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Studies conducted in cell lines indicate that cyclophilin A (CypA) is a component of HIV type 1 (HIV-1) virions, and that when CypA incorporation into virions is inhibited by treatment of infected cells with the immunosuppressive agent cyclophilin A (CsA), HIV-1 infection also is inhibited. Because HIV-1 particles assemble along a different pathway and incorporate different host proteins in macrophages than in other cell types, we investigated CypA and CsA activities in HIV-1-infected primary human macrophages, compared with primary human lymphocytes. We tested virus protein production, virion composition and infectivity, and progress through the virus life cycle under perturbation by drug treatment or mutagenesis in infected cells from multiple donors. Our findings from both primary cell types are different from that previously reported in transformed cells and show that the amount of CypA incorporated into virions is variable and that CsA inhibits HIV-1 infection at both early and late phases of virus replication, the stage affected is determined by the sequence of HIV-1 Gag. Because the cell type infected determines the identity of host proteins active in HIV-1 replication and can influence the activity of some viral inhibitors, infection of transformed cells may not recapitulate infection of the native targets of HIV-1. The Journal of Immunology, 2006, 177: 443–449.

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3 Abbreviations used in this paper: HIV-1, HIV type 1; CypA, cyclophilin A; CsA, cyclosporin A; MDM, monocyte-derived macrophage.

Materials and Methods

Cells

Human PBLs and monocytes were obtained by centrifugal elutriation from blood collected from >20 healthy individuals under an exemption from Institutional Review Board review. CEM and 293T cells were obtained from the AIDS Research Reagent Repository (Rockville, MD). PBLs were stimulated with 5 μg/ml PHA (Sigma-Aldrich) in RPMI 1640 medium containing 10% FBS (Omega Scientific) and 1 ng/ml IL-2 (R&D Systems) for 48 h and then cultured without PHA. Monocytes were induced to differentiate to macrophages by culture in 10% human serum (Cambrex) and 10% giant cell tumor-conditioned medium (BioVeris) in DMEM for 5 days and thereafter were cultured in DMEM containing 10% FBS.
Viruses

NL-3 was propagated in CEM cells and isolated during acute infection. Subtype A/E CM235 was obtained from Dr. N. Michael (Walter Reed Army Institute of Research, Rockville, MD) through the AIDS Research Reagent Repository and was propagated in PBLs and isolated during acute infection of PBLs from donor 224E, a plasmid-resistant mutant of NL-4.3 was a gift from Dr. J. Luban (Columbia University Medical Center, New York, NY). NLHXADA-GP was a gift from Dr. L. Ratner (Washington University, St. Louis, MO). The plasmids were propagated in DH5α cells and transfected into 293T cells using LipofectAMINE (Invitrogen Life Technologies) according to the manufacturer’s instructions, and infectious virions were isolated from supernatant 48 and 72 h after transfection. ADA was a gift from Dr. H.E. Gendelman (University of Nebraska Medical Center, Omaha, NE) and was propagated exclusively in primary macrophages and isolated from their supernatants. A mutant of NLHXADA-GP carrying the A224E mutation was prepared by PCR, carrying out the single point mutation of C to A so as to change the amino acid from alanine to glutamic acid at position 224 of the gag protein, and the mutation was confirmed by sequencing. The mutation was conducted by amplifying the 4.3-kb fragment of the plasmid between SpH I and EcoRI with the mutation in the forward primer MUT5 (5'-TCTAGTCATGACGGGCTATT GAACAGCCGATAGTACG-3'). The reverse primer NL5727 (5'-TTGT TGCAAGAATCTTAGCTCCAC-3') contained the EcoRI site. The restriction sites are underlined, and the point mutation is mentioned in bold letters in the primers. The PCR was conducted using AccuPrime Pfx DNA polymerase (Invitrogen Life Technologies) by denaturing the template at 95°C for 2 min initially and 30 s in the subsequent cycles, annealing at 55°C for 30 s and polymerization at 68°C for 4.5 min for 30 cycles, followed by a 7-min extension at 68°C. The PCR product was digested with SpH I and EcoRI and ligated with similarly digested and purified NLHXADA-GP. The new plasmid carrying the point mutation was called GP-M. The mutation was confirmed by sequencing. The p24 content of all virus stocks was determined by ELISA using the HIV-1 p24 Ag assay (Beckman Coulter), according to the manufacturer’s instructions. For analysis of infection by HIV-1 DNA levels by PCR, the virus inoculum was treated with DNase I as described before infection (3).

