Cyclophilin A Is Required for the Replication of Group M Human Immunodeficiency Virus Type 1 (HIV-1) and Simian Immunodeficiency Virus SIV$_{CPZ}$GAB but Not Group O HIV-1 or Other Primate Immunodeficiency Viruses

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The human immunodeficiency virus type 1 (HIV-1) Gag polyprotein binds to cyclophilin A and incorporates this cellular peptidyl prolyl-isomerase into virions. Disruption of cyclophilin A incorporation, either by *gag* mutations or by cyclosporine A, inhibits virion infectivity, indicating that cyclophilin A plays an essential role in the HIV-1 life cycle. Using assays for packaging of cyclophilin A into virions and for viral replication sensitivity to cyclosporine A, as well as information gleaned from the alignment of Gag residues encoded by representative viral isolates, we demonstrate that of the five lineages of primate immunodeficiency viruses, only HIV-1 requires cyclophilin A for replication. Cloned viral isolates from clades A, B, and D of HIV-1 group M, as well as a phylogenetically related isolate from chimpanzee, all require cyclophilin A for replication. In contrast, the replication of two outlier (group O) HIV-1 isolates is unaffected by concentrations of cyclosporine A which disrupt cyclophilin A incorporation into virions, indicating that these viruses are capable of replicating independently of cyclophilin A. These studies identify the first phenotypic difference between HIV-1 group M and group O and are consistent with phylogenetic studies suggesting that the two HIV-1 groups were introduced into human populations via separate zoonotic transmission events.

The Gag polyprotein of human immunodeficiency virus type 1 (HIV-1) binds to members of a family of proteins known as cyclophilins (CyPs) (29), which are expressed in all organisms from bacteria to primates (13, 45, 53). All CyPs share a conserved core of about 110 amino acids, and individual family members are distinguished from one another by unique extensions that function in subcellular localization. CyPs were originally discovered as a result of their ability to bind the immunosuppressive drug cyclosporine A (CsA) (18). The CsA-CyP complex disrupts T-cell signal transduction pathways by inhibiting the calcium-dependent phosphatase calcineurin (43). In the absence of CsA, CyPs catalyze the isomerization of peptidyl-prolyl bonds, a rate-limiting step in protein folding (11, 30). CsA blocks the isomerase activity of CyP and disrupts the folding of several proteins in vivo (27, 30, 46). Several properties of the CyPs, including a broad subcellular distribution reminiscent of that of the heat shock proteins, induction of CyP mRNA transcription by heat shock, and decreased survival of yeast with disrupted CyP genes following heat shock (47, 54), suggest that CyPs also function as chaperones. The best evidence for a specific chaperone function is provided by a *Drosophila* CyP mutant, in which transit through the endoplasmic reticulum of specific isoforms of rhodopsin is blocked (3).

Several mammalian CyPs have been cloned, one of which, CyPA, is an abundant, cytosolic protein found in all tissues examined (17–19). The HIV-1 Gag polyprotein is also expressed in the cytoplasm, and it interacts directly with CyPA in cells and incorporates CyPA into nascent virions (12, 49). CsA and nonimmunosuppressive analogs, as well as specific, engineered mutations in *gag*, disrupt Gag binding to CyPA in vitro (12, 29), disrupt CyPA incorporation into virions (12, 49), and block the replication of HIV-1 in tissue culture (4, 12, 26, 41, 49, 52). Disruption of the Gag-CyPA interaction leads to the production of virions, the infectivity of which is blocked at an early step in the retroviral life cycle (49), following receptor binding and membrane fusion but preceding reverse transcription (7). These results suggest that CyPA functions as a Gag foldase or chaperone essential for the disassembly of HIV-1 virion cores.

In contrast to HIV-1, several *Oncoviridae* and non-HIV-1 primate immunodeficiency viruses encode Gag polyproteins which do not bind to CyPA in vitro and which do not package CyPA into virions (12, 29, 49). The replication of these same viruses is not inhibited by CsA (5, 49), consistent with the conclusion that the replication of these viruses does not require CyPA. In response to these observations we undertook a systematic analysis of the known primate immunodeficiency viruses for replication dependence on CyPA. HIV-1 isolates, together with the closely related simian immunodeficiency virus (SIV$_{CPZ}$) isolate from a chimpanzee, constitute one of five viral lineages that have been identified from alignments of primate immunodeficiency virus sequences (23, 32, 44). The other four lineages comprise HIV-2 and the closely related SIV$_{SM}$ from sooty mangabeys and SIV$_{MAC}$ from captive macaques, SIV$_{AGM}$ from African Green monkeys, SIV$_{SYK}$ from the Sykes' monkey, and SIV$_{MND}$ from mandrills. Within the HIV-1 lineage, viruses can be divided into two groups, M (main) and O (outlier) (8, 31, 44). The M group constitutes the

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vast majority of HIV-1 isolates and can be further subdivided into subgroups of genetically similar viruses called clades.

Here we demonstrate that of the five phylogenetic lineages of primate immunodeficiency viruses, only members of the HIV-1/SIV$_{CPZ}$ lineage require CyPA for replication. This is true of representative HIV-1 isolates from three different clades within the M group, as well as of an SIV isolate from a chimpanzee. In contrast, two isolates from the more distantly related HIV-1 group O do not require CyPA for replication. These studies provide the first identification of a phenotypic distinction between HIV-1 group M and group O isolates.

