Vpr Cytopathicity Independent of G₂/M Cell Cycle Arrest in Human Immunodeficiency Virus Type 1-Infected CD4⁺ T Cells[∇]

Diane L. Bolton[†] and Michael J. Lenardo^{*}

Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892-1892

Received 18 January 2007/Accepted 30 May 2007

The mechanism of CD4⁺ T-cell depletion in human immunodeficiency virus type 1 (HIV-1)-infected individuals remains unknown, although mounting evidence suggests that direct viral cytopathicity contributes to this loss. The HIV-1 Vpr accessory protein causes cell death and arrests cells in the G₂/M phase; however, the molecular mechanism underlying these properties is not clear. Mutation of hydrophobic residues on the surface of its third alpha-helix disrupted Vpr toxicity, G₂/M arrest induction, nuclear localization, and self-association, implicating this region in multiple Vpr functions. Cytopathicity by virion-delivered mutant Vpr protein correlated with G₂/M arrest induction but not nuclear localization or self-association. However, infection with whole virus encoding these Vpr mutants did not abrogate HIV-1-induced cell killing. Rather, mutant Vpr proteins that are impaired for G₂/M block still prevented infected cell proliferation, and this property correlated with the death of infected cells. Chemical agents that inhibit infected cells from entering G₂/M also did not reduce HIV-1 cytopathicity. Combined, these data implicate Vpr in HIV-1 killing through a mechanism involving inhibiting cell division but not necessarily in G₂/M. Thus, the hydrophobic region of the third alpha-helix of Vpr is crucial for mediating G₂/M arrest, nuclear localization, and self-association but dispensable for HIV-1 cytopathicity due to residual cell proliferation blockade mediated by a separate region of the protein.

Human immunodeficiency virus type 1 (HIV-1) infection is characterized by a progressive and inexorable decline in circulating CD4⁺ T cells, resulting in susceptibility to the lethal opportunistic infections seen in AIDS. Kinetic modeling and quantitative image analyses support a model in which peripheral blood CD4⁺ T cells turn over rapidly in a cycle of infection, elimination, and replenishment in HIV-1-infected individuals (21, 27, 70). Recent evidence suggests that the mucosal tissue harboring naive and memory T cells suffers a 30 to 60% loss of CD4⁺ T cells infected with the virus within 2 weeks during the early acute stages of simian immunodeficiency virus (SIV) and HIV-1 infection (7, 38). Several direct and indirect causes have been proposed to account for this loss, including effects on both T-cell destruction and production (22, 39). Although bystander death of uninfected cells, as well as cell-mediated cytotoxicity against HIV-1-infected cells, may contribute to T-cell depletion, viral replication within CD4⁺ T lymphocytes causes direct viral cytopathicity in these cells by a necrotic form of cell death (5, 8, 34). Therefore, elucidating the mechanism of direct HIV-1 cytopathicity may help to clarify, and ultimately uncover means to prevent, the decline in CD4⁺ T cells in infected individuals.

The HIV-1 accessory protein viral protein R (Vpr) is an important determinant of viral cytopathicity and can independently kill cells in the absence of viral infection (45, 55, 60).

Mutation of the *vpr* homologues, *vpr* and *vpx*, results in severe attenuation of SIV replication in rhesus monkeys, indicating that Vpr is essential for replication and pathogenesis in vivo (18). Moreover, Vpr is highly conserved among primate lentiviruses with significant sequence and functional homology across primate lentiviral groups (66). Vpr also induces remarkable cell cycle arrest in the G₂/M phase characterized by tetraploid DNA content (23, 29, 52, 53). Several other properties have been described for Vpr, including nuclear translocation of the viral preintegration complex (24), enhancement of transcriptional activity of the HIV-1 LTR promoter by severalfold (1, 15, 19, 20, 68), disruption of nuclear membrane integrity (13), and induction of centrosomal abnormalities (10, 41, 69) and apoptosis (26, 33, 57, 65, 72). It is thought that many, if not all, of these effects may be due to the propensity of Vpr to localize to the nucleus.

