Establishment of Latent HIV-1 Infection of Resting CD4\(^+\) T Lymphocytes Does Not Require Inactivation of Vpr

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The introduction of highly active antiretroviral therapy (HAART) for the treatment of HIV-1 infection has allowed dramatic reductions in plasma virus levels to below the limit of detection in many patients. However, latently infected CD4\(^+\) memory T lymphocytes persist as an important reservoir for the virus in the presence of this aggressive therapy and represent a major barrier to HIV-1 eradication with HAART. The mechanism through which the latent compartment is formed has not yet been established. It may involve actively proliferating CD4\(^+\) T-cell intermediates that are infected with HIV-1 and revert back to a resting state, carrying integrated provirus at some low frequency. The HIV-1 accessory protein Vpr, which mediates G\(_2\) cell cycle arrest in host cells, may interfere with the formation of the latently infected T cells by preventing them from exiting the cell cycle to return to a resting state. To investigate the role of the Vpr in the formation of latently infected memory T cells, we cloned and characterized vpr genes from viruses in the latent reservoir. Both sequence analysis and functional assays demonstrated that the vpr gene products of the viruses isolated from the latent pool did not differ significantly from those of a functional Vpr (NL4-3). These results indicate that the generation of resting G\(_2\)-memory T lymphocytes that carry latent HIV-1 provirus occurs despite the G\(_2\) arrest function of the vpr gene product. © 2000 Academic Press

Key Words: HIV latency; Vpr; reservoir; cell cycle; resting T lymphocytes; HAART.

INTRODUCTION

Advances in antiretroviral therapy, particularly the introduction of highly active antiretroviral therapy (HAART), allow control of viral replication in patients with HIV-1 infection. These regimens, which consist of combinations of reverse transcriptase and protease inhibitors, successfully reduce plasma HIV-1 RNA levels to below the limit of detection of current assays in many patients (Gulick et al., 1997; Hammer et al., 1997; Perelson et al., 1997). However, it now appears that there is a low level of continued virus production in most patients on HAART (Dornadula et al., 1999; Furtaldo et al., 1999; Sharkey et al., 2000; Zhang et al., 1999). In addition, HIV-1 persists in stable reservoirs in successfully treated patients whose plasma virus levels have fallen to undetectable levels (Chun et al., 1997; Finzi et al., 1997; Wong et al., 1997). The major viral reservoir for which long-term persistence was previously demonstrated consists of latently infected resting CD4\(^+\) T lymphocytes (Chun et al., 1997; Finzi et al., 1997, 1999; Perelson et al., 1997; Persaud et al., 2000; Wong et al., 1997). These latently infected CD4\(^+\) T lymphocytes have stably integrated provirus (Chun et al., 1995, 1997), but show no active virus production until they are activated in vitro (Chun et al., 1995). Latent infection is established during primary infection, and establishment is not blocked by early antiretroviral treatment (Chun et al., 1998; Finzi et al., 1997, 1999).

Given the fact that this latent reservoir represents a major barrier to virus eradication in patients on HAART, it is important to study the mechanism through which the latent infection is established. Latent HIV-1 is found mainly in resting CD4\(^+\) T cells from the memory subset (Chun et al., 1995; Pierson et al., 2000), and the establishment of HIV-1 latency most likely occurs during the generation of memory T cells. It is generally believed that memory T cells are produced when a small portion of the activated T cells that respond to a particular antigen revert to a resting state and survive as memory cells capable of rapid activation upon reencountering the antigen. Latency may thus be established through a mechanism in which activated HIV-1-infected CD4\(^+\) T cells revert to a resting G\(_2\) state, carrying an integrated provirus that becomes transcriptionally silent in the resting cell. Alternatively, latency may be established through direct infection of resting memory T cells. However, the exact mechanism through which the latently infected resting T lymphocytes are generated has not yet been established.

The HIV-1 accessory gene product viral protein R (Vpr) interferes with cell cycle progression, and causes a G\(_2\) arrest in infected host cells (Jowett et al., 1995; Rogel et al., 1995). Vpr is a virion-associated 14-kDa protein which, in addition to perturbing the cell cycle, also plays a role in nuclear import of the preintegration complex.

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(PIC) in nondividing cells (Heinzinger et al., 1994) such as terminally differentiated macrophages. Vpr may also be involved in the induction of apoptosis in infected host T cells that have undergone cell cycle arrest at $G_2$ (Stewart et al., 1997).