MATERIALS AND METHODS

Plasmid DNAs, recombinant proteins, and viruses. Plasmid DNAs were propagated in *Escherichia coli* DH5α by standard methods (42). To minimize the occurrence of unwanted deletions, proviral DNAs were propagated in JM109 clone 3226, a gift from Frederick Bloom (Life Technologies, Inc., Gaithersburg, Md.) at 30°C (24). Supercoiled plasmids for use in transfection experiments were purified by using the Plasmid Maxi kit (Qiagen, Chatsworth, Calif.).

pNL4-3 is an infectious clone of HIV-1 (2). Proviral DNA clones HIV-1$_{LAI}$, HIV-1$_{ELI}$, and HIV-1$_{MAL}$ (36) were gifts from Keith Peden (Food and Drug Administration, Bethesda, Md.), as was HIV-2$_{ROD10}$. The proviral clones SIV$_{AGM}$GRI-1 and SIV$_{AGM}$155-4 (22) were gifts from Vanessa Hirsch (National Institute of Allergy and Infectious Diseases, Rockville, Md.). An infectious molecular clone of amphotropic murine leukemia virus strain 4070A (9) was provided by Sisir Chattopadhyay and Janet Hartley (National Institutes of Health [NIH], Bethesda, Md.). A cell culture supernatant containing viral isolate SIV$_{CPZ}$GAB (21) and HIV-1 group O isolate HIV-1$_{CA9}$ (34) was provided by David Ho (Aaron Diamond AIDS Research Center, New York, N.Y.). A cell culture supernatant containing HIV-1 group O isolate HIV-1$_{MVP-5180}$ (16) was obtained from the NIH AIDS Research and Reference Program.

Partial CA coding sequences were amplified by PCR from genomic DNA of peripheral blood mononuclear cells (PBMC) infected by HIV-1$_{CA9}$. Design of the primer pair was based on published sequences for other group O isolates (16, 51): 5′-GCATGGGTAAAGGCAGTAGAAG-3′ and 5′-GTCCCTGCCTAAT ATCTAAGATGC-3′. PCR products were blunt-end cloned into pBS-KS− (Stratagene), and both strands were sequenced by standard dideoxy methods (42).

Cell culture, transfections, and infections. Human fibroblast 293T cells were maintained in Dulbecco's modified Eagle's medium-F12 (1:1) supplemented with 10% fetal bovine serum. Human lymphocyte lines, Jurkat (55) and CEM-SS (33), were obtained from the NIH AIDS Research and Reference Program and maintained in RPMI 1640 supplemented with 10% fetal calf serum. Human PBMC were prepared by standard methods (36) and kindly provided by Paul Simonelli. PBMC were maintained in RPMI with 10% fetal calf serum and 5 μg of phytohemagglutinin (Pharmacia) per ml. After 48 h, delectinized human interleukin-2 (Advanced Biotechnologies, Inc.) was added to 10%.

Viral proteins were expressed transiently by calcium phosphate transfection of 10 μg of supercoiled proviral DNA into 293T cells by using the Mammalian Cell Transfection Kit (Specialty Media, Lavellette, N.J.). Viral infections were initiated in 10^7 Jurkat or CEM-SS cells with 2 μg of proviral DNA and 250 μg of DEAE-dextran (Pharmacia Biotech, Inc., Piscataway, N.J.) per ml in 1 ml of serum-free RPMI for 20 min at room temperature. Cells were washed in serum-free medium, resuspended in 3 ml of conditioned medium with 6 ml of fresh medium, and split into 3-ml cultures with the addition of drugs at the concentrations indicated below. Every 2 days, the supernatant was harvested and frozen, and the cells were passaged. At the conclusion of the experiment, the frozen samples were thawed and analyzed for reverse transcriptase activity as described below.

For experiments in which infection was initiated with exogenous virus, viral stocks were prepared by transfection of proviral DNAs into 293T cells (10^4 infectious units per ml) or, in experiments utilizing uncloned virus, by collection of the supernatant from previously infected Jurkat T cells or PBMC. All stocks utilized in a given experiment were normalized for virion content with reverse transcriptase. For infection of PBMC, 2×10^7 cells were prepared and maintained in culture for 48 h as described above. Cells were pelleted and resuspended in 1 ml of RPMI containing interleukin-2, phytohemagglutinin, and 10^3 infectious units of virus. After 1 h, the culture was expanded to 9 ml and divided into thirds for the addition of the indicated drugs. Infection of Jurkat cells with viral stocks was initiated similarly, except that fewer cells were utilized and interleukin-2 and phytohemagglutinin were left out of the medium.

CsA was obtained from Sandoz Pharmaceuticals Corporation (East Hanover, N.J.). Prior to the addition to tissue culture medium, the drug powder was dissolved in ethanol to make stock solutions of less than 2 mM. All cultures within a given experiment received the same volume of ethanol.

Reverse transcriptase assay. The cell culture supernatant (10 μl) (viral replication assays) or 10 μl of precleared, filtered supernatant (transient transfections of proviral DNA) was added to 50 μl of reverse transcriptase cocktail {60 mM
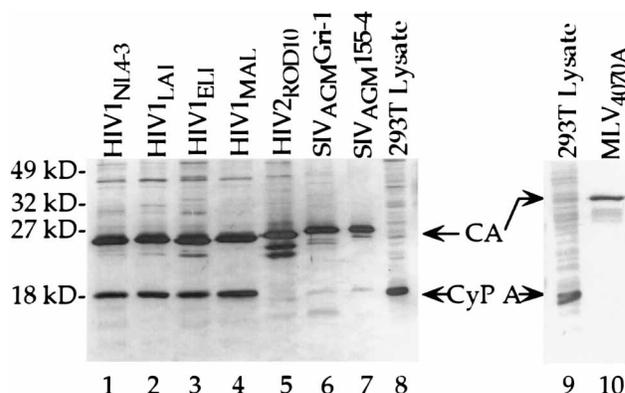

FIG. 1. CyPA incorporation into virions released from 293T cells transfected with proviral DNAs. Lanes: 1 to 7 and 10, Western blot of proteins associated with virions purified from the supernatant of cells transfected with the indicated proviral DNAs; 8 and 9, soluble 293T-cell lysate. The details of virion purification and immunoblotting are described in Materials and Methods. Samples in all lanes were probed with a primary antibody against CyPA; lanes 1 to 8 were probed simultaneously with a primary antibody that recognizes CA of different primate lentiviruses (12), and lanes 9 and 10 were probed simultaneously with an anti-MLV CA antibody. The positions of molecular size markers (in kilodaltons [kD]), CyPA, and the CA proteins of the different retroviruses are indicated.

