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Infection of CD4\(^+\) Memory T Cells by HIV-1 Requires Expression of Phosphodiesterase 4\(^1\)

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Using PCR to monitor HIV-1 RNA genome reverse transcription and nuclear import of preintegration complexes, we found that memory, but not naive, CD4\(^+\) T cells could support transport of HIV-1 DNA to nuclei upon TCR/CD3 and IL-2 stimulation. Moreover, memory CD4\(^+\) T cells, unlike naive CD4\(^+\) T cells, express high levels of phosphodiesterase 4 (PDE4) constitutively. Selective blocking of PDE4 activity inhibited IL-2R expression and thereby led to abolishing HIV-1 DNA nuclear import in memory T cells; however, full-length viral DNA synthesis was not affected. Thus, blocking PDE4 prevents initiation of HIV-1 DNA circle formation in T cells. The fact that PDE4 is expressed constitutively at higher levels in memory vs naive CD4\(^+\) T cells may help HIV-1 readily infect memory T cells. The Journal of Immunology, 2000, 165: 1755–1761.

Cyclic AMP is a key second messenger that plays a pivotal role in regulation of a wide range of cellular functions. The intracellular level of cAMP is controlled by two distinct enzyme superfamilies: the adenylyl cyclases, which use ATP as a substrate to synthesize cAMP; and the cAMP-specific phosphodiesterases (PDEs),\(^3\) which catalytically hydrolyze cAMP to its biologically inactive adenosine 5‘-monophosphate. Increasing cAMP levels by cAMP analogues, cAMP-elevating drugs, or PDE inhibitors results in inhibition of T cell activation, proliferation, and cytokine production (1–6). Currently, PDEs are grouped into 11 broad families; each PDE differs from the others in its substrate specificity, inhibitor sensitivity, tissue distribution, and regulation of activity (7–12). So far, at least three cAMP-specific PDEs have been reported in T cells: PDE3, PDE4, and PDE7 (13, 14). Among them, PDE4 is thought to be the predominant regulator of degradation of cAMP (10). Selectively blocking PDE4 activity has been shown to inhibit inflammatory responses and HIV-1 replication in CD4\(^+\) T cells (15–17).

According to their activation stage and biological function, CD4\(^+\) T cells can be divided into CD45RA\(^-\) naive and CD45RO\(^+\) memory cell subsets (18, 19). CD4\(^+\) memory T cells differ from naive T cells in their responses to SIV and HIV-1 infection. Compared with naive T cells, memory T cells are more easily targeted and their functions are impaired earlier and more severely during SIV and HIV infection (20–25). There is substantial evidence that turnover of CD4\(^+\) memory T cells is faster than naive T cells (26–29), suggesting that HIV-1 replicates optimally in memory T cells. Indeed, highly purified CD4\(^+\) memory T cells from HIV-infected individuals harbored 4- to 10-fold higher viral DNA than in naive T cells (24). Furthermore, recent studies have shown that temporally labile postfusional HIV-1 complexes exist in memory T cells longer than in naive T cells (30). These observations indicate that memory T cells may serve as a principal reservoir in HIV-1 infection.

It is well known that establishment of SIV/HIV infection requires T cell activation (31–39). After virus entry, the processes required for completion of SIV or HIV-1 reverse transcription and nuclear import of preintegration complexes (PICs) are not active in resting T cells (35, 36). Two T cell activation signals are required to overcome these blockages: one signal through the TCR, which regulates cell passage through G0 to G1, induces the completion of reverse transcription of SIV or HIV-1 RNAs; the second signal, through CD28 or the IL-2 receptor complex (IL-2R), controls the entry of virus PICs to the nuclei (35, 36). Moreover, the c-Myc oncoprotein plays a key role in regulation of this process (39). Given the fact that the activation requirements are less stringent for memory T cells than for naive T cells (40–43), it is perhaps not surprising that CD45RO\(^+\) memory are more susceptible to HIV-1 infection than CD45RA\(^-\) naive T cells (24, 44–46). However, the mechanisms by which HIV-1 preferentially infects CD4\(^+\) memory T cells are unknown.