The discovery that Vpr arrests host cells at $G_2$ raised the question of how infected T cells could escape $G_2$ arrest and revert to a resting $G_0$ state to form the latently infected memory T cell compartment. One hypothesis is that only viruses containing mutant forms of the $vpr$ gene could establish latency. This hypothesis is based on the observations that the $vpr$ gene prevents cell proliferation during chronic infection of T-cell lines and thus they typically have inactivating $vpr$ mutations (Planelles et al., 1995; Rogel et al., 1995). To assess the functional status of Vpr in latent virus, we cloned $vpr$ genes from viral isolates obtained from the latent reservoir of patients who were successfully being treated with HAART. Both sequence data and the functional studies demonstrated that the $vpr$ genes from latent HIV-1 did not have mutations that render them nonfunctional. These results indicate that the generation of resting $G_0$ memory T lymphocytes that carry latent HIV-1 provirus occurs despite the $G_2$ arrest function of the $vpr$ gene product.

RESULTS

Sequence analysis of $vpr$ genes from viruses obtained from latently infected cells

Vpr genes from viruses in the latent reservoir were studied in four patients who had undetectable levels of plasma virus as a result of long-term HAART. Previous studies showed that, in such patients, HIV-1 persists in integrated form in resting CD4$^+$ T cells (Finzi et al., 1997; Chun et al., 1997; Perelson et al., 1997; Persaud et al., 2000; Wong et al., 1997). Viruses were isolated by stimulation of highly purified resting CD4$^+$ T cells under limiting dilution conditions (Finzi et al., 1997). These viruses represent replication-competent biological clones derived from the latent reservoir. The isolates were used to infect CD4$^+$ lymphoblasts in short-term cultures, and the $vpr$ genes were amplified by PCR using genomic DNA from infected cells as a template. PCR products were cloned and sequenced. To look for mutations that render $vpr$ nonfunctional, sequences of these clones were compared with the HIV-1 clade B consensus sequence and that of the reference strain HIV-1 NL4-3 (which is known to have a functional $vpr$ gene) (Fig. 1). The sequences all contained open $vpr$ reading frames with no insertions or deletions that would affect the reading frame. Each of the patient sequences differed both from the others and from the reference sequences as a result of distributed single nucleotide substitutions. Each of these substitutions was previously observed in at least one naturally occurring HIV-1 isolate. Inactivating mutations demonstrated by mutagenesis studies, e.g., mutations at residues A30, R73, or R80 (Di Marzio et al., 1995; Mahalingam et al., 1997), were not observed. Taken together, these data suggest that latent HIV-1 does not contain previously observed inactivating mutations.

$vpr$ gene products from patient isolates were functional in causing $G_2$ arrest

To determine whether the $vpr$ gene products from the patient isolates were functional in inducing $G_2$ cell cycle arrest, we carried out high-efficiency cotransfections of 293T cells with constructs containing $vpr$ genes from patient isolates along with a vector expressing GFP. Flow cytometry was performed to determine transfection efficiency, as indicated by GFP expression, while cell cycle arrest was measured by DNA content. A representative experiment is shown in Fig. 2. Transfection efficiencies were typically very high (Fig. 2A). They ranged from 70% to 95% in different experiments but were consistent among all samples within each experiment. In some experiments, GFP-positive cells were sorted to 99% purity before cell cycle analysis, but cell sorting did not significantly affect the ratio of cells in $G_2$ vs cells in $G_1$ (data not shown). After analysis of transfection efficiency, the transfected cells were subjected to PI staining to detect DNA content. In Fig. 2B, the cells chemically arrested at $G_2$ by nicodazol as a positive control showed a high proportion of cells with 2N DNA content. Figure 2C shows DNA content profile of cells transfected with only the pCl vector, and only a small fraction of cells in $G_2$ were seen. A construct carrying a nonfunctional $vpr$ from the HXB2 isolate (Mahalingam et al., 1997) typically induced only a background level of $G_2$ arrest (Fig. 2D). A control construct carrying a functional $vpr$ gene from the reference NL4-3 isolate induced a modest but significant level of $G_2$ arrest in transfected cells (Fig. 2E). FACS profiles of all the patient samples looked very similar to that of the wild-type Vpr, in that there were modest but significant increases in the number of cells at $G_2$ (Fig. 2F). The ratios of cells in $G_2 + M$ vs those in $G_1 + G_0$ were calculated by dividing the percentage of cells in $G_2 + M$ by that in $G_1 + G_0$, indicated on top of the panels in Fig. 2. The ratio was typically 1.5-fold greater in cells transfected with wild-type Vpr than with nonfunctional Vpr.
(0.58 in wild-type; 0.38 in nonfunctional vpr). These vpr gene products from the patient isolates had G2:M : G1 + G0 ratios very close to that of the wild type.

