Naturally Occurring Capsid Substitutions Render HIV-1 Cyclophilin A Independent in Human Cells and TRIM-cyclophilin-resistant in Owl Monkey Cells*

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In this study, we asked if a naturally occurring HIV-1 variant exists that circumvents CypA dependence in human cells. To address this issue, we sought viruses for CypA independence using Debio-025, a cyclosporine A (CsA) analog that disrupts CypA-capsid interaction. Surprisingly, viral variants from the Main group replicate even in the presence of the drug. Sequencing analyses revealed that these viruses encode capsid substitutions within the CypA-binding site (V86P/H87Q/I91V/M96I). When we introduced these substitutions into viruses that normally rely on CypA for replication, these mutants no longer depended on CypA, suggesting that naturally occurring capsid substitutions obviate the need for CypA. This is the first demonstration that isolates from the Main group naturally develop CypA-independent strategies to replicate in human cells. Surprisingly, we found that these capsid substitutions render HIV-1 capable of infecting Owl monkey (OMK) cells that highly restrict HIV-1. OMK cell resistance to HIV-1 is mediated via TRIM-Cyp, which arose from a retrotransposition of CypA into the TRIM5α gene. Interestingly, saturation experiments suggest that the Pro90/Gln89/Ile92/Va19 capsid core is “invisible” to TRIM-Cyp. This study demonstrates that specific capsid substitutions can release HIV-1 from both CypA dependence in human cells and TRIM-Cyp restriction in monkey cells.

HIV-1 specifically recruits the abundant host protein, cyclophilin A (CypA), to optimally replicate in human cells (1, 2). It is thought that CypA binds HIV-1 capsid, a major structural component that forms the core that surrounds the viral genome (3–5). Genetic and structural studies showed that HIV-1 binds CypA via a Gly89-Pro90 peptide within the unique exposed loop of capsid (1, 5). CypA-capsid interaction can be competitively disrupted by the immunosuppressive drug cyclosporine A (CsA), which binds to the hydrophobic pocket of CypA (2, 6, 7). Preventing CypA-capsid interaction, either by the introduction of mutations in the CypA binding region of capsid or by the addition of CsA, decreases and delays HIV-1 infectivity in human cells (1–7). Furthermore, HIV-1 infection is attenuated in human T cells homozygous for a deletion of the gene encoding CypA (8) or in cells treated with small interference RNA that target the CypA gene (9, 10). Recent studies suggest that the CypA in target cells rather than the CypA in virus producer cells is required for optimal HIV-1 infectivity in human cells (10–13). To date, the block of infectivity has been attributed to post-entry events prior to integration (14, 15). Although the CypA-mediated enhancement of HIV-1 infectivity in human cells was discovered more than a decade ago (1, 2), its role in the HIV-1 life cycle as well as the mechanisms that govern it remain obscure. Because CypA catalyzes the cis-trans isomerization of the Gly89-Pro90 peptide bond of capsid (16) and the capsid core likely undergoes an ordered uncoating in order to deliver the viral genome into the cytosol of target cells, it has been proposed that CypA orchestrates HIV-1 uncoating in human target cells (17), although no experimental evidence has been reported to support this model. However, observations in monkey cells may help us to understand the role of CypA in HIV-1 infection. Paradoxically, recent findings point to a detrimental role for CypA in HIV-1 infection in monkey cells. Towers et al. (18) found that CypA-capsid interactions are responsible for the inability of HIV-1 to infect owl monkey (OMK) cells. OMK cells are normally highly refractory to HIV-1 infection; however, CsA renders them permissive to HIV-1 (18). Moreover, the introduction of a mutation in the CypA binding region of capsid (G89V), which abolishes CypA-capsid contact, renders the mutant virus capable of infecting OMK cells (1). Supporting the hypothesis that CypA or CypA-like proteins are responsible for the refractivity of OMK cell to HIV-1, two studies elegantly showed that OMK cell resistance to HIV-1 is mediated via a TRIM-Cyp chimeric protein, which arose from a retrotransposition of CypA into the TRIM5α gene (19, 20). Thus, CypA and CypA-like proteins possess the capacities to influence either positively (human cells) or negatively (OMK cells) HIV-1 infectivity of pri-mates. The mechanisms that dictate whether CypA has a beneficial or detrimental effect on HIV-1 infection in primate cells are totally unknown. In the present study, we asked if a naturally occurring HIV-1 variant exists that circumvents both CypA dependence in human cells and TRIM-Cyp sensitivity in OMK cells. The identification of which molecular feature that renders HIV-1 susceptible or resistant to CypA

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‡‡The abbreviations used are: HIV-1, human immunodeficiency virus, type 1; X-gal, 5-bromo-4-chloro-3-indolyl-D-galactopyranoside; GFP, green fluorescent protein; CypA, cyclophilin A; CsA, cyclophilin A; OMK, Owl monkey cells; VSVG, vesicular stomatitis virus G protein.
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dependence is imperative to shed light on the functions of CypA in HIV-1 infection in primate cells.

