Altered HIV-1 Gag Protein Interactions with Cyclophilin A (CypA) on the Acquisition of H219Q and H219P Substitutions in the CypA Binding Loop*

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From the 1Experimental Retrovirology Section, HIV and AIDS Malignancy Branch, NCI, National Institutes of Health, Bethesda, Maryland 20892, the 2Departments of Hematology and Infectious Diseases, Kumamoto University School of Medicine, Kumamoto 860, Japan, and the 3Department of Chemistry, University of Illinois, Chicago, Illinois 60607

HIV-1 Gag protein interaction with cyclophilin A (CypA) is critical for viral fitness. Among the amino acid substitutions identified in Gag noncleavage sites in HIV-1 variants resistant to protease inhibitors, H219Q (Gatanaga, H., Suzuki, Y., Tsang, H., Yoshimura, K., Kavlick, M. F., Nagashima, K., Gorelick, R. J., Mardy, S., Tang, C., Summers, M. F., and Mitsuya, H. (2002) J. Biol. Chem. 277, 5952–5961) and H219P substitutions in the viral CypA binding loop confer the greatest replication advantage to HIV-1. These substitutions represent polymorphic amino acid residues. We found that the replication advantage conferred by these substitutions was far greater in CypA-rich MT-2 and H9 cells than in Jurkat cells and peripheral blood mononuclear cells (PBM), both of which contained less CypA. High intracellular CypA content in H9 and MT-2 cells, resulting in excessive CypA levels in virions, limited wild-type HIV-1 (HIV-1WT) replication and H219Q introduction into HIV-1 (HIV-1H219Q), reduced CypA incorporation of HIV-1, and potentiated viral replication. H219Q introduction also restored the otherwise compromised replication of HIV-1P222A in PBM, although the CypA content in HIV-1H219Q/P222A was comparable with that in HIV-1P222A, suggesting that H219Q affected the conformation of the CypA-binding motif, rendering HIV-1 replicative in a low CypA environment. Structural modeling analyses revealed that although hydrogen bonds are lost with H219Q and H219P substitutions, no significant distortion of the CypA binding loop of Gag occurred. The loop conformation of HIV-1P222A was found highly distorted, although H219Q introduction to HIV-1P222A restored the conformation of the loop close to that of HIV-1WT. The present data suggested that the effect of CypA on HIV-1 replicative ability is bimodal (both high and low CypA content limits HIV-1 replication), that the conformation of the CypA binding region of Gag is important for viral fitness, and that the function of CypA is to maintain the conformation.

Combination antiretroviral therapy has brought about improved quality of life and extended survival in patients with HIV-1 infection. However, the emergence of HIV-1 variants resistant to anti-HIV-1 therapeutic agents, including reverse transcriptase inhibitors and protease inhibitors (PIs), has limited the efficacy of chemotherapy (1). HIV-1 develops resistance mainly by substituting amino acids in the target viral enzyme or component; however, recent studies have revealed that certain polymorphic amino acid residues also contribute to the viral resistance (2, 3). We recently found that multiple amino acid substitutions emerged in noncleavage sites of the Gag protein, which were associated with the development of HIV-1 resistance against PIs (4). Among such amino acid substitutions, H219Q, occurring in the cyclophilin A (CypA) binding loop in the p24 Gag protein, conferred the greatest replication advantage on HIV-1 (4). CypA binds to p24 Gag protein, resulting in the packaging of ~200 copies of CypA into each HIV-1 virion (5, 6), and is thought to perform an essential role early in the HIV-1 replication cycle (7, 8), perhaps by destabilizing the capsid (p24 Gag protein) shell during viral entry and uncoating (9) and/or by performing an additional chaperon function, facilitating correct capsid condensation during viral maturation (10, 11).

