CHAPTER III – NEGATIVE EFFECTS OF CSA ON VIRUS ASSEMBLY

Competitive inhibitors of the CypA-CA interaction inhibit gp120 incorporation into the viral particles. Virions produced in the presence of CsA do not show any structural abnormalities as determined by electron microscopy (21). However, CypA binding drugs could affect the stoichiometry of viral proteins by inhibiting synthesis of viral proteins or by interfering with protein incorporation into the viral particles, thus inflicting changes not necessarily reflected in the morphology of the virions. We therefore decided to investigate whether the disrupted infectivity of virions produced in the presence of CsA is due to a defect in the viral protein responsible for the first contacts with the target cell: the envelope glycoprotein gp160.

Fully infectious HIV-1_{NL4-3} virions were produced from 293T cells in drug free media, or in presence of CsA, $MeIle^4$-CsA or Sanglifehrin, respectively. Virion containing supernatants were harvested 48h post transfection, pelleted through a 25% sucrose cushion, resuspended in cell culture medium and normalized by RT activity. The virion stocks were subjected to Western blotting utilizing antibodies against gp120 and gp41. As shown in Fig. 3.1, both HIV-1 wild-type and HIV-1/G89V virions produced in the presence of any of the drugs showed significantly decreased amounts of gp120 and gp41, as compared to virions produced in drug-free media. Immunoblotting using a anti-CA antibody confirmed equal sample loading. As expected, virions produced in the presence of a drug did not incorporate CypA. These findings indicate that CsA exerts an inhibitory effect on HIV-1 infectivity by interfering with Env function. Disruption of Env incorporation in the presence of Sanglifehrin appeared somewhat lower as compared

to CsA and MeIle^4-CsA, correlating with the previous observation that disruption of HIV-1 infectivity by Sanglifehrin during virus production is less pronounced then the two other drugs (Fig. 2.5). This can be explained by lower cell permeability of the drug (39).

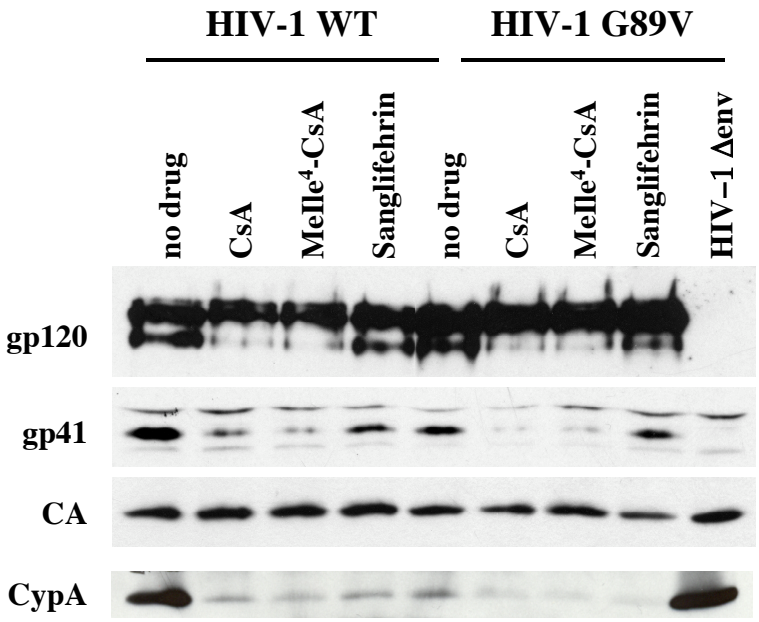

Figure 3.1. CsA disrupts Env incorporation into HIV-1 virions. HIV-1_{NL4-3} virions were produced in the presence or absense of the indicated drugs, pelleted through 25% succrose cushion, normalized by RT activity, and subjected to Western blotting using the α-gp120 and α-gp41 antibodies. α-CA antibody was used to confirm equal sample loading. α-CypA antibody was used to confirm drug effectiveness. HIV-1 Δ env has a deletion in *env* and was used as a negative control.