Here we show that CD4\(^+\) memory T cells produce more HIV-1 DNA than CD4\(^+\) naive T cells in response to CD3 mAb together with IL-2. Most strikingly, translocation of HIV-1 DNA to the nuclei occurred only in CD4\(^+\) memory T cells, but not in CD4\(^+\) naive T cells under the same stimulation. High levels of PDE4 were constitutively present in CD4\(^+\) memory T cells. Selectively blocking PDE4 activity resulted in inhibition of nuclear import of HIV-1 DNA in memory T cells, whereas full-length viral DNA synthesis was not affected. In addition, blocking PDE4 activity abolished induction of IL-2Rα-chain and c-Myc expression, suggesting that PDE4 may regulate HIV-1 infection by interfering with IL-2R.

Materials and Methods

Reagents

Purified mAbs to human CD8 (G10-1, IgG2a), CD16 (FC-2, IgG2b), CD20 (1F5, IgG2a), and HLA-DR (HB10a, IgG2a) were produced by us and used...
with negative selection to purify human primary CD4⁺ T cells as described (36, 39). Biotin-conjugated CD45RA (3AC5, IgG2a) or CD45RO (UCHL-1, IgG2a) Abs were also prepared by us and used to isolate CD4⁺ naïve and memory T cells. Streptavidin-magnetic microbeads and goat anti-mouse IgG conjugated to magnetic microbeads were purchased from Miltenyi Biotec (Sunnyvale, CA). mAbs to human CD3 (64.1, IgG2a), CD28 (9.3, IgG2a), and IL-2 (Cetus) were used to activate CD4⁺ T cells. Dr. M. D. Houslay (University of Glasgow, Scotland, U.K.) kindly provided the affinity-purified rabbit antiserum (651) against PDE4A. Phospho-ERK1 and -ERK2 (Thr202 and Ser204) polyclonal Ab was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), CAT-c-Myc mAb (9E10, IgG1) and rabbit polyclonal anti-ERK1 (c-16) serum were purchased from Sigma (St. Louis, MO), respectively.

**Purification of CD45RA⁺ and CD45RO⁺ T cell subsets**

CD4⁺ T cells were prepared from peripheral blood samples from healthy, HIV-seronegative donors as described (36, 39). Highly purified CD4 T cells were further fractionated by negative selection using magnetic beads. The purity of isolated CD45RA⁺ and CD45RO⁺ T cells was 90–95% and 95–98%, respectively, as monitored by flow cytometry. In some experiments, more than one treatment with negative selection was required to remove contaminating cells. Cells were cultured in RPMI 1640 medium supplemented with 5% heat-inactivated human AB serum, 2 mM glutamine, 10 U/ml penicillin, 10 mg/ml streptomycin, 1 mM pyruvate, and nonessential amino acids.

**HIV-1 infection and PCR assays**

HIV-1-Lai strain was prepared as described (36). Cells were infected with HIV-1 at a multiplicity of infection of 0.01 per cell. DNA was extracted from HIV-1-infected cells as described (36). Primers and the thermal cycling reaction for monitoring initiation, elongation of HIV-1 DNA synthesis, and virus DNA nuclear import have been described (36, 39).

**RT-PCR assay for PDE4 mRNA expression**

Total RNA was extracted using TRIzol (Life Technologies, Grand Island, NY) according to the manufacturer’s instructions. Primers used to amplify PDE4A were as described (6). Reverse transcription was performed as follows: 1 μg of RNA with 1 μl of random primer (1 μg/μl) (Promega, Madison, WI) were incubated at 70°C for 10 min. After incubation, the mixture was immediately cooled on ice and the following reagents added: 23 U avian myeloblastosis virus reverse transcriptase (Promega), 40 U RNAsin (Promega), 500 μM dNTP, 500 μM DTT, and 4 μl 5× avian myeloblastosis virus buffer. The final volume was 20 μl. The cDNA synthesis reactions were conducted at 37°C for 1 h, following by heating of the samples at 95°C for 5 min to stop the reactions. Thermal cycling reaction conditions were the same as described (39).