The ability to cause cell cycle blockade is conserved among Vpr proteins from a wide variety of HIV and SIV isolates (62). Controversy surrounds the role of cell cycle arrest in Vpr-induced death. Compounds that alleviate Vpr G₂/M arrest, such as caffeine, can reduce apoptosis induced by Vpr (75), while analysis of amino acid substitutions in Vpr showed a correlation between cell cycle arrest and a reduction in viable cells (60, 71). However, other evidence suggests that cell cycle blockade and cytopathicity are independent functions. For example, Vpr death was intact when the G₂/M arrest was abrogated by the drug pentoxifylline (60), and some Vpr point mutants with cell cycle arrest activity fail to induce apoptosis (11, 47). Still other experiments indicate that the cytostatic effect of Vpr serves to prevent cell death and, by some accounts, protects against apoptotic stimuli (4, 12). It has been difficult to reconcile these disparate results. Vpr toxicity may depend on protein abundance within the cell, since low-level Vpr expression is generally associated with antiapoptotic ac-

^{*} Corresponding author. Mailing address: Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bldg. 10, Rm. 11N311, 10 Center Dr., Bethesda, MD 20892-1892. Phone: (301) 496-6753. Fax: (301) 402-8350. E-mail: lenardo@nih.gov.

[†] Present address: Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892.

[∇] Published ahead of print on 6 June 2007.

tivity (12, 16). A more thorough examination of the significance of Vpr G₂/M blockade for cytopathicity in T cells is warranted. In addition, it remains to be determined whether accumulation in G₂/M phase is required for HIV-1-mediated cell death.

Recent determination of the structure for full-length Vpr by nuclear magnetic resonance (NMR) could offer new insights into Vpr function, including its cytopathic effects (43). Vpr forms three well-defined alpha-helices with two separate hydrophobic patches exposed on the outer surface of the first and third helices. A series of hydrophobic residues orientated toward the aqueous cytoplasmic environment would be expected to physically destabilize a protein, unless another molecule binds these regions. Thus, the exposed hydrophobic patches likely mediate Vpr interaction with itself and/or other proteins. Evidence for Vpr self-association has come from several different *in vitro* systems, including dimeric Vpr by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (48), multimeric complexes of recombinant Vpr by gel filtration and dynamic light scatter analysis (25, 73), and homodimerization of Vpr C-terminal fragments (amino acids 52 to 96) by circular dichroism and yeast two-hybrid analysis (58). Together, these data indicate that Vpr likely self-associates within living cells, but the significance of Vpr self-association and its regulation in live cells remain unknown. Thus, a better understanding of the relationship between Vpr dimerization and cell cycle blockade and cytotoxicity could be useful in the development of antiretroviral strategies targeting Vpr function.

We used the structural information to design Vpr mutants that test the involvement of the third-helix superficial hydrophobic residues in Vpr activity and assess the interdependence of the known functions of Vpr. By targeting residues with an outward-facing orientation we aimed to minimize the likelihood of compromising the predicted global conformation of Vpr, such as the folding of the helices around the hydrophobic core. Alteration of the hydrophobic residues impaired the cell cycle arrest and cytotoxic functions of the protein. In addition, nuclear localization and Vpr self-association were markedly reduced upon mutation of this region. Strikingly, the active mutant I70S, which retained the ability to block cells in G₂/M and cause cell death, did not localize to the nucleus or oligomerize normally, suggesting these Vpr functions are executed from the cytoplasm by monomeric protein. Unexpectedly, infection with HIV-1 virus encoding Vpr mutants with attenuated G₂/M arrest ability was as lethal as WT virus, suggesting blockade in G₂/M is not required for viral cytopathicity. Moreover, HIV-1 cytopathicity proceeded unabated in cells chemically arrested in the G₁ phase. These data suggest that Vpr causes infected cell death independent of the third alpha-helix hydrophobic region and G₂/M cell cycle block and that a previously unappreciated inhibition of proliferation, irrespective of any particular cell cycle stage, may be a key component of HIV-1 cytopathicity.