MATERIALS AND METHODS

Viruses and Cells—All HIV-1 isolates were obtained from the AIDS Research and Reference Reagent Program. The pNL4.3-GFP plasmid was provided by C. Aiken and D. Gabuzda. Capsid mutants were generated as described previously (21). To obviate specific cell entry requirements, all pNL4.3-GFP viruses were pseudotyped with the VSVG envelope generously provided by D. Trono. TZM-bl cells (contributed by J. C. Kappes, X. Wu, and Tranzyme, Inc.) were obtained through the AIDS Research and Reference Reagent Program. CCR5+ Jurkat T cells were provided by M. Emerman, whereas OMK cells were provided by G. Towers and C. Aiken. CsA and Debio-025 were provided by Sigma and Debiopharm, respectively. Capsid NL4.3 (R9) and pNL4.3-GFP mutant viruses were generated by PCR mutagenesis and by liposome-mediated transfection of 293T cells using Genejuice (Novagen). Viral supernatants were harvested 48 h post-transfection and filtered through a 0.2-μM pore size filter to remove cellular debris. Viral inoculum was standardized by exoRT assay or p24 enzyme-linked immunosorbent assay (PerkinElmer Life Sciences).

Infections—TZM-bl and OMK cells (80,000 or 20,000 cells/well/ml) were seeded for 24 h pre-infection in 24-well plates. Cells were exposed to HIV-1 in the presence of 5 μg/ml polybrene. CsA or Debio-025, at the concentrations indicated, were added 15 min prior to virus addition. Infected cells were analyzed by fluorescence-activated cell sorting (GFP content), by X-gal staining (counting blue foci) or by β-galactosidase activity 48 h post-infection. For β-galactosidase activity, infected TZM-bl cells were washed twice with 1 ml of phosphate-buffered saline and lysed in 100 mM potassium phosphate, pH 7.8, containing 0.2% Triton X-100. Plates were stored at −80 °C for 16 h and thawed on ice, and 20 μl of lysate was transferred to a 96-well plate for detecting β-galactosidase activity. Galacton-Star substrate (Applied Biosystems, Bedford, MA) was diluted 1:50 in the reaction buffer diluent (100 mM sodium phosphate pH 7.5, 1 mM MgCl2, 5% Sapphire-IITM enhancer) to make the reaction buffer. 100 μl of reaction buffer was added to 20 μl of lysate, and the light emission was measured over 1 s in a microplate luminometer after 30 min. For saturation experiments, OMK cells were initially exposed to increasing amounts of wild-type or mutant NL4.3 (R9) viruses that do not encode GFP and subsequently (5 min later) exposed to wild-type or mutant NL4.3 viruses that do encode GFP.

Western Blot Analysis—Purification and immunoblot analysis of viruses produced from 293T-transfected cells were conducted as previously described (21). CsA and Debio-025 were added 24 h post-infection, and viruses were collected 48 h post-transfection. Rabbit anti-capsid and anti-CypA rabbit antibodies were generated as described previously (21). To obviate specific cell entry requirements, all pNL4.3-GFP viruses were pseudotyped with the VSVG envelope generously provided by D. Trono. TZM-bl cells (contributed by J. C. Kappes, X. Wu, and Tranzyme, Inc.) were obtained through the AIDS Research and Reference Reagent Program. CCR5+ Jurkat T cells were provided by M. Emerman, whereas OMK cells were provided by G. Towers and C. Aiken. CsA and Debio-025 were provided by Sigma and Debiopharm, respectively. Capsid NL4.3 (R9) and pNL4.3-GFP mutant viruses were generated by PCR mutagenesis and by liposome-mediated transfection of 293T cells using Genejuice (Novagen). Viral supernatants were harvested 48 h post-transfection and filtered through a 0.2-μM pore size filter to remove cellular debris. Viral inoculum was standardized by exoRT assay or p24 enzyme-linked immunosorbent assay (PerkinElmer Life Sciences).

