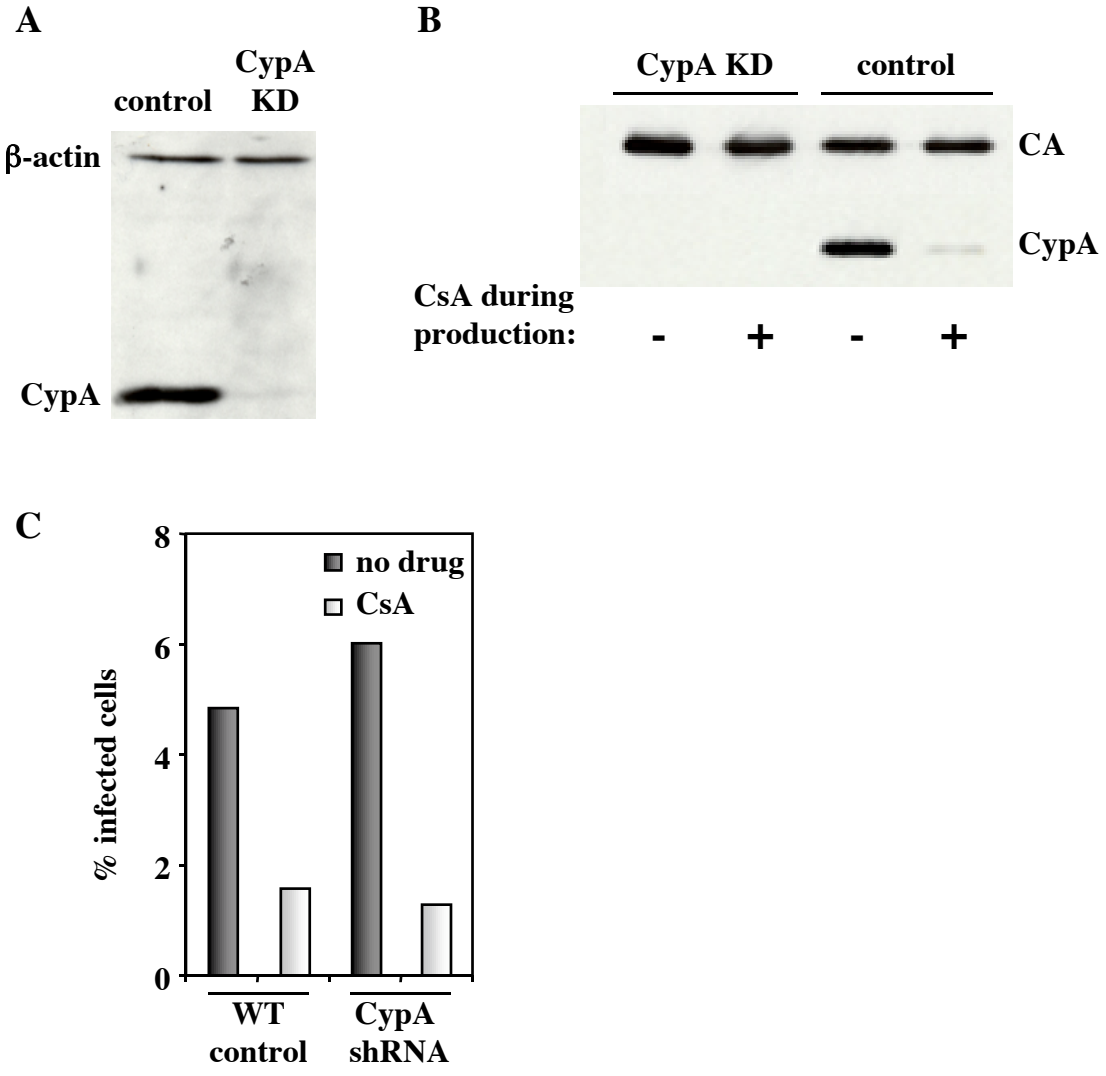


Figure 2.6. Producer cell CypA has no effect on HIV-1 virion infectivity. HeLa cells were cloned after transduction with an MLV-based vector delivering an shRNA expression construct specific for CypA mRNA. After normalization by cell number and screening by immunoblot for CypA and β-actin expression, clones with knockdown of CypA protein (CypA KD) or with the original level of CypA protein (control) were selected for further analysis (A). Virions were produced by transfection of CypA KD or control HeLa cells with pNL4-3 in the presence of 2.5 μM CsA, as indicated (B). Virions were purified as in Fig. 2.1, normalized by RT activity, probed in western blot with antibodies against CypA and CA (B) and used to infect Jurkat cells in drug free media (C); the percentage of Jurkat cells infected was determined by flow cytometry after immunostaining for CA (C).

The target cell determines cell line-specific effects of CsA on the replication of HIV-1 CA variants. Infectivity of most HIV-1 isolates is inhibited by CsA. However, some HIV-1 group O isolates, such as HIV-$1_{MVP5180}$ or HIV-1_{CA9}, are CsA-resistant in Jurkat cells or PBMCs (22). In addition, continuous propagation of wild-type HIV-1 in the presence of the drug gives rise to spontaneous mutations in the region of HIV-1 CA (1). One such CA mutant, A92E, has lost CsA-sensitivity despite the retained ability to bind CypA. Additionally, it has acquired CsA dependence (1). Interestingly, the CypA independence/CsA resistance phenotypes of the A92E mutant are cell-type specific. Thus, HIV-1/A92E replicates in Jurkat cells at the levels of wild-type HIV-1 and is uninhibited by CsA (133). In a different human T cell line, H9 cells, the same CA mutant is incapable of replication, and addition of CsA rescues its replication