Infection

PBLs or monocyte-derived macrophage (MDMs) were infected with various HIV-1 at 0.1 pg p24 per cell for 1–2 h at 37°C, washed, and then cultured in the absence or presence of 2.5 μM CsA (Sigma-Aldrich or Calbiochem). Cells and cell supernatants were harvested at the times indicated for evaluation of p24 and CypA content, HIV-1 DNA or RNA, or infectious HIV-1.

RT and PCR

Cells were collected at the indicated time points, and genomic DNA was prepared using DNazol (Invitrogen Life Technologies). DNA content was standardized by amplification of the single-copy cellular 28S rRNA gene using primers as described (3). To detect HIV-1 DNA, a 115-bp region of the gag gene was amplified using primers SK38 (5'-TTTGGTCTTGTCTTATGCCA-3') and SK39 (5'-TAGGAGAAAT-3') using primers as described (3). To detect HIV-1 DNA, a 115-bp region of the gag gene was amplified using primers SK38 (5'-TTTGGTCTTGTCTTATGCCA-3') and SK39 (5'-TAGGAGAAAT-3') using primers as described (3).

FIGURE 1. CypA content in virions produced by HIV-1-infected primary macrophages and lymphocytes

We investigated the CypA incorporation into virions isolated from NL-4.3-infected PBLs or ADA-infected MDMs by Western blot. In this study, all samples for Western blot are first standardized by p24 content by ELISA to facilitate a direct comparison of samples. MDMs obtained from three donors and PBLs from two of the same donors were infected in culture, and virions isolated at different times after infection were compared for CypA content (Fig. 1). The CypA incorporation into virions was variable, depending on the cell donor and time after infection, and virions from MDM contained less CypA than those from PBLs. In Fig. 1B, CypA content appeared to decrease over the course of infection of MDM but increase over the course of infection of PBLs (see Fig. 2B). This variable incorporation suggested that the association of CypA with Gag is not tightly regulated in primary cells and may differ from that in transformed cells (16).

