Cellular Restriction Targeting Viral Capsids Perturbs Human Immunodeficiency Virus Type 1 Infection of Nondividing Cells[▽]

Masahiro Yamashita and Michael Emerman*

Division of Human Biology, Fred Hutchinson Cancer Research Center, Seattle, Washington 98109

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The ability of human immunodeficiency virus (HIV) to infect nondividing cells is a fundamental property by which HIV replicates in critical target cells, such as macrophages and resting CD4⁺ T cells. Recent studies have revealed that the capsid (CA) protein is a dominant factor that determines retrovirus infectivity in nondividing cells, and several mutations in HIV type 1 (HIV-1) CA abrogate the ability of HIV-1 to infect nondividing cells. We present evidence for a connection between cellular restriction against viral capsids and the resistance of nondividing cells to retrovirus infection. TRIM proteins that are able to target incoming viral capsids restrict HIV-1 more potently in nondividing cells than in dividing cells, thus rendering HIV-1 infection dependent on cell division. Moreover, cyclophilin A, another cellular protein that binds to HIV-1 CA, regulates HIV-1 infection of nondividing cells. Together, these data demonstrate the importance of capsid-binding cellular proteins in the control of the cell cycle independence of HIV-1. We propose that cellular restrictions to retroviral infections are themselves cell cycle dependent.

The physiological status of host cells has an enormous influence on the outcome of retrovirus infection (19, 43). For example, cells that exit from the cell cycle are not susceptible to infections by many retroviruses. However, human immunodeficiency virus (HIV) and lentiviruses are exceptional in that they can propagate independently of the cell division of host cells (23, 40). This property expands the host cell range of HIV type 1 (HIV-1), which includes a variety of nondividing cell types, such as tissue macrophages, partially activated (noncycling) CD4⁺ T cells, and often naïve resting CD4⁺ cells (8). Therefore, the ability of HIV to infect nondividing cells is a fundamental property of this virus, and understanding of this mechanism has broad implications for AIDS research (14).

One of the central questions in HIV-1 biology is which viral and cellular elements confer on HIV-1 this ability to infect cells during interphase. Previous studies in this field were directed at HIV-1-encoded molecules (e.g., matrix, Vpr, integrase, and cPPT) that harbor nuclear import activity (9, 39). However, our laboratory demonstrated that none of the previously reported karyophilic elements encoded by HIV-1 is essential for the cell cycle independence of HIV infection (44). Instead, we found that transfer of the capsid (CA) protein from murine leukemia virus (MLV), which does not infect nondividing cells, is sufficient to eliminate the cell cycle independence of HIV-1 (42). This suggests that the CA protein is a dominant viral determinant for retroviral infection of nondividing cells. Consistent with this finding, we and others identified mutations in HIV CA protein that cause the loss of HIV infectivity in nondividing cells (30, 45, 48).

It remains unclear exactly how nondividing cells resist infections by MLV and by these HIV-1 CA mutants. One poten-

tially relevant observation is that MLV contains much larger amount of CA proteins in its intracellular virus complexes than HIV-1 (4, 13, 15, 18, 20, 27). Similarly, there are several lines of evidence that HIV-1 CA mutants do not disassemble with kinetics similar to those of wild-type (WT) HIV-1 strains (10, 45). Thus, one hypothesis is that the quantitative or qualitative differences in the uncoating of the viral core between HIV-1 and MLV determine the ability to infect nondividing cells. Another possibility is that nondividing cells express a restriction factor that blocks some retroviruses (34).

A number of host cellular factors are known to bind to CA proteins soon after entry into the infected cell. For example, the cytoplasmic protein TRIM5 α from rhesus monkeys can directly recognize specific retroviral capsid cores and accelerates the uncoating of incoming viral capsids, which impedes viral DNA synthesis (37, 38). Rhesus TRIM5 α also seems to be able to restrict HIV-1 after viral DNA synthesis when proteasome activity is suppressed by treatment with proteasome inhibitors (41). In addition, cyclophilin A (CypA), another protein that specifically binds HIV-1 and other lentiviral capsids (24, 26), is also a critical factor in the restriction of HIV by Old World monkey TRIM5 α proteins (2) at a step after reverse transcription (25).

In the current study, we investigated whether or not CA-binding molecules can influence the cell cycle preference of HIV-1. Indeed, we find that TRIM family proteins and CypA can modulate the ability of HIV-1 to infect nondividing cells. That is, TRIM proteins that bind CA can block HIV-1 infection more strongly in nondividing cells than in dividing cells. These results lend additional evidence to the hypothesis that CA is a major determinant of the ability of HIV to infect nondividing cells. In addition, we propose that one of the mechanisms of the cell cycle dependence of retroviruses is a consequence of cellular restrictions that are strong or weak depending on the cell cycle stage.