up to the wild-type levels (133). While the infectivity of the HIV-1/A92E mutant in H9 is dependent upon CsA, it was surprising to find that wild-type HIV-1 has lost its CsA sensitivity in these cells (133). Screening several human cell lines revealed that the HIV-1/A92E mutant is CsA resistant/CypA independent (Jurkat phenotype) in the majority of the human cell lines tested, including human PBMC, and is CsA dependent (H9 phenotype) in a human cervical cancer cell line HeLa (Sokolskaja E. and Luban J., unpublished data). Since these cell-type specific phenotypes are determined by CypA, we next assessed the relative contribution of target cell CypA and producer cell CypA to the phenotypes of these CA variants.

Alignment of the CypA binding regions of two CypA-independent HIV-1 Group O isolates HIV-$1_{MVP5180}$, HIV-1_{CA9} revealed sequence similarities to the NL4-3/A92E mutant (Fig. 2.7). The alanine residue in position 92 is changed from the original alanine

in NL4-3 to a proline in both Group O isolates. In addition, both viruses contain additional mutations upstream of A92 but within the CypA binding motif HAGPIA (Fig. 2.7). We therefore wanted to investigate whether these two naturally occurring isolates would exhibit the same CsA independence/resistance that is observed with the A92E mutant in Jurkat and H9 cells.

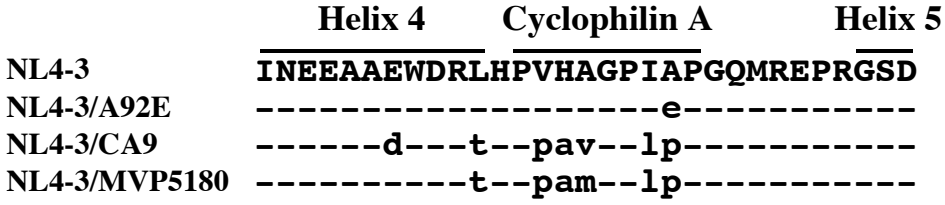


Figure 2.7. Amino acid sequence alignment of the CypA binding regions from wild-type HIV-1_{NL4-3}, the A92E mutant, the NL4-3/CA9 chimera, and the NL4-3/MVP5180 chimera. Residues constituting the CypA-binding site and alpha-helices 4 and 5 are indicated. Dashed lines indicate residues identical to HIV-1_{NL4-3}. Residues that differ from HIV-1_{NL4-3} are indicated with lower case letters. Note that in all three variants the A92 residue is altered.