MATERIALS AND METHODS

Cell lines. Jurkat and SupT1 T cells were maintained in RPMI 1640 (Cambrex, East Rutherford, NJ) supplemented with 10% fetal calf serum, 100 U of penicillin-streptomycin/ml, 2.4 mM L-glutamine, and 50 μ M β -mercaptoethanol. All Jurkat experiments were conducted by using a CD4^{hi} Jurkat subclone, Jurkat 1.9, generated by single-cell sorting of a low CD4-expressing parental JAK3 cell line

(5). 293T cells were maintained in Dulbecco modified Eagle medium supplemented as described above. Drugs added to cultures included the following: 3 mM caffeine (Sigma-Aldrich), indirubin-3'-monoxime (A.G. Scientific, Inc., San Diego, CA), and piceatannol (Calbiochem, San Diego, CA).

Transfection. Transient transfection of Jurkat cells with Vpr expression constructs was performed by electroporation using an Electro Cell Manipulator (BTX, Holliston, MA) apparatus. A total of 4×10^6 cells resuspended in 0.4 ml of growth medium and 10 to 15 μ g of DNA were electroporated in a 4-mm gap cuvette (Bio-Rad Laboratories, Hercules, CA) at 260 V and 1,060 μ F, followed by recovery in growth medium for 2 to 4 days. Transfection efficiency was routinely between 60 and 80% as determined by flow cytometric analysis of green fluorescent protein (GFP)-expressing cells 48 h posttransfection. Typically, pEGFP-N1 (Clontech, Mountain View, CA) was cotransfected with other expression plasmids at a ratio of 1:3.3 (e.g., 3 μ g of pEGFP-N1 with 10 μ g of pCDNA3-hVpr) as a marker of transfected cells. Vpr expressed by transient transfection off of a plasmid is referred to as Vpr_r.

HIV virus stock and infections. HIV viral plasmids were obtained from the National Institutes of Health AIDS Research and Reference Reagent Repository unless otherwise indicated. HIV-1 viral stocks of NL4-3_{n-GFP} (pNLnEGFP-Kp; a gift from H. Akari [17]) were prepared from cell-free supernatant from 293T cells transfected with plasmid by using ExGen (Fermentas, Hanover, MD) according to the manufacturer's recommendations and as described previously (5). Briefly, all infections were conducted with *env* mutant derivatives of NL4-3_{n-GFP} (NL4-3_{e-n-GFP}) pseudotyped with vesicular stomatitis virus G protein (VSV-G). Virus titers were assessed by functional multiplicity of infection (MOI) determination based on the Poisson distribution, in which the predicted frequency of 63.2% infected cells is expected after inoculation with an MOI equal to 1 in a single-round infection. Additional viral strains included a Vif mutant derivative of NL4-3_{e-n-GFP}, NL4-3_{e-n-GFP}^{Vif} (55), and Vpr mutant derivatives of NL4-3_r, including deleted Vpr (NL4-3_r^{Δ22-86}, with an amino acid deletion from 22 to 86), and substitution mutants L67E, I70S, L74E, and R80A. Viral stocks of NL-EGFP (no HIV genes expressed) (42) were generated by cotransfection with the encapsidation mutant HIV-1 strain, CMVΔR8.2ΔVpr (44). Virion Vpr (Vpr_r) was delivered to Jurkat cells by using a reverse transcriptase inactive mutant (D186N, RT-; a gift from E. Freed, National Cancer Institute, National Institutes of Health) derivative of NL4-3. To package mutant Vpr into virions, the coding sequence for Vpr was deleted (amino acids 22 to 86; data not shown) from RT- NL4-3_{e-n-GFP}, and this construct was cotransfected with a mutant Vpr expression plasmid. RT- viral stocks were normalized by delivery of HIV-1 p24 capsid protein to target cells by Western blotting. Typically, Jurkat T cells were inoculated at an MOI between 2 and 5 in 12-well (8×10^5 cells/well) or 24-well (4×10^5 cells/well) plates in the presence of Polybrene (5 μ g/ml; Sigma-Aldrich). Virus was adsorbed for 30 min at 37°C in 5% CO₂, followed by centrifugation for 30 min at 800 $\times$ g. Cultures were maintained at 5×10^5 to 10×10^5 cells/ml.