We then used pharmacological intervention with CsA to inhibit the Gag–CypA association. Cell viability was tested during culture of both uninfected and HIV-1-infected PBLs and MDMs in the absence and presence of CsA. CsA was not toxic under the conditions used in this study in which it is only added to cells after loading 10–50 ng of p24; the bands were transferred to a polyvinylidene difluoride (Millipore) membrane. Bands were visualized by Western blotting using standard procedure and staining with anti-CypA Ab (Affinity BioReagents), HRP-conjugated anti-human Ab, and the detection by chemiluminescence using p-coumaric acid (4-hydroxycinnamic acid; Sigma-Aldrich) and luminol (5-aminooxy-1,4-phthalaldehydine; Sigma-Aldrich). The membrane was stripped with 0.2 M sodium hydroxide for 5 min, washed with PBS, and used to detect p24 protein using a mouse monoclonal anti-HIV-1 CA (catalog no. 183-H12-5C) provided by Dr. B. Chesebro (Rocky Mountain National Laboratories, Hamilton, MT) and Dr. K. Wehrly (Rocky Mountain National Laboratories, Hamilton, MT) through the AIDS Research Reagent Repository.
culture in mitogen or differentiation factors (not shown). We exposed MDMs to ADA or to NLHXADA-GP, a molecular clone that carries the ADA V3 region on the background of NL4–3 Gag–Pol (17) for 2 h, washed the cells, and then cultured them in the presence of CsA; this experimental format was designed to focus on postbinding events in virus replication. During infection in culture, MDMs retain most of the capsid protein inside cells, so we tested the levels of both intracellular and extracellular p24 (Table I). The replication of both viruses was inhibited up to 5000-fold by CsA. We then tested the generality of this observation by using MDMs from four different donors for infection by ADA, as modulated by culture in CsA (Table II). In each case, CsA was a potent inhibitor of infection. Using cells from >10 different donors and multiple HIV-1 stocks, we have found that CsA consistently inhibits p24 expression by primary MDMs in culture (data not shown). The extent of inhibition of viral protein production by infected MDMs was significantly greater than that previously reported for infected transformed cell lines, which was on the order of 3- to 10-fold (12) or that previously reported for infected PBLs using the CsA analog, SDZ MIN 811, that inhibited 4- to 20-fold (18). This consistent inhibition of HIV-1 replication by CsA was not reflected in the variable levels of CypA found in virions from infected primary cells (Fig. 1), raising the possibility that CypA incorporation is not the only event in virus infection sensitive to CsA. To directly link these physiological findings to virion composition, the CypA content of virions isolated from infected PBLs or MDMs that had been cultured in the presence or absence of CsA was determined. Cells were obtained from two different donors. Virions from infected transformed CEM cells were run in parallel, all samples were standardized by p24 content (Fig. 2A). Consistent with previous studies, culture of infected CEM cells in CsA significantly inhibited CypA incorporation into virions. In contrast, there was little to no inhibition of CypA incorporation into virions derived from primary cells. Despite standardization by p24 content, virions from highly viable MDMs seemed to show enhanced incorporation of CypA after CsA treatment. To evaluate a more highly purified population of virions for CypA content, we repeated this experiment using PBLs from another donor and subjected virions to sucrose density gradient sedimentation (Fig. 2B). Based on p24 content, sucrose gradient-purified virions had more CypA than the starting virion population. However, CsA treatment of virion-producing cells increased rather than decreased CypA content, and gradient purification further increased the relative CypA content of virions. These studies from a total of five different macrophage and five different PBL donors indicate the CypA incorporation into virions by primary lymphocytes and macrophages is regulated differently than those of transformed cells.

Many previous studies have shown that the CypA–Gag association, and thus CypA virion incorporation, is blocked by CsA and that this reduction in the CypA content of virions renders them less infectious (10, 12, 13). Our finding in primary cells that CsA is a potent inhibitor of HIV-1 infection but does not decrease CypA content of virions is inconsistent with this observation. To directly test the proposal, we isolated virions from infected cells cultured in the presence or absence of CsA and tested their infectivity by

### Table I. CsA stably inhibits infection of MDM using two different strains of HIV-1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 7</th>
<th>Day 9</th>
<th>Day 11</th>
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<td></td>
<td>ex</td>
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<tr>
<td>ADA</td>
<td>$2.4 \times 10^6$</td>
<td>$4.6 \times 10^3$</td>
<td>$1.1 \times 10^6$</td>
</tr>
<tr>
<td>ADA + CsA</td>
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<td>$7.3 \times 10^3$</td>
<td>$1.8 \times 10^6$</td>
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<tr>
<td>GP</td>
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<tr>
<td>GP + CsA</td>
<td>$3.2 \times 10^4$</td>
<td>$5.0 \times 10^4$</td>
<td>$240$</td>
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* MDM were infected with ADA or NLHXADA-GP and then cultured in the absence or presence of CsA. The levels of p24 obtained from ELISA of cells and supernatants harvested at the indicated times after infection are shown, designated intracellular (int) or extracellular (ex), respectively.
MAGI assay of single cycle infection or by assay of p24 production by infected PBL (Fig. 3). When standardized by p24 levels, several different HIV-1 produced in the presence of CsA were equally as infectious as those produced without inhibitor. CsA derived HIV-1 of both subtype B and A/E or R5 and X4 tropism were found to be infectious. Similar results have been obtained using three different cell donors and virus stocks. To better understand this apparent CypA independence but CsA sensitivity of HIV-1 infection of primary cells, we used an HIV-1 mutated in the CypA binding region.