* Corresponding author. Mailing address: Division of Human Biology, Fred Hutchinson Cancer Research Center, 1100 Fairview Ave. N., P.O. Box 10924, Seattle, WA 98109. Phone: (206) 667-5058. Fax: (206) 667-6523. E-mail: memerman@fhcrc.org.

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MATERIALS AND METHODS

Plasmid constructs. The HIV-1 reporter construct pLai3 Δ Env-GFP3 used in our study expresses green fluorescent protein (GFP) but lacks the *env* and *nef*

genes (42). All of the HIV-1 CA mutants were generated previously based on this HIV-1 reporter construct (24, 45), except for two CA mutants, in which the A92E and G94D mutations were introduced into HIV-1 reporter constructs encoding GFP or luciferase (42). A retroviral vector expressing the fusion protein between TRIM19 and CypA was constructed based on a previous publication by Yap et al. (47). Basically, the RBCC domains of TRIM19 and CypA were PCR amplified using the human cDNA prepared from 293T cells and pLPCX-TRIMCyp, respectively. The overlapping PCR technique was used to create the fusion fragment between these two genes, which were cloned into the EcoRI-NotI-digested pLPCX vector to create pLPCX-TRIM19Cyp. Retroviral constructs expressing rhesus TRIM5 α (pLPCX-rhTRIM5 α) or owl monkey TRIMCyp have been described previously (24, 37). Plasmid pBABE-puro-shRNA-CypA is a retroviral vector for short hairpin RNA (shRNA) expression that targets CypA under the control of the H1 promoter and was created by inserting the target sequence of CypA (5'-GGGTTCCTGCTTTCACAGA-3' [35]) into the original retrovirus construct (28) by utilizing two unique sites (BglII and EcoRI).

Cells. All cells were maintained in Dulbecco's modified Eagle's medium with 7% bovine growth serum (HyClone). OMK is an owl monkey epithelial cell line from kidney tissue. Telo-RF is a rhesus monkey fibroblast cell line immortalized by the catalytic subunit of telomerase (21). HeLa cells stably expressing TRIM proteins or shRNA that targets CypA were generated by transduction of MLV carrying the corresponding vector. Transduced cells were selected in the presence of 0.4 or 0.8 μ g/ml of puromycin for 2 weeks.

Virus production. We produced VSV-G-pseudotyped viruses by transient transfection of 293T cells by using the TransIT-LT1 reagent (Mirus Bio). Plasmid DNA used for virus production has been described previously (42) (45), except for SIVmac239, which was created by using an *env*-defective GFP-reporter virus (17). Culture supernatants of transfected cells were harvested 2 and 3 days after transfection, cleared of cell debris, filtered with 0.2- μ m-pore-size syringe filters (Nalgene), and stored at -80°C until use. If necessary, the virus was concentrated by ultracentrifugation as described previously (42).

Viral infections. Single-cycle replication assays were performed by using HeLa and its derivative cell lines expressing TRIM proteins or shRNA targeting CypA. In order to have equal numbers of dividing and nondividing cells at the time of infection, three times more cells were plated in wells containing 2 μ M of aphidicolin (Sigma-Aldrich) than in wells without the drug. The integrase inhibitor 118-D-24, obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, was used at 300 μ M. To block the interaction between CypA and incoming virus capsids, cells were pretreated with cyclosporine (CsA; Sigma-Aldrich) at a concentration of 1 or 2 μ M. CsA was kept in culture medium throughout the infections except for the time course experiments. Infections were initiated by spinoculation and the addition of DEAE-dextran (20 μ g/ml). For GFP viruses, 2 days after infection, the infected cells were fixed with 1% paraformaldehyde for an hour and were then analyzed by fluorescence-activated cell sorting. For luciferase-encoding viruses, the infected cells were lysed by using the luciferase cell culture lysis 5X reagent (Promega) 2 days after infection, and luciferase activity was measured on a luminometer.

Western blot analysis. Total-cell lysates were extracted by using NP-40-DOC buffer (44) and were analyzed by Western blotting. Subcellular fractionation was performed as described previously (44). The antibodies used in the present study were an anti-hemagglutinin antibody (Babco), an anti-CypA antibody (Biomol), an anti-lactate dehydrogenase antibody (Cortex Biochem), and an anti-lamin B antibody (Calbiochem).

