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Human Immunodeficiency Virus Type 1 Cell Cycle Control: Vpr Is Cytostatic and Mediates G_2 Accumulation by a Mechanism Which Differs from DNA Damage Checkpoint Control

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Vpr is a 96-amino-acid protein encoded by human immunodeficiency virus type 1 (HIV-1) that prevents proliferation of infected cells. We have established a system for infection of 100% of a T-cell population with HIV and use this system to show that within the context of HIV-1 infection, Vpr is primarily cytostatic rather than cytotoxic. Vpr acts upstream of dephosphorylation of the mitotic cyclin-dependent kinase, and causes infected cells to accumulate in the G_2 stage of the cell cycle. However, some HIV-1 infected cells increase in ploidy and size, accumulating DNA to an 8N level. Furthermore, the mechanism of the Vpr mitotic block is qualitatively different from that of G_2 DNA damage checkpoint control.

Several viruses encode proteins which alter the cell cycle progression of the host cell. For example, human papillomavirus type 16, adenovirus, and simian virus 40 encode proteins that target p53 and pRB function (for reviews, see references 26, 28, and 40), and herpesvirus saimiri encodes a cyclin D homolog (17). Investigating the interactions between these viral proteins and cell cycle machinery has been instrumental in understanding viral replication strategies and important to our understanding of how these cellular proteins function in regulating the cell cycle.

Human immunodeficiency virus type 1 (HIV-1) also encodes a protein, Vpr, which alters the cell cycle of its host cell. HIV-1 disease is characterized by the loss of $CD4^+$ cells. Whether the destruction of $CD4^+$ cells is primarily due to direct killing by HIV-1 or is immune mediated is currently not clear, but several viral proteins, including Vpr (16, 34), have been proposed as effectors of the cell loss. Expression of Vpr causes primary $CD4^+$ lymphocytes and other cells to accumulate in G_2 (12, 16, 33, 34) and causes terminal differentiation in some other cell types (24). Recent results indicate that the $p34^{cdc2}$ kinase is hyperphosphorylated in HeLa cells that express Vpr (12, 32, 33). Vpr is a virion-incorporated protein (22, 30) and localizes to the nucleus of the infected cell (25).

In this study we investigated the mechanism of Vpr-mediated accumulation of cells in G_2/M. We developed a protocol to infect 100% of a T-cell population with HIV and therefore could determine that Vpr is primarily cytostatic rather than cytotoxic. We also show that HIV-infected T cells are prevented from entering mitosis because the cyclin B-$p34^{cdc2}$ kinase is inactive because of hyperphosphorylation. However, we observed that increased percentages of Vpr-expressing cells accumulated levels of DNA greater than those observed in G_2, suggesting that some cells are not arrested in the cell cycle but rather undergo an additional round of DNA synthesis, thus becoming tetraploid without undergoing an intervening mitosis stage. Although eukaryotic cells can also delay in G_2 as a result of DNA damage, we show that the mechanism by which Vpr prevents mitosis is qualitatively different from the DNA damage checkpoint control because it is independent of p53, independent of the ataxia-telangiectasia (AT) gene, and unresponsive to methylxanthines. Our results also suggest a selective advantage that a block to mitosis could confer on HIV in vivo by maximizing virus production in cells with DNA contents of more than 2N.

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MATERIALS AND METHODS

Cells. Jurkat T cells were maintained in RPMI medium with 10% fetal calf serum. A549 cells were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium and F12 medium with 10% calf serum. HeLa and 293T cells were grown in Dulbecco's modified Eagle's medium containing 10% calf serum. All cell lines were propagated at 37°C and 5% CO_2 in a humidified chamber. AT cells were given to us by Steve Friend (Fred Hutchinson Cancer Research Center). All other cells were obtained from the American Type Culture Collection.

Plasmids. All proviral plasmids were derived from pLai (31) and have been previously described (34). The vesicular stomatitis virus (VSV) G expression vector was given to us by J. Victor Garcia (St. Jude's Children's Hospital) and subsequently modified for higher levels of expression by replacing the cytomegalovirus promoter with the HIV-1 long terminal repeat.

Virus stocks and infections. To prepare virus stocks, 293T cells were transfected with 2.5 μg of proviral DNA and 1 μg of VSV G DNA per well of a six-well plate by the modified calcium phosphate method. The cells were washed 1 day after transfection, and cell-free supernatant was collected on day 2. The viral stocks were concentrated by centrifugation at 23,000 rpm at 4°C for 1.5 h in a Beckman SW28 rotor. The viral pellet was resuspended in RPMI medium–10% fetal calf serum by adding 1/100 of the original volume of medium and incubating the mixture at 4°C for 1 h. The concentrated virus stocks were aliquoted and stored at −70°C. The titers of the viral stocks were determined by the MAGI assay as previously described (20).

Nonadherent cells were infected by pelleting 1×10^6 to 2×10^6 cells and resuspending the cell pellet in a 100-μl final volume which contained virus at a multiplicity of infection (MOI) of 10 and DEAE-dextran at 10 μg/ml. Adherent cells were infected at an MOI of 10 in either 300 or 600 μl (12- or 6-well plate, respectively) of medium with 10 μg of DEAE-dextran per ml. After 2 h, the nonadherent cells were brought to a concentration of 5×10^5 cells per ml and adherent cells were cultured in 1- or 2-ml volumes for 12- and 6-well plates, respectively.