CsA added during production disrupts HIV-1 infectivity in a dose-dependent manner. To further study the inhibitory effect of CsA during HIV-1 virion production, HIV-1_{NL4-3} virions were produced in 293T cells in presence of increasing CsA concentrations (0, 2.5, 5, 7.5, 10 and 12.5 μM). After virion purification and normalization by RT activity, the infectivity of produced virions was assayed in Jurkat target cells. As depicted in Fig. 3.2, the HIV-1 infectivity is inversely proportional to the

CsA concentration present during virion production, indicating that CsA interferes with viral assembly in a dose-dependent manner.

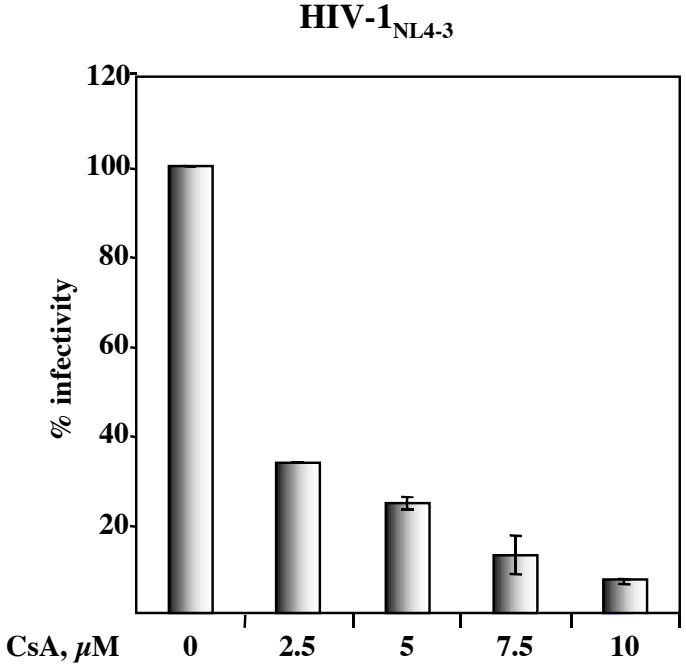

Figure 3.2. Treatment of producer cells with CsA disrupts infectivity of gp160 pseudotyped HIV-1 virions in a dose-dependent manner. gp160 pseudotyped NL4-3_{GFP} virions were produced from 293T cells in the presense of indicated CsA concentrations, purified by filtration and centri through 25% succrose, normalized by RT activity and used to infect Jurkat cells. The percentage of infected cells was determined by flow cytometry.

CsA addition during viral production results in disrupted infectivity of viruses bearing Envs from different lentiviruses. To investigate whether the inhibitory effect of CsA on viral production is specific to HIV-1_{NL4-3} Env, an HIV-1_{NL4-3} proviral construct with an inactivating deletion in *env* and containing a GFP reporter cDNA (NL4-3_{GFP}) was used to produce HIV-1 virions pseudotyped with HXb2, IIIb, VCP and SIV_{mac259} Envs. HXb2 and IIIb are HIV-1-derived Envs, whereas VCP is an Env from a CD4-independent strain of HIV-2. SIVmac259 Env is derived from a closely related SIV. Virion stocks were normalized by RT activity and used to infect Jurkat target cells. As determined by the infectivity assay, all envelope glycoproteins used here were affected by the presence of CsA during production of the virus (Fig.3.3).