Quantitative PCR. For the quantitative PCR assay, 3.3×10^5 cells per well were plated in a 6-well plate as actively dividing cells while 5×10^5 cells were plated in the presence of aphidicolin as nondividing cells 1 day before infection. Genomic DNA was extracted from infected cells 24 h after infection or at different time points by using a QIAamp DNA blood minikit (Qiagen). Subcellular fractionation was performed as described previously (44). We performed real-time PCR based on the method developed by the Bushman laboratory (5).

RESULTS

Cell cycle-dependent restriction of HIV-1 by TRIM proteins.

We wanted to test whether cellular proteins that bind incoming HIV-1 capsids could modulate the ability of HIV to infect nondividing cells. To do this, we first tested whether restriction of HIV-1 by the TRIM19-cyclophilin fusion protein (TRIM19Cyp) depends on cell cycle progression. We chose TRIM19Cyp because it blocks HIV-1 after the comple-

tion of reverse transcription (47), a step at which both MLV and HIV-1 CA mutants are blocked in nondividing cells. To this end, we artificially arrested the cell cycle progression of HeLa cells expressing TRIM19Cyp at the early-S phase of the cell cycle. Consistent with previous observations (47), TRIM19Cyp expression conferred strong (~ 50 -fold) antiviral activity against HIV-1 on HeLa cells over a wide range of viral inputs (Fig. 1A). However, the cell cycle arrest of HeLa cells stably expressing TRIM19Cyp resulted in dramatically more potent antiviral activity ($>1,000$ -fold) against HIV-1 (Fig. 1A). Viral infectivity was much more strongly reduced by TRIM19Cyp in nondividing cells than in dividing cells, and the number of GFP-positive cells was not significantly above background levels in nondividing cells at any dilution.

Next, we examined the cell cycle dependence of the following naturally occurring TRIM proteins for their activity against HIV-1: the owl monkey TRIM5-CypA fusion protein (omk-TRIMCyp) (29, 32) and the rhesus TRIM5 α (rhTRIM5 α) protein. TRIMCyp binds HIV-1 via its cyclophilin A domain, while rhTRIM5 α binds HIV-1 CA via the B30.2 (PRY/SPRY) domain (33, 38), and both of these proteins have strong antiviral activity against HIV-1. Importantly, these molecules block virus replication mainly before reverse transcription (37) but also have the potential to act at stages after reverse transcription (41). Consistent with previous observations, expression of omkTRIMCyp in HeLa cells introduced a significant decrease in virus infectivity (Fig. 1B). Moreover, as with HeLa cells expressing TRIM19Cyp, antiviral activities against HIV-1 by omkTRIMCyp were higher when target cells were arrested in the cell cycle than when target cells were actively dividing (Fig. 1B). A similar but more modest enhancement of restriction was also observed in HeLa cells expressing rhTRIM5 α (Fig. 1C). This phenomenon is not specific to HeLa cells, since we obtained similar findings by using HOS cells expressing either omkTRIMCyp or TRIM19CypA (data not shown).

CypA is a cellular protein that positively affects HIV-1 replication through its ability to bind to HIV-1 CA proteins (26). However, this CypA-CA interaction becomes detrimental when incoming HIV-1 capsids encounter TRIM5 α from Old World monkeys (2). Our laboratory recently demonstrated that the CypA-dependent restriction by rhTRIM5 α occurs after the completion of reverse transcription (25). Based on this observation, we reasoned that the CypA-CA interaction is necessary for rhTRIM5 α to impair HIV-1 infection of nondividing cells. We tested this idea by infecting either control or rhTRIM5 α -expressing HeLa cells with HIV-1 bearing the G89V mutation in CA, which blocks interaction between CypA and CA (49). Unlike the WT virus (see Fig. 1C), the G89V mutant, although still restricted by rhTRIM5 α , is not further restricted in nondividing cells (Fig. 1D). We confirmed this finding by using CsA. CsA is an immunosuppressive drug that binds to CypA and disrupts the CypA-CA interaction. HeLa cells expressing rhTRIM5 α were infected with HIV or MLV in the presence or absence of cell cycle arrest (with or without aphidicolin) and in the presence or absence of CsA. The cells were infected with the amount of WT virus that causes $\sim 10\%$ of dividing cells to become infected (Fig. 1E). Cell cycle arrest of rhTRIM5 α -expressing HeLa cells decreased susceptibility to HIV infection, resulting in ~ 10 -fold fewer virus-infected cells ($\sim 1\%$) (Fig. 1E, left). However, when we blocked the