HIV-1 vectors were engineered that encode either the CA A92E mutant or NL4-3 chimeric CAs bearing the CypA binding regions of the Group O isolates, HIV-$1_{MVP5180}$ or HIV-1_{CA9} (Fig. 2.7). VSV-G-pseudotyped virions produced by transfection of 293T cells with the engineered HIV-1 CA variants, as well as the wild-type, were used to infect Jurkat cells. When CsA was present during infection, the titer of the wild-type vector was reduced 5-fold (Fig. 2.8-A). The A92E mutant, the NL4-3/CA9 chimera, and the NL4-

3/MVP5180 chimera each exhibited relative resistance to CsA (Fig. 2.8-A).

When the same virus stocks produced in 293T cells were used to infect HeLa cells very different results were obtained. The A92E mutant and the CA chimeras showed significantly reduced titers, as compared to the wild-type (Fig 2.8-B). CsA administration during infection of the HeLa cells had minimal effects on the titer of the wild-type, but the titers of the CA variants were stimulated by as much as 20-fold (Fig. 2.8-B).

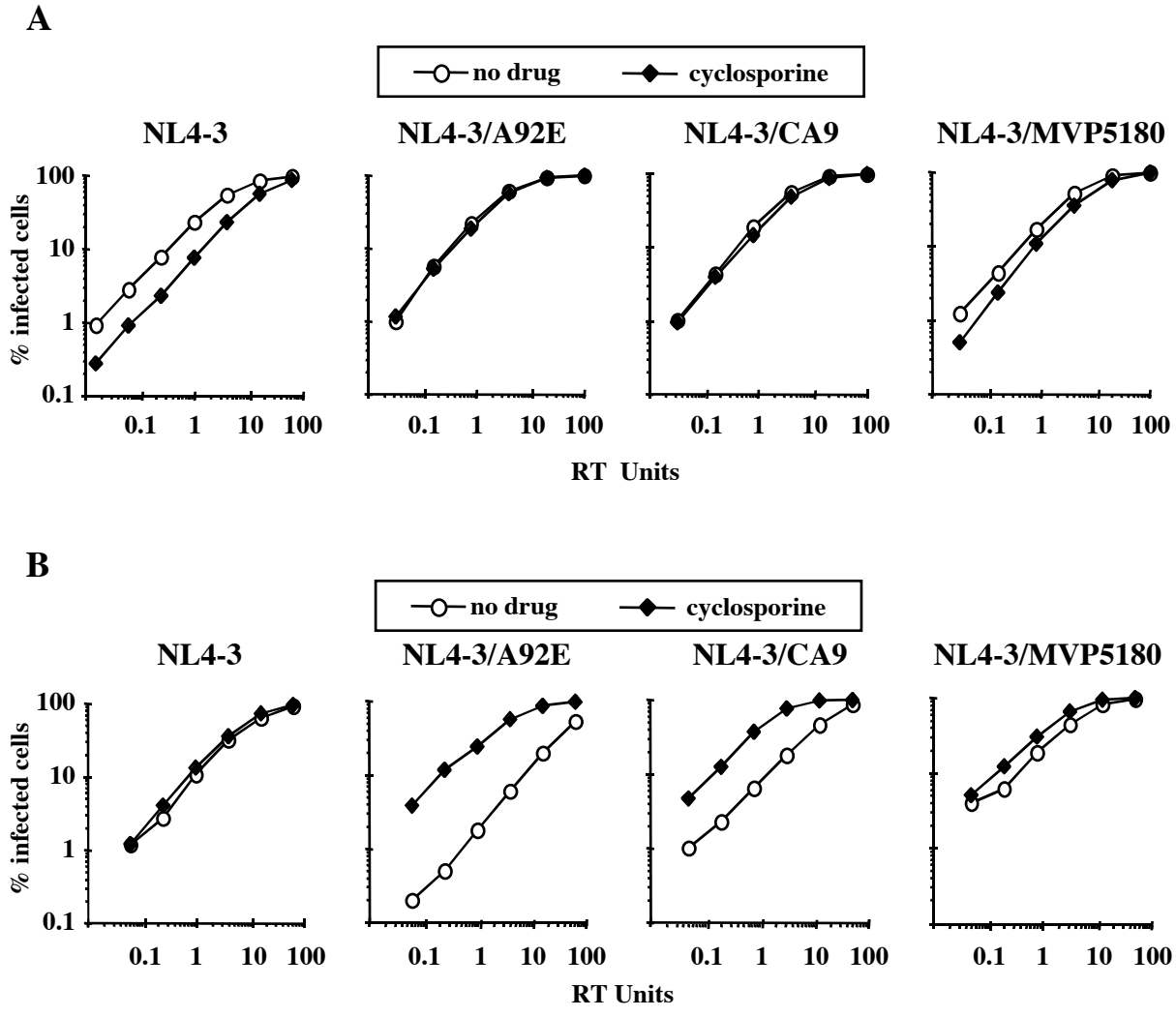


Figure 2.8. Cell-type specific effects of CsA on HIV-1 replication are determined by the target cell. VSV-G pseudotyped HIV_{GFP} vectors, either wild-type, CA mutant A92E, CA chimera NL4-3/CA9, or CA chimera NL4-3/MVP5180, were produced by transfection of 293T cells, normalized by RT activity and used to infect Jurkat cells (A) or HeLa cells (B). Where indicated, 2.5 μM cyclosporine was added to the media during infection.

All vectors used in Fig. 2.8 were produced by transfection of 293T cells. Identical results were obtained if the vectors were produced in HeLa cells (Fig. 2.9-A). Also, addition of CsA to the producer cells had no effect on the phenotype of these viruses (Fig. 2.9-B). Taken together, these results indicate that these CypA-dependent, CA-specific, and cell-type specific phenotypes are completely determined by the target cell, not by the producer cell.