Assays for viral production, cell viability, and cell cycle. HIV-1 cytopathicity was assessed by flow cytometric forward and side light scatter or forward scatter and propidium iodide (PI; Sigma-Aldrich) profiles of at least 10,000 live cells daily throughout the course of infection (FACScalibur; Becton Dickinson, Franklin Lakes, NJ). All fluorescence-activated cell sorting (FACS) data analysis was performed by using FlowJo software (Tree Star, Inc., Ashland, OR). Simultaneously, these samples were measured for HIV-1 provirus expression by either staining with anti-mouse HSA (CD24a; BD Pharmingen) or by GFP fluorescence. Infected viable cell number counts were measured by collection for a constant period of time per sample. To measure DNA content, cells were fixed in 1% paraformaldehyde (in phosphate-buffered saline [PBS]) for 10 min, washed once in PBS, resuspended in 70% ethanol with gentle mixing, incubated at -20°C for 30 min or overnight, washed in PBS, and resuspended in 200 μ l of DNA staining solution (5 μ g of PI/ml, 50 μ g of RNase/ml, and 0.45 mg of sodium citrate/ml in PBS) for 30 min at 25°C. Stained cells were processed on a FACScalibur or FACScan (Becton Dickinson) using the doublet discriminator module. Bromodeoxyuridine (BrdU) staining was performed by using the BD Pharmingen BrdU flow kit according to the manufacturer's instructions. Briefly, cells were pulsed for 60 min with 5 mM BrdU (Sigma-Aldrich), fixed with BD Cytofix/Cytoperm buffer, treated with 300 μ g of DNase/ml, and stained with anti-BrdU conjugated with Alexa Fluor 647 (Molecular Probes, Invitrogen), followed by DNA labeling with 7-amino-actinomycin D (7-AAD). BrdU-stained cells were processed on an LSR II (Becton Dickinson), and doublets were excluded by using a linear 7-AAD height-versus-area gate for analysis.

Expression plasmids. The HIV-1 NL4-3 *vpr* sequence was subcloned using the HindIII and BamHI restriction sites into pECFP-C1 and pEYFP-C1 (Clontech) for fluorescence resonance energy transfer (FRET) analysis. *vpr* was placed C terminal to the fluorophore because tests of an N-terminal position showed this inactivated Vpr cell cycle arrest function and FRET-detectable self-association. To increase *vpr*

expression in the Jurkat T-cell transient-transfection system, we optimized the NL4-3 *vpr* sequence for human codon expression (*hVpr*; GenScript Corp., Piscataway, NJ) by generating the following nucleotide sequence: ATGGAACAGGCTC CTGAAGATCAGGGCCCTACGCGGGAGCCCTACACGAATGGACCCTG GAGCTGCTCGAGGAAGTGAAGGCAAGCTGTGCGGCACCTCCCTCG GATCTGGCTCCACAACTGGGCCAGCACATCTACGAACCTACGGCG ATACCTGGGCCGGCTGGAAGCCATCATCAGAATTCTGCAGCAGCTG CTCTTCATCCACTTCCGGATCGGCTGCCGGCAGCCGGATCGGCGT GACCCGGCAGCGCGGGCTCGGAACGGCGCTAGCCGGTCC. The *hVpr* gene was subcloned into the pcDNA3.1 expression vector (Invitrogen). *vpr* point mutations and deletions were generated by using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol using either PfuTurbo or PfuUltra polymerase (Stratagene).