In the present study, we asked how H219Q and H219P substitutions occurring within the viral CypA binding loop conferred replication advantage to HIV-1, and we examined whether these substitutions affected the conformation and interaction of p24 Gag protein and CypA during HIV-1 propagation in various host cells. We also attempted to better understand the functional role of CypA in HIV-1 replication. To that end, we determined the virological and biochemical properties of a variety of recombinant infectious clones and their CypA contents. We also carried out molecular modeling analyses of the wild-type and mutated p24 Gag complex with CypA. The data demonstrate that both H219Q and H219P enhance HIV-1 replication by reducing viral CypA contents in daughter virions as produced in CypA-rich cells, but not in cells that has a low CypA content. We suggest that the effect of CypA on HIV-1 replicative ability is bimodal, both high and low contents of CypA limit HIV-1 replication. The data also suggest that the conformation of p24 Gag is strongly correlated with viral fitness.

**Experimental Procedures**

*Antiviral Agents—Three PIs, KNI-272, JE-2147, and UIC-94003, were synthesized as described previously (12–16). Three newly synthesized PIs, UIC-00041, UIC-00142, and UIC-00145 (Fig. 1), were also*
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A. K. Ghosh and K. A. Hussain, personal communication.

The crystal structure of human CypA that was bound to the amino-terminal domain of p24 Gag (residues 1–151 (CA151), Protein Data Bank code 1AK4) (9) was analyzed to determine the conformation of the p24CA151-CypA complex. The crystal structure was used to model the interaction of the p24CA151-CypA complex with p24 amino acid substitutions. The crystal structures were used to perform molecular dynamics calculations using the OPLS2003 force field and to study the interaction of the p24CA151-CypA complex with peptides.

Generation of HIV-1 Variants Resistant to PIs—The wild-type infectious clone, HIV-1WT (14), was propagated in human CD4+/H9262 p24 Gag was used for the analysis of virion-associated CypA, the culture supernatants of chronically HIV-1-infected H9 or Jurkat cells were centrifuged and passed through a 0.22-μm pore-size filter to remove cellular debris; the filtrates were normalized with p24 contents measured with radioimmunoassay and were ultracentrifuged to pellet virions (4). The pelleted virions were lyzed in lysis buffer. The resultant samples were processed with SDS-polyacrylamide gradient gels, and CypA was visualized by Western blot analysis using an anti-CypA antiserum (Biomol, Plymouth Meeting, PA). An anti-p24 Gag antiserum (Advanced Biotechnologies, Inc., Columbia, MD) served as a control to ensure that appropriate amounts of the samples were loaded. The signal density of CypA and p24 Gag was analyzed on a Windows computer by using the ImageJ Program (developed at National Institutes of Health, rsb.info.nih.gov/ij/) as published previously (19).

Molecular Modeling of the p24CA151-CypA Complex with p24 Amino Acid Substitutions—The crystal structure of human CypA that was bound to the amino-terminal domain of p24 Gag (residues 1–151 (CA151), Protein Data Bank code 1AK4) (9) was analyzed to determine the changes with H219Q or H219P substitution. There were two CA-CypA complexes in the structure. Chain A of CypA and chain D of CA form one complex and were retained for the calculation. Identical results should be obtained with the other complex (chain B of CypA and chain C of CA) because the conformations of both CypA and CA in these complexes are quite similar. Structural modifications, visualization, and analysis were performed utilizing the Maestro interface from Schrödinger (Maestro 7.0, Schrödinger, LLC, New York). OPLS2003 force field (20) was used to study the interaction of the p24CA151-CypA complex with peptides. The OPLS2003 force field was used to study the interaction of the p24CA151-CypA complex with peptides. The OPLS2003 force field was used to study the interaction of the p24CA151-CypA complex with peptides.

Generation of HIV-1 Variants Resistant to PIs—The wild-type infectious clone, HIV-1WT (14), was propagated in human CD4+/H9262 (PerkinElmer Life Sciences). An enzyme-linked immunosorbent assay kit (Beckman Instruments, Fullerton, CA) was also used for the determination of p24 Gag amounts as needed.