Tris-HCl (pH 8.0), 180 mM KCl, 6 mM MgCl$_2$, 6 mM dithiothreitol, 0.6 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid] 0.12% Triton-X 100, 6 μg of oligo(dT) per ml, 12 μg of poly(rA) per ml, 0.05 mM [α-^{32}P]dTTP (800 Ci/mmol)} for 1 h at 37°C. For Moloney murine leukemia virus, (MLV) MnCl$_2$ was substituted for MgCl$_2$. A 2-μl aliquot was spotted onto DE-81 paper and washed three times with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). A PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.) was used to quantitate the radioactivity incorporated, as a relative indication of reverse transcriptase activity.

Virion purification. The supernatant collected from 293T cells at 48 h post-transfection with proviral DNAs, or from infected Jurkat cells at the peak of reverse transcriptase activity, was centrifuged at 1,000 rpm for 5 min and passed through a 0.45-mm-pore-size filter to remove the cellular debris. The filtrate was gently layered on top of a step gradient consisting of 2 ml of 25% sucrose over 2 ml of 45% sucrose in TNE (10 mM Tris-HCl [pH 7.5], 100 mM NaCl, 1 mM EDTA) and subjected to 80,000 × g for 2 h in a Beckman SW41 rotor. The virion-containing interface was harvested and brought up to 6 ml with TNE to dilute the sucrose, and the virions were pelleted at 80,000 × g for 2 h in an SW41 rotor. The pellet was resuspended in 50 μl of 2× sodium dodecyl sulfate (SDS) sample buffer for Western blot (immunoblot) analysis as described below.

Antibodies and Western blots. The murine monoclonal anti-HIV-1 p24 antibody (NEA-9306) was obtained from Dupont NEN. Murine monoclonal antibody (no. 740) against HIV-2 CA was obtained from the NIH AIDS Research and Reference Program. Rabbit anti-CyPA antibody was a gift from Louis Henderson (National Cancer Institute, Frederick, Md.). Goat anti-MLV CA antibody (79S 804) was obtained from the National Cancer Institute.

Virion-associated proteins normalized for reverse transcriptase activity were size separated by SDS-polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose membranes with a mini-blotting apparatus (Bio-Rad Laboratories, Hercules, Calif.). Antibody binding was detected following incubation with secondary biotinylated anti-immunoglobulin antibody with an alkaline phosphatase reaction with a Vectastain Elite kit (Vector Laboratories, Burlingame, Calif.).

Nucleotide sequence accession number. The HIV-1$_{CA9}$ Gag capsid protein partial coding sequence presented in this report was submitted to GenBank and assigned accession number U53175.

RESULTS

Non-HIV-1 retroviruses do not package CyPA into virions. The HIV-1 Gag-CyPA interaction was initially identified by using the two-hybrid system in yeast cells (29). Gag polyproteins encoded by distantly related retroviruses, including two members of the subfamily *Oncovirinae*, Mason-Pfizer monkey virus and MLV (29), and the lentivirus feline immunodeficiency virus FIV$_{34TF10}$ (48), do not interact with CyPA in the same assay. Subsequently, it was demonstrated that CyPA is

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                    205                                      241
HIV-1_LAI/HXB2    INEEAAEWDRVHPVHAGPIAPGQMREPRGSDIAGTTS
HIV-1_NL4-3       ----------l--------------------------
HIV-1_ELI         ----------l--------------------------
HIV-1_MAL         ------d---v-------p------------------
SIV_CPZ           ----------l--t---------l-------------
HIV-1_MVP-5180    ----------t--pam--lp---i---t---------
HIV-1_CA9         ------d---t--pav--lp---i---t---------
HIV-2_ROD         ---------vq--.ip--lpa--l-------------
SIV_MAC239        ------d--lq--.qpa-.qq--l---s---------
SIV_SM PBJ        ------d--lq--.qp---p---l-------------
SIV_AGM SAB384    ------d--lr--ppqq-paq-vl-d-q---------
SIV_AGM 155-4     ------q--vt--pp---lpa--l-d-g---------
SIV_AGM GRI-1     ---------thr-.p---lpa--l-d-t---------
SIV_MND           ---q-----lt--qqqpaqpg-gl-t-s--------
SIV_SYKES         ----g-a--le--qpqq-apqagl-d-sa-------
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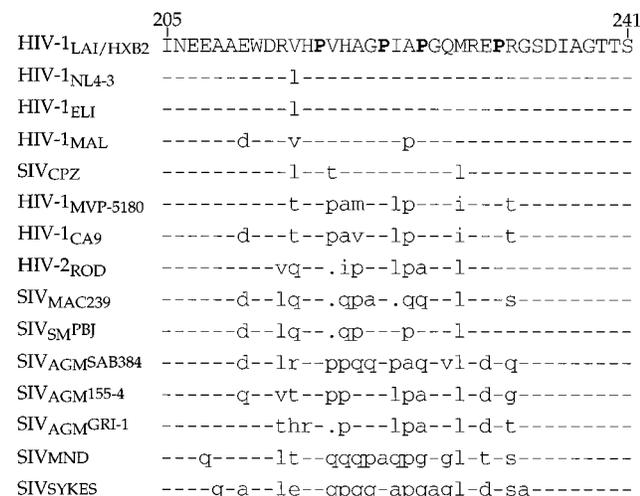
FIG. 2. Amino acid sequence alignment of Gag polyproteins encoded by representative isolates of HIV-1 and other primate lentiviruses. The proline-rich region required for CyPA binding and virion incorporation (12) encompasses amino acid residues 205 to 241 (numbering is with respect to the amino terminus of the majority of clade B HIV-1 Gag polyproteins). Proline residues in the top sequence are shown in bold type. Conserved residues are indicated by dashes, and sequence gaps are indicated by dots.