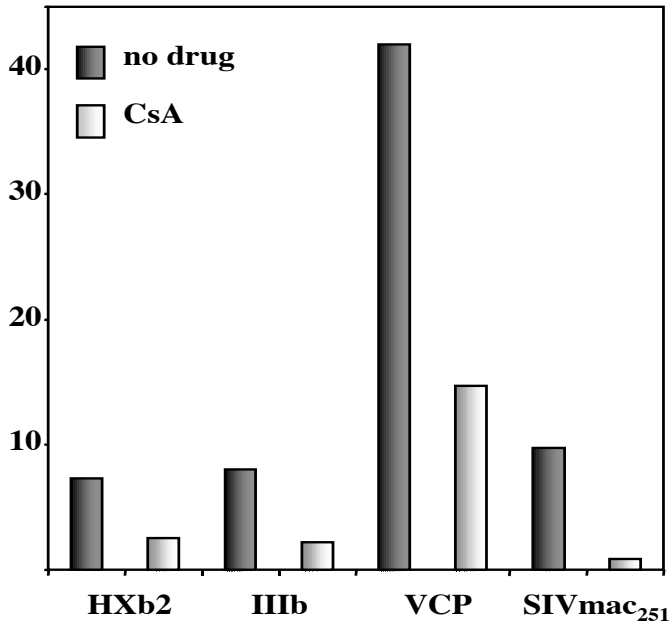

Figure 3.3. Envs from different lentiviruses are inhibited by CsA. $NL4\text{-}3_{GFP}$ virions pseudotyped with Envs from different lentiviruses were produced in presence of 10 μM CsA, purified as in Fig. 6, normalized by RT activity and used to infect Jurkat target cells. The percentage of infected cells was determined by flow cytometry.

VSVG-pseudotyping renders HIV-1 virion assembly significantly less sensitive to CypA-binding drugs. Vesicular stomatitis virus belongs to the family of Rhabdoviridae and is unrelated to HIV-1. It utilizes the endocytic pathway for entry into the target cells, mediated by its envelope glycoprotein VSVG (vesicular stomatitis virus protein G). HIV-1 normally enters the cell by direct fusion with the target cell membrane. To investigate whether the inhibitory effect of CsA on the envelope glycoprotein function was dependent on the way of viral entry, HIV-1 virions pseudotyped with gp160 or with VSVG were produced from 293T cells in presence of increasing CsA concentrations. As shown in Fig. 3.4, infectivity of both gp160 and VSVG pseudotyped virions was disrupted by CsA in a dose-dependent manner. However, the magnitude of inhibition was much greater for gp160, resulting in an up to 13-fold infectivity reduction at the highest dose of the drug, while the infectivity of VSVG pseudotyped virions at the highest dose of CsA was disrupted only by 2-fold.

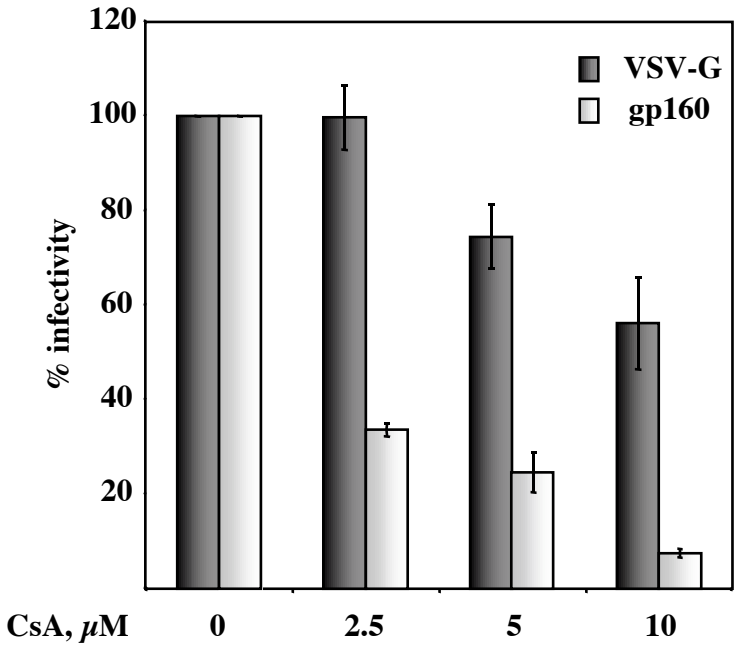

Figure 3.4. CsA disrupts infectivity of HIV-1 virions pseudotyped with gp160, but not with VSV-G. gp160 or VSV-G pseudotyped NL4-3_{GFP} virions were produced in 293T cells in presence of indicated CsA concentrations, purified as in Fig. 1, normalized by RT activity and used to infect Jurkat target cells. The percentage of infected cells was determined by flow cytometry.