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RESULTS

Amino Acid Substitutions Identified in p24 Gag Protein in HIV-1 Variants Resistant to PI—We have reported previously that several amino acid substitutions were seen in common in the Gag protein at noncleavage sites among HIV-1 variants that acquired in vitro a high multtitude of resistance to PIs such as APV, JE-2147, KNI-272, and UIC-94003. In an attempt to corroborate and extend our previous observations and to determine how often such amino acid substitutions develop in the Gag protein, we examined four more HIV-1 variants that were selected in vitro against various PIs, including three novel PIs, UIC-00041, UIC-00142, and UIC-00145. Table 1 depicts the properties of eight PI-resistant HIV-1 variants (including four HIV-1 variants reported previously). Seven of the eight variants were selected against PIs in MT-2 cells, although one variant was selected in H9 cells. The strains used were HIV-1NL4-3 and HIV-1LAI, and 3–6 amino acid substitutions were identified in the protease. It was noted that all eight variants had in common an amino acid substitution at position 219 in p24 Gag protein, seven variants had H219Q substitution and one had H219P substitution. The His219 residue is located within the CypA binding loop and is thought to play a role in the p24 Gag interactions with CypA through a hydrox bonding and hydrophobic contact(s) (9). It is worth noting that when HIV-1NL4-3 was propagated in the absence of PI in MT-2 cells, the virus also acquired H219Q, V218M, or A224V mutation by passage 10 (4). It should be noted that two amino acids, Val218 and Ala224, are also located within the CypA binding loop of the p24 Gag protein and are also known to interact with CypA through hydrophobic contacts (9). These data, taken together, strongly suggest that the amino acids interacting with CypA, in particular His219, are prone to undergo substitutions under certain circumstances and are associated with viral replication fitness. Indeed, in the HIV Sequence Compendium 2000 (22), of 88 different HIV-1 strains, 65 had histidine at the position 219, whereas 13 had glutamine, and 4 had proline at the position 219, indicating that both Gln219 and Pro219 represent polymorphic amino acid residues. Hence, the present data suggest that these two polymorphic amino acids are associated with viral fitness and possibly to the acquisition of resistance to certain PIs.

Effects of Gag Mutations at Position 219 on HIV-1 Replication—In order to examine the effects of the Gag mutation at position 219 on HIV-1 replication, we generated two infectious HIV-1 clones, HIV-1H219Q and HIV-1H219P and we assessed their virologic properties. In MT-2 cells, HIV-1H219Q rapidly replicated compared with the wild-type HIV-1WT (Fig. 2A), in agreement with our previous observation (4). HIV-1H219Q also replicated more rapidly than HIV-1WT in H9 cells (Fig. 2B). HIV-1H219Q replicated as rapidly as HIV-1H219Q suggesting that the amino acid at position 219 is critical for the replication fitness of HIV-1. When we examined the fitness of the three infectious clones (HIV-1WT, HIV-1H219Q, and HIV-1H219P) in CD4+ Jurkat cells and PHA-PBM, there was no significant difference seen in their replication fitness (Fig. 2, C and D).

Competition HIV-1 Replication Assays for H219Q Mutation in PHA-PBM—As shown above, although HIV-1H219Q exhibited a greater replication capability when propagated in MT-2 and H9 cells compared with HIV-1WT, there was no apparent difference in the replication profiles of HIV-1H219Q and HIV-1WT as propagated in PHA-PBM (Fig. 2, A, B, and D). In order to evaluate the possible biological relevance of the replication kinetic data generated by using immortalized and long term cultured MT-2 and H9 cells, we conducted a modified competitive HIV-1 replication assay (CHRA) (18), in which freshly prepared PHA-PBM served as host cells. HIV-1H219Q readily and uniformly overgrew HIV-1WT in CHRA regardless of three different ratios of paired clones used in the assay (Fig. 2, E–G). These data indicated that the H219Q substitution conferred replication advantages on HIV-1 when propagated in immortalized CD4+ T cells as well as PHA-PBM. It should be noted, however, that the replication advantage of HIV-1 acquired with the H219Q substitution was limited in PHA-PBM and was detected only when assessed with CHRA.