specifically incorporated into HIV-1 virions (12, 49). Here we demonstrate that CyPA is not detected in purified virions produced by the amphotropic virus MLV_{4070A} (Fig. 1, lane 10), a result consistent with the findings in the two-hybrid system and with experiments examining CyPA incorporation into HIV-1/MLV chimeric viruses (49).

We next determined which of the primate immunodeficiency viruses package CyPA into virions. Previously, we demonstrated that members of the HIV-2/SIV_{SM} lineage, including SIV_{MAC239}, SIV_{BK28}, SIV_{SMMPBJ}, and HIV-2$_{ROD2}$, encode Gag proteins which do not bind to CyPA in vitro and which do not package CyPA into virions (12). Here we extend this finding to another clone from this lineage, HIV-2$_{ROD10}$ (a gift from Keith Peden), showing that it also does not package CyPA into virions (Fig. 1, lane 5).

Analysis of HIV-1$_{HXB2}$ gag mutants identified a proline-rich region in the CA domain of the Gag polyprotein which is necessary for binding to CyPA (12, 29). Alignment of the proline-rich region of HIV-1$_{HXB2}$ with those of other primate immunodeficiency viruses demonstrated that representative isolates of the SIV_{SYK} (20) and SIV_{MND} (50) lineages encode proteins which are significantly more divergent from HIV-1$_{HXB2}$ in this region than are viruses of the HIV-2/SIV_{SM} lineage (Fig. 2). We conclude, therefore, that viruses from the SIV_{SYK} and SIV_{MND} lineages are unlikely to produce virions which package CyPA.

In contrast to those of SIV_{SYK} and SIV_{MND}, the amino acid sequences of several SIV_{AGM} isolates are relatively closely related to HIV-1 in the proline-rich region. On examination, however, cloned viruses isolated from the grivet monkey, SIV_{AGM}GRI-1, and from the vervet monkey, SIV_{AGM}155-4 (22), did not incorporate CyPA into virions (Fig. 1, lanes 6 and 7). Of the known primate immunodeficiency virus lineages, then, only members of the HIV-1/SIV_{CPZ} lineage encode a Gag protein which packages CyPA into virions.

A range of genetically disparate isolates from the HIV-1/SIV_{CPZ} lineage incorporate CyPA into virions. Next we sought to determine if virions produced by all members of the HIV-1/SIV_{CPZ} lineage package CyPA. The vast majority of HIV-1

isolates lie within group M, which has been divided into phylogenetically defined subtypes called clades. The cloned proviruses HIV-1$_{HXB2}$ and HIV-1$_{NL4-3}$ encode Gag polyproteins capable of binding to CyPA and incorporating this cellular protein into virions (12, 29). The closely related clone HIV-1$_{LAI}$ also incorporates CyPA into virions (Fig. 1, lane 2). All three of these viruses are members of clade B. To determine if CyPA incorporation is a property unique to clade B we obtained HIV-1 clones from other clades. HIV-1$_{ELI}$ (clade D) and HIV-1$_{MAL}$ (clade A by gag sequence) were both found to incorporate CyPA into virions as efficiently as the clade B viruses (Fig. 1, lanes 3 and 4).

SIV_{CPZ}GAB, a virus isolated from a chimpanzee (21), is a member of the HIV-1 lineage, though it does not fit within phylogenetic group M or O. To determine if this virus packages CyPA, virions were purified from the cell culture supernatant following infection of the Jurkat T-cell line. Virions produced by SIV_{CPZ}GAB were found to incorporate CyPA at levels comparable to that of HIV-1$_{NL4-3}$ produced by the same cell line (Fig. 3, lanes 1 and 2).

The HIV-1 group O viruses are phylogenetically more distantly related to group M viruses than is SIV_{CPZ}GAB. We then tested two group O HIV-1 isolates, HIV-1$_{CA9}$ (34) and HIV-1$_{MVP-5180}$ (16), for the ability to package CyPA. We were unable to detect replication of HIV-1$_{CA9}$ in Jurkat, CEM-SS, or Molt-4-clone 8 T-cell lines, but this virus replicates well in PBMC. Although we detected CyPA in HIV-1$_{CA9}$ virions produced in PBMC (data not shown), we were not confident that our virion preparations from PBMC were free from contamination with cellular proteins. Since HIV-1$_{CA9}$ has not been cloned, and we were unable to propagate this virus in any T-cell lines that we used, we were unable to determine unequivocally if HIV-1$_{CA9}$ packages CyPA into virions.