CypB is not required for proper processing and incorporation of Envs into the virus particles. As a highly glycosylated membrane protein, gp160 is synthesized at the rough endoplasmic reticulum (ER), where it undergoes folding and trimerization (29). Cyclophilin B (CypB) is a human cyclophilin that resides in the ER and represents a potential binding target for CsA. To investigate whether the negative effect of CsA on HIV-1 virion production results from CypB inhibition, RNAi was used to downregulate CypB expression. HeLa cells were transduced with a retroviral-based vector delivering an shRNA construct targeting human CypB and a puromycin resistance cassette, generating the HeLa-CypB-shRNA cell line. After selection of the transduced cell population in puromycin, CypB was undetectable by Western blotting (Fig. 3.5-A). When HIV-1_{NL4-3} virions were produced from HeLa-CypB-shRNA cells, no infectivity reduction was

observed as compared to virions produced from control cells (Fig. 3.5-B), indicating that CypB does not play a role in HIV-1 assembly. If CsA was present during virion production, infectivity of virions produced from HeLa-CypB-shRNA cells was reduced to the same magnitude as for HIV-1 produced from control cells, confirming that CsA negative effect on virion assembly does not result from inhibition of CypB.

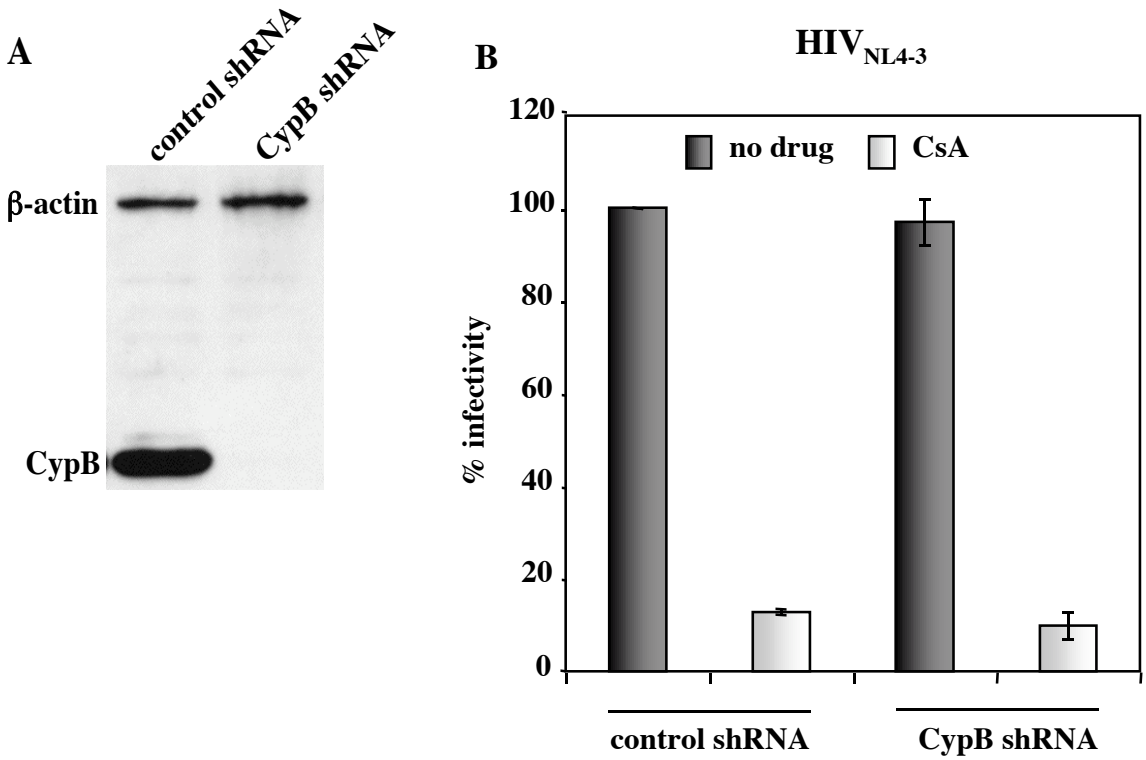

Figure 3.5. CypB does not regulate gp160 processing. HeLa cells were transduced with a vector delivering an shRNA construct specific for CypB. CypB knockdown was confirmed by Western blotting using an α-CypB antibody (A) . HIV-1_{NL4-3} virions were produced from HeLa CypB shRNA cells in presense or in absence of 10 μM CsA, normalized by RT activity, and used to infect Jurkat cells (B). Wild-type HeLa cells were used as a control for virion production. The percentage of infected cells was determined by flow cytometry.