**Western blotting**

The cell lysates (equivalent to 1×10⁶ cells) were electrophoresed by 8% SDS-polyacrylamide gel and then transferred to nitrocellulose membranes (Schreicher & Schuell, Dases, Germany). Western blot analysis was performed as described (39).

**PDE4 activity assay**

Ten to 20 million cells were homogenized as described (6) and sonicated. Freshly prepared lysates were processed as described (47) with some modifications: cell lysates were incubated with 1 μM [³²P]ATP in the presence or absence of 10 μM rolipram, a PDE4 inhibitor, at 30°C for 15 min. The reactions were stopped by placing tubes in a boiling water bath for 1 min. After the addition of snake venom, the samples were incubated at 30°C for 5 min and then applied to DEAE A-25 ion exchange resin columns. Eluted [³²P]adenosine from the resin was measured by a beta counter.

**PKA activity assay**

PKA activity was measured using a PKA assay system (Life Technologies) according to manufacturer’s instruction. Briefly, 2×10⁶/ml CD4⁺ T cells were incubated in the presence or absence of PKA rabbit peptide inhibitor, Rp (5 μM), and stimulated with 1 μg/ml CD3 Ab and 10 U/ml IL-2 for 24 and 48 h. Cellular protein was extracted by repeated freeze/thaw cycles in extraction buffer provided by the company. PKA activity results in the transfer of ³²P-labeled phosphate from [γ-³²P]ATP to the substrate-biotinylated Kemptide (Biomol, Plymouth Meeting, PA). The phosphorylated Kemptide was then captured on a streptavidin matrix, and incorporated radioactivity was determined by a beta counter.

**Results**

CD4⁺ CD45RO⁺ memory T cells are more readily infected by HIV-1 compared with CD4⁺ CD45RA⁻ naïve T cells

To study whether CD4⁺ naïve vs memory T cells are equally susceptible to HIV-1 infection upon TCR ligation in combination with either IL-2R or CD28 cross-linking, we infected these two subsets with HIV-1-Lai for 3 days. PCR was performed to monitor early (LTR/LTR) and late (LTR/gag) products of virus reverse transcription (36, 39), which represent initiation and completion of HIV-1 RNA genome reverse transcription, respectively, and also to monitor formation of the HIV-1 LTR circle (indicating nuclear import of HIV-1 PICs). Initiation of reverse transcription occurred in both T cell subsets at equivalent levels without any stimulation, suggesting that HIV-1 can enter both T cell subsets equally well.