Such functional assays were repeated multiple times and three data points were collected for each patient sample as well as the controls. The G2 + M : G1 + G0 ratios of the functional Vpr NL4-3 were used as a standard for vpr-induced cell cycle arrest (set to be 100% arrest) in each experiment and all patient samples and other controls were expressed as a percentage of arrest seen in NL4-3. An average of the three data points is shown in Fig. 3 with standard deviations. ANOVA and Tukey–Kramer multiple comparison tests were performed to determine the statistical significance of the arrest. The results showed that there is no statistically significant difference between the amount of G2 arrest induced by wild-type Vpr (NL4-3) and the Vpr from patient isolates (P < 0.05). This level of arrest was highly significant (P < 0.05) compared to the much smaller G2 + M : G1 + G0 ratios seen in cells transfected with the nonfunctional Vpr (HXB-2) and pCI alone. Thus the patient vpr genes were functionally equivalent to those of the wild type in inducing cell cycle arrest.

To determine whether the partial G2 arrest observed was the result of inadequate levels of Vpr expression, we transfected 293T cells with a HA-tagged wild-type vpr construct that was previously shown to induce G2 arrest (data not shown). Immunoblotting was used to detect Vpr expression in the transfected cells. As shown in Fig. 4, in pCI-HA-VPR-transfected cell lysate, a 14-kDa Vpr-size
Viruses isolated from latently infected resting CD4+ T lymphocytes are thought to arise from infected activated T cells that revert to a resting G0 state. The HIV-1 accessory gene product Vpr is known to cause G2 arrest in host cells. In principle, the G2 arrest function of Vpr could prevent the activated T lymphocytes from reverting to memory cells after HIV-1 infection. Therefore, we hypothesized that the vpr genes might be mutated in the integrated proviruses in the latently infected T lymphocytes, as is seen in chronically infected T-cell lines. These Vpr mutations would give rise to altered Vpr proteins that could not cause G2 arrest in the host cells.

To test our hypothesis, we cloned the vpr genes from viruses isolated from latently infected resting CD4+ T cells of patients on HAART. Primary sequences were analyzed to identify mutations that may result in inactivation of Vpr. There were no obvious inactivating mutations among any of the four isolates. To extend this analysis, we then tested the clones functionally to determine whether their ability to arrest cells at G2 was compromised. Our studies demonstrated that the vpr genes from all four patient isolates were capable of inducing arrest to levels seen in using a wild-type vpr (from NL4-3).

The vpr genes in these latent proviruses were functional. Interestingly, in one of the patient isolates, there is a single amino acid deletion close to the C-terminus. As shown in our functional assays, this single amino acid deletion does not inactivate Vpr function. This observation suggests that there was even some selective advantage to preserving the reading frame of vpr.

In light of the finding that vpr genes from viruses in the latent reservoir can induce G2 arrest, several models for the generation of latently infected memory T cells can be considered (Fig. 5). One model suggests that latency is established after productive infection of activated CD4+ T cells. A small fraction of productively infected CD4+ T cells survive the cytopathic effects of the virus and the host cytolytic effector mechanisms and revert to a quiescent state. The absence of the requisite host transcription factors in resting cells allows the virus to enter a latent state of infection, in which there is minimal or no viral gene expression, and no new virus is produced by the cells. However, there remains the question of how infected T cells escape the Vpr-induced G2 arrest and proceed into a resting memory state (at G0). We considered several possibilities. First, Vpr-induced G2 arrest might not be complete, i.e., not all cells that express vpr are arrested at G2. In fact, this was the case in our functional assay, in which the level of arrest induced by vpr expression was much smaller than that induced by the drug nicodazole. As demonstrated by our data and work in other laboratories, Vpr-induced G2 arrest varies drastically in magnitude in different systems, from 47% (Gummuluru and Emerman, 1999) to 91% (Poon et al., 1998) of the cell population in G2, but is never absolute. Thus, it is reasonable to imagine that some infected cells would not be arrested at G2 and could revert to the resting memory state. A second possibility is that the Vpr-induced G2 arrest might not be permanent. Cells could arrest at G2 for a period of time and then proceed through the cell cycle (Goh et al., 1998). In this case, cells arrested transiently could eventually revert to G0. Either of these possibilities would allow certain productively infected T cells to revert to a memory state, despite the cell cycle arrest of Vpr.

A second model suggests that latency arises from the infection of a T cell during a narrow window of time, in which T cells are reverting to a G0 memory state (Steven-son, 1997). In this model, the fate of all productively infected T cells is death. However, if a cell is infected when it is already in the process of returning to a resting state, it may be permissive for virus entry, reverse transcription, and integration, but not for activation of viral gene expression from the LTR, resulting in the generation of a latently infected cell. In this case, Vpr would not be expressed during the time when viral latency is es-
tablished and, thus, would not interfere with the formation of the latent reservoir. Although virion-associated Vpr can induce cell cycle arrest (Poon et al., 1998), the cell might have already exited the cell cycle to G_0. Therefore, nonfunctional Vpr would not be selected in latency.