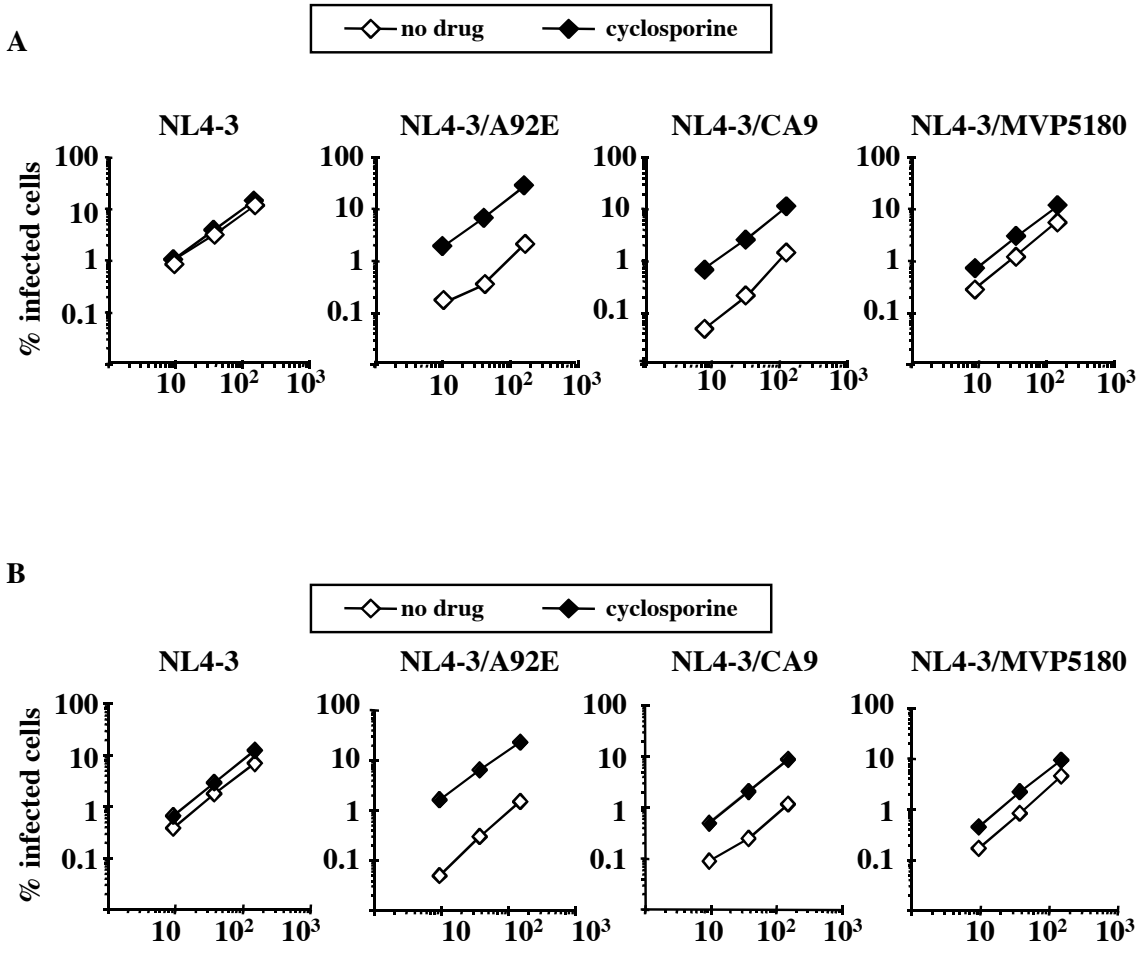


Figure 2.9. Cell-specific phenotypes on HIV-1 replication are not determined by the producer cell. CA mutant A92E, CA chimera NL4-3/CA9, or CA chimera NL4-3/MVP5180, were produced by transfection of HeLa cells in drug-free media (A) or in the presence of CsA (B), normalized by RT activity, and used to infect or HeLa cells in the presence or absense of CsA, as indicated. Percentage of infected cells was determined by flow cytometry.

CsA phenotypes of HIV-1 CA variants result from effects of the drug on target cell CypA. The human genome encodes at least 15 human cyclophilins, each of which is a potential CsA ligand (23). We therefore tested the hypothesis that the CsA-resistance in Jurkat, or CsA-dependence in HeLa, exhibited by the CA variants, is due to effects of the drug on CypA, as opposed to effects on other cyclophilin family members. VSV-G-pseudotyped HIV-1 vectors were used to infect Jurkat cells homozygos for CypA gene deletion (*Ppia*$^{-/-}$ Jurkat) (23) or CypA KD HeLa cells (Fig. 2.10). In Jurkat cells, the effect of CypA gene disruption mirrored the effect of CsA in wild-type Jurkat. In particular, replication of A92E and NL4-3/CA9 was relatively resistant to the CypA-deficient condition (Fig. 2.10-A). Similarly, the effect of CypA KD in HeLa cells mirrored the effect of CsA in control HeLa cells: replication of the CA variants was stimulated by the knockdown of CypA expression (Fig. 2.10-B) as it was by CsA (Fig. 2.8-B, 2.9-B). Consistent with this observation, CsA did not stimulate A92E infectivity in the CypA KD HeLa cells (Fig. 2.11-B).