**FIGURE 1.** CD3 and IL-2R ligation induce nuclear import of HIV-1 DNA in CD4⁺ memory T cells but not in naïve CD4⁺ T cells. A. Freshly isolated human CD4⁺ CD45RA⁻ naïve and CD4⁺ CD45RO⁺ memory T lymphocyte subsets were infected with HIV-1_Lai and were either unstimulated (Media) or activated with soluble anti-CD3 (1 μg/ml) plus IL-2 (10 U/ml) (αCD3+IL-2) or PMA (10 ng/ml) plus ionomycin (50 ng/ml) (PMA+Iono). Heat-inactivated HIV-1 controls were infected in parallel. At day 3, DNA was extracted and the levels of initiation of reverse transcription (LTR/LTR), full-length virus DNA (LTR/gag), and formation of LTR circular DNA (LTR circle) were detected by PCR, as described (36, 39). Serial diluted HIV-1-infected CEM DNA was used to monitor the PCR condition under the linear range of amplification. β-globin was used to standardize input of DNA. Similar results were obtained in three additional experiments. B. Naïve and memory T cells were infected with HIV-1 in the absence or presence of CD3 and CD28 mAbs. At day 3, DNA was extracted and subjected to PCR to monitor LTR/LTR, HIV-1 gag, and LTR circles. One of three experiments.
FIGURE 2. PDE4 is constitutively expressed at high levels in CD4⁺ memory T cells but at low levels in naive T cells. A, RT-PCR analysis of PDE4A gene expression in memory and naive CD4⁺ T cells. One microgram of total RNA was subjected to reverse transcription. The reverse transcription product was diluted as indicated, and PDE4A was quantified by PCR. Data are from one of three representative experiments. B, Western blot analysis of PDE4A protein. The same blot was probed with an anti-extracellular signal-related kinase serum as a protein loading control. Induction of both PDE4A mRNA (C) and protein (D) in CD4⁺ naive T cells after costimulation with soluble CD3 (1 μg/ml) and soluble CD28 (10 μg/ml) mAbs. The cells were stimulated for the indicated times. E, Time course of PDE4 activity (1 μM cAMP as substrate). Total CD4⁺ T cells were stimulated by soluble CD3 mAb (1 μg/ml) and IL-2 (10 U/ml) for the indicated times. Total PDE activity was measured in the presence or absence of 10 μM rolipram. The numbers in the open bars represent rolipram-sensitive activity (PDE4), and the dotted area indicates rolipram-resistant PDE activity. F, PDE4 activity in CD4⁺ naive vs memory T cells. Two CD4⁺ T cell subsets from three different donors were subjected to PDE4 activity analysis as described in Materials and Methods. Induction of PDE4 activity in naive and memory T cells by different stimulation. Naive and memory T cells were stimulated as indicated for 1 h (G) or for 4 h (H). PDE4 activity was measured as described above in E. The numbers in the open bars represent activity of rolipram-sensitive PDE4. Data are from one of three representative experiments.
PDE4 IS REQUIRED FOR HIV-1 INFECTION OF CD4⁺ MEMORY T CELLS

PDE4 is essential for HIV-1 infection of memory T cells

The correlative results above suggested that PDE4 may play a role in the formation of HIV-1 circles. To test whether expression of PDE4 is required for infection of CD4⁺ memory T cells, we examined whether selective blockade of PDE4 activity could inhibit nuclear import of HIV-1 DNA in CD4⁺ memory T cells. As shown in Fig. 3, blocking PDE4 activity by rolipram, a PDE4-specific inhibitor, abolished HIV-1 DNA nuclear import in memory T cells, but full-length viral DNA synthesis was not affected. The same result was obtained using forskolin, a cAMP-elevating drug (Fig. 3). Thus, PDE4 appears to be required for a step after...
viral DNA synthesis leading to translocation of HIV-1 PICs to the nucleus. A. CD4+ T cells were activated by CD3 and CD28 mAbs and infected with HIV. Either rolipram or a combination of rolipram and a PKA inhibitor, Rp (20 μM) or Rp-cAMP (500 μM), was added in the defined groups. LTR circle formation was detected by PCR. Induction of c-Myc expression requires PDE4 (B) but is independent of the cAMP-PKA pathway (C). CD4+ T cells were activated by CD3 and CD28 mAbs as described in Fig. 2D in the presence of different doses of rolipram for 24 h (B) or the PKA inhibitor Rp (5 μM) was added. In C, 10 μM of rolipram and 20 μM of Rp were used. Expression of c-Myc was detected by Western blot. Data are from one of three representative experiments.

FIGURE 5. A cAMP-PKA-dependent pathway does not regulate movement of HIV-1 DNA to the nucleus. A. CD4+ T cells were activated by CD3 and CD28 mAbs and infected with HIV. Either rolipram or a combination of rolipram and a PKA inhibitor, Rp (20 μM) or Rp-cAMP (500 μM), was added in the defined groups. LTR circle formation was detected by PCR. Induction of c-Myc expression requires PDE4 (B) but is independent of the cAMP-PKA pathway (C). CD4+ T cells were activated by CD3 and CD28 mAbs as described in Fig. 2D in the presence of different doses of rolipram for 24 h (B) or the PKA inhibitor Rp (5 μM) was added. In C, 10 μM of rolipram and 20 μM of Rp were used. Expression of c-Myc was detected by Western blot. Data are from one of three representative experiments.