Sites of restriction by CsA treatment of HIV-1-infected primary cells as a function of Gag sequence

To clarify the link between CypA inhibition and CypA in HIV-1-infected primary cells, we used A224E, an NL4-3 variant that emerged after propagation in the presence of CsA but that still encapsidates CypA into virions. This point mutation replaces an uncharged residue with a charged residue in a CypA binding region of Gag and has been shown to render HIV-1 infection in transformed Jurkat cells resistant to CsA and infection in transformed CEM or MAGI cells dependent on CsA (12, 13, 16). We first tested the replication of A224E, compared with NL4-3 by culture of infected PBLs in the absence and presence of CsA and assay of extracellular p24 over time (Fig. 4A). Surprisingly, A224E replication in PBLs was as sensitive to CsA inhibition as that of wild-type NL4-3. Unlike CEM or MAGI cells, PBL infection by A224E was neither dependent nor enhanced by CsA; unlike Jurkat, PBL infection was not resistant to CsA. Similar results were obtained using PBLs from three other donors (data not shown). Analogous studies performed in MDMs using an A224E mutation in NLHXADA-GP yielded similar findings (Fig. 4B) in MDMs from some, but not all, donors, so further studies here will be restricted to A224E replication in PBL. The results of A224E infection reveal a CsA-sensitive postentry phase in HIV-1 replication in primary cells absent from transformed T cell lines.

To investigate the basis of this inhibition by CsA, we tested viral DNA synthesis over time after infection by PCR, amplifying a

FIGURE 4. Efficiency of infection of PBLs or MDMs by wild-type or A224E mutated HIV-1 as perturbed by culture in CsA. PBLs were infected with NL4-3 or A224E (A), and MDMs were infected by NLHXADA-GP or NLHXADA-GP/A224E (B) and then cultured in the absence or presence of CsA. Cells or supernatants were harvested at the indicated times after infection, supernatants were replaced by fresh medium, and the extracellular p24 content from PBLs or the intracellular p24 content of MDMs was determined by Elisa.

FIGURE 5. Viral DNA synthesis by HIV-1-infected PBLs or MDMs as perturbed by culture in CsA. PBLs were infected by DNase-treated NL4-3 or A224E (A) or MDMs were infected by DNase-treated ADA (B) and then cultured in the absence or presence of CsA. Cells were harvested at the indicated times after infection, DNA was isolated and subjected to PCR amplification of regions in the cellular β-globin gene or the HIV-1 gag gene. The β-globin amplicon was visualized by ethidium bromide staining, and the gag amplicon was visualized by hybridization with radiolabeled probe.
region in gag from NL4-3- and A224E-infected PBLs or ADA-infected MDMs cultured in the absence or presence of CsA (Fig. 5). Consistent with inhibition of synthesis of viral protein, NL4-3 DNA synthesis in PBLs and ADA DNA synthesis in MDMs was inhibited by CsA treatment. In contrast, A224E DNA synthesis was not affected by CsA treatment. To probe the next phase of the A224E life cycle in PBL, we tested viral RNA production by RT-PCR, amplifying the singly spliced \textit{vif} mRNA in PBL infected and cultured under the same conditions (Fig. 6). Once again, there was a clear distinction between NL4-3 infection and A224E infection; NL4-3 RNA production was almost completely inhibited by CsA treatment, but A224E transcription was less sensitive to CsA, declining only at the later time points relative to untreated infected cells. These findings narrow the possible activities of CsA in A224E-infected cells either to inhibition of viral protein production or to impairment of the infectivity of progeny virus. To distinguish a block in virus assembly or export from a block in virus protein production, we repeated the experiment shown in Fig. 4A and measured both extracellular and intracellular p24 in infected PBLs (Fig. 7). We found that CsA inhibited A224E and NL4-3 p24 accumulation in both compartments, ruling out a block to virion export, but consistent with a block at or near the level of translation. We tested the infectivity of A224E virions produced in the absence and presence of CsA, with NL4-3 run in parallel for reference, standardizing infectious dose by p24 content (Fig. 8). Although CsA is a potent inhibitor of HIV-1 infection in PBLs and greatly reduces the production of progeny virions, it had little effect upon the infectivity of the progeny virus produced. It should be noted that CsA was shown to enhance A224E replication in MAGI cells (12, 13, 16), as confirmed in the assay performed in this study. Taken as a whole, the findings reported in this study indicate that CsA inhibits an early phase in HIV-1 replication in primary cells before reverse transcription, and that this sensitivity is controlled by Gag. Mutants in Gag that evade the first block are subject to a second phase of inhibition by CsA at the level of viral protein production.