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RESULTS

Pharmacological Prevention of CypA-Capsid Interactions—To identify HIV-1 variants refractory to both human and OMK CypA-mediated activities, we first searched for viruses that do not rely on CypA to optimally infect human cells. To address this issue, we sought conditions that abrogate CypA-capsid interactions in human cells. CsA binds CypA and prevents interaction between CypA and HIV-1 capsid, but it also exhibits an immunosuppressive activity, which complicates the interpretation of its effect on HIV-1 replication. To examine the effect of CypA-capsid interactions on HIV-1 replication exclusively, we sought CsA analogs, which prevent CypA-capsid interactions, but are not immunosuppressive. We used a CsA analog, Debio-025, which lacks the immunosuppressive effect usually associated with CsA but nevertheless retains an inhibitory activity against HIV-1. The non-immunosuppressive CsA analog Debio-025 is a derivative of the non-immunosuppressive CsA analog, SDZ-811, which prevents CypA-capsid interactions and HIV-1 infectivity (22–24). We first compared the efficiencies of CsA and Debio-025 to prevent CypA-capsid interactions by examining their potential to inhibit CypA incorporation into virions. 293T cells were transfected with proviral HIV-1 NL4.3 plasmid in the presence of Debio-025 or CsA (0.25–8 μM). Forty-eight hours post-infection, infection was scored by X-gal staining. Results are expressed in number of blue foci per nanogram of p24. This experiment is representative of three independent experiments.

 Naturally Occurring HIV-1 Isolates Derived from the Main Group Replicate in Human Cells Independently of CypA—To identify HIV-1 isolates that do not rely on CypA to infect human cells, we used TZM-bl HeLa cells that express β-galactosidase after infection with HIV-1. To determine if HIV-1 requires CypA-capsid interactions to infect TZM-bl
cells, Debio-025 was added to target cells 15 min prior to addition of virus. By adding Debio-025 to target TZM-bl cells, we anticipate that the drug, by saturating cytosolic CypA, prevents CypA recruitment onto the incoming viral capsid and therefore decreases HIV-1 infectivity. Supporting this hypothesis, Debio-025, even at low concentrations, inhibits HIV-1 infectivity (Fig. 2). It is critical to emphasize that the 2- to 3-fold decrease of infectivity, although modest, is always consistent between experiments using TZM cells. Note that we obtained similar results using CCR5/H11001 Jurkat cells (data not shown); however, given that the assay using TZM-bl cells is quicker (48 h instead of 2 weeks) than the assay using CCR5/H11001 Jurkat cells that do not contain a reporter gene, we preferred using TZM-bl cells as targets to screen a large panel of isolates.

Forty-two viruses from different clades and with different co-receptor usage were used to infect TZM-bl cells in the presence of Debio-025. We found that a majority of viruses are highly sensitive to Debio-025 and possess a mean 50% inhibitory concentration (IC\textsubscript{50}) of 0.097 \(\mu M\) (TABLE ONE). Furthermore, we found that a few viruses are partially resistant to Debio-025 with IC\textsubscript{50} values of 1.98 \(\mu M\) (highlighted in green in TABLE ONE). Importantly, we found that two viruses derived from the Main group (M group) are highly resistant to Debio-025 with IC\textsubscript{50} values of 8.5 \(\mu M\) (highlighted in pink in TABLE ONE). The definitions of “partially resistant” and “highly resistant” viruses are arbitrary. The “highly resistant viruses” are the two viruses that exhibit an IC\textsubscript{50} of \(2.5 \mu M\) Debio-025; similarly “partially resistant viruses” are the ten viruses that possess IC\textsubscript{50} values between 1.5 and 2.5 \(\mu M\) of Debio-025. All the other viruses that exhibit an IC\textsubscript{50} of \(0.17 \mu M\) are termed “sensitive viruses”. Amounts of Debio-025 necessary to decrease the infectivity of the 42 viruses are lower (TABLE ONE) than those used to decrease NL4.3 infectivity in HeLa cells (Fig. 2), because we used less virus (100 pg instead of 1 ng). We obtained similar results for the resistant viruses, using Debio-025-treated CCR5/H11001 Jurkat cells, by monitoring viral replication by p24 enzyme-linked immunosorbent assay over a period of 3 weeks (data not shown). Thus, we identified for the first time HIV-1 isolates from the