In contrast to HIV-1$_{CA9}$, HIV-1$_{MVP-5180}$ replicates well in the Jurkat T-cell line, and we were able to obtain purified virion preparations from cells infected with this virus. Like other HIV-1 isolates, HIV-1$_{MVP-5180}$ packages significant quantities of CyPA, and, as previously demonstrated with two clade B isolates (7, 49), this incorporation is inhibited by CsA
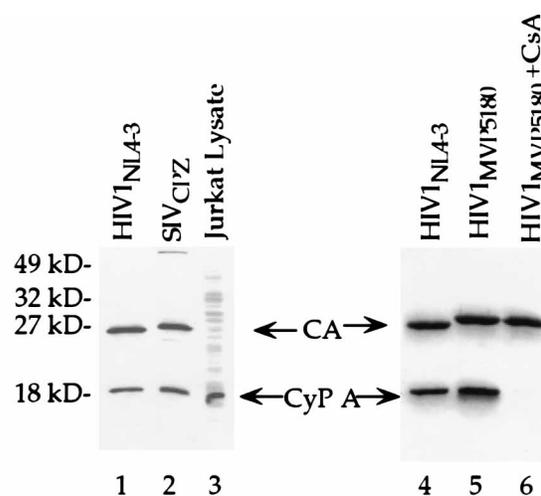

FIG. 3. CyPA incorporation into virions released from the Jurkat T-lymphocyte cell line. Western blots of virion-associated proteins following infection with stocks of the indicated viruses are shown (lanes 1, 2, 4, 5, and 6). In lane 6, virions were produced in the presence of 2.5 μM CsA. A soluble Jurkat cell lysate is shown (lane 3). All lanes were probed with primary antibodies as described in the legend to Fig. 1. The positions of molecular size markers (in kilodaltons [kD]), CyPA, and the CA proteins of the different retroviruses are indicated.
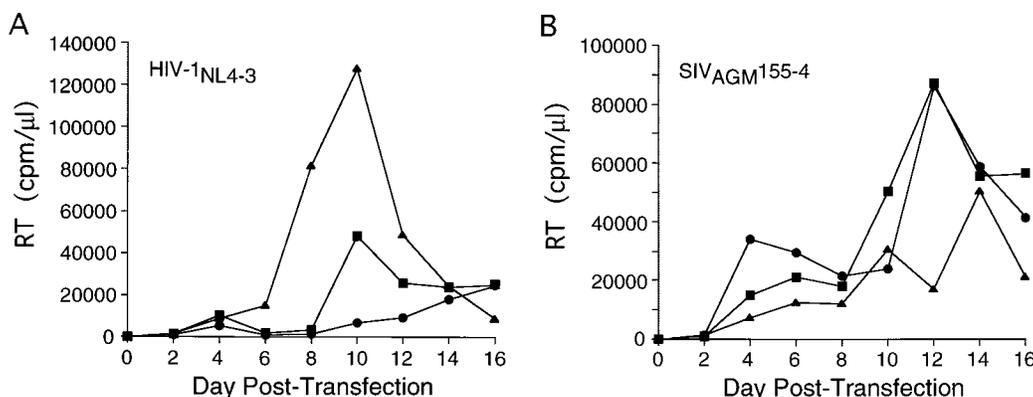
FIG. 4. CsA does not inhibit the replication of an SIV$_{AGM}$ isolate. The CEM-SS T-cell line was infected by transfection of proviral DNAs pNL4-3 (A) and SIV$_{AGM}$155-4 (B). CsA was maintained in the cell culture medium at concentrations of 0.5 μM (■) and 2.5 μM (●) or 0 (no drug) (▲). The accumulation of extracellular virions in the culture supernatant resulting from virus spread through the culture is indicated by the reverse transcriptase (RT) activity in the supernatant (ordinate) at the indicated times posttransfection (abscissa).

(Fig. 3, lanes 5 and 6). These results indicate that CyPA incorporation is a property shared by a wide range of genetically diverse HIV-1 isolates.

Replication of non-HIV-1 primate immunodeficiency viruses is not inhibited by CsA. Previously, it was demonstrated that the replication of SIV$_{MAC239}$ and HIV-2 is resistant to inhibition by CsA (5, 49): effects on HIV-2 replication by CsA are not observed until doses 10-fold higher than the required for inhibition of HIV-1 are attained, and such doses almost certainly have toxic effects on the host cells. These data indicate that CyPA is not required for the replication of members of the HIV-2/SIV$_{SM}$ lineage. To extend the analysis we tested the replication sensitivity to CsA of a member of the SIV$_{AGM}$ lineage. The CEM-SS T-cell line was used for these replication studies since it is one of the few cell lines in which SIV$_{AGM155}$ replicates (22). HIV-1$_{NL4-3}$ also grows well in CEM-SS cells with kinetics and CsA sensitivity similar to those observed in Jurkat cells (Fig. 4A) (see below). SIV$_{AGM155}$ also replicated quite well, but CsA did not inhibit its replication (Fig. 4B); on the contrary, we observed repeatedly that CsA has a stimulatory effect on the replication of this virus. Thus, primate immunodeficiency viruses of the HIV-2/SIV$_{SM}$ and SIV$_{AGM}$ lineages are not sensitive to inhibition by CsA.

Replication of HIV-1 group M viruses and SIV$_{CPZ}$GAB is inhibited by CsA. To test the effect of CsA on the replication of different HIV-1 isolates, the Jurkat T-cell line was used. Drug doses of 0.5 and 2.5 μM were chosen to maximize the inhibition of viral replication while preserving the capacity of the host cell to replicate; at doses greater than 5 μM, the replication of Jurkat cells slowed, which is indicative of drug toxicity. When no drug was added, virus accumulated, with a peak at 10 days posttransfection of HIV-1$_{NL4-3}$ proviral DNA (Fig. 5A). However, when CsA was added to the culture medium, the kinetics of viral replication was delayed and the height of the reverse transcriptase peak was attenuated; the magnitude of the inhibition was dependent on the concentration of the drug. At a CsA dose of 2.5 μM, extracellular virions never accumulated above 10% of the virus peak without the drug. Similar results were obtained whether infection was initiated by transfection of proviral DNA or by incubation of T cells in virion-containing supernatants (Fig. 5A and 6A).