**Discussion**

Our results demonstrate that HIV-1 replication in primary cells is sensitive to the multifunctional inhibitor CsA during two processes: before reverse transcription and at protein production, the phase affected depends on sequences within Gag. Inhibition of virus production appears to be independent of CypA, and CypA itself is variably associated with virions. These findings are quite different from earlier studies of HIV-1 infectivity regarding a requirement for incorporation of CypA into virions for its function (19), but are, in part, consistent with recent studies using transformed cells that place the major function for CypA at early but not late stages of HIV-1 replication (20, 21). Our work extends these studies by suggesting that the interaction of Gag with cellular proteins in

**FIGURE 6.** Viral RNA synthesis by HIV-1-infected PBLs as perturbed by culture in CsA. PBLs were infected by NL4-3 or A224E and then cultured in the absence or presence of CsA. Total cellular RNA was isolated at the indicated times, and a fixed amount of RNA was subjected to RT-PCR, amplifying a region in the singly spliced viral \textit{vif} mRNA. The \textit{vif} amplicon was visualized by hybridization with a radiolabeled probe.

**FIGURE 7.** Efficiency of viral protein export from PBLs infected by wild-type or A224E-mutated HIV-1 as perturbed by culture in CsA. PBLs were infected by NL4-3 (A and C) or A224E (B and D) and then cultured in triplicate in the absence or presence of CsA. Cells and supernatants were harvested at the times indicated and extracellular (A and B) or intracellular p24 (C and D) were measured by ELISA. Means ± SDs are shown.
many transformed cell lines does not predict these interactions in primary cells and opens the possibility that antiviral interventions proceed in the native target cells of HIV-1 differently than in transformed cells.

HIV-1 assembly and virion content are different in infected macrophages than other cell types. Virions assemble in late endosomes in macrophages and contain cellular proteins characteristic of this compartment, LAMP-1, CD81, and CD82 rather than cell surface markers like CD11a or CD14 (22, 23); virions exit the cell through exocytosis. In contrast, in primary and transformed T cells, virion components assemble at and bud directly from the plasma membrane. We investigated whether CypA incorporation may also distinguish HIV-1 virions produced by infected macrophages from those produced by lymphocytes. Contrary to expectation, both primary lymphocytes and macrophages produced virions with low and variable levels of CypA; a virion composition different from that observed from infection of transformed cells in culture (10–13). Our observation that PBL- or MDM-derived infectious HIV-1 virions sometimes contain little to no CypA is supported by recent studies showing that transformed cells lacking CypA can produce infectious HIV-1 virions (21).

These results question the involvement of CypA in HIV-1 infection of primary cells, however they pertain only to late stages of virus assembly. CsA is a multifunctional immunosuppressive agent (24) that was shown to disrupt the association between Gag and CypA and has frequently been used to probe this protein interaction. We used untreated virus for infection and then cultured infected PBLs or MDMs in the absence or presence of CsA to investigate the sensitivity of early events in HIV-1 replication. This approach revealed a profound block at or before reverse transcription of wild-type HIV-1 in both lymphocytes and macrophages, consistent with the block described in transformed cells (19). Although the extent of the defect is not precisely quantified, CsA appears to exert a greater effect upon infection of primary cells than the 3- to 10-fold reduction observed in most transformed cells (13, 19). In the absence of efficient viral DNA synthesis, later synthesis of viral RNA and protein also was greatly reduced in primary cells. However, CsA treatment of infected primary cells not only did not reduce the CypA content of virions, but also, in some cases, increased CypA content of virions, dissociating the inhibitory activity of CsA from the CA-driven incorporation of CypA into virions.