MT-2 and H9 Cells Contain Greater Amounts of CypA—The Gag mutations H219Q and H219P conferred significant replication advantage on HIV-1 as propagated in MT-2 cells and H9 cells, but such advantage was limited in PHA-PBM. Considering that His219 is located within the CypA binding loop and that HIV-1 replication is known to be affected by intracellular CypA contents (6, 7), we examined intracellular CypA content in each cell preparation by using Western blot analysis. As shown in Fig. 3, A and B, the CypA contents in 10⁴ MT-2 (relative density, 100%) and 10⁴ H9 cells (79.6%) appeared to be greater than those in 5 x 10⁴ Jurkat cells (55.4%) and 5 x 10⁴ PHA-PBM (42.9%), suggesting that MT-2 and H9 cells contained 6–12 times as much CypA per cell as Jurkat cells and PHA-PBM. As normalized with total cellular protein amounts, the CypA content in 1 μg of MT-2 and H9 cellular protein preparations was comparable with that in 2–4 μg of Jurkat protein preparations and that in 4 μg of PHA-PBM protein preparation, suggesting that the former two cell preparations contained 2–4-fold greater amounts of CypA per cellular protein than the latter two cell preparations. These data showed that MT-2 and H9 cells have greater CypA amounts than Jurkat T cells and PHA-PBM.

Decreased CypA Incorporation into HIV-1H219Q Virions—Considering that the data from crystal structure analyses by Gamble et al. (9) of p24 Gag protein complexed with CypA showing that His219 binds to Asn73 and Gln111 of CypA through a hydrox bonding and hydrophobic contact(s), respectively, we postulated that H219Q and H219P substitutions cancel or weaken such hydrox bonds, resulting in the reduction of p24 binding to CypA and of CypA incorporation into daughter virions, leading to increased HIV-1 replication in CypA-rich MT-2 and H9 cells. We then examined the virion-associated CypA amounts in

| PI          | Strain       | Cell | Passage |
|-------------|--------------|------|---------|
| KNI-272     | HIV-1NL4-3   | MT-2 | 27      |
| KNI-272     | HIV-1LAI     | H9   | 55      |
| APV         | HIV-1NL4-3   | MT-2 | 31      |
| JE-2147     | HIV-1NL4-3   | MT-2 | 33      |
| UIC-94003   | HIV-1NL4-3   | MT-2 | 62      |
| UIC-00041   | HIV-1NL4-3   | MT-2 | 73      |
| UIC-00142   | HIV-1NL4-3   | MT-2 | 84      |
| UIC-00145   | HIV-1NL4-3   | MT-2 | 80      |

* PI-resistant HIV-1 variants described previously (4) are shown.
three infectious HIV-1 clones containing HIV-1WT, HIV-1H219Q, and HIV-1H219P, employing Western blotting analysis using anti-p24 Gag and anti-CypA antisera. The culture supernatants of chronically HIV-1-infected H9 cells were prepared to contain the same amount of p24. The virions in each supernatant thus prepared were pelleted by ultracentrifugation and subsequently subjected to SDS-PAGE. Direct sequencing of cellular DNA confirmed that no unintended mutations developed during the culture. As shown in Fig. 3C-1–3, the experiment was performed three times. Percent densities of the CypA signal relative to each p24 Gag signal (making each signal 100%) were 11.1, 7.38, and 6.01% (Fig. 3C-1), 6.99, 4.99, and 4.71% (Fig. 3C-2), and 18.1, 12.0, and 11.2% (Fig. 3C-3) for HIV-1WT/H9, HIV-1H219Q/H9, and HIV-1H219P/H9, respectively.

These data demonstrated that H219Q and H219P substitutions increased HIV-1 replication in CypA-rich cells, which was associated with the substantial reduction of CypA incorporation into daughter virions.

p24 Gag Binding to CypA Affects HIV-1 Replication Kinetics—Yin et al. (23) have reported that the addition of CsA (0.5 μM) reduces CypA incorporation into daughter virions and increases replication rates of HIV-1NL4-3 in H9 cells, suggesting that excessively high intracellular CypA contents may reduce HIV-1 replication rates. We then examined the effects of CsA on the replication rates of HIV-1WT (Fig. 4, A–D) and HIV-1H219Q (Fig. 4, E–H) in four different cell preparations.