To demonstrate that the effects of CsA on HIV-1 replication were not secondary to toxic effects of the drug on the host cell, the effect of CsA on the replication of cloned amphotropic MLV in Jurkats was tested in parallel. Since the Gag polypro-

tein of MLV does not bind to CyPA (29) and does not package CyPA into virions (Fig. 1, lane 10), we hypothesized that this virus would not be inhibited by CsA. Even in the presence of 2.5 μM CsA, no noticeable effect on MLV replication was observed (Fig. 5B).

HIV-1$_{ELI}$ was capable of replicating in Jurkat cells (Fig. 5C), but with a much delayed kinetics compared with that of HIV-1$_{NL4-3}$ (Fig. 5A). Despite this lag, however, when CsA was maintained in the medium at 0.5 or 2.5 μM, there was a dose-dependent inhibition of HIV-1$_{ELI}$ replication, as evidenced by the delayed appearance and decreased magnitude of the reverse transcriptase activity accumulating in the media. Thus, we were able to demonstrate inhibition of viral replication by CsA using HIV-1 isolates exhibiting either fast or slow replication kinetics.

Viral stocks of SIV$_{CPZ}$GAB produced in PBMC were capable of initiating and propagating infection in Jurkat T cells. The appearance of the peak of reverse transcriptase activity was much delayed compared with that of HIV-1$_{NL4-3}$, but SIV$_{CPZ}$GAB replication was also inhibited in a dose-dependent manner by CsA (Fig. 6B).

As previously reported, HIV-1$_{MAL}$ was unable to replicate in Jurkat T cells (36) whether infection was initiated by DNA transfection or with cell-free virus. Therefore, the effect of CsA on the replication of this virus in PBMC was studied. Infection was initiated with a viral stock produced by transfection of proviral DNA into 293T cells. First, it was demonstrated that HIV-1$_{NL4-3}$ infected PBMC with kinetics very similar to that in Jurkat T cells (Fig. 7A); HIV-1$_{NL4-3}$ replication in PBMC was also inhibited by CsA, although the drug was significantly more effective than in Jurkat T cells (Fig. 7A). HIV-1$_{MAL}$ was able to replicate in PBMC, and its replication was also sensitive to CsA (Fig. 7B). We conclude that four genetically disparate members of the HIV-1/SIV lineage all package CyPA into virions and exhibit CsA sensitivity, indicating that these viruses are dependent on CyPA for their replication.

Replication of HIV-1 group O isolates is not inhibited by CsA. Lastly, we tested the CsA sensitivity of the most genetically disparate members in our collection of viruses from the HIV-1/SIV lineage, the group O isolates. HIV-1$_{MVP-5180}$ replicated in Jurkat T cells with a kinetics similar to that of HIV-1$_{NL4-3}$, but unlike the other HIV-1 isolates that we tested, the replication of this virus was not inhibited by CsA (Fig. 6C).

As stated above, we were unable to detect replication by the group O isolate HIV-1$_{CA9}$ in Jurkat, CEM-SS, or Molt-4-clone
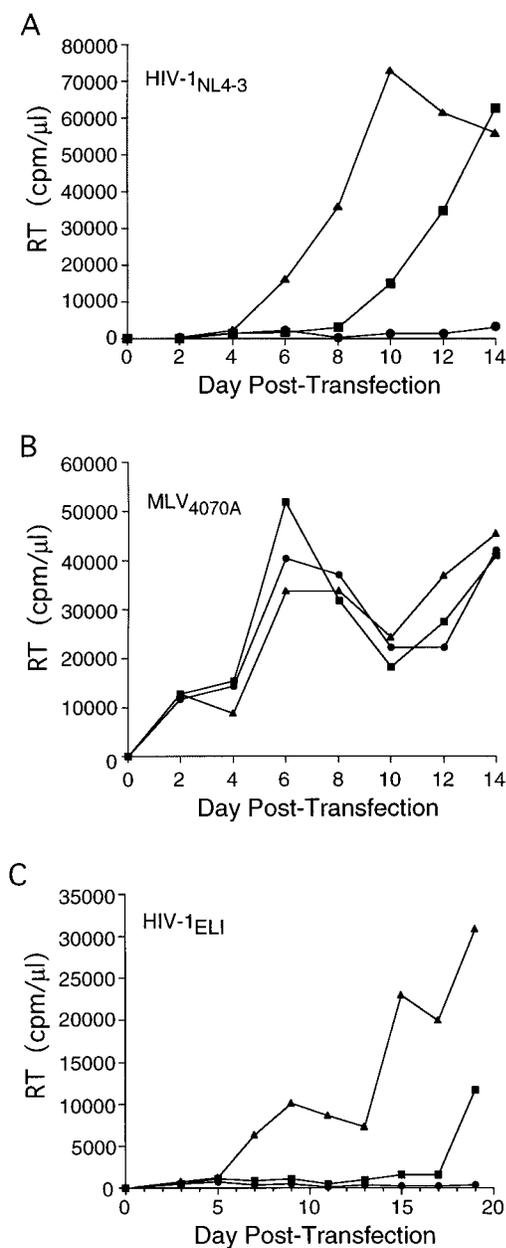
FIG. 5. Effect of CsA on retroviral replication in the Jurkat T-cell line. Infection was initiated by transfection of infectious proviral DNAs HIV-1$_{NL4-3}$ (A), MLV$_{4070A}$ (B), or HIV-1$_{ELI}$ (C). CsA was maintained in the cultures at concentrations of 0.5 μM (■) and 2.5 μM (●) or 0 (no drug) (▲). Data are reported as described in the legend to Fig. 4.
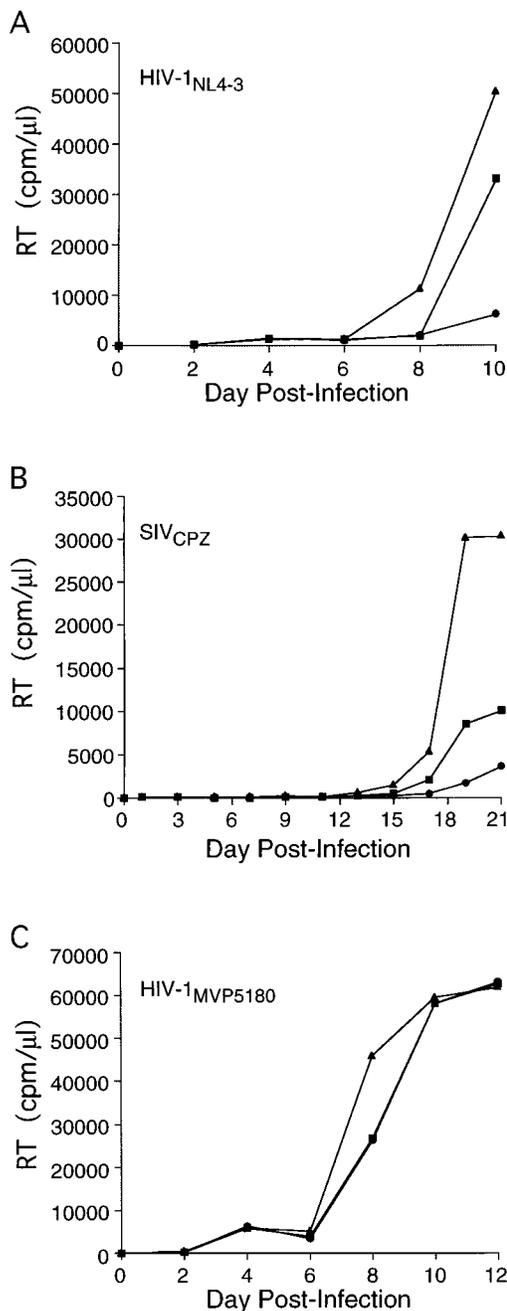