Although CsA treatment greatly reduces the quantity of virions produced by PBLs or MDMs by inhibiting events before wild-type viral DNA transcription, the inhibition is not absolute, and the small quantity of progeny virus that is produced has unimpaired infectivity. This is a clear demonstration that the major effect of CsA on wild-type HIV-1 is felt in the target cell and not in the virus-producing cell. Results reaching the same conclusion by different means have been obtained in some transformed cells (20, 21). Our studies do not permit us to unambiguously identify the CsA-sensitive step early in infection. Virion uncoating has been proposed as a CypA-dependent, CsA-sensitive step in HIV-1 replication in transformed cells by some investigators (19) but has been ruled out by others (25, 26). Several different cell and in vitro systems were used for these studies. Because viral mutants in the CypA binding region of Gag have been shown to behave differently in different transformed cells, this distinction between experimental approaches can be biologically significant (13, 16, 21). An independent indication of fundamental differences among model systems is the observation that a viral vector incorporating Gag from a macrophage-tropic HIV-1 has reduced sensitivity to CsA in certain human and simian cell lines (27), while the present study shows that two macrophage-tropic HIV-1 are profoundly inhibited by CsA during replication in primary human macrophages.

Our studies strongly indicate that there are intrinsic differences between HIV-1 virion infectivity and production in primary lymphocytes and those in transformed cells; this is most clearly seen in infection by the Gag mutant A224E. A224E infection of PBLs can be inhibited by CsA at the stage of production of viral protein. Viral DNA and RNA synthesis are unaffected by treatment of infected cells with CsA. This behavior is strikingly different from that described during infection or transfection of transformed cells, in which A224E is resistant to CsA (12, 13, 16) and also is different from PBL infection by wild-type NL4-3 in which an early event in virus replication is sensitive to CsA. These findings indicate that factors that distinguish primary from transformed lymphocytes govern a critical interaction with HIV-1 Gag during viral protein production. In addition, the demonstration of CsA sensitivity of A224E replication, deemed CsA resistant (12, 13, 16), indicates that the activity of HIV-1 inhibitors is best evaluated in primary host cells of the virus.

Indeed, natural experiments are consistent with the ability of CsA to block HIV-1 infection in its human host. Because of the widespread use of CsA as an immunosuppressive agent during organ transplant, data have been collected on the course of HIV-1 infection in patients under CsA treatment. HIV-1-infected transplant recipients receiving CsA were found to have a significantly reduced risk of progression to AIDS, compared with similar patients not treated with CsA (28). It is possible that this protective
effect is due to the block in virus infection of primary cells demonstrated in this study.

The present study, like many others, indicates that cell type-specific factors can greatly influence the outcome of HIV-1 infection. One such factor is APOBEC3G, which was shown to antagonize the activity of HIV-1 Vif; its expression was postulated to underscore the difference among some target cells lines in their susceptibility to Vif-negative HIV-1 (29). Curiously, although HIV-2 Vif can complement Vif-negative HIV-1 (30), it is poorly antagonized by APOBEC3G, and cell lines expressing APOBEC3G can be susceptible to Vif-negative HIV-2 but not to Vif-negative HIV-1 (31). Different species-specific host restriction factors have been proposed to interact with the CypA-binding domain of CA during early phases of HIV-1 infection of certain nonpermissive simian cells (21, 32, 33). The analogous human factor, TRIM 5-α, was also postulated to act through CypA, but recent studies demonstrate its independence from CypA in human cells (34). This observation underscores the difficulties of extrapolating HIV-1 behavior from model systems to human lymphocytes. Our findings of the activities of CypA and CsA during early and late stages of HIV-1 infection of primary lymphocytes and macrophages recommend reevaluation of the interactions of viral proteins with hosts in the native targets of HIV-1 infection.

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Disclosures

The authors have no financial conflict of interest.

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