In MT-2 and H9 cells, 0.5 μM CsA increased the replication rate of HIV-1WT, whereas at a higher concentration (2.5 μM) its replication rate...
showed a substantially decreased replication rate (Fig. 5C). In PHA-PBM, HIV-1P222A was virtually replication-incompetent (Fig. 5D). As expected, with the H219Q substitution added, HIV-1H219Q/P222A exhibited an improved replication rate, which, however, yet remained below that of HIV-1WT, both in Jurkat cells and PHA-PBM (Fig. 5, C and D).

These data suggested either that the added H219Q substitution enabled HIV-1P222A to incorporate more CypA into daughter virions or that H219Q altered or restored a conformation of the CypA binding domain of p24 Gag protein, thereby rendering the virus replication-competent without increasing CypA content.

H219Q Substitution Improves HIV-1P222A Fitness without Increasing CypA Content—In order to ask whether H219Q improved the otherwise compromised CypA incorporation caused by the P222A substitution into virions or H219Q rendered HIV-1P222A replication-competent without increasing CypA content, we examined the virion-associated CypA amounts by employing Western blotting analysis.

As can be seen in Fig. 5E–I, as HIV-1 virions in the culture supernatants of chronically HIV-1-infected H9 or Jurkat cells were pelleted and subjected to Western blotting analysis for the determination of virion-associated CypA amounts using anti-p24 Gag and anti-CypA antisera, the percent densities of the CypA signal relative to each p24 Gag signal (making each p24 signal 100%) were 33.8, 8.03, 6.92, and 6.48% in Fig. 5E–I; 26.3, 14.1, 6.93, and 6.19% in Fig. 5E–J; and 6.8, 4.68, 5.72, and 5.51% in Fig. 5E–F for HIV-1WT/H9, HIV-1WT/Jurkat, HIV-1P222A/H9, and HIV-1H219Q/P222A/H9, respectively, although HIV-1H219Q/P222A had a greater replication rate compared with HIV-1P222A (Fig. 5, A–E).

These data demonstrated that HIV-1WT produced by Jurkat cells and HIV-1P222A Produced by H9 cells contained approximately less than half and approximately one-fourth of the CypA amount detected in HIV-1WT produced by H9 cells, respectively. The data strongly suggested that H219Q altered the conformation of the CypA binding domain of p24 Gag protein without affecting the CypA incorporation into HIV-1P222A virions, thus rendering HIV-1P222A replication-competent independently of CypA. It is possible that H219Q substitution not only decreased the incorporation of CypA into HIV-1 but also altered the conformation of Gag protein, thus leading to increased HIV-1 replication especially when HIV-1 is produced in CypA-rich cells. The latter effect of H219Q substitution is apparently viable in the presence of P222A substitution because increased HIV-1H219Q/P222A replication was seen without significant changes in CypA content in virions (Fig. 5, A–E).