FIG. 1. TRIM proteins block HIV-1 more potently in nondividing cells than in dividing cells. (A to C) Control or TRIM protein-expressing HeLa cells were infected with increasing amounts of the WT HIV-1 GFP reporter virus. Open symbols represent dividing cells, while filled symbols represent nondividing (aphidicolin-treated) cells. Orange lines and symbols represent infections in HeLa cell lines expressing TRIM19Cyp (A), omkTRIMCyp (B), or rhTRIM5 α (C), while black lines and symbols represent infections in control HeLa cells. Aph, aphidicolin. (D) An HIV GFP reporter virus carrying the G89V mutation was used to infect control or rhTRIM5 α -expressing HeLa cells. At least two independent experiments were performed with similar results, and representative data from one experiment are shown. (E) Rhesus TRIM5 α -expressing HeLa cells were infected with an HIV-1 or MLV GFP reporter virus in the presence (2 μ M) or absence of CsA. Both dividing (open bars) and nondividing (filled bars) cells were used for infectivity assays. Infections were performed in triplicate. Error bars, standard deviations. (F) Western blot analysis was performed to examine the steady-state levels of rhTRIM5 α in dividing and nondividing cells. (Top) Rhesus TRIM5 α proteins were detected by an anti-hemagglutinin antibody. (Bottom) Levels of actin proteins were also examined as a loading control. Lysates of nondividing cells were extracted 1 day after incubation with 2 μ g of aphidicolin per ml.

interaction between CypA and CA (by addition of CsA), HIV-1 reacquired the ability to infect nondividing cells; in the presence of CsA, HIV-1 infected nondividing cells as efficiently as dividing cells (Fig. 1E, left). In contrast, CsA had no effect on MLV infections (Fig. 1E, right). These findings indicate that a CypA-CA interaction is required for the enhanced restriction of HIV due to rhTRIM5 α that is observed in nondividing cells. Finally, these data are not due to enhanced expression of rhTRIM5 α in nondividing cells, since the steady-state level of rhTRIM5 α in nondividing cells is actually somewhat lower in aphidicolin-treated cells than in actively proliferating cells (Fig. 1F).

To confirm that the enhanced restriction in nondividing cells by TRIM proteins is specific to the virus blocked by restriction factors, we assessed the infectivities of viruses that are not sensitive to rhTRIM5 α both in dividing and in nondividing cells. For this purpose, we used NB-tropic MLV (NB-MLV) and SIVmac, both of which are resistant to rhTRIM5 α -mediated restriction. Infection of nondividing cells with NB-MLV was poor regardless of cell type, and this phenotype was not

FIG. 2. Control and rhTRIM5 α -expressing HeLa cells were infected with either MLV or SIVmac. Open bars, dividing cells; filled bars, nondividing cells. CTRL, control (infected HeLa cells that have not been manipulated); +rhT5 α , infected HeLa cells that stably express the rhesus TRIM5 α gene, introduced via a retroviral vector. Aph, aphidicolin. Two independent experiments were performed with similar results, and representative data from one experiment are shown.

overtly affected by the presence of rhTRIM5 α (Fig. 2). On the other hand, SIVmac exhibited similar levels of virus infectivity in dividing and nondividing cells, and this phenotype also was not altered by the presence of rhTRIM5 α (Fig. 2). These results indicate that the more-dramatic restriction of HIV-1 by TRIM proteins in nondividing cells (Fig. 1A to C) is caused by specific and selective recognition of incoming viral capsids by restriction factors. Overall, these data demonstrate that the extent of the antiviral effects of TRIM proteins depends both on the recognition of the CA by the restriction factor and on the cell cycle progression of target cells.