The *vpr* gene was cloned into a murine leukemia virus retrovirus vector based on LXSH (27). To produce the retrovirus vector stocks, a plasmid encoding murine leukemia virus gag/pol was engineered under control of the HIV-1 long terminal repeat to achieve very high level expression in the presence of the HIV-1 Tat gene. Likewise, the VSV G gene was placed under control of the HIV-1 long terminal repeat. These two plasmids, along with LXSH-Vpr and a plasmid encoding HIV-1 Tat, were cotransfected into 293T cells. Virus was collected 2 days after transfection and concentrated 100-fold by ultracentrifuga-

tion as described above. HeLa cells were then infected with the high-titer virus and selected under high concentrations of hygromycin (0.75 mg/ml) for 3 days to obtain pure populations of infected cells.

Flow cytometry. Cells were prepared for flow cytometry as previously described (34). Briefly, 5×10^5 cells were pelleted and resuspended in 0.2 ml of phosphate-buffered saline (PBS) and then fixed by the addition of 1 ml of 95% ethanol and incubation at 4°C for at least 15 min. The cells were washed once with PBS and then treated with 0.5 ml of PBS containing RNase A (180 U/ml) and propidium iodide (PI) (50 μg/ml) for at least 30 min before flow cytometric analysis. The DNA profiles were analyzed by the Multicycle AV program (Phoenix Flow Systems, San Diego, Calif.). For intracellular $p24^{gag}$ staining, the cells were fixed as described above, washed once with PBS, and then incubated with an anti-p24 monoclonal antibody for 30 min on ice. The cells were washed and incubated with goat anti-mouse fluorescein isothiocyanate for 30 min on ice. The cells were washed with PBS and analyzed by flow cytometry.

To isolate nuclei for PI staining, ethanol-fixed cells were pelleted and resuspended in 0.08% pepsin and incubated at 37°C for 20 min. The nuclei were pelleted and resuspended in RNase-PI solution and examined by flow cytometry.

Flourescence microscopy. Cells were prepared for fluorescence microscopy by fixing in 1% paraformaldehyde in PBS overnight. The cells were washed with PBS, resuspended in PI (10 μg/ml) for 15 min, washed with PBS, and resuspended in 50% glycerol containing 1 μg of PI per ml. The cells were mounted on a slide and examined on a Bio-Rad (Richmond, Calif.) MRC-600 scanning laser confocal microscope.

Histone H1 kinase assay. Infected Jurkat cells were pelleted, resuspended in 0.5% Nonidet P-40 lysis buffer as described previously (36), and vortexed for 1 min. A total of 1 μg of anti-cyclin B1 monoclonal antibody (Oncogene Sciences, Cambridge, Mass.) was added to 8×10^5 cell equivalents for 20 min on ice, and this was followed by addition of 50 μl of a 1:1 protein A-Sepharose bead (Pharmacia) slurry and incubation for an additional 60 min. A total of 1 ml of lysis buffer was then added and incubated for 5 min on ice before being spun at $16{,}000 \times g$ for 15 s at 4°C. The beads were washed one more time with lysis buffer and then an additional three times with H1 wash buffer (10 mM $MnCl_2$, 1 mM dithiothreitol). Immune precipitates were resuspended in 25 μl of H1 kinase buffer (50 mM Tris-HCl [pH 7.5], 10 mM $MnCl_2$, 1 mM dithiothreitol, 70 mM NaCl, 10 μM ATP, 80 mM β-glycerol phosphate, 50 mM NaF, 4 μg of histone H1 per reaction mixture, and 0.01 μCi of [γ-^{32}P]ATP per reaction mixture) and incubated for 35 min at 37°C. The samples were prepared for gel electrophoresis by adding 10 μl of 4× sample buffer, and the reactions were run on a sodium dodecyl sulfate–12% polyacrylamide gel. The gel was dried and exposed to X-ray film for 2 h at room temperature.

For the Cdc25 activation assay, cyclin B complexes were immunoprecipitated as described above. The beads were washed twice with lysis buffer and then three times with Cdc25 assay buffer (100 mM Tris [pH 8.2], 250 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol). After the final wash, the beads were resuspended in 25 μl of Cdc25 assay buffer, and glutathione *S*-transferase (GST)-Cdc25A was then added to some samples and incubated for 30 min at 30°C. GST-Cdc25A was a kind gift of Jim Roberts (Fred Hutchinson Cancer Research Center). The beads were then washed once with Cdc25 assay buffer and twice with H1 wash buffer. The samples were then assayed for histone H1 kinase activity as described above.

RESULTS

Characterization of cell cycle effect of HIV-1 with high-titer virus. In order to examine the effects of Vpr on the infected cell in the context of the complete provirus during a single round of infection, we established a system for generating high-titer pseudotyped HIV-1 stocks that would allow 100% of a cell culture to be infected without virus spread. We used the VSV G protein to pseudotype HIV-1 particles with deletions of envelopes because VSV G pseudotypes have a very wide host range and because the association of the VSV G protein to the retroviral virion is stable to ultracentrifugation conditions. Therefore, infectious virus can be concentrated to extremely high titers (3, 42). We routinely obtain titers of 10^7 infectious units or greater from cell-free supernatants after the transient cotransfection of 293T cells with proviruses with deletions of envelopes (Vpr^+ or Vpr^-) and VSV G expression plasmids. Upon concentration, the titers reached 10^9 infectious units or more for both Vpr^- and Vpr^+ HIV proviruses (Fig. 1A). The pseudotype HIV-1 stocks were used to infect Jurkat cells (a T-cell line) at an MOI of 10. At day 2 postinfection, the cells were stained for intracellular p24 expression and analyzed by flow cytometry to determine the percentage of cells infected. Nearly 100% of the cells expressed $p24^{gag}$ regardless of the presence of Vpr (Fig. 1B). These results were verified by immunofluorescent-antibody staining and fluorescence microscopy (data not shown). Cells infected with the high-titer pseudotypes were as sensitive to zidovudine as were those infected with wild-type HIV-1 (Table 1), which indicates that reverse transcription was needed for expression of viral proteins in the target cells.