Molecular Modeling of the p24 Gag CA151–CypA Complex with H219Q or H219P—We finally carried out molecular modeling studies to better understand the following two aspects: the reason for less viral incorporation of CypA with H219Q, H219P, and P222A substitutions, and the rescue of viral replication with the H219Q/P222A double mutation. The crystal structure determined by Gamble et al. (9) revealed the sequence-specific interactions of p24 Gag (CA151) with CypA (Fig. 6A). Those interactions include seven hydrogen bonds between residues 219 and 223 (excluding the bonds mediated through bridging water molecules) and various hydrophobic contacts, all of which appear to stabilize the interactions between p24 Gag and CypA (Fig. 6B). Our molecular dynamics calculations for 1 ns show valuable insights into the changes in interaction between wild-type and mutated p24 Gag and CypA. Initially, the change in conformation of the wild-type structure as well as the fluctuation of the hydrogen bonds between CA and CypA at intervals of 50 ps up to 1 ns was analyzed. The backbone conformation essentially remains the same even though there is loss of the hydrogen bonds between His219 and Asn71CypA during this dynamics calculation. The
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hydrogen bonds between Pro\textsuperscript{222} and Arg\textsuperscript{55} \textsubscript{CypA}, Gly\textsuperscript{221}–Asn\textsuperscript{102} \textsubscript{CypA}, and Ala\textsuperscript{220}–Gln\textsuperscript{63} \textsubscript{CypA} are the most invariant. Theses data suggest that the backbone conformation of the CypA binding region of p24 Gag is maintained with the mutation at position 219. For the mutated structures, the loop conformations at the end of 1 ns of molecular dynamics calculation were compared with the wild-type crystal structure. The weighted root mean square differences of the structures were calculated after a best fit of residues from Val\textsuperscript{218} to Ala\textsuperscript{224}. The root mean square differences of CA\textsubscript{H219Q} from the wild-type CA\textsubscript{151} was only 0.99 Å. Even though there is loss of hydrogen bond interactions with H219Q substitution, the loop conformations that depend on the overall conformational contact between CA and CypA do not undergo significant change (Fig. 6, C and D). The loss of the hydrogen bond between residue 219 of CA and CypA reduces the strong interaction between CA and CypA.
and in a CypA-rich environment helps in improving viral replication. As shown in Fig. 7A, in the ribbon diagram of CAP222A superimposed on that of CAWT, CAP222A causes a significant distortion of the loop structure. The value of weighted root mean square deviation of the structure of CAP222A from CAWT structure is 3.16 Å. In comparison, as can be seen in the ribbon diagrams of the structures of the CypA binding region of various mutant CA species (Fig. 7B), generated with 1-ns dynamics calculations and superimposed on the structure of CAWT, H219Q and H219P substitutions do not significantly affect the conformation of the CypA binding region of CAWT.

Effects of H219Q and P222A Substitutions on the Conformation of the CypA Binding Loop—It is of note that P222A not only results in less CypA incorporation but the significant change in loop conformation is probably responsible for reduction in viral replication. That the loop conformation is responsible for viral fitness is further evidenced by analysis of the structures with concurrent H219Q and P222A substitutions. These two concurrent substitutions recovered the distortion of the loop conformation that was associated with P222A mutation alone (Fig. 7A). Fig. 7B illustrates that the conformations of the CypA binding region of CAWT and mutant CA species structurally resemble each other. The
FIGURE 6. Loss of hydrogen bonds in CAWT-CypA complex with H219Q or H219P substitution. A, there are two p24 Gag CAWT-CypA complexes in the asymmetric unit (9, 8) in the area of the CypA binding loop shown there are seven hydrogen bonds, which are between His219–Asn71, Ala220–Gln63, Ala220–Gly72, Gly221–Asn71, Pro222–Arg55, and Ile223–Trp121. These hydrogen bonds along with hydrophobic contacts are responsible for maintaining the optimum relative conformations of p24 and CypA. H219Q substitution results in the loss of the following three hydrogen bond interactions: Gln219–Asn71, Ala220–Gly72, and Ile223–Trp121. CypA and Ile223–Trp121 cause a significant conformational change of the contact region of the CypA binding loop (see D). 0, changes in the complex configurations with H219Q substitution. Note that in the mutated structure Gln219 (wire) is located far apart from Asn71 (stick), although in the wild-type structure His219 (stick) forms a tight hydrogen bond with Asn71 (stick). CAWT is shown by a green ribbon and the complexed CypA by a pink ribbon; CAH219Q is shown by a red tube and the complexed CypA by a purple tube. The loss of hydrogen bonds results in reduced CypA incorporation, but there persists sufficient interaction so that there are minimal alterations in the conformation of the CypA binding loop of CAWT.

As noted above, when HIV-1 NL4-3 was propagated in the absence of PI in MT-2 cells, the virus also acquired A224V mutation by passage 10 (4). It should be noted that Ala224 is also located within the CypA binding loop of p24 Gag protein and is also known to interact with CypA through hydrophobic contacts (9). Therefore, we also examined the highly intriguing findings by Braaten and co-workers (24) that the addition of A224E substitution rescued the replication of an otherwise poorly replicating HIV-1 without increasing the level of viral CypA incorporation that had been reduced by P222A substitution. As shown in Fig. 7B, the loop conformation, with the two amino acid substitutions, is restored closely to that of the wild-type conformation. The values of weighted root mean square deviation of CAH219Q/P222A have the conformation of the CypA binding loop region restored close to that of CAWT in comparison with CAH219Q/CAP222A.