Regulation of IL-2R expression by PDE4 in naive vs memory T cells

IL-2/IL-2R interaction provides necessary signaling for translocation of SIV PICs to the nucleus of macaque CD4+ T cells (35). Moreover, increasing cAMP levels by either cAMP-elevating drugs or with a PDE4 inhibitor can suppress IL-2R signals (6, 7, 50). Thus, it seemed appropriate to test whether preferential infection of memory T cells by HIV-1 upon TCR and IL-2R cross-linking results from differential expression of IL-2R. IL-2R is composed of three subunits, α, β, and γc. Of these, IL-2Rα (CD25) is required for high-affinity IL-2 binding and is not expressed on resting T cells, but is potently induced after T cell activation (51, 52). Stimulation of naive T cells with soluble CD3 mAb and IL-2 failed to induce IL-2Rα expression (Fig. 4). However, the same stimulation could up-regulate IL-2Rα-chain expression in memory T cells (Fig. 4). Selectively blocking PDE4 activity by rolipram or increasing cAMP by forskolin inhibited the induction of IL-2Rα expression (Fig. 4).

Control of HIV-nuclear import by PDE4 through a cAMP-PKA-independent mechanism

Elevation of cAMP results in activation of cAMP-dependent protein kinase, PKA. In addition, the cAMP-PKA pathway is involved in regulation of T cell proliferation (5, 53). To explore the involvement of PKA in the regulation of HIV-1 DNA nuclear import, we preincubated CD4+ T cells with rolipram in the presence or absence of the PKA inhibitors, Rp or Rp-cAMP, followed by TCR and CD28 ligation. Selectively inhibiting PDE4 activity led to a block of HIV-1 DNA nuclear import in CD4+ T cells upon costimulation with CD3 and CD28 mAbs (Fig. 5A). However, neither PKA inhibitor restored nuclear import of HIV-1 DNA in CD4+ T cells exposed to rolipram. In parallel experiments, the same dose of Rp-cAMP restored proliferation of CD4+ memory T cells blocked by PDE7 antisense oligonucleotides (6).

We have found that the oncoprotein c-Myc is essential for translocation of HIV-1 DNA to the nuclei of virus-infected T cells (39). Therefore, we tested whether PDE4 was involved in regulation of c-Myc expression. As shown in Fig. 5B, rolipram indeed inhibited c-Myc induction in CD4+ T cells in a dose-dependent manner. Consistent with the results of HIV-1 infection, the PKA inhibitor failed to restore c-Myc expression in CD4+ T cells exposed to rolipram (Fig. 5C), although 80% of PKA activity could be inhibited by Rp (data not shown).

Discussion

Nuclear import of virus PIC is a key step for establishment of HIV-1 infection in target cells. In this study, we found that both CD4+ naïve and memory T cells are equally susceptible to HIV-1 infection after both TCR and CD28 ligation at the preintegrated stage of the HIV-1 life cycle. However CD4+ memory T cells, but not naïve T cells, can support the process of HIV-1 DNA nuclear translocation after TCR/CD3 and IL-2 stimulation (Fig. 1). These data indicate that the activation requirements for establishment of HIV-1 infection are less stringent for memory T cells than for naïve T cells. Upon entry into the nuclei of the target memory cells, some linear HIV-1 DNA integrates into the host genome to form proviruses. Therefore, these results may explain, at least in part, why CD4+ memory T cells serve as a principal reservoir of HIV-1 in infected individuals.

Although T cell activation is essential for establishment of HIV-1 infection in CD4+ T cells, the molecular basis of why memory T cells are infected readily by HIV-1 is not well understood. All RT-PCR, Western blot, and enzymatic activity assays clearly showed the constitutive presence of high levels of PDE4 mRNA, protein, and activity in CD4+ memory T cells. More importantly, we found that expression of PDE4 is essential for the nuclear import of HIV-1 DNA in memory T cells. To date, the only known function of PDE4 is to hydrolyze cAMP in cells and thereby eliminate cAMP’s effects. It is established that cAMP elevating agents, such as forskolin, PGE2, or dibutyryl cAMP, can