TRIM proteins act both before and after reverse transcription to limit HIV-1 in nondividing cells. We next determined the replication step(s) of HIV-1 that is modulated by TRIM proteins specifically in nondividing cells by measuring the amount of newly synthesized viral DNA as well as the number of two-LTR circles by quantitative real-time PCR. Viral infectivity was also measured in parallel (Fig. 3A and B, left). We observed that although TRIM19Cyp causes a 500-fold drop in infectivity in nondividing cells compared to a 30-fold drop in dividing cells (Fig. 3A, left), equivalent amounts of reverse transcription products accumulate in control and TRIM19Cyp-expressing HeLa cells regardless of cell proliferation conditions (Fig. 3A, center). This observation is in line with the previous finding that TRIM19Cyp acts after reverse transcription to block HIV-1 (47). In dividing cells, TRIM19Cyp increases the number of two-LTR circles ~ 10 -fold over that in control HeLa cells (Fig. 3A, right); however, this is probably due to the fact that when viral DNA fails to integrate, it enters into a nonproductive pathway of two-LTR circles (11). More importantly, in the presence of TRIM19Cyp, we saw three- to fourfold reductions in the number of two-LTR circles in nondividing cells from that in dividing cells (Fig. 3A, right). Given that TRIM19Cyp causes a ~ 15 -fold-greater drop in viral infectivity in nondividing cells than in dividing cells (Fig. 3A, left), this partial reduction in the number of two-LTR circles suggests multiple blocks before and after nuclear entry of viral DNA in nondividing cells.

In contrast to TRIM19Cyp, omkTRIMCyp can block HIV-1 infection before reverse transcription in actively proliferating cells (Fig. 3B, center) (1). Consistent with this fact, cell cycle arrest of HeLa cells expressing omkTRIMCyp, which causes a 10-fold decrease in virus infectivity from that in proliferating cells, causes a decrease in cDNA production (2.4- to 4.5-fold) and two-LTR circle formation (2.4- to 3.2-fold). Thus, we conclude that the more-pronounced restriction of HIV-1 by TRIM proteins in nondividing cells is caused by a block(s) that occurs not only before but also after viral DNA formation.

Endogenous levels of restriction factors restrict HIV-1 more potently in nondividing cells than in dividing cells. We also examined whether the endogenous levels of restriction factors could affect HIV infection of nondividing cells by examining cell types in which the restriction factors are normally expressed. We used two simian cell lines, owl monkey kidney (OMK) cells, which naturally express TRIMCyp, and telomerase-immortalized rhesus fibroblasts (telo-RFs), which naturally express rhTRIM5 α . As shown in Fig. 4A, restriction of OMK cells against WT HIV-1 is more severe in nondividing cells than in actively dividing cells. This difference is specific to WT virus; there was no difference in viral infectivity when the

FIG. 3. The replication defect specific to nondividing cells occurs at steps during and after reverse transcription. Dividing HeLa cells (open bars) and nondividing HeLa cells treated with aphidicolin (Aph) (filled bars) were infected with an HIV-1 reporter virus encoding GFP. Virus infectivity was examined by quantifying GFP-positive cells 1 day after infection. Total DNA was extracted 1 day after infection. Five microliters out of 100 μ l of DNA was used in quantitative PCR to measure the copy number of late products of reverse transcription (late RT) as well as that of two-LTR circles. The viral DNA copy number is shown as the number per 5 μ l of DNA. Infections were done in triplicate. Error bars, standard deviations. At least two independent experiments were performed with similar results. The results of one representative experiment are shown here. (A) Control (CTRL) and TRIM19CypA-expressing HeLa cells; (B) control and omkTRIMCyp-expressing HeLa cells.

G89V mutant virus, which is not restricted in OMK cells, was used to infect OMK cells. Similarly, infection of telo-RFs, a rhesus macaque fibroblast cell line, with WT HIV-1 results in enhanced restriction in nondividing cells (Fig. 4B). SIVmac, which is not well targeted by rhTRIM5 α , did not show this enhanced restriction (Fig. 4B), demonstrating that the effect is specific. Thus, these data confirm the findings obtained from experiments using ectopic expression systems and demonstrate that natural levels of restriction factors that target HIV CA can modulate its ability to infect nondividing cells.

CypA is also required for the loss of the cell cycle independence of HIV-1 CA mutants. We previously identified CA mutations that render HIV-1 dependent on cell cycle progression for efficient infection (45). Since some of the HIV-1 CA mutants were previously shown to infect cells better in the presence of CsA (16, 35, 46), we examined the CsA sensitivities

FIG. 4. Simian cells modulate the ability of WT HIV-1 to infect nondividing cells. (A) The OMK cell line was infected with either WT HIV-1 or the G89V mutant HIV-1 reporter virus construct. The experiments were performed in the absence or presence (2 μ g per ml) of aphidicolin (Aph) in order to compare virus infectivity between dividing (open circles) and nondividing (filled circles) cells. (B) Rhesus monkey fibroblasts (telo-RFs) were infected with HIV-1 or SIVmac. Both dividing (open bars) and nondividing (filled bars) cells were used for infectivity assays. Infections were performed in triplicate, and error bars indicate standard deviations. At least two independent experiments were performed with similar results, and representative data from one experiment are shown.