Cyp60 is not required for proper processing and incorporation of gp120/gp41 into the viral particles. Human cyclophilin Cyp60 was shown to be implicated in processing of membrane proteins such as CD147/EMMPRIN (94). CD147 is an integral membrane protein known to regulate cellular adhesion (62), and downregulation of Cyp60 by RNAi was found to decrease surface expression of CD147 (94). Thus, Cyp60 represents another potential CsA-sensitive regulator of HIV-1 gp160. To assess its role in gp160 processing, shRNA was used to downregulate Cyp60 expression in HeLa cells by transduction with a retroviral vector expressing shRNAs specific for human Cyp60, and a puromycin resistance cassette. After selection in puromycin, downregulation of Cyp60 expression was confirmed by Western blotting (Fig. 3.6-A). When HIV-1 was produced from cells with knock-down Cyp60 expression, virion infectivity was equal to that of HIV-1 produced in control cells with wild-type Cyp60 expression (Fig. 3.6-B). CsA presence during virion production resulted in an infectivity reduction of the same magnitude in both Cyp60-shRNA and control cells (Fig. 3.6-B). This finding indicates that Cyp60 is not involved in gp160 cell surface expression.

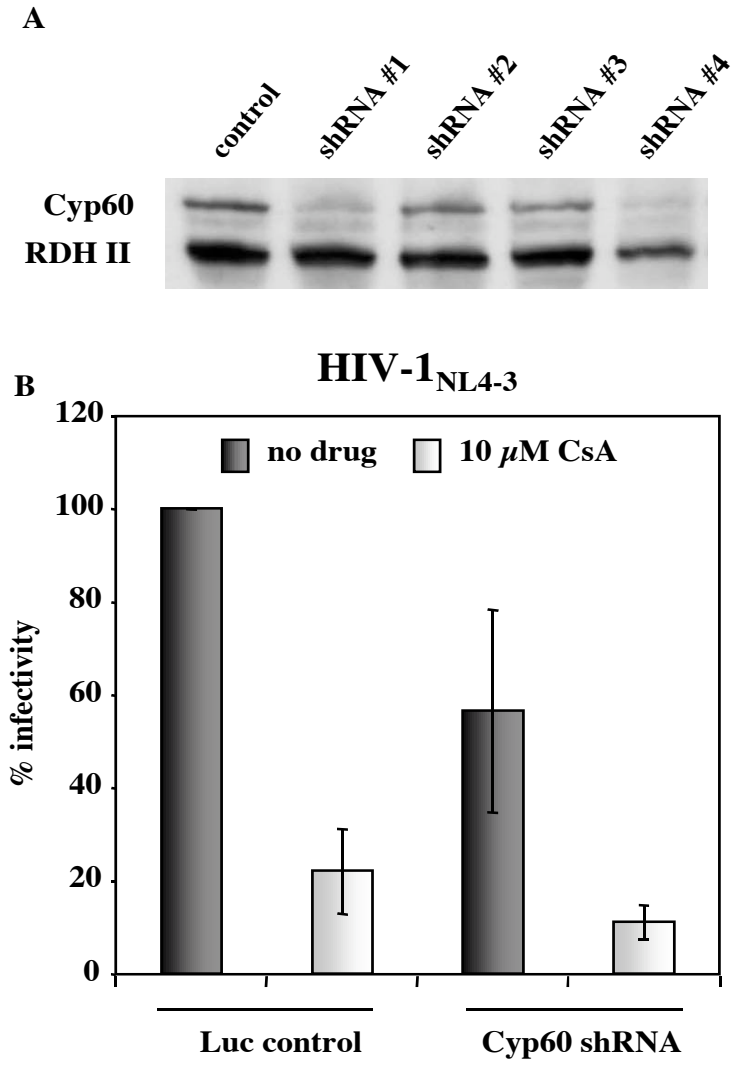

Figure 3.6. Cyp60 is not involved in processing of gp160. (A) HeLa cells were transduced with a retroviral vector delivering several shRNA construct specific for Cyp60. Cells were normalized by cell number and Cyp60 knockdown was confirmed by Western blotting using an α-Cyp60 antibody. RDH II is cross-recognized by the same antibody and serves here as a loading control. HIV-1_{NL4-3} viruses were produced from HeLa Cyp60 shRNA cells (shRNA#4), purified as in Fig. 2.1, RT normalized, and used to infect Jurkat cells (B). Luc shRNA HeLa were used as a control for virus production. The percentage of infected cells were determined by flow cytometry.

DISCUSSION

Producer cell CypA is not required for HIV-1 infectivity. The CA-CypA interaction was discovered with the Gag polyprotein (46, 77), but it also binds the mature CA (24, 30, 77). The findings of most subsequent studies were consistent with the hypothesis that producer cell CypA is important for HIV-1 replication. CypA binds the Gag polyprotein stronger than it binds mature CA (24, 30). CypA is incorporated into HIV-1 virions or Gag particles with a fixed stoichiometry and disruption of CypA incorporation into virions by *gag* mutants correlated with decreased infectivity (46, 85, 120). The hypothesis seemed especially secure when it was found that, like the *gag* mutants, competitive inhibitors that block CypA incorporation also inhibited virion infectivity (21, 45, 120). These drugs had no effect on the infectivity of related viruses such as HIV-2 and SIV_{MAC239} (21, 45, 120), viruses that do not incorporate CypA into particles. Finally, the most convincing proof was provided by a Jurkat T-cell line homozygous for the deletion of the CypA gene. HIV-1 virions produced from these cells exhibited delayed replication kinetics, suggesting that producer cell CypA is required for the virions to gain their full infectivity (23).