The infected cell cultures were then analyzed for DNA content by flow cytometry at 2 days postinfection. Jurkat T cells infected with Vpr^+ virus but not Vpr^- virus display a dramatic redistribution of their cell cycle profile (Fig. 1C). Whereas the cell cycle profile of cells infected with Vpr^- virus is indistinguishable from that of uninfected cells (43% of the cells were in G_1 and 26% were in G_2/M), Jurkat T cells infected with the Vpr^+ virus accumulated in the G_2/M stage of the cell cycle; only 21% of the cells were in G_1, while 58% of the cells were in G_2/M. Therefore, by infecting and analyzing an unsorted population of T cells, we showed that HIV infection is able to mediate the accumulation of cells in G_2/M in a process that is dependent on the presence of the *vpr* gene, a result previously demonstrated only by sorting for minor populations of cells expressing Vpr (12, 16, 33, 34).

Vpr is primarily cytostatic and not cytotoxic. The increased percentage of cells in G_2/M could result either from the loss (death) of cells in G_1 or a block of the cell cycle at the G_2/M stage, and because previous analyses of Vpr activity relied on sorting for minor populations of cells expressing Vpr (12, 16, 33, 34), these two possibilities could not previously be formally distinguished. However, since the entire cell culture is infected in our experimental system, we were able to distinguish between these two possibilities by viable cell counts at successive days postinfection. In addition, since the HIV-1 pseudotypes could not encode *env*, cell death by gp120/gp41 (37) was not a complicating factor in the analysis. We found that while cells infected with the Vpr^- virus continued to grow and divide, the number of cells in the culture infected with Vpr^+ virus remained constant during the three days of the experiment (Fig. 2A). Moreover, we were also able to examine the kinetics of the Vpr-mediated cell accumulation in G_2/M during a single round of infection. No significant increase in the percentage of cells in G_2/M was observed after 1 day with the Vpr^+ virus (Fig. 2B). However, by 2 days postinfection a large percentage (52%) of the cells infected with Vpr^+ virus had accumulated in the G_2/M stage of the cell cycle, with a corresponding decrease in the number of cells in the G_1 and S stages. The redistribution of the cell cycle profile was even more pronounced at 3 days postinfection, with 59% of the cells in G_2/M. Cell cultures infected with Vpr^- virus had no alterations in their cell cycle profile through the three days of infection (data not shown). These results demonstrate that T cells infected with wild-type HIV accumulate in G_2/M because of progression of cells from G_1 and S into G_2/M and not because of the death of cells in G_1. Moreover, since our results indicate that Vpr is cytostatic rather than cytotoxic in the context of viral infection for 3 days (Fig. 2A), it is unlikely that Vpr is directly responsible for the short half-life (<2.5 days) of infected cells in vivo (14, 41).

The cyclin B-$p34^{cdc2}$ kinase complex regulates the entry of cells from G_2 into mitosis and is itself regulated by cyclin-activating kinase-mediated phosphorylation on Thr-161 and inactivating phosphorylation at Thr-14 and Tyr-15. Activation of the mitotic cyclin-dependent kinase (CDK) prior to mitosis depends on dephosphorylation of Thr-14 and Tyr-15 by the Cdc25C phosphatase (for a review, see reference 6). To determine the state of the mitotic CDK in infected T cells, lysates of cells infected with Vpr^+ or Vpr^- virus were prepared. Nocodazole-treated cells, which are blocked in mitosis, were used as a