of the HIV-1 CA mutants that had lost the ability to infect nondividing cells. Moreover, recent publications have indicated that some of the HIV-1 CA mutants are restricted more strongly when the target cells are not dividing (30, 48). To this end, we infected either dividing or nondividing HeLa cells with HIV-1 CA mutants in the presence or absence of CsA (Fig. 5). As shown in Fig. 5 (left), CA mutations (E45A, Q63A/Q67A, A92E, and G94D) abrogated the ability of HIV-1 to infect nondividing cells. However, blocking of the CypA-CA interaction by CsA completely restored the abilities of these HIV CA mutants to infect nondividing cells (Fig. 5, right). In contrast to these HIV-1 CA mutants, WT HIV-1 infects nondividing cells as efficiently as dividing cells in both cases, where CypA binds or does not bind to HIV-1 capsids. These results indicate the existence of a molecular mechanism by which CypA enhances the restriction of replication of HIV-1 CA mutants in nondividing cells.

CypA acts after reverse transcription but before integration to limit the replication of the HIV-1 CA G94D mutant in nondividing cells. We next investigated the molecular mechanism by which CypA enhances the restriction against HIV-1 CA mutants in nondividing cells. First, we examined viral DNA synthesis in the presence or absence of functional interaction between CypA and viral capsid. We used the G94D mutant for these experiments, since this mutant displays the most dramatic difference in infectivity between the presence and absence of CypA-CA interaction and between dividing and

FIG. 5. The loss of the ability of HIV-1 CA mutants to infect nondividing cells is also mediated by the binding of cyclophilin A to incoming viral capsid. Normal HeLa cells were infected with HIV-1 GFP reporter viruses carrying different mutations, as indicated, in the presence (1 μ M) or absence of CsA. Open circles represent dividing cells; filled circles, nondividing cells treated with aphidicolin (Aph).

nondividing cells (Fig. 5). We reduced the level of CypA expression by stably transducing shRNA that targets CypA (Fig. 6A). In fact, CypA knockdown by shRNA increased the virus titer of the G94D mutant nearly 100-fold in nondividing cells (0.7% of control HeLa cells and 60% of shRNA-expressing cells were virus infected [Fig. 6B, left]). A real-time PCR assay showed that the restricted cells (HeLa cells without shRNA-CypA) supported reverse transcription of the viral genome as efficiently as the cells with shRNA-CypA (Fig. 6B) with similar kinetics (Fig. 6C).

We further investigated whether nuclear entry of intracellular virus complexes was affected for the G94D mutant by the disruption of the CypA-CA interaction. Initially, we tested the formation of two-LTR circles, a dead-end product of viral integration that has been used as a marker for the nuclear migration of viral DNA. We found that inhibition of the CypA-CA interaction had little effect on the synthesis of two-

FIG. 6. Cyclophilin A blocks the HIV-1 CA G94D mutant in non-dividing cells at a step after reverse transcription. (A) Western blots with increasing amounts of cell lysates (fivefold increment) from control HeLa cells or HeLa cells with CypA shRNA (CypA-KD) were probed for anti-CypA antibodies. The HeLa CypA-KD cells have 5- to 25-fold less CypA than the HeLa control cells. (B) Effects of CypA knockdown on virus infectivity, reverse transcription, and nuclear import. Aphidicolin-treated HeLa cells were infected with an HIV-1 reporter virus carrying the G94D mutation in the CA protein. + CypA, control HeLa cells; - CypA, HeLa cells stably expressing shRNA that targets CypA. Normalized virus was used to infect these two cell lines, and virus infectivity was measured 1 day after infection by counting GFP-positive cells. Total DNA was extracted 1 day before infection and was used as a template for quantitative PCR to measure the amounts of both late reverse transcription products (late RT) and

LTR circles (Fig. 6B, right). The number of two-LTR circles produced by the CA G94D mutant was slightly lower (three-fold) in control cells than in cells in which CypA had been knocked down; however, this degree of drop does not explain the severely blocked virus replication in control cells (100-fold decrease). To complement the assay monitoring two-LTR circles, we also followed the migration of viral DNA more directly by separating the cytoplasmic and nuclear fractions (Fig. 6D). The results showed that the nuclei of control HeLa cells contained levels of viral DNA similar to those in the nuclei of HeLa cells in which CypA had been knocked down. Together, these results indicate that CypA prevents a step after nuclear entry, thus preventing the G94D mutant from infecting non-dividing cells.