### TABLE ONE

**Resistance of HIV-1 to Debio-025**

100,000 TZM cells were infected with 100 pg of HIV-1 in the presence of Debio-025 (0.25 to 8 \(\mu M\)). Forty-eight hours post-infection, infection was scored by X-gal staining. Results are expressed in micromolar Debio-025 necessary to inhibit 50% of HIV-1 infectivity in the absence of the drug (IC\textsubscript{50}). On the right side of the table, amino acid sequences of the CypA-binding site are presented. Some capsid sequences are missing due to a lack of viral replication or due to our inability to sequence the CypA binding region. The “highly resistant” and “partially resistant” viruses are highlighted in pink and green, respectively.

<table>
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<tr>
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<td></td>
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<td>R5</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td>R5</td>
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Cyclophilin A – Binding Site

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FIGURE 3. Alignment of the CypA binding region of HIV-1 capsid from different subtypes.

Main Group that do not rely on CypA to optimally infect human cells. Interestingly, among the 42 isolates tested, only R5 and R5X4 viruses were found to partially or highly resist to Debio-025 (TABLE ONE).

HIV-1 CypA-independent Infection of Human Cells Correlates with the Presence of Specific Residues within the CypA-binding Site of Capsid—We then asked if this CypA independence correlates with specific residues in the CypA binding region of HIV-1 capsid. To address this issue, we sequenced the CypA binding region of capsid for a majority of the 42 viruses amplified in CCR5+ Jurkat T cells (TABLE ONE). Interestingly, we found that four capsid residues in positions 86, 87, 91, and 96, which are normally well conserved among all HIV-1 subtypes (Fig. 3), were frequently substituted in viruses that are partially or highly resistant to Debio-025 (TABLE ONE). The most commonly occurring capsid sequence contains residues Val86, His87, Ile91, and Met96 (Fig. 3). Interestingly, the two viruses that are highly resistant to Debio-025, RU570 and RU132, have all four residues mutated: Pro86, Gln87, Val91, and Ile96 for RU570 and Gln86, Gln87, Phe91, and Ile96 for RU132 (TABLE ONE). It is also interesting to note that two consensuses contain frequent substitutions at these positions: consensus G that contains Gln86, Gln87, Ile91, and Ile96 and consensus O that contains Pro86, Ala87, Leu91, and Ile96 (Fig. 3). Supporting the possibility that substitutions at these specific positions of capsid mediate CypA independence of HIV-1, we found here that the two highly Debio-025-resistant RU570 and RU132 viruses are derived from consensus G (TABLE ONE). Moreover, previous work showed that two isolates from the Outlier group (group O) infect human cells even in the presence of cyclosporine A (25, 26). Altogether these data indicate that naturally occurring mutations in capsid can substitute for the need for CypA for optimal HIV-1 infection of human cells.

To determine if these residues govern CypA independence, we introduced them in the capsid region of a virus, which is normally highly sensitive to Debio-025 and NL4.3 (TABLE ONE) and which contains the most commonly occurring capsid residues (Val86, His87, Ile91, and Met96) (Fig. 3), and asked whether these mutations confer CypA independence. Using the highly resistant RU570 capsid sequence as a template, we created single, double, triple, and quadruple mutations in the context of the proviral molecular clone NL4.3. Viruses were produced from 293T-transfected cells and used to infect TZM-bl cells as above. Debio-025 significantly reduced the infectivity of wild-type NL4.3 virus (Fig. 4A). As a control, we used a capsid mutant, which cannot bind CypA (NL4.3 G89V) (1). This mutant, like the CsA-treated wild-type virus, exhibited a significant defect in infectivity, further reinforcing the notion that CypA is necessary for optimal HIV-1 infectivity in human cells. We found that single and double mutants are still sensitive to Debio-025 (Fig. 4A). In sharp contrast, the infectivity of the triple or quadruple mutant viruses is weakly influenced by Debio-025 (Fig. 4A). This strongly suggests that a combination of three to four capsid substitutions (TABLE ONE) renders HIV-1 CypA independent in human cells. We obtained similar Debio-025 resistance results using physiological peripheral blood mononuclear as target cells (data not shown).