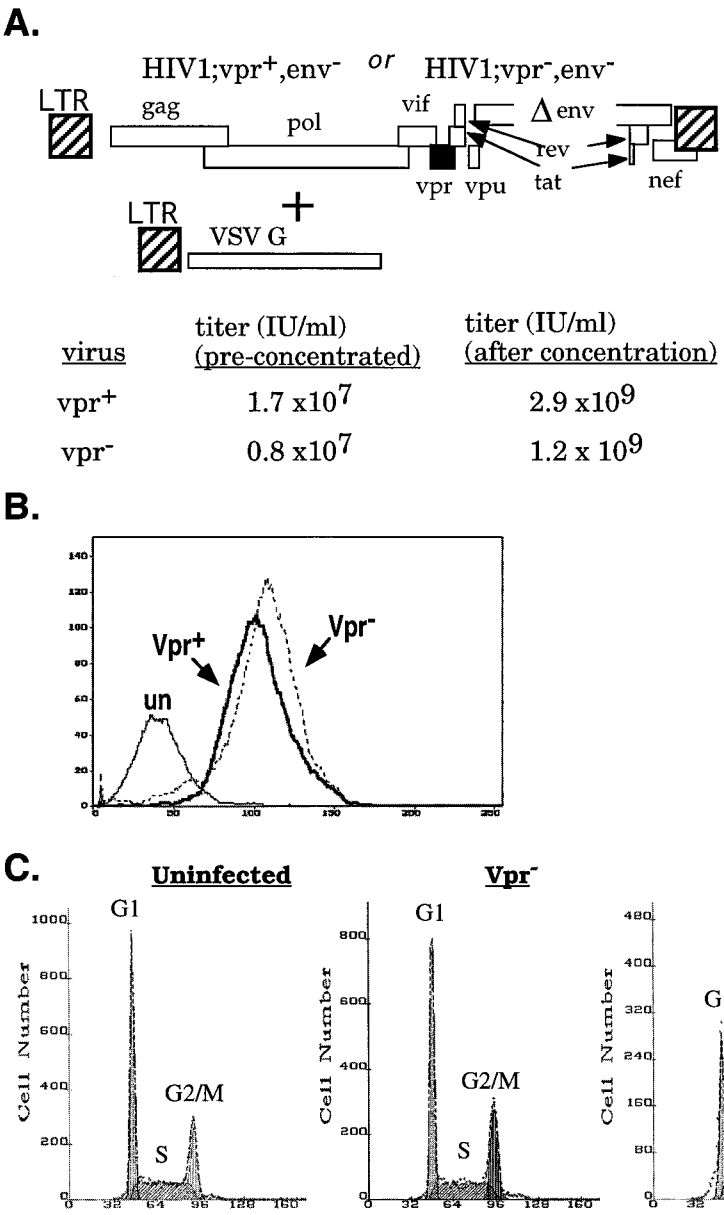

FIG. 1. HIV-1 (VSV G) pseudotypes cause G2 accumulation in infected T cells. (A) An HIV provirus with a deletion in *env* (env$^-$) and either a wild-type copy of *vpr* (vpr$^+$) or a frameshift mutation in *vpr* (vpr$^-$) was cotransfected into 293T cells with the VSV G gene driven by the HIV-1 long terminal repeat (LTR) promoter. Two days after transfection, about 100 ml of supernatant was collected and the titer of an aliquot was determined by the MAGI assay (20). The virions were pelleted, resuspended in 1-ml volumes, and retitered. Titers of HIV (VSV G) pseudotypes before and after concentration are shown. (B) Jurkat T cells were infected with the Vpr$^-$ and Vpr$^+$ HIV-1 (VSV G) pseudotypes at an MOI of 10. Two days after infection, cells were stained for intracellular p24^{gag} expression and analyzed by flow cytometry. Essentially 100% of the cells were infected with the pseudotype express viral antigen, as shown. un, uninfected cells. (C) Jurkat T cells (10^6) were infected with 10^7 infectious units (IU) of HIV (VSV G) pseudotyped virus. The virus stocks were either Vpr$^-$ or Vpr$^+$. Two days after infection, cells were analyzed for DNA content (fluorescence intensity; shown on the *x* axis) by PI staining of nuclei. The positions of G_1, S, and G_2/M are marked. This panel shows that infection of Jurkat cells causes an accumulation in G_2/M that is dependent on the presence of Vpr.

positive control. The cyclin B-p34^{cdc2} complex was immunoprecipitated from the extracts with antibody to cyclin B, and the associated kinase activity was examined by using histone H1 as a substrate. Cells infected with the Vpr$^+$ virus contained less kinase activity than cells infected with the Vpr$^-$ virus, even though more of the Vpr-expressing cells were at the G_2 stage of the cell cycle (Fig. 3A). This demonstrates that Vpr-expressing cells accumulate in G_2 because Vpr acts upstream of events that cause activation of the mitotic CDK.

To verify that the cyclin B-p34^{cdc2} complex was inactive because of hyperphosphorylation in infected T cells, the cyclin B-associated kinase was treated with recombinant Cdc25 and then tested for kinase activity (Fig. 2C). Indeed, the cyclin B-p34^{cdc2} complex could be activated by dephosphorylation in vitro by recombinant Cdc25 (Fig. 2C). This indicates that the cyclin B-p34^{cdc2} kinase is present in T cells arrested in G_2 by Vpr but is inactive because of the failure of Cdc25C to dephosphorylate the complex in vivo.

Accumulation of tetraploid DNA in T cells infected with Vpr$^+$ HIV. While performing cell cycle analysis of Jurkat cells

TABLE 1. Sensitivities of HIV-1 types to a reverse transcriptase inhibitor

Virus[a]	Titer at AZT concn (μM)[b]		Reduction of infectivity (fold)
	0	50	
HIV-1 (HIV *env*)	2.9×10^4	5.0×10^1	580
HIV-1 (VSV G)[c]	1.6×10^8	2.6×10^5	615

[a] Virus stocks were made by transfection of 293T cells with a provirus that either contained a wild-type HIV-1 *env* gene or had a deleted *env* gene and were cotransfected with the VSV G gene to create a pseudotype.

[b] The titer was measured by counting the number of β-galactosidase-positive cells at limiting dilutions (20) by the MAGI assay. AZT, zidovudine.

[c] The HIV-1 (VSV G) virus stock was concentrated 50-fold by ultracentrifugation before use.

infected with the Vpr^+ psuedotyped virus, we observed that a large percentage of the cells contained 8N amounts of DNA; in the culture infected with Vpr^+ virus, 18% of the cells contained 8N amounts of DNA at 2 days postinfection, which increased to 32% of the culture by 3 days postinfection (Fig. 3A). Although uninfected cells and cells infected with Vpr^- virus also had a small percentage of cells with 8N amounts of DNA (<8% of the total culture), the percentage of 8N cells did not increase with time. This indicates that Vpr may induce the accumulation of additional DNA in the HIV-infected cells.