The experiments presented here render the original hypothesis unlikely and show that only target cell CypA interaction with HIV-1 CA is important for viral infectivity. We were, in fact, unable to detect any contribution to the infectivity of HIV-1 virions by producer cell CypA. There are perhaps several technical reasons why this result was not apparent before. The simultaneous analysis of CypA function using the large number of experimental tools that have accumulated over the years, including CA mutants, three

different competitive inhibitors of the CA-CypA interaction, and cell lines in which CypA expression was disrupted by two different genetic methods, permitted us to detect effects that were not apparent previously. Jurkat cells engineered to be CypA-deficient by gene targeting were valuable to prove the importance of CypA for spreading infection (23) but these cells are difficult to transfect or otherwise manipulate to produce virus for single-cycle assays. It is therefore likely that attenuated production of virus in these cells resulted in a virus stock with reduced infectivity for reasons other then lack of CypA, and that these changes in the virion infectivity were not reflected in the amount of RT activity. The recent development of RNA interference in mammalian cells permitted us to efficiently disrupt CypA expression in transfectable cell lines, and to more readily assess the role of CypA. Finally, more sensitive and accurate assays for quantitating HIV-1 infectivity in single-cycle assays are now available. This last point is critical since the effects of CypA on HIV-1 replication in human cells are modest in magnitude and MOI-dependent (123).

These new conclusions are consistent with most reports in the literature. Aside from minimal effects on the kinetics of *gag* processing or virion release (113, 128), disruption of CypA incorporation into virions has no effect on biochemical or ultrastructural characteristics of HIV-1 virions, including endogenous reverse transcription (21, 23, 46, 67, 120, 128). We had proposed that virion-associated CypA might promote virion disassembly (76), but the stability of virion cores is not detectably altered by CypA disruption (128), and structural models place CypA on the outside of the core (51, 73) where it would seem unlikely to disrupt CA-CA interactions.