One possibility why naturally occurring HIV-1 variants derived from the Main group did not rely on CypA to infect human cells is that, like HIV-2 and simian immunodeficiency virus, they do not bind CypA, explaining their resistance to CsA and CsA-analogs (2, 25). However, we found that all Debio-025-resistant HIV-1 viruses, including the variant that contains the four capsid substitutions (Pro86/Gln87/Val91/Ile96), incorporate wild-type levels of CypA (data not shown and Fig. 4B). Moreover, Debio-025 prevented CypA incorporation into these viruses at a degree similar to that in the wild-type virus (Fig. 4B). This indicates that these naturally occurring HIV-1 variants derived from the Main group bind CypA well but do not rely on it to optimally infect human cells. Altogether these data suggest that HIV-1 variants, which naturally pre-exist in the Main group, contain specific residues in the unique exposed loop of capsid that permit infectivity of human cells independently of the CypA recruitment onto the capsid core in the cytosol of target cells.

Cyclophilin A and Capsid Contribution to HIV-1


FIGURE 4. Specific capsid substitutions render HIV-1 CypA-independent. A, 100,000 TZM cells were infected with 5 ng of p24 of virus in the presence of Debio-025 (0.5 μM). Forty-eight hours post-infection, infection was scored by β-galactosidase assay. This experiment is representative of two independent experiments.

B, 293T cells were transfected with pNL4.3-GFP constructs, Debio-025 (0.5 μM) was added 24 h post-transfection, viruses were collected 48 h post-transfection, standardized by exoRT assay and analyzed for CypA and capsid content by Western blot using anti-capsid and anti-CypA rabbit antibodies.
partly) to the CypA-mediated OMK restriction. Interestingly, the infectivity of Pro86/Gln87/Val91/Ile96 NL4.3 perfectly mirrored the infectivity of the wild-type virus in the presence of Debio-025 (Fig. 5), suggesting that the Pro86/Gln87/Val91/Ile96 NL4.3 escapes the block mediated by Debio-025-sensitive inhibitory factors in OMK cells (i.e. TRIM-Cyp). However, Debio-025 further enhances the capacity of Pro86/Gln87/Val91/Ile96 NL4.3 to infect OMK cells (3-fold) (Fig. 5). This suggests that the Pro86/Gln87/Val91/Ile96 capsid core is not totally impervious to Debio-025-sensitive OMK inhibitors (i.e. TRIM-Cyp). Supporting this notion, the infectivity of G89V NL4.3 was superior to that of Pro86/Gln87/Val91/Ile96 NL4.3 in the absence of Debio-025 (Fig. 5). Nonetheless, the rescue in infectivity of wild-type NL4.3 by Debio-025 was superior to that of Pro86/Gln87/Val91/Ile96 NL4.3 (10-fold versus 3-fold). Together, these data strongly suggest that the wild-type capsid core is highly vulnerable to OMK inhibitors (i.e. TRIM-Cyp), whereas the Pro86/Gln87/Val91/Ile96 capsid core possesses intrinsic capacities to partly circumvent these factors.

Capsid That Permits HIV-1 CypA Independence in Human Cells Does Not Saturate OMK Restriction Factors—When OMK cells are exposed to a viral challenge (using virus-like particles), they become more susceptible to a subsequent viral challenge (18). This observation led researchers to postulate that OMK cells contain inhibitory factors that are saturable (18). One possibility to explain why the Pro86/Gln87/Val91/Ile96 NL4.3 virus is more competent to infect OMK cells than wild-type virus is that its capsid core is less well recognized by the saturable inhibitory factors. To test this hypothesis, OMK cells were pre-challenged with either wild-type or Pro86/Gln87/Val91/Ile96 viruses and subsequently exposed to wild-type HIV-1. As above, in the absence of pre-challenge, wild-type HIV-1 barely infects OMK cells. In contrast, pre-challenging OMK cells with increasing amounts of wild-type virus (100, 500, and 1000 ng of p24) allows wild-type HIV-1 capable of infecting these cells (Fig. 6, right panel). This is in accordance with the hypothesis that an initial viral challenge can saturate inhibitory factors present in OMK cells (18–20). Surprisingly, pre-challenging OMK cells with increasing amounts of the Pro86/Gln87/Val91/Ile96 virus renders target cells minimally more permissive to a subsequent wild-type virus exposure (Fig. 6, left panel). Similarly, we found that a pre-challenge with G89V NL4.3 virus does not affect the refractoriness of OMK cells as previously reported (18) (data not shown). Our observation that the Pro86/Gln87/Val91/Ile96 viral pre-challenge does not alleviate the block in OMK cells suggests that the Pro86/Gln87/Val91/Ile96 capsid core cannot saturate the OMK inhibitory factors. This strongly suggests that the Pro86/Gln87/Val91/Ile96 capsid core is less well recognized by the OMK inhibitory factors than wild-type capsid core. This capsid core “invisibility” may explain the enhanced capacity of the Pro86/Gln87/Val91/Ile96 virus to infect OMK cells. Together, our data reveal that naturally occurring HIV-1 variants exist that circumvent both CypA dependence in human cells and TRIM-Cyp sensitivity in OMK cells.