Cells with 8N levels of DNA could contain one nucleus with 8N DNA or multiple nuclei with 2N or 4N DNA if nuclear division proceeded in the absence of cell division. To distinguish between these two possibilities, nuclei were isolated from infected cell cultures at day 2 after infection, stained with PI, and analyzed for DNA content by flow cytometry (Fig. 4A). A comparison of DNA content and forward scatter (measure of relative size) demonstrates that individual nuclei contain 8N DNA and show a corresponding increase in size (Fig. 4A). In addition, the 8N nuclei incorporated BrdU when labeled between days 1 and 2 after infection (data not shown). To confirm that cells infected with Vpr^+ virus contained single enlarged nuclei, we examined infected cells by fluorescence microscopy. Infected Jurkat cells were fixed and labeled with PI at 2 days postinfection. Very large nuclei are readily apparent in cultures infected with Vpr^+ virus (Fig. 4B). The majority of the nuclei in cultures infected with Vpr^- virus are small (Fig. 4B) and appear indistinguishable from those in uninfected cultures (data not shown). Taken together, these results suggest that cell cycle control by Vpr causes some cells that do not pass through mitosis to become tetraploid.

The effect of Vpr on cell accumulation in G_2 is independent of DNA damage checkpoint control. Cells respond to DNA cell cycle progression by delaying in the cell cycle in both G_1 and G_2. Although p53 has been implicated in both G_1 and G_2 checkpoints (1, 35), in an asynchronized mammalian cell population the cell cycle progression of most cells halts at the G_1 checkpoint in response to DNA damage (18). Because Jurkat cells have a mutated p53 gene, we wanted to determine if the presence of a G_1 checkpoint would influence Vpr-mediated G_2 and accumulation of DNA to 8N levels. We infected A549 cells, which have wild-type p53 function (23), with the HIV-1 pseudotyped stocks. To confirm that the division of A549 cells was able to halt in the G_1 stage of the cell cycle, uninfected cells were irradiated with 1,000 rads to cause DNA damage, and, as expected, the majority of A549 cells stopped dividing in G_1, with very few cells in S phase and only a small proportion of cells stopping in G_2 (Fig. 3B, left side). Infected A549 cells were analyzed 2 days after infection for DNA content. Similar to what we observed with Jurkat T cells, the A549 cells infected with the Vpr^+ virus accumulated in the G_2 stage of the cell cycle (Fig. 3B). In addition, a large population of cells with more-than-4N DNA were observed in the Vpr^+- but not Vpr^--infected A549 cultures. By 2 days after infection, at least 22% of the A549 cells infected with the Vpr^+ virus were 8N (Fig. 3B). Because uninfected A549 cells, unlike the Jurkat T cells, had no detectable 8N cells (Fig. 3B, top right), this result suggests that the increase in cell ploidy is not due to expansion of a preexisting tetraploid population. Moreover, the accumulation of cells with 4N and 8N DNA content is independent of the presence of the G_1 checkpoint.

Cells from patients with AT are unable to arrest in the cell cycle at either G_1 or G_2 checkpoints in response to DNA damage (20, 31). To determine whether Vpr acted through the G_2 DNA damage checkpoint, we infected AT cells with pseudotyped Vpr^+ or Vpr^- HIV and examined their DNA profiles 2 days after infection (Fig. 5A). We found that Vpr^+ pseudotype HIV-1 induces AT cells to accumulate in G_2. Therefore, it is unlikely that Vpr induces DNA damage as a means of inducing the mitotic block.

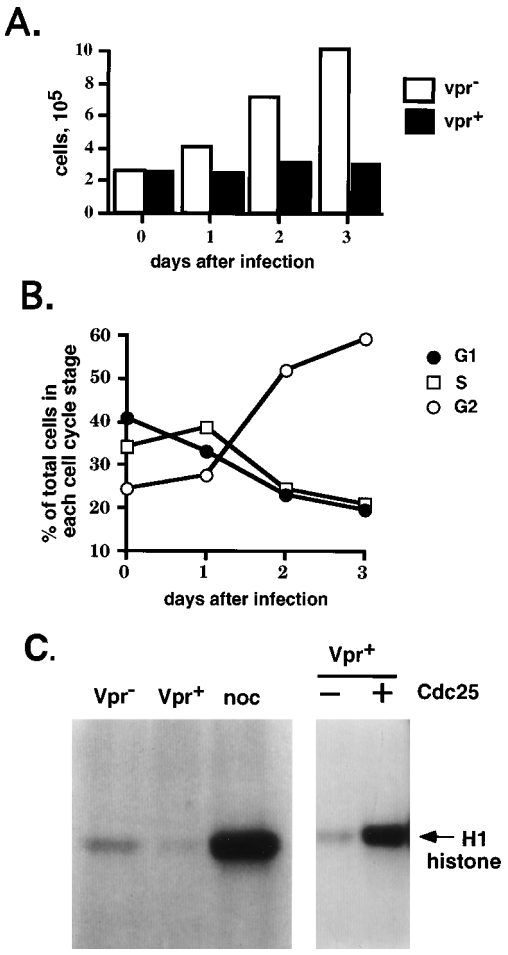

FIG. 2. HIV infected T cells accumulate in G_2 because of progression of cells from G_1 and S. (A) The number of total viable cells in the culture infected with each virus (shown in panel B) was counted each day. (B) Jurkat T cells were infected with a Vpr^+ HIV (VSV G) pseudotype at an MOI of 10, and the percentages of cells in G_1, S, and G_2 were determined at 0, 1, 2, and 3 days after infection. (C) Extracts from 8×10^5 cells infected with Vpr^- virus or Vpr^+ virus or treated with nocodazole (noc) as a positive control were immunoprecipitated with cyclin B antibodies and tested for histone H1 kinase activity (left gel). The cyclin B-associated kinase was also was incubated with (+) and without (−) recombinant GST-Cdc25A (right gel).