FIG. 6. Effect of CsA on HIV-1 replication in Jurkat T-cells. Infection was initiated by infection with the following viral stocks normalized for virion-associated reverse transcriptase activity: HIV-1$_{NL4-3}$ (A), SIV$_{CPZ}$GAB (B), and HIV-1$_{MVP-5180}$ (C). CsA was maintained in the cultures at concentrations of 0.5 μM (■) and 2.5 μM (●) or 0 (no drug) (▲). Data are reported as described in the legend to Fig. 4.

8 T-cell lines, but this virus replicated in PBMC; the peak of reverse transcriptase was observed later than that with HIV-1$_{NL4-3}$ but earlier than that with HIV-1$_{MAL}$ (Fig. 7C). As with HIV-1$_{MVP-5180}$, CsA had no effect on the replication of this virus, even with drug doses which completely inhibited the replication of HIV-1$_{NL4-3}$ (Fig. 7C). Since the replication of neither HIV-1$_{MVP-5180}$ nor HIV-1$_{CA9}$ is inhibited by CsA, at doses which disrupt CyPA incorporation into virions (Fig. 3, lane 6), we conclude that the replication of HIV-1 group O viruses is not dependent on CyPA.

DISCUSSION

By directly testing a panel of representative viral clones and isolates (Table 1), in combination with an examination of viral sequences in the database (Fig. 2), we have demonstrated that, of the five primate immunodeficiency virus lineages, the HIV-1/SIV$_{CPZ}$ lineage is the only one likely to contain viruses which require CyPA for replication. Although we have not tested representatives from all HIV-1 group M clades, we
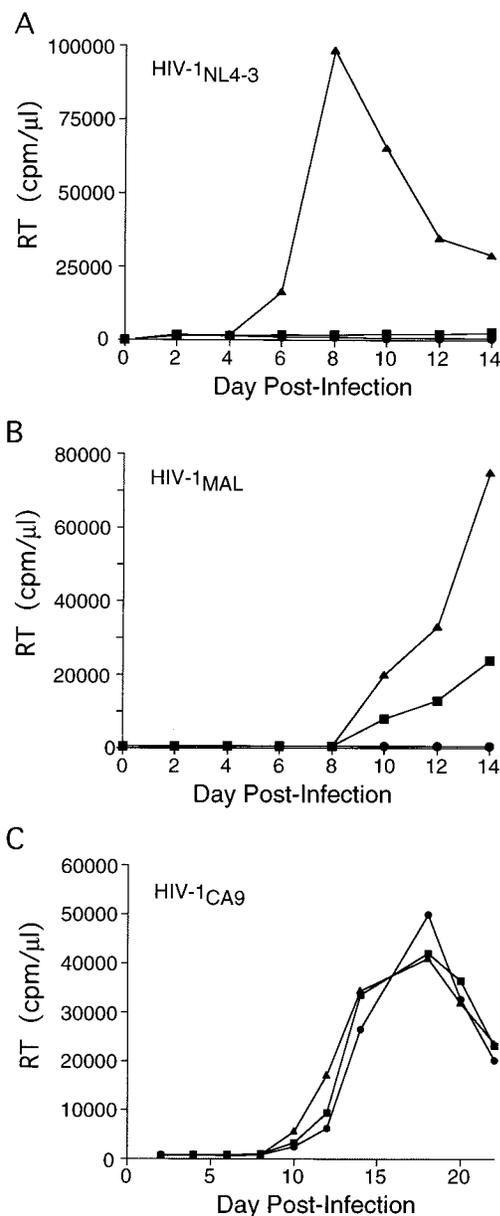
FIG. 7. Effect of CsA on the replication of HIV-1 in PBMC. Infection was initiated by infection with the following viral stocks normalized for virion-associated reverse transcriptase activity: HIV$_{NL4-3}$ (A), HIV$_{MAL}$ (B), and HIV$_{CA9}$ (C). CsA was maintained in the cultures at concentrations of 0.5 μM (■) and 2.5 μM (●) or 0 (no drug) (▲). Data are reported as described in the legend to Fig. 4.