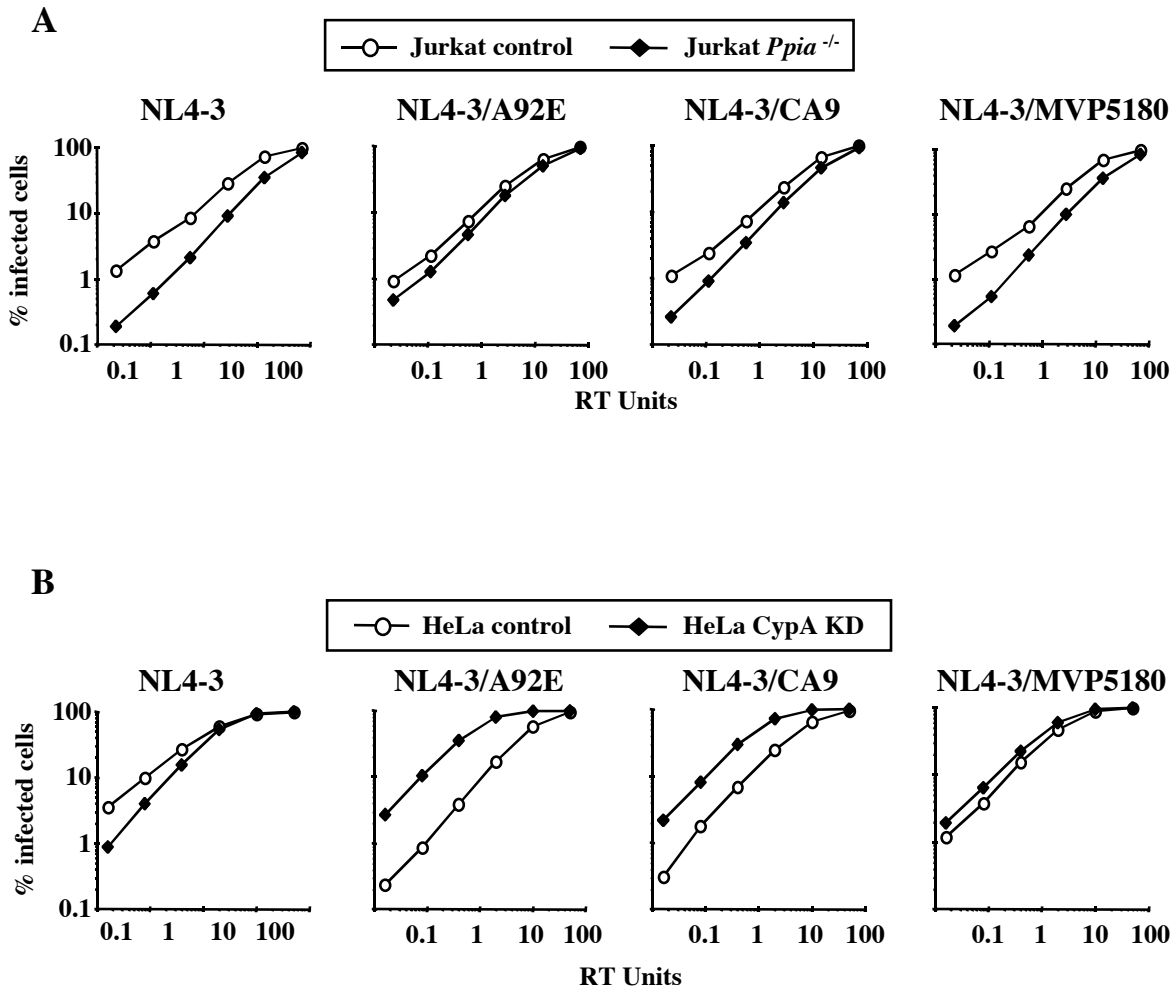


Figure 2.10. Target cell CypA regulates the cell-type specific effects on HIV-1 replication. HIV_{GFP} transducing vectors, either wild-type, CA mutant A92E, CA chimera NL4-3/CA9, or CA chimera NL4-3/MVP5180, were produced by transfection of 293T cells, normalized by RT activity, and used to infect Jurkat cells (A) or HeLa cells (B). The Jurkat cells were wild-type (control) or homozygous for a deletion of the gene encoding CypA (*Ppia*$^{-/-}$), as indicated (A). HeLa cells with wild-type levels of CypA (control) or with knocked down CypA expression (CypA KD) were used, as indicated (B).

To demonstrate that the effect of RNAi transduction on A92E replication in CypA KD HeLa cells was indeed due to disruption of CypA protein, CypA protein was restored by transduction of non-targetable CypA cDNA (nt-CypA) bearing silent mutations that render it resistant to the RNAi construct used here. When CypA protein was restored (Fig. 2.11-A), A92E infection was again inhibited, and the replication efficiency of the mutant was restored by CsA (Fig. 2.11-B). Transduction of ntCypA cDNA encoding the active site mutant R55A which does not bind HIV-1 CA (20) failed to restore the A92E phenotype (Fig. 2.11-A and B). These studies indicate that the cell-type dependent effects of CsA are dependent upon the CA-CypA interaction.