CsA inhibits HIV-1 virion production via mechanisms independent of CA. We also provided evidence that CsA inhibits infectious HIV-1 virion production and entry via independent mechanisms. The magnitude of inhibition was greater if CsA was administered during entry than during production and the effects were additive if the drug was present at both times (Fig. 2.3-A). Consistent with the fact that the G89V mutation already abolishes CypA binding to CA, infectivity of HIV-1_{NL4-3} G89V mutant was not decreased further if CsA was added during infection of target cells. However, CsA added during virion production decreased infectivity of the HIV-1_{NL4-3} G89V mutant to the same degree as for the wild-type virus (Fig. 2.3-B). These results indicate that the inhibitory effect of CsA during virion production is independent of the CA interaction with CypA. Finally, virions produced from cells with CypA knockdown by RNAi are as infectious as virions produced from cells with wild-type CypA expression, demonstrating that the disruptive effect of CsA on virion production is independent of CypA. The inhibition of virion production by CsA in the context of cells with knocked down CypA was of equal magnitude as compared to wild-type cells (Fig. 2.6-C). Thus, the inhibitory effect of CsA on virion assembly is CypA-independent. CA variants that exhibit cell specific CsA resistance/dependence provide another evidence that only target cell CypA is relevant for HIV-1 infectivity. Our experiments demonstrate that only alteration in target cell CypA expression modulate infectivity of these vectors, while producer cell CypA is irrelevant for their phenotype (Fig. 13, 14, 15).

CsA inhibits HIV-1 Env function. Additional indication that CsA has CA-independent effects was provided by the finding that VSV-G pseudotyping suppresses the effects of CsA on virion production but not the effects of CA mutants (4). Here, we extended these findings further by showing that CsA administration during virion production results in inhibition of proper Env function. This effect was shown to be specific for several lentiviral Envs tested here, regardless of whether the Envs were expressed in *trans* or in the context of the provirus. Consistent with previous studies, the VSVG envelope appeared to be less sensitive against CsA treatment (4), since the infectivity of VSVG-pseudotyped envelopes was reduced only by two-fold at the highest CsA concentration (Fig. 3.4). How could CsA affect Env function? The fact that both virion-associated gp120 and gp41 levels are reduced suggests that decreased infectivity does not result from increased spontaneous dissociation of gp120 from the Env complexes (shedding), indicating that CsA affects gp160 earlier in the viral life cycle. Also, western blot analysis of virion protein content rules out proteolytic cleavage failure as the reason for this malfunction, since there is no significant accumulation of the uncleaved gp160 on the virions as compared to virions produced in drug-free media (Fig. 3.1).

Possible mechanisms of CsA action include interference with proper synthesis of the precursor protein gp160, or inhibition of processing such as folding and/or glycosylation. Alternatively, CsA might affect trafficking of gp160 to the cell membrane either directly by binding and trapping gp160 in a wrong compartment, or by inhibiting cellular factors mediating this process. The observation that MeIle^4-CsA had the same effect as CsA (Fig. 3.1) indicates that inhibition of calcineurin phosphatase activity (97) is not required for the anti-viral effect. Sanglifehrin also has the same effect (Fig. 3.1).

The only property that Sanglifehrin shares with the other drugs is the ability to bind to cyclophilin family members (100, 135). This suggests, then, that inhibition by CsA may involve targeting of one of the cyclophilin family members. Our knockdown data rules out CypA (Fig. 2.6), CypB (Fig. 3.5) and Cyp60 (Fig. 3.6), but there are 13 more cyclophilins to test (23). Consistent with the CypA-independence of the drug effect, there was no correlation between the ability of the drugs to disrupt CypA binding to CA and their effectiveness at inhibiting virion infectivity. For example, Sanglifehrin was a less potent inhibitor of infectivity but all three drugs abrogated CypA incorporation into virions with comparable efficiency (Fig 2.4). Lastly, CsA could be interfering with the assembly steps of the viral life cycle, for example inhibiting Env incorporation into viral particles. Further work will be required to determine which cyclophilin family member and which process in viral replication is targeted by CsA during virion production.