FRET. 293T human embryonic kidney cells were transiently transfected using ExGen (Fermentas) according to the manufacturer's instructions. pECFP-C1 and pEYFP-C1 plasmids (Clontech) were transfected alone, as a CFP-YFP fusion protein, or as fusion proteins with wild-type (WT) or mutant Vpr. The CFP and YFP plasmids were transfected at a 1:1 ratio, and cells were harvested 24 to 48 h posttransfection. Detection of FRET by flow cytometry was performed as described by Siegel et al. (59), and all analyses were performed on CFP and YFP double-positive cells.

Immunofluorescence imaging. Jurkat cells were washed once in PBS and fixed in 4% paraformaldehyde for 30 min at 4°C. A total of 2×10^5 cells in 0.2 ml were cytospun onto glass slides (Electron Microscopy Sciences, Hatfield, PA) and permeabilized for 5 min with 0.05% Triton X-100 (Sigma-Aldrich) in PBS, followed by three washes with PBS. Mounted cells were then saturated with 10% fetal calf serum in PBS for 30 min at 25°C, washed twice in PBS, incubated in primary antibody against Vpr (K. Strebel, NIAID) at a 1:200 dilution in 0.5% bovine serum albumin-PBS, washed three times in PBS, incubated in Texas red-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA) diluted 1:200, and washed again. All antibody stainings were conducted at 25°C for 45 min. Nuclei were stained with Hoechst 33342 (40 ng/ml; Invitrogen Molecular Probes, Carlsbad, CA) at 1:10,000 in PBS for 5 min, followed by three washes with PBS. Slides were mounted in 5 μ l of Fluoromount-G (Southern Biotech, Birmingham, AL) with a glass coverslip (Fisher Scientific, Pittsburgh, PA). Cells were imaged by confocal microscopy on a Leica TCS-NT/SP1.

Immunoblotting. For standard Western blotting, whole-cell protein extracts were prepared by lysis for 30 min on ice in modified Laemmli buffer (60 mM Tris [pH 6.8], 10% glycerol, and 2% sodium dodecyl sulfate [SDS]), followed by DNase (Benzonase nuclease; Novagen, San Diego, CA) treatment for 30 min. Complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) was included in all lysis buffers. Protein concentration in lysates was estimated by bicinchoninic acid assay (Pierce, Rockford, IL). Equal protein mass was electrophoresed on a 4 to 20% Tris-glycine-SDS gel (Invitrogen Novex) and blotted onto nitrocellulose using a semidry transfer apparatus (Bio-Rad).

Cytoplasmic and nuclear separation extracts were prepared by cell lysis in electrophoretic mobility shift assay buffers as previously described (63), with modifications. Briefly, 5×10^6 cells were gently lysed in buffer A for 5 min and centrifuged to collect the cytoplasmic supernatant. Pelleted nuclei were lysed in buffer C for 10 min on ice, and the nucleoplasm was harvested after centrifugation at $12,000 \times g$ for 10 min. Lysates were separated by SDS-polyacrylamide gel electrophoresis as described above.

Blots were blocked with 2.5% nonfat dry milk in 0.1% PBS-Tween 20 (PBS-T), probed with primary antibody, followed by horseradish peroxidase-conjugated secondary antibody diluted 1:10,000, with three 5-min washes in PBS-T after each 30-min incubation. All antibody probes were performed in 2.5% nonfat dry milk and 0.1% PBS-T. Blots were stripped with Re-Blot Plus Mild (Chemicon International, Temecula, CA) for 20 min and blocked again before reprobing. Bands were imaged with enhanced chemiluminescent substrate (Amersham, Piscataway, NJ) or SuperSignal West Dura (Pierce). The antibodies used with corresponding catalog numbers were as follows: β -actin (A5441; Sigma-Aldrich), PARP (P76420; BD Transduction Laboratories), and HIV-1 Vpr (K. Strebel).