We next asked whether HIV-1 CA mutants are blocked before or after integration. In order to address this question, we took advantage of the fact that the inhibition of HIV-1 CA mutants can be reversed by the addition of CsA (48). We find that this is also true when cells are arrested in the cell cycle with aphidicolin (Fig. 7). For example, addition of CsA at the time of infection enhanced infection of the G94D mutant in nondividing cells >50-fold over that without CsA (Fig. 7, compare condition 1 to condition 2). We also found that CsA could be added up to 12 h after infection, and enhanced infection could still be observed (Fig. 7, condition 3), although no effect was seen when CsA was added at later time points (24 hpi, 36 hpi, and 48 hpi [data not shown]). This finding indicates that CypA-mediated inhibition does not immediately destroy the intracellular virus complex, which can stay infectious for ~12 h after infection.

Next, we determined whether CsA acts before or after integration by adding integrase inhibitor at the time of CsA addition. We reasoned that if the G94D virus is blocked before integration in nondividing cells due to its CypA interactions, then the addition of an integrase inhibitor together with CsA would prevent the rescue of virus infectivity by CsA. However, if the virus is blocked after integration, then the addition of an integrase inhibitor would allow the rescue of the G94D mutant by CsA. We added the integrase inhibitor at 12 h after infection, because most of the integration events are already completed by this time in normal infections (data not shown), and because this is the latest time point at which we could rescue infection with CsA. As shown in Fig. 7, addition of the inte-

two-LTR circles. All the infections were performed in triplicate, and error bars indicate standard deviations. (C) Infections were performed under conditions similar to those for the experiments for which results are shown in panel B, but DNA was extracted at different time points. Open circles, control HeLa cells; filled circles, HeLa cells expressing shRNA targeting CypA. The viral copy number for late-RT products was determined as for Fig. 3. (D) (Top) Similar infection experiments were done with control HeLa cells or HeLa cells expressing shRNA targeting CypA in the presence of aphidicolin (2 μ g/ml). One day after infection, virus-infected cells were subjected to subcellular fractionation. Late-RT products were measured in DNA extracted from the nuclear fraction. (Bottom) Western blot analysis was performed to ensure the integrity of subcellular fractionation by using a cytoplasmic protein, LDH 1, and a nuclear protein, lamin B. Tot, total cell lysates; Cyt, cytoplasmic extract; Nuc, nuclear lysates.

FIG. 7. The HIV-1 CA G94D mutant is blocked before integration. Nondividing HeLa cells (treated with aphidicolin) were infected with the HIV-1 CA mutant G94D virus carrying the luciferase gene. CsA was added to the cells at the indicated time points. The virus was added at 15°C by spinoculation, and infections were synchronized by washing off the inocula and adding medium at 37°C. Two days after infection, the cells were lysed, and luciferase (Luc) activity was measured with the luminometer. The cells were treated with CsA (1 μ M) alone or with the integrase (IN) inhibitor 118-D-24 (300 μ M) at the indicated time points. RLU, relative light units. Representative data from two independent experiments with similar results are shown.

grase inhibitor (condition 4; no drug, followed by CsA plus the integrase inhibitor) prevents the CsA-mediated rescue of virus infectivity shown under condition 3 (no drug, followed by CsA alone). This result clearly indicates that the binding of CypA to the G94D mutant prevents integration from occurring in nondividing cells. These findings provide evidence for the cell cycle-dependent modulation of viral infectivity by CA-binding proteins, underscoring a dominant role for the CA protein in regulating retroviral infection in nondividing cells that occurs after nuclear entry but before proviral integration.

DISCUSSION

Among retroviruses, HIV-1 and other lentiviruses possess an exceptional ability to efficiently infect nondividing cells. However the viral elements that confer this ability on HIV-1 have been under intensive debate. Our laboratory previously demonstrated that the capsid protein is a dominant viral determinant for HIV infection of nondividing cells (42, 45). The present study demonstrates that cellular proteins with the ability to bind to the incoming viral capsid are capable of manipulating the cell cycle preference of HIV-1. TRIM proteins render HIV-1 more dependent on cell division for infection, while CypA abrogates the ability of HIV-1 CA mutants to infect cells independently of mitosis. These findings lend further support to the idea that the CA protein plays a key role in the infection of nondividing cells by HIV.