TABLE 1. Characterization of retroviral clones and isolates for interactions with CyPA and replication sensitivity to CsA

Proviral clone	Gag polyprotein binding to CyPA[a]	CyPA incorporation into virions[b]	Replication inhibited by CsA[c]
HIV-1$_{HXB2/LAI}$	+	+	+
HIV-1$_{NL43}$	+	+	+
HIV-1$_{ELI}$	ND[d]	+	+
HIV-1$_{MAL}$	ND	+	+
SIV$_{CPZ}$	ND	+	+
HIV-1$_{MVP-5180}$	ND	+	−
HIV-1$_{CA9}$	ND	ND	−
HIV-2$_{ROD}$	−	−	−
SIV$_{MAC239}$	−	−	−
SIV$_{BK28}$	−	−	ND
SIV$_{SMMPBj}$	−	−	ND
SIV$_{AGM}$SAB384	−	ND	ND
SIV$_{AGM}$155-4	ND	−	−
SIV$_{AGM}$GRI-1	ND	−	ND
FIV$_{34TF10}$	−	ND	ND
MPMV$_{6A}$	−	ND	ND
MLV$_{4070A}$	−	−	−

[a] Data were generated previously (except for SIV$_{SMMPBj}$ and FIV$_{34TF10}$) by using the two-hybrid system (29) and/or affinity chromatography with recombinant proteins (12).
[b] The results were demonstrated in this study or previously (12) by Western blot of virion-associated proteins.
[c] The results for HIV-1$_{HXB2}$ (49), SIV$_{MAC239}$ (49), and HIV-2$_{ROD}$ (5) were reported previously. The results for the other viruses were demonstrated in this study.
[d] ND, not done.

cates that this adaptation must provide a significant replication advantage for the virus in vivo.

To understand what advantage the dependence on CyPA provides for HIV-1 will most likely require elucidation of the function of CyPA in HIV-1 replication. Over the course of the retrovirus life cycle, *gag*-encoded proteins have several functions, including directing the assembly of nascent virions, establishing and maintaining the structure of the mature virion, and uncoating the virion core in such a way that reverse transcription is successfully accomplished. Recent studies indicate that disruption of CyPA incorporation into the virion correlates with a reduction in the initiation of reverse transcription after infection of a new host cell (7). Since CyPs are thought to function as protein foldases or as protein chaperones (15, 45), one can imagine that CyPA might provide a kinetic advantage in viral replication by functioning as a chaperone of virion uncoating.

In contrast to the group M HIV-1 isolates and SIV$_{CPZ}$GAB, the HIV-1 group O viruses do not require CyPA for replication. On the basis of similarities among the *gag* coding sequences of the group O isolates (16, 28, 51), we believe that the replication of all group O viruses will occur independently of CyPA. Differences between group M and group O viruses, either with respect to pathogenicity or replication requirements, have not been reported previously. Our studies of CyPA are the first to identify a phenotypic difference between these two groups of viruses.

The HIV-1 group O virus phenotype indicates that CyPA binding can be separated from an unidentified function which is CyPA dependent. Although this function could be encoded by another viral element, the identification of *gag* point mutations which confer CsA resistance to an HIV-1 group M virus (1, 6) suggests not only that *gag* encodes the CyPA-packaging factor but also that *gag* encodes the CyPA-dependent function. Therefore, it seems likely that the ability of the group O viruses

believe that all group M viruses require CyPA for replication. This deduction is based on alignment of the sequences of the HIV-1 group M viruses contained within the database with the part of HIV-1$_{HXB2}$ *gag* sequence shown to be required for CyPA binding (Fig. 2) and on the fact that disruption of the Gag-CyPA interaction inhibits the replication of SIV$_{CPZ}$GAB, an isolate phylogenetically distinct from the group M viruses. The extraordinary genetic diversity of HIV-1, whether a result of HIV-1 reverse transcriptase infidelity (39, 40) or the enormous rate of virus replication in vivo (10), allows the virus to readily adapt to selective pressures by generating endless genetic variants and escape mutants. That a range of genetically diverse HIV-1 isolates all require CyPA for replication indi-

to replicate independently of CyPA is due to differences in the *gag* coding sequence which distinguish them from the other HIV-1 isolates.

The high prevalence of immunodeficiency virus infection in diverse African primate populations (23) suggests that HIV-1 was originally transmitted to humans from other primate species. For example, 20 to 40% of wild African green monkeys (25, 35) and more than half of a colony of sooty mangabey monkeys (14) were found to be infected with immunodeficiency viruses. The identification of members of the HIV-1 lineage in a wild-born chimpanzee, SIV$_{CPZ}$, is especially intriguing in this context (21, 37, 38) and suggests that HIV-1 may have been transmitted to humans from chimpanzees.

Further study of the interaction between HIV-1 and CyPA may help to shed light on the origin of HIV-1 in human populations. The fact that group M viruses and SIV$_{CPZ}$GAB require CyPA for replication suggests that these viruses arose from a common ancestral virus which had adapted to require the host CyPA prior to introduction into human populations. Group O viruses, in contrast, do not require CyPA for replication and most likely evolved from a different ancestral virus which lacked this adaptation. Although group O viruses do not require CyPA for replication, the fact that they package CyPA into virions suggests that they evolved from a virus which was at one time CyPA dependent. We conclude that our data support the hypothesis that the group M and O viruses were transmitted to humans on two separate occasions from nonhuman primates, as has previously been suggested (31).

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