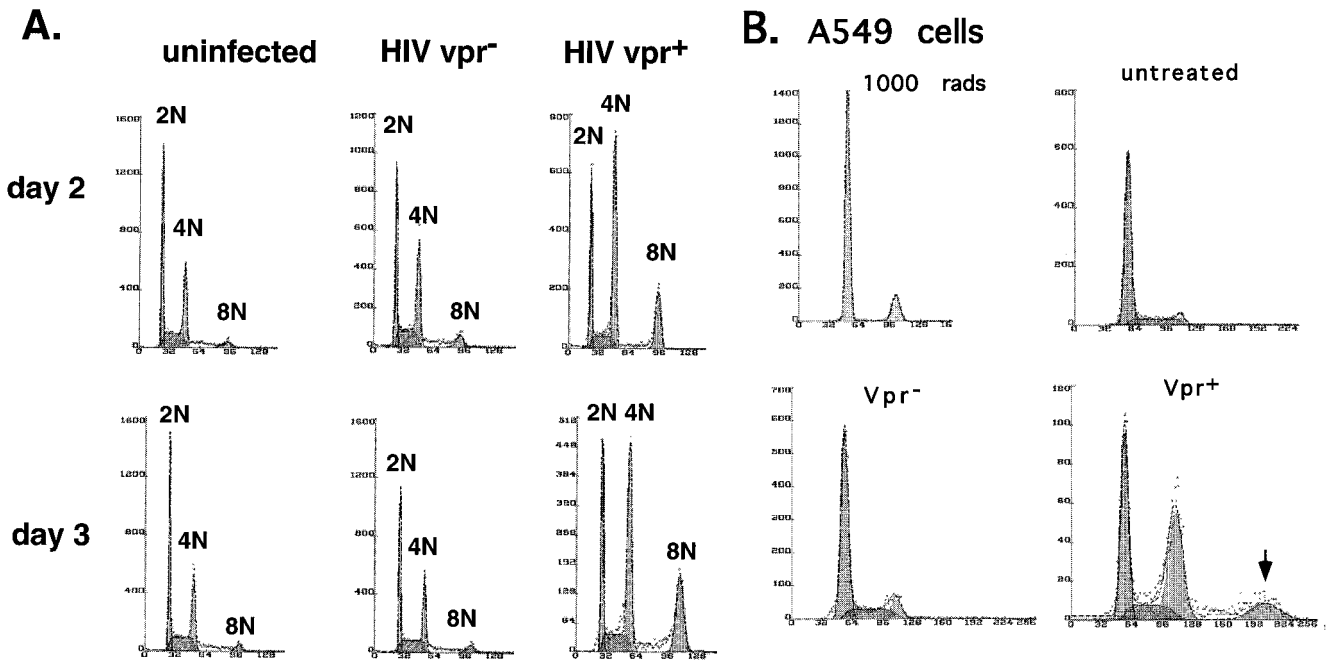

FIG. 3. Progression of Vpr-arrested T cells to tetraploidy. (A) Jurkat T cells were infected with the Vpr^- and Vpr^+ HIV-1 (VSV G) pseudotypes at an MOI of 10, and DNA profiles were analyzed 2 and 3 days after infection. DNA content is given on the *x* axis (fluorescence intensity), and the number of events is given on the *y* axis. The 2N, 4N, and 8N populations are marked. The number of 8N cells accumulated from day 2 to 3 in the Vpr^+-infected cultures but not Vpr^--infected or uninfected cultures. (B) A549 cells, which express wild-type p53, were infected with Vpr^- or Vpr^+ HIV (VSV G) pseudotypes, and DNA profiles were analyzed. Uninfected A549 cells were also irradiated (1,000 rads from a cesium source) to verify the presence of a functional G_1 checkpoint. The arrow on the graph for Vpr^+-infected cells indicates the 8N population.

To further characterize the differences between the DNA damage G_2 checkpoint and Vpr-mediated G_2 block, Jurkat T cells were treated with gamma irradiation and compared with cells infected with Vpr^+ HIV. There were two obvious differences in the arrest of Jurkat cells due to DNA damage and the accumulation of Jurkat cells in G_2 due to Vpr (Fig. 5B). First, in contrast to Jurkat cells infected with wild-type HIV (Fig. 3A), Jurkat cells arrested in G_2 by irradiation did not accumulate 8N DNA. Rather, the tetraploid population was the same as that in nontreated cells (Fig. 5B). Secondly, in contrast to the Vpr-mediated accumulation in G_2, the irradiated cells contained a cyclin B-p34^{cdc2} kinase that was active when tested in vitro (Fig. 5B). This indicates that while the cyclin B-p34^{cdc2} kinase in Vpr-expressing cells is not active unless dephospho-