RESULTS

Glutamic acid substitutions in the exposed hydrophobic residues of Vpr helix three impair G_2/M arrest function. NMR structure revealed that full-length Vpr consists of at least three α -helices that fold around a hydrophobic core, with flexible N and C termini and a possible fourth helix in the C terminus

(43). The third α -helix has been shown to be important for Vpr cell cycle arrest function (14, 36). We hypothesized that the hydrophobic residues in helix three—I63, L67, I70, and I74—oriented to the outside of the Vpr protein (Fig. 1A) may serve as a protein-protein binding region critical for Vpr interaction with either a cellular protein or itself in order to mediate its functions. We therefore studied the requirement for the third helix hydrophobic patch in Vpr cell cycle arrest, cytotoxicity, and self-association after substituting each of these residues with alanine, serine, or glutamic acid. Delivery of WT or mutant Vpr protein via HIV-1 virions (Vpr_v) using a reverse transcriptase mutant derivative of NL4-3_{e-n-GFP} (Fig. 1B and data not shown) showed that Vpr G_2/M blockade was dramatically attenuated when these residues were changed to glutamic acid (Fig. 1C, left). The ratio of cells in G_2/M to G_1 (R) induced by WT Vpr_v was 6.2 (medium dose), whereas substitution of I63, L67, or I74 with glutamic acid reduced the blockade ratio to 2.1, 1.0, and 1.0, respectively. By comparison, complete deletion of Vpr gave a ratio of 0.2. Thus, the L67E and I74E substitutions had the greatest effect. I70E was not efficiently delivered by the virion (data not shown). Expression and stability of the mutants were verified by Western blot, which showed all Vpr mutants present at equal levels in the target Jurkat cells, indicating that each had been appropriately packaged into the virion and delivered (Fig. 1C, right). The amount of virion-delivered viral capsid protein was also comparable, indicating that a similar amount of virus was applied to the cells. Substitution of I70 with serine did not impair G_2/M blockade activity (Fig. 1C, R = 5.7) but did disrupt other Vpr properties as described below. The R80A mutant, which fails to be phosphorylated at S79 and completely lacks G_2/M blockade function (74), did not cause any accumulation in G_2/M (R = 0.3), as expected. Only mild effects were noted with serine or alanine replacements of individual hydrophobic residues (data not shown), suggesting that the negative charge imparted by glutamic acid disrupts Vpr activity. Substitution of multiple residues to alanine or serine resulted in poor Vpr expression, presumably due to compromised protein stability (data not shown).

We also evaluated these mutants by transfection directly in Jurkat cells to characterize endogenously expressed protein (Vpr_i) (Fig. 1D). These experiments yielded similar results, and the G_2/M block by I70S was actually enhanced relative to WT Vpr, suggesting that the isoleucine at this site may function as a negative regulator of cell cycle arrest activity. The cell cycle arrest activity of the Vpr mutants after virion delivery (three experiments) and transfection (one experiment) is summarized in Fig. 2A. Interestingly, cell cycle arrest was not as potent when the protein was endogenously expressed in T cells by transfection compared to delivery by a virion, despite significantly greater Vpr abundance upon transfection (data not shown). In summary, mutation of three of the exposed hydrophobic residues of the third helix to a negatively charged residue interfered with Vpr-induced G_2/M arrest.