There is a third model for the generation of latently infected memory T cells, which involves direct infection of T cells in the resting memory state. In this model, viral genes are not expressed during the establishment of latency and the cells are not cycling. The evidence for this model is limited. It is generally believed that, although HIV-1 isolates that utilize CCR5 can enter the subset of resting memory CD4^+ T cells that express low levels of CCR5 (Pierson et al., 2000), integration of the provirus does not occur as a result of a failure in nuclear import of the preintegration complex in resting cells (Stevenson et al., 1990). However, recent work by Unutmaz et al. (1999) demonstrates that cytokine-mediated signals may overcome this block and allow for the integration of the HIV-1 genome following the infection of a resting T cell (Unutmaz et al., 1997) and represents a biological clone. Genomic DNA from infected cultures was then isolated using the Puregene Genomic DNA purification kit (Gentra Systems, Minneapolis, MN).

**MATERIALS AND METHODS**

**Viral isolates**

Viral isolates from the latent reservoir were obtained from patients successfully treated with highly active antiretroviral therapy (HAART) with plasma virus levels below the limit of detection (50 copies/ml). Viral isolates were amplified for 4 days on CD8-depleted phytohemagglutinin (PHA)-activated peripheral blood mononuclear cells (PBMCs). Four different isolates from four previously characterized patients (patients 8, 11, 13, and 50; see Finzi et al., 1999) were studied. Each viral isolate was derived at limiting dilution as described by Finzi et al. (1997) and represents a biological clone. Genomic DNA from infected cultures was then isolated using the Puregene Genomic DNA purification kit (Gentra Systems, Minneapolis, MN).

**Cloning of vpr gene from latent proviruses**

A sensitive PCR amplification strategy was designed to amplify the entire vpr ORF from the genomic DNA derived from cultures of biological clones of the virus from the latent compartment. Primers were designed to anneal to regions flanking vpr (corresponding to 5512 to 5536 and 5956 to 5979 on HIV-1 HXB2) that are highly conserved in clade B HIV-1 viruses (sense primer: 5'-GATAGACGGCTAAGAGATAAACCCACTTTTGCTAGTGC-3'; antisense primer: 5'-CCTTGTCGCGCCCGCGGCTTC-
CTGCCATAGGAGATGCCTAAG-3'). Approximately 200 ng of genomic DNA was used in a reaction containing 1 μM each primer, 200 μM dNTPs, 1.5 mM MgCl₂, and 3.5 units of Expand High Fidelity PCR system (Boehringer Mannheim GmbH, Germany). The reaction was cycled between 94°C (30 s) and 68°C (30 s) for 30 cycles. Products were analyzed by agarose gel electrophoresis. The desired fragment was gel purified (Qiagen, Valencia, CA) and cleaved with NotI and MluI for insertion into the multiple cloning site of pCI vector to generate vector pCI-VPR. The plasmid vectors were transformed into bacteria, and the positive pCI-VPR clones were picked and sequenced using standard cycle sequencing. The vpr genes were also PCR amplified from vectors pNL4-3 (functional vpr) and pHBX2 (truncated vpr) using the primers mentioned above. In addition, N-terminal hemagglutinin (HA)-tagged vpr were also PCR amplified from vectors pNL4-3 and pHXB2 using a sense primer with HA-tag sequence added to the 5’ end and the antisense primer discussed above. These PCR products were then cloned into vector pCI. HA-tagged vpr clones were designated pCI-HA-VPR.

Transfection

293T cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum at 37°C and 5% CO₂. Cells (4 × 10⁵ in 24-well plates) were cotransfected with 2 μg of pCI-VPR and 1 μg of pCI-GFP (green fluorescent protein [GFP] cloned into multiple cloning site of pCI) using the Lipofectamine 2000 transfection system (GibcoBRL, Gaithersburg, MD).

Cell cycle analysis

Cells were harvested 48 h after transfection and tested for transfection efficiency using flow cytometric analysis of GFP expression on a FACSCAN (Becton Dickinson, San Jose, CA). The cells were then stained with propidium iodide (PI) for DNA content. The staining solution, consisting of 1% sodium citrate, 0.1% Triton X-100, and 50 μg/ml PI, was added to the cells, which were then incubated on ice in the staining solution for 10 min before analysis on a FACSCAN. Expression of GFP and PI analysis were measured sequentially because the PI staining procedure causes leakage of GFP from the cell, resulting in a reduction in fluorescence signal. Nontransfected cells and cells transfected with pCI alone were used as negative controls, and cells chemically arrested at G₀ (by incubating with 2 μg/ml nicodazole for 24 h) were used as a positive control.

Immunoblot analysis

Protein lysates from the transfected cells (40 μg each lane) were separated by a precast 10–20% gradient Tricine SDS–PAGE gel (Novex, San Diego, CA). The proteins were then transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA) and blotted with a 1:1000 dilution of an HRP-conjugated anti-HA antibody (Boehringer Mannheim).

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