A.

B.

C.

D.

FIGURE 7. Loop conformations of CAWT and CAMT. A, ribbon diagram of CAH219Q in magenta, and CAH219P in cyan. Residues 218–223 of CAWT are shown as red sticks. The values of weighted root mean square deviation of CAH219Q, CAH219P, CAH219Q/P222A, and CAH219Q/P222A/CAP222A structures from CAWT structure were 0.99, 1.77, 1.24, and 1.48 Å, respectively. H219Q and H219P substitutions do not significantly affect the conformation of the CypA binding region of CAH219Q/CAP222A. Note that the conformations of the CypA binding region of CAMT and mutant CA species structurally resemble each other. Also note that CAH219Q/P222A and CAMT structures from CAWT structure are 0.99, 1.77, 1.24, and 1.48 Å, respectively. H219Q and H219P substitutions do not significantly affect the conformation of the CypA binding region of CAWT.

DISCUSSION

It should be noted that certain polymorphic amino acid residues seen in HIV-1 strains are associated with HIV-1 drug resistance (2, 3). It is also known that certain drug resistance-conferring amino acid substitutions found in one subtype HIV-1 isolated from patients under therapy may be detected in HIV-1 of other subtypes from individuals having received no therapy (25, 26). Moreover, a recent study by Colson et al. (27) has revealed that HIV-2 strains harbor specific patterns of natural polymorphism and resistance. It is particularly of note that out of 88 different HIV-1 strains compiled in the HIV Sequence Compendium 2000 (22), 65 had histidine at position 219, whereas 13 had glutamine, and 4 had proline. Hence, as studied in this work both Gin219 and Pro219 represent polymorphic amino acid residues, and it is thought that these
two polymorphic amino acids are associated with the acquisition of resistance to certain PIs.

In the present study, we found that the two substitutions H219Q and H219P were closely associated with replication advantages when propagated in CypA-rich MT-2 and H9 cells. The same advantage was seen in PHA-PBM containing a smaller amount of CypA, but only in a limited fashion (Fig. 2 and Fig. 3, A and B). We also found that these substitutions reduced CypA incorporation into virions (Fig. 3C), which is compatible with previous reports by two groups (28, 29). It was therefore postulated that MT-2 and H9 cells contained high CypA amounts, thereby compromising HIV-1 WT replication. However, H219Q and H219P substitutions apparently reduced the viral interaction with CypA, resulting in enhanced HIV-1 replication (Figs. 2 and 3). Most interestingly, when HIV-1H219Q was exposed to CsA (0.5 μM), its replication was suppressed, unlike that of HIV-1 WT, and the higher CsA concentration (2.5 μM) further suppressed HIV-1H219Q replication in all the cell preparations examined (Fig. 4). It is noteworthy that Braaten et al. (5) reported that virion-associated CypA amounts were reduced by 50 and 75% when HIV-1 was propagated in the presence of 0.5 and 2.5 μM CsA, respectively. Although the exact role of CypA in HIV-1 replication is as yet unclear, CypA seems to play a critical role early in the HIV-1 replication cycle (5, 8, 30) by destabilizing the capsid (p24)-capsid interactions, thereby promoting disassembly of the viral core (9). An excessive depletion of CypA may tighten capsid-capsid binding, thereby interfering with virion uncoating and reducing HIV-1 replication rates, although such a sequel is speculative at present. On the other hand, higher amounts of CypA may greatly destabilize capsid-capsid interactions, thereby rendering the virion core unstable and likewise decrease HIV-1 replication (23). Thus, it was thought that the CsA-induced HIV-1H219Q replication reduction in MT-2 and H9 cells was because of an excessive depletion of CypA. It is also of note that our observation that CypA potentiated HIV-1 WT replication in MT-2 and H9 cells, although it failed to boost HIV-1H219Q replication, makes our view more plausible that H219Q substitution is directly responsible for the increased viral replication and the reduction of CypA content in daughter virions.