Vpr G_2/M arrest activity correlates with induction of cell death. To determine whether G_2/M blockade correlated with Vpr cytopathicity, we further tested the mutants for their ability to kill Jurkat T cells upon virion delivery. Vpr_v-induced cell death was proportional to the extent of G_2/M blockade caused by each of the mutants, with R80A, L67E, and I74E showing a

FIG. 1. Exposed hydrophobic residues in the third alpha-helix of Vpr are important for Vpr cell cycle arrest activity in T cells. (A) Cn3D ribbon diagram of the NMR structure of HIV-1 Vpr depicting the relative orientation of the three principle alpha-helices. Residues within the well-defined α -helical structures are colored in green, the flexible N- and C-terminal domains and internal loops are depicted in blue, and the hydrophobic residues (yellow) exposed on the outer surface of Vpr's third alpha-helix are indicated. A side view of the protein is shown on the left, and an end-on view of the helices is shown on the right (arrow indicates the hydrophobic patch; N-terminal domain not shown). (B) Schematic of the NL4-3 HIV-1 molecular clone, NL4-3_{e-n-GFP}, used here. Further derivatives are described in Materials and Methods. (C) hVpr (codon-optimized) was delivered into Jurkat cells by infection with HIV-1 (NL4-3_{e-n-GFP}) RT- virions (Vpr_v) as WT (wt) or mutant protein (mutants indicated in the single-letter amino acid code; stp8,11 contains stop codons at residues 8 and 11). Virions containing WT Vpr (2) were titrated (low [lo], medium [md], and high [hi]) to offer a matched WT Vpr protein control for the mutant Vpr samples. Histograms of cell cycle blockade by Vpr_v at 28 h postinfection (left) show DNA content measured by flow cytometry on PI-stained cells. G₁, S, and G₂ populations were modeled using a Watson Pragmatic cell cycle model, and the ratio of G₂/M to G₁ cells (R) is indicated in boldface type. Western blot of the Jurkat cells (right) shows Vpr_v protein delivery for each of the mutants (top). The same membrane was probed for the HIV-1 capsid protein (p24) (middle) and β -actin as a cellular protein loading control (bottom). (D) Jurkat T cells were cotransfected with pcDNA3.1-hVpr (Vpr_i) expression plasmids encoding WT or mutant Vpr and pEGFP-N1 as a transfection marker at a 3:1 ratio. DNA content analysis was performed on GFP⁺ cells (left), and Western blotting (right) of Vpr (top) and β -actin (bottom, loading control) was performed on transfected cell lysates 3 days posttransfection. The data are representative of three independent experiments.

large reduction in cytopathicity (Fig. 2B; the corresponding cell cycle data are shown in Fig. 2A, series b). The 25 to 40% decrease in cell viability caused by WT Vpr_v is consistent with other reports of virion-associated Vpr killing (61). I63E, which exhibited an intermediate G₂/M arrest phenotype, caused minimal death, whereas I70S, which induced a strong G₂/M arrest, induced the most death of any of the mutants. Thus, G₂/M blocking activity of Vpr mutants correlated directly with the propensity to kill T cells.

Strong nuclear localization is neither required nor sufficient for Vpr G₂/M arrest. Since G₂/M blockade preceded cell death, we explored how Vpr accomplishes cell cycle arrest to gain potential insight into the death mechanism. Vpr nuclear local-

ization was evaluated in the panel of mutants using indirect immunofluorescence. WT Vpr and the cell cycle arrest dead mutant, R80A, showed a predominant nuclear localization with distinct exclusion from nucleoli in transfected Jurkat cells (Fig. 3A) (14, 35). In contrast, the strong G₂/M blocking mutant, I70S, as well as the other helix three mutants were predominantly cytoplasmic with most cells exhibiting a staining pattern surrounding the nucleus (Fig. 3A and B). Thus, mutations in the exposed hydrophobic residues impaired the nuclear localization of Vpr. Fractionation of Jurkat cells into cytoplasmic and nuclear extracts and immunoblotting also revealed that I70S mutation reduced nuclear expression of Vpr (lane 2 compared to lanes 1 and 3, Fig. 3C). Together, these