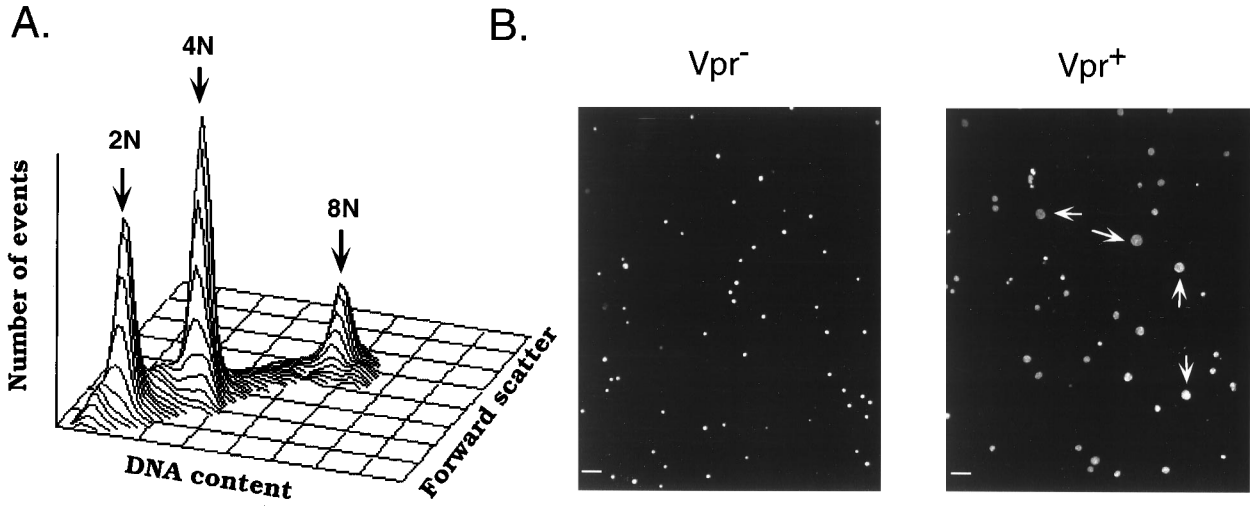

FIG. 4. Single nuclei from Vpr-expressing T cells increase in size and ploidy. (A) DNA content and forward scatter for isolated nuclei were analyzed 2 days after infection of T cells with the Vpr^+ virus. To isolate nuclei for PI staining, ethanol-fixed cells were pelleted and resuspended in 0.08% pepsin and incubated at 37°C for 20 min. The nuclei were pelleted and resuspended in RNase-PI solution and examined by flow cytometry. (B) PI staining of nuclei in Vpr^-- and Vpr^+-infected T-cell cultures. Arrows point to a few of the enlarged nuclei in the Vpr^+-infected culture. Cells were prepared for fluorescence microscopy by fixing in 1% paraformaldehyde in PBS overnight. The cells were washed with PBS, resuspended in PI (10 μg/ml) for 15 min, washed with PBS, and resuspended in 50% glycerol containing 1 μg of PI per ml. The cells were mounted on a slide and examined on a Bio-Rad MRC-600 scanning laser confocal microscope. Bars, 50 μm.

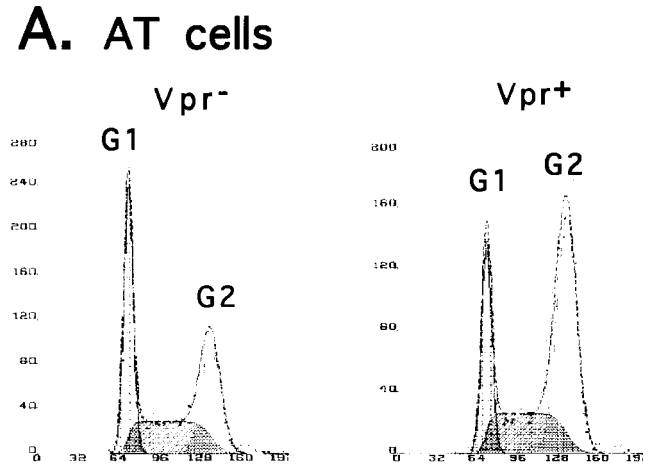

FIG. 5. Vpr-induced G_2 accumulation is different from the G_2 DNA damage checkpoint control. (A) AT cells (from Steve Friend and Chris Kemp, Fred Hutchinson Cancer Research Center) were infected with Vpr^- or Vpr^+ HIV (VSV G) pseudotypes, and DNA profiles were analyzed 2 days after infection. (B) Jurkat T cells were irradiated with 600 rads from a cesium source, and the DNA profile was analyzed after 24 h. Note that the majority of cells are in G_2, and only a small percentage of cells (the same number as that for untreated cells in Fig. 3) are tetraploid. Extracts from 10^6 Jurkat cells that were either irradiated or infected with the Vpr^+ virus were used in a cyclin B-dependent histone kinase assay.

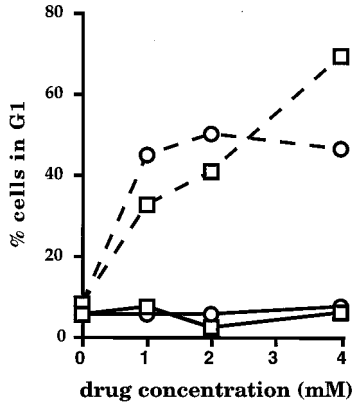

FIG. 6. Vpr-arrested cells do not respond to methylxanthines. HeLa cells were either infected with a retrovirus vector that expresses Vpr or irradiated with 1,000 rads from a cesium source. Irradiated cells were allowed to accumulate in G_2 (18 h) before being treated with 0, 1, 2, or 4 mM caffeine or pentoxifylline and were analyzed for cell cycle progression into G_1 6 h later. Infected cells were treated with hygromycin (0.75 mg/ml) 16 h after infection and were trypsinized and replated at 72 h. At 90 h postinfection, cells were treated with 0, 1, 2, or 4 mM caffeine or pentoxifylline, and they were analyzed for cell cycle progression into G_1 6 h later. The percentage of the total population that passed from G_2 to G_1 is shown on the *y* axis. Dashed lines, irradiated cells; solid lines, Vpr-expressing cells; squares, caffeine treatment; circles, pentoxifylline treatment.

rylated in vitro (Fig. 2C), the control of mitosis in irradiated Jurkat cells is under some other negative control.