How do these CA-binding host factors alter the cell cycle preference of HIV-1? Although it has been proposed that TRIM proteins inhibit reverse transcription by accelerating the uncoating step (38), this does not seem to be the sole case for the greater restriction against the virus in nondividing cells, since reverse transcription does not seem to be overtly affected in nondividing cells expressing TRIM19Cyp. Rather, these data suggest a post-reverse transcription block in nondividing cells. Consistent with this post-reverse transcription block by TRIM proteins, CypA-dependent restriction occurs primarily after reverse transcription (25), and our current data reveal that the CypA-CA interaction is necessary for the more-pro-nounced restriction of HIV-1 in nondividing cells. It is not clear precisely how TRIM proteins affect virus replication after

reverse transcription; however, it has been suggested that the proteasome-independent restriction of TRIM5 α may occur by sequestration of intracellular virus complexes within cytoplasmic bodies (6). Given that most of the members of the TRIM protein family self-assemble to form multimeric complexes (including the formation of nuclear bodies by PML, an alternate designation for TRIM19) (31), we propose a model in which HIV-1 particles that have completed reverse transcription are trapped in cytoplasmic/nuclear bodies during interphase, and mitosis liberates these trapped viral particles from the restriction to complete the replication step, since mitosis affects the integrity and composition of these intracellular structures (12).

Recent studies have indicated that HIV-1, but not MLV, seems to depend on the cellular nuclear import factor TNPO3, probably for transporting the viral genome into the nucleus (3, 7, 22). These findings raise the possibility that the differential use of TNPO3 by retroviruses may control the ability of viruses to infect nondividing cells. It was also suggested that the critical target for TNPO3-mediated nuclear entry is the HIV-1 integrase protein (7). However, we previously demonstrated that an HIV-1 based chimeric virus with MLV integrase, thus lacking the ability to interact with TNPO3, infects nondividing cells as efficiently as dividing cells (44). One possible explanation for this discrepancy may be that HIV-1 dependence on TNPO3 for nuclear entry is manifested both in macrophages (nondividing cells) and in actively proliferating cells. Consistent with this explanation, previous work demonstrated the effects of small interfering RNA knockdown of TNPO3 not only in nondividing macrophages (7) but also in actively proliferating cells (22). Thus, while it is true that viral and cellular factors involved in nuclear entry must be essential for HIV-1 infection of nondividing cells, these factors do not affect the cell cycle independence of HIV-1, since the abrogation of these factors affects viral infectivity both in dividing and in nondividing cells. In contrast, as shown in this and previous studies, mutations of the capsid protein, as well as modulation by capsid-binding proteins, can affect viral infectivity distinguishably between dividing and nondividing cell types (30, 45, 48). Importantly, these steps appear to occur after nuclear entry, as shown in Fig. 6 and 7, a finding that is also consistent

with previous publications (30, 45, 48), and thus, a step subsequent to TNPO3-mediated nuclear import may be involved.

Our study also reveals a connection between the resistance of nondividing cells to retroviruses and cellular restriction aimed at incoming virus capsids. In the presence of known antiviral factors (TRIM proteins) that bind to the capsid proteins, nondividing cells were more resistant to the virus than dividing cells. This observation indicates that the restriction activity is cell cycle regulated and that this cell cycle-dependent restriction causes the cell cycle-dependent infection. Therefore, it is tempting to speculate that the inability of some of the retroviruses, such as HIV-1 CA mutants and MLV, to infect nondividing cells may be due to cellular restriction targeting viral capsids.

Recent publications have shown that CypA-CA interactions are necessary for the loss of the cell cycle independence of many HIV-1 CA mutants (30, 48). One mutant, however, the T54A/N57A mutant, did not change its phenotype in nondividing cells in the absence of a CypA-CA interaction (30). We confirmed this observation with additional CA mutants and showed that the majority of HIV-1 CA mutants (except for the T54A/N57A mutant [data not shown]) regain the ability to infect nondividing cells when CypA is prevented from binding incoming viral capsids. It is not clear why this particular mutant (the T54A/N57A mutant) is independent of CypA for its loss of cell cycle independence, but our previous study (45) suggests that the mutant keeps its capsid longer than WT virus after entry. Thus, this mutation may cause a defect in a specific stage that is independent of CypA action during viral uncoating, which results in the selective loss of viral infectivity in nondividing cells. Our data also extend the importance of CypA-CA interactions to restriction by rhTRIM5 α , which blocks WT HIV-1 in a cell cycle-dependent manner. This striking similarity argues for the existence of rhTRIM5 α -like restriction systems in human cells (36) that specifically recognize and restrict the replication of HIV-1 CA mutants in a CypA-dependent and cell cycle-dependent manner.

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