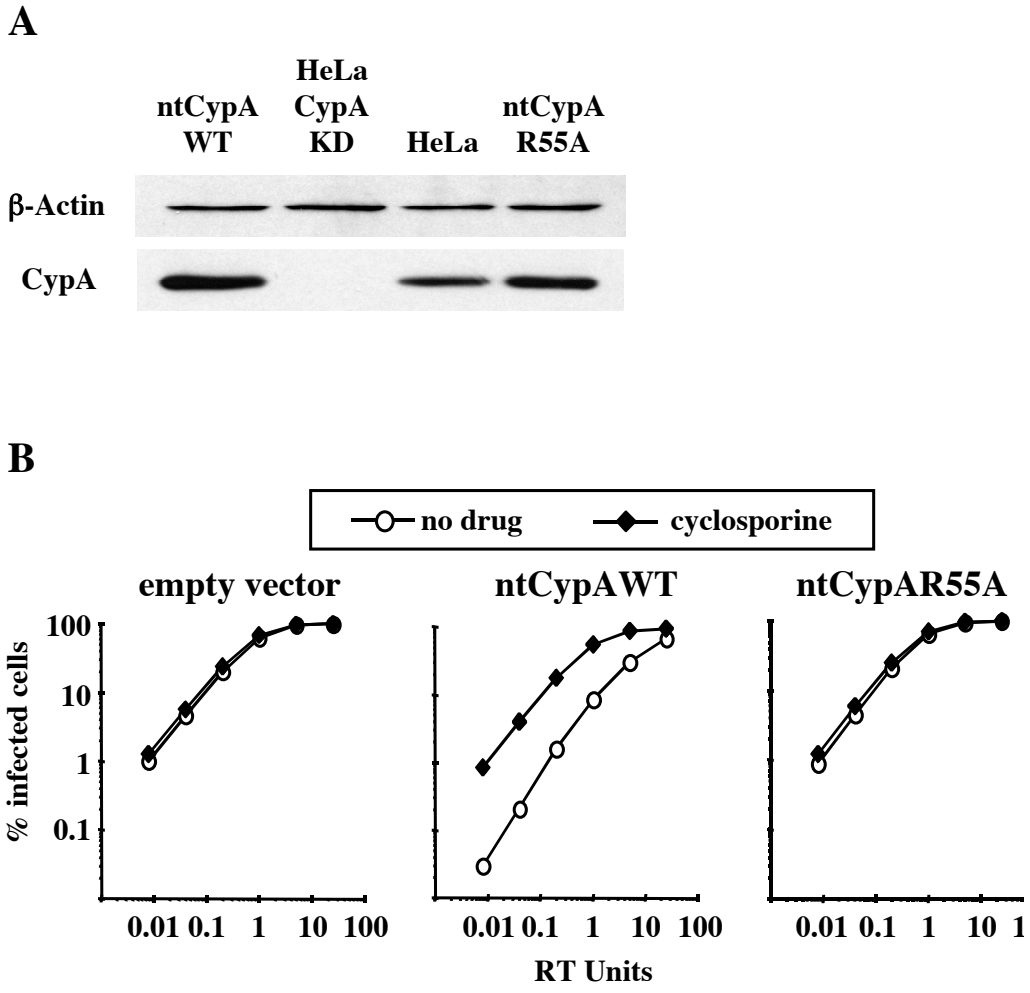


Figure 2.11. CypA binding to HIV-1 CA is required for HeLa resistance to HIV-1 A92E. HeLa CypA KD cells were retrovirally transduced with CypA cDNAs bearing silent mutations (nt-CypA) that confer resistance to the RNAi targeting CypA in these cells. Lysates from CypA KD cells transduced with empty vector, nt-CypA WT, or nt-CypA bearing the active site mutant R55A, were probed in western blots with anti-CypA or anti-actin antibodies (A). Lysate from wild-type HeLa are shown as a control. (B) The transduced cell populations in (A) were challenged with VSV-G pseudotyped HIV-1-GFP vector bearing CA mutant A92E. Where indicated, 2.5 μM CsA was added to the media during infection.