DISCUSSION

In this study, we identified naturally occurring HIV-1 isolates from the Main group that do not depend on CypA for optimal infection of human cells. Sequencing analyses revealed that these viruses contain specific residues at specific positions in the CypA-binding site of capsid. To determine whether these residues mediate CypA independence, we introduced them into viruses that normally highly depend on CypA. Importantly, these viruses no longer depend on CypA for infection after the introduction of these capsid substitutions. These findings demonstrate that naturally pre-existing residues located at specific positions within capsid can render HIV-1 capable of optimally infecting human cells even in the absence of CypA.

An analysis of 2599 HIV-1 capsid sequences available from the Los Alamos Data base indicates that 3.35% of the capsid sequences contain the V86P substitution, 19.74% contain H87Q, 27.36% contain I91V, and 15.31% contain M96I (Fig. 7). We also found that 46.44% (1207/2599) have no change from the His87/Ile91/Met96 consensus, whereas 7% (182/2599) of the capsid sequences have a change in all three locations. Moreover, 45.48% (1182/2599) have no change from the Val86/His87/Ile91/Met96 consensus, whereas 3.89% (101/2599) of the capsid sequences have a change in all four locations. The two consensus G and O contain frequent substitutions at these positions (Fig. 3). Supporting the possibility that substitutions at these specific positions of capsid mediate CypA independence, the two highly Debio-025-resistant RU570 and RU132 viruses are derived from consensus G (TABLE ONE) and two isolates from the consensus O, viruses 5180 and 9435, that infect human cells even in the presence of CsA (25, 26) contain Pro86/Ala87/Leu91/Ile96 and Gln87/Ala87/Leu91/Ile96 capsid substitutions, respectively (26).

During the course of this study, Ikeda et al. (13) also found that substitutions within capsid correlate with CsA resistance in human cells. Specifically, Ikeda et al. (13) transferred the full-length gag gene of eleven HIV-1 isolates into a CsA-sensitive NL4.3 backbone and tested the resulting chimeric viruses for their resistance to CsA in human cells. They found that the gag region of four viruses (89.6, Gun WT, Gun V, and 93BR) also confers CsA resistance in human cells (13). They also identified a triple Pro86/Gln87/Val91 capsid substitution that renders
HIV-1 partially resistant to CsA. We confirmed these data by showing that the Pro86/Gln87/Val91 resistance to Debio-025 is very similar to those of the two variants that we identified in this study, the triple Gln87/Val91/Ile96 and quadruple Pro86/Gln87/Val91/Ile96 HIV-1 variants (Fig. 4A). Interestingly, Kootstra et al. identified another CsA-resistant HIV-1 variant (Pro86/Gln87/Pro88/Val91) (11), which is distinct from that of Ikeda et al. (Pro86/Gln87/Ala88/Val91) (13) and of ours (Val86/Gln87/Ala88/Val91/Ile96 and Pro86/Gln87/Ala88/Val91/Ile96/Ile96). Given that we showed that the Gln87/Val91, Gln87/Ile96 and Val91/Ile96 double mutations do not suffice to render HIV-1 resistant to CsA or Debio-025, altogether these data suggest that a combination of three to four capsid substitutions allows HIV-1 capable of escaping the drug pressure and of replicating in a CypA-independent manner. Ikeda et al. (13) also presented evidence that the His87 substitution alone renders NL4.3 more resistant to CsA. However, we found in this study that H87Q alone or in combination with I91V or M96I (double substitutions) does not suffice to render HIV-1 resistant to CsA or Debio-025, suggesting that these artificially induced capsid muta-

**FIGURE 6.** The capsid that renders HIV-1 CypA-independent in human cells does not saturate OMK TRIM-Cyp. 20,000 OMK cells were pre-challenged with 0, 100, 500, and 1000 ng of p24 of VSVG-pseudotyped wild-type or Pro86/Gln87/Val91/Ile96 NL4.3 viruses that do not encode GFP and immediately exposed to 10 or 25 ng of p24 of VSVG-pseudotyped wild-type pNL4.3-GFP. Forty-eight hours post-infection, infection was quantified by GFP content by fluorescence-activated cell sorting. This experiment is representative of two independent experiments.