block IL-2-dependent signaling at multiple levels, including down-regulation of IL-2 and IL-2R (6, 7), reducing JAK3 levels (50) and c-Myc oncogene expression (50, 54). Presumably, the presence of high levels of PDE4 activity in memory T cells and a subsequent decrease in a pool of intracellular cAMP facilitates IL-2R expression in this subset upon TCR/CD3 ligation (Fig. 4). When IL-2 binds to IL-2R, c-Myc expression is induced, and this is required for translocation of HIV-1 PIC to the nuclei (39). Because blocking PDE4 did not affect synthesis of viral DNA, it is likely that IL-2 signals are not required for initial HIV-1 DNA synthesis. The completion of HIV-1 DNA synthesis does require a TCR/CD3 signal, while CD28 or IL-2 signals are responsible for transport of viral DNA to nuclei (35, 36). Inhibiting IL-2 signaling (35) blocks CD28-dependent viral DNA circle formation. Thus, we hypothesize that when memory T cells are activated by CD3 mAb and IL-2, the TCR/CD3 signal induces HIV-1 DNA synthesis, as well as IL-2R expression via a PDE4-dependent pathway. Then IL-2/IL-2R signaling initiates viral DNA transport to nuclei via a c-Myc-dependent pathway. Consistent with this model, after TCR ligation, native T cells fail to express IL-2R (Fig. 4), probably due to a lack of PDE4, and therefore cannot support transport of HIV-1 DNA to nuclei. Although this model suggests that induction of PDE4 is essential for HIV-1 DNA circle formation, it by no means suggests that an increase in PDE4 alone is sufficient for this process. In fact, unstimulated CD4+ memory T cells do not support transport of HIV-1 DNA to nuclei even though PDE4 is constitutively expressed in these cells.

Most recently, two subsets of memory T cells with distinct homing potentials and effector functions were identified by their differential expression of CCR7, a chemokine receptor (55). CCR7 memory T cells display immediate effector function and are constitutively more sensitive to anti-CD3 in the presence or absence of costimulation. CCR7+ memory T cells can efficiently stimulate dendritic cells and differentiate into CCR7+ T cells. The susceptibility of the two memory T cell subsets to HIV-1 infection is not known; further studies are also required to define the relationship between susceptibility to HIV-1 infection and PDE4 expression in these two memory subsets. As mentioned earlier, besides PDE4, other two cAMP-specific PDEs, PDE3 and PDE7, are also present in peripheral T cells (13). We will address the constitutive expression of PDE7 in CD4+ memory T cells and its role in the regulation of this subset’s functions elsewhere. Investigating the key role of different PDE families in HIV-1-infected T cells, or other HIV-1-targeted cells, such as monocytes/macrophages or dendritic cells, may provide potential intracellular targets for the treatment of AIDS.

Both PKA-dependent and -independent pathways are reported to be involved in cAMP-mediated signaling (6, 50, 56–58). In our experiments, PKA inhibitors had no effect on restoration of c-Myc expression and HIV-1 LTR circle formation in CD4+ T cells exposed to rolipram. Thus, the data suggest that cAMP-mediated suppression of HIV-1 DNA translocation to the nuclei may be PKA independent.

Previous studies have shown that specific blockade of PDE4 by rolipram inhibits provirus transcription and p24 Ag release from both acutely and chronically HIV-1-infected T cells (16, 17). The mechanism leading to these effects was first hypothesized to be due to prevention of TNF-α production by rolipram (16). However, later studies conducted by Navarro et al. (17) did not support this model, because treatment of HIV-1-infected T cells with TNF-α could not restore rolipram inhibition of p24 production. Moreover, a PKA inhibitor prevented the inhibition of TNF-α secretion but not that of HIV-1 replication caused by rolipram. Instead, inhibition of NF-κB and NF-AT activation is more likely to contribute to blockage of HIV-1 replication by rolipram (17, 59, 60). Taken together, these results suggest PDE4 may affect HIV-1 infection at both pro- and postintegration stages.

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References