Cells arrested in G_2/M by DNA damage can be pushed into mitosis by treatment with methylxanthines, such as caffeine and pentoxifylline (35). We examined whether cells which had accumulated in G_2 because of Vpr could also be forced to enter mitosis following exposure to methylxanthines (Fig. 6). Eighty-nine percent of the untreated irradiated cells were in G_2, with 7.9% in G_1. Of the untreated Vpr-expressing cells, 87% had accumulated in G_2 and 5.5% remained in G_1. The treatment of the irradiated cells with caffeine resulted in 32, 40, and 69% of the cells being pushed through the cell cycle and back to G_1 for 1, 2, and 4 mM drug concentrations, respectively. Similar results were obtained with the treatment of irradiated cells with pentoxifylline: 45, 50, and 46% of the cells were in G_1 after treatment with 1, 2, and 4 mM drug concentrations, respectively. In contrast, the treatment of Vpr-expressing cells with caffeine or pentoxifylline did not result in the progression of cells through the cell cycle (Fig. 6). Rather, the number of cells in G_1 remained constant, with 7, 2, and 5.6% of the cells in G_1 for 1, 2, and 4 mM caffeine treatment, respectively, and 5.4, 5.3, and 7% of the cells in G_1 for 1, 2, and 4 mM pentoxifylline treatment, respectively. These results demonstrate that the Vpr-mediated accumulation of cells in G_2 is distinct from the arrest of cells in G_2 due to DNA damage.

DISCUSSION

Here we demonstrate that the Vpr protein of HIV-1 initiates dramatic changes in the cell cycle of infected T cells. Vpr causes cells to accumulate in G_2 because of an inhibition of mitosis that is mediated through the inability of Vpr-expressing cells to dephosphorylate the cyclin B-$p34^{cdc2}$ mitotic CDK. However, the primary effect of Vpr is cytostatic rather than cytotoxic and therefore it is unlikely that Vpr expression is responsible for the short half-life of infected cells in vivo. In addition, the block to mitosis is qualitatively different from G_2 arrest mediated by DNA damage checkpoint control because it is independent of p53, can be manifested in cells from AT patients, differs in levels of activated mitotic CDK activity, and is unresponsive to methylxanthines. Although mitosis is blocked, HIV-infected T cells display increases in nuclear size and ploidy.

Mutations in components of the mitotic CDK in *Schizosaccharomyces pombe* cells cause these cells to increase in nuclear size and ploidy by decoupling S phase from mitosis (2, 11). The ability to resume S phase after a G_2 block has also been observed in rat cells treated with a tyrosine kinase inhibitor (39). At present we cannot distinguish between the possibilities that Vpr induces additional S phases in the absence of mitosis or that the accumulation of 8N cells is the result of a mitotic block in a cycling population of aneuploid cells. However, similar to what we observed with Vpr, the simian virus 40 T antigen can also induce cells to acquire amounts of DNA greater than those observed in G_2. A temperature-sensitive mutant of T antigen which retains the ability to stimulate S phase but does not induce cells to obtain amounts of DNA

greater than those observed in G2 has been isolated. Therefore, the induction of tetraploid DNA levels is a distinct function of T antigen (8). As in Vpr-expressing cells, the cyclin B-p34^{cdc2} kinase is inactive in T antigen-expressing cells and the T antigen-expressing cells increase in ploidy without an intervening mitosis stage (36). The observation that proteins from two different viruses can mediate similar effects underscores the potential importance of this function.

It may seem counterintuitive for an HIV gene to encode a product that would prevent the proliferation of infected cells. However, in simian immunodeficiency virus-infected monkeys Vpr$^+$ viruses have a selective advantage over Vpr$^-$ viruses (21), and while Vpr is not essential for the induction of AIDS in simian immunodeficiency virus-infected monkeys, it can accelerate the disease (9, 15). Moreover, in cell cultures mutation of *vpr* decreases virus production (5, 29, 32). We speculate that HIV has subverted the cell cycle in order to increase virus production in the face of the rapid destruction of infected cells in vivo (5, 29, 32). Indeed, our preliminary observations suggest that viral expression is increased in cells in G_2. In light of the short half-life of infected cells in vivo, it would be advantageous for virus production to spend the maximum amount of time possible at a stage in the cell cycle when virus expression is highest.

It is noteworthy that in HIV-infected people the majority of cells undergoing apoptosis are uninfected, and only rarely have HIV-infected cells been observed to be apoptotic (7). Apoptosis induced by cytotoxic T-cell lysis is mediated by granzyme B, which induces apoptosis in target cells by premature activation of the mitotic CDK (4, 10). Therefore, as another explanation for the selection of Vpr during primate lentivirus evolution (38) and in in vivo infection, we are also exploring the hypothesis that Vpr protects HIV-infected cells from apoptosis by cytotoxic T-cell lysis by preventing activation of the mitotic CDK.

The Vpr protein is also important in targeting the preintegration complex of HIV-1 into the nucleus of nonproliferating cells such as terminally differentiated macrophages (13). However, for HIV-2, which contains a Vpr protein and a related Vpx protein, it appears that the two functions of Vpr of HIV-1 may have been split between Vpr and Vpx (19). Therefore, it is likely that the inhibition of mitosis is a discrete function of Vpr. Determining the molecular mechanism by which Vpr mediates an inhibition of mitosis will not only be important in the study of the cell cycle but will also be crucial to understanding the role of Vpr in HIV-1 replication and in the development of therapeutic agents to inhibit the activity of Vpr.

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