The presence of Pro at position 222 in p24 Gag protein has been shown to be a primary determinant of CypA binding (9), and its substitution to Ala (P222A) decreases viral CypA incorporation, causing reduced HIV-1 replication in Jurkat cells (5, 6). In this regard, HIV-1P222A was originally reported to have a severely compromised infectivity in Jurkat cells (5, 6), but it was later reported that CypA-rich H9 and CEM cells could support the replication of HIV-1P222A (23, 31). In the present study, we also found that the P222A substitution reduced CypA incorporation by HIV-1 by ~75% (Fig. 5E) and significantly reduced HIV-1 replication in Jurkat cells and PHA-PBM, which contained relatively low CypA amounts (Fig. 3, A and B). We presumed that the introduction of H219Q substitution, which decreases p24 Gag protein binding to CypA (Fig. 3C), to HIV-1P222A would further decrease viral CypA incorporation and thereby replication. It was intriguing that the H219Q substitution added to HIV-1P222A potentiated HIV-1 replication as examined in all the cell preparations used (Fig. 5, A–D). These data suggest either that the added H219Q substitution enabled HIV-1P222A to incorporate more CypA into daughter virions or altered a conformation of the CypA binding domain of p24 Gag protein, thereby rendering the virus relatively independent of CypA. In fact, the virus-associated CypA amount in HIV-1H219Q/P222A was less than or comparable with that in HIV-1P222A (Fig. 5E). It is worth noting that a substitution at position 224 of p24 Gag protein from Ala to Gln (A224E) recovers the compromised replication of HIV-1P222A in Jurkat cells but does not alter the viral CypA incorporation (24). It is also worth noting that even though all the amino acid substitutions examined here decreased viral CypA incorporation, only P222A substitution decreased viral replication. We showed that the loop conformation of the CypA binding region of CAWT, CAH219Q, and CAH219P was quite similar to each other, whereas the conformation of CAH222A sustained the most distortion (Fig. 7, A and B). It should be noted that CAH219Q/P222A and CAH222A/A224E (24) not only improve viral replication over CAH222A but also restored their replication (Fig. 5, A–D, and Fig. 7, A and B). We postulate that the conformation of the CypA binding region of CAH219Q is strongly correlated with viral fitness, and the functional role of CypA is to maintain the conformation of CAH219Q for viral replication.

It should be noted that HIV-1 infection of simian cells is restricted at an early post-entry step by the presence of simian TRIM5a (tripartite motif 5a) (32). In this respect, replacement of HIV-1 capsid protein with simian immunodeficiency virus capsid sequence significantly reduced the simian TRIM5a-mediated restrictions, demonstrating that the capsid protein of HIV-1 is a critical viral determinant for susceptibility to post-entry restriction in simian cells (33). Most interestingly, H219Q substitution of HIV-1 capsid is reported to be associated with the reduction of simian TRIM5a-mediated restriction (34, 35), suggesting that conformational change of CypA binding loop by H219Q, as we described in this work, might reduce the recognition by simian TRIM5a. It is also possible that the effects of H219Q observed in this study involve alterations of the interaction of TRIM5a and/or TRIM5 cofactors with the HIV-1 capsid. It is noteworthy that human TRIM5α, which has been shown to partly restrict HIV-1 infection (32), may be contributing to the effects on HIV-1 replication that occur when CypA is not able to bind the capsid protein of HIV-1 (36).

In summary, our study suggests that the H219Q substitution increases HIV-1 replication through (i) maintaining the loop conformation of CypA binding region and (ii) providing favorable conditions for viral replication by reducing viral CypA incorporation. The present data also show that the replication of HIV-1 with CAH219Q/P222A, and that with CAH222A/A224E, as studied elsewhere (24), were restored as compared with the otherwise compromised replication of HIV-1 with CAH222A by restoring the loop conformation without increasing CypA content. We believe that an optimal concentration of CypA, which is neither excessively high nor excessively low, is critical for viral fitness and that the functional role of CypA is to maintain the conformation of CA151.

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