**FIGURE 7.** Polymorphisms at amino acids 86, 87, 91, and 96 of HIV-1 capsid. Counts and percentages for the various combinations of polymorphisms at amino acids 86, 87, 91, and 96 based on the 2599 sequences from the Los Alamos data base.
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tions do not pre-exist in the HIV-1 population. Because CypA-independent viruses are poorly represented among the HIV-1 population and that a majority of simian immunodeficiency virus isolates do not require CypA (at least to infect human cells), this suggests that CypA recruitment represents a major advantage for HIV-1 infection in human cells. Nevertheless, our present findings suggest that HIV-1 may exploit CypA-dependent and -independent strategies to guarantee optimal infection of human cells.

After revealing the existence of naturally occurring HIV-1 variants that circumvent CypA dependence in human cells, we asked whether these variants could also escape the TRIM-Cyp-mediated infectivity block in OMK cells. In contrast to wild-type HIV-1, which fails to infect OMK cells even at a high inoculum, we found that the CypA-independent Pro86/Gln87/Val91/Ile96 virus infects OMK cells significantly. During the course of this study, two studies showed that viruses containing the H87Q substitution also exhibit a higher capacity to infect OMK cells than that of wild-type virus (13, 31). This further suggests that the CypA-binding site of capsid represents a major locus for TRIM-Cyp action. In contrast to wild-type virus, we found that the CypA-independent virus cannot saturate the OMK restriction. Given that it is thought that primate restrictions target the incoming virus at a post-entry step and that the locus for restriction resides in the viral capsid, our observations suggest that TRIM-Cyp fails to recognize the Pro86/Gln87/Val91/Ile96 incoming capsid core. This is intriguing given that native CypA likely binds Pro86/Gln87/Val91/Ile96 capsid. Indeed, we showed that CypA is packaged at wild-type level in the Pro86/Gln87/Val91/Ile96 capsid. However, we cannot exclude the possibility that TRIM-Cyp binds differently to HIV-1 Gag than to the incoming capsid core. We tried to determine if TRIM-Cyp binds HIV-1 Gag by co-transfecting 293T cells with NL4.3 and OMK TRIM-Cyp and by looking for TRIM-Cyp incorporation into nascent viruses. Although TRIM-Cyp was abundantly expressed in transfected cells, we failed to detect TRIM-Cyp in pelleted particles by Western blot analysis (data not shown). This suggests that either TRIM-Cyp cannot bind NL4.3 Gag or that its subcellular localization prevents its packaging. Nevertheless, if CypA binds Pro86/Gln87/Val91/Ile96 capsid, we would expect TRIM-Cyp capable of recognizing the incoming Pro86/Gln87/Val91/Ile96 capsid core in the cytosol of OMK cells. Our observations, that Pro86/Gln87/Val91/Ile96 infectivity in OMK cells is lower than that of the G89V virus that cannot bind CypA and that Debio-025 boosts Pro86/Gln87/Val91/Ile96 infectivity, suggest that TRIM-Cyp recognizes, at least partially, the incoming Pro86/Gln87/Val91/Ile96 capsid core.

Although further work is required to determine how these capsid substitutions render HIV-1 Debio-025-resistant and CypA-independent in human cells as well as competent for OMK infection, it is likely that these residues subtly modify the capsid loop structure in a way that circumvents CypA-mediated activities on the capsid core. Highlighting the importance of our findings, Debio-025 is currently being tested in a clinical trial for its anti-HIV-1 efficacy. By identifying naturally occurring primary isolates (and not VSVG-pseudotyped HIV-1-based vector-encoding mutated capsid) that resist to Debio-025, our study suggests that the use of Debio-025 or CsA analogs as therapeutic agents may fail in patients infected with viral variants that contain the capsid substitutions identified in this study (i.e. subtype G and O variants). Thus, our present data provide a molecular context for the interpretation of the results of this trial.

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Naturally Occurring Capsid Substitutions Render HIV-1 Cyclophilin A Independent in Human Cells and TRIM-cyclophilin-resistant in Owl Monkey Cells


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