FIG. 2. Vpr G₂/M cell cycle arrest activity correlates with death-inducing function. (A) Summary of mutant Vpr cell cycle arrest activity in a transfection-based assay (Vpr_i [▨]) and the virion-delivered system (Vpr_v) from two independent experiments: from Fig. 1C (■) (a) and at 26 h postinfection (□) (b) and 66 h postinfection (▨). The ratio of G₂/M to G₁ is plotted on the x axis. (B) Kinetics of Vpr_v cytopathicity after virion delivery of hVpr mutants or no Vpr (ΔVpr) for samples shown in panel A, series b. Viability was measured by flow cytometric detection of PI-negative, forward-scatter large cell events, and the percentage of viable cells is plotted over time. The data are representative of three independent experiments.

data provide strong evidence for a cytoplasmic mode of Vpr-induced cell cycle arrest, although it is also possible that a small amount of nuclear Vpr is sufficient to mediate the G₂/M block. Moreover, nuclear localization is not sufficient for cell cycle arrest given the robust nuclear presence of R80A.

Vpr oligomerization can be detected in real time, but multimers are not sufficient or required for G₂/M arrest. We next investigated whether Vpr-Vpr binding occurs in live human cells using flow cytometric FRET to detect proximity at 100 Å or less (59). Vpr-fluorophore fusion proteins were transfected into 293T cells, and significant FRET was detected between WT Vpr molecules (Fig. 4, change in mean fluorescence intensity [ΔMFI] = 9.7 relative to the CFP-Vpr and YFP co-transfected control), indicating that Vpr binds to itself in living cells. Hydrophobic patch mutants, I63E and I70S, however, both failed to self-associate (ΔMFI < 1.0). Since I70S causes G₂/M block, Vpr self-binding is apparently not required for cell cycle arrest activity. In fact, self-association may negatively regulate G₂/M blockade, since transfected I70S often induced a stronger G₂/M block than WT Vpr (Fig. 2A, Vpr_i). A lack of such regulation for the I63E mutant could also explain why it is not more severely attenuated in cell cycle blockade and killing. Conversely, basic residue mutants in the C-terminal loop that attenuate cell cycle arrest, R80A and R87/88A, showed increased self-association (ΔMFI = 14.3 and 26.9, respectively, but 100 and 50% reduction in cell cycle block, respectively, Fig. 2A and data not shown). A Vpr deletion mutant lacking residues 22 to 87 generated little FRET signal (ΔMFI = 1.8), confirming that the Vpr signal is specific (Fig. 4). In summary, the hydrophobic residues in the third helix are important for Vpr self-association. However, self-association

of Vpr is not required for cell cycle arrest and may even reduce it. For the mutants studied, nuclear localization did correlate with self-association, suggesting that oligomerized Vpr may expedite or facilitate nuclear entry.

Mutant Vpr retains cytopathic activity during HIV-1 infection. To examine further whether cell cycle arrest correlates with cytopathicity in T cells, we tested the third-helix hydrophobic patch mutants in the context of an HIV-1 infection. Because the HIV-1 accessory protein, Vif, also has G₂/M blocking activity and induces cytopathicity (55), we tested the mutants in an NL4-3 strain lacking Vif (NL4-3_{e-n-GFP}). Viral cytopathicity is markedly reduced when both Vif and Vpr are removed from NL4-3, although some death is still apparent when an MOI of 2 is used to achieve ≥90% infection (Fig. 5A) (55). Unexpectedly, we found that Vpr mutants with severe attenuation in G₂/M blockade, L67E and I74E, were as lethal as WT Vpr (40% viable 100 h postinfection; Fig. 5A) when the MOI was matched to achieve the same percentage of infected cells (Fig. 5B). The mutants were still impaired for G₂/M arrest activity, since infected cells showed a decrease in G₂/M accumulation relative to WT Vpr (Fig. 5C). The defect in G₂/M blockade was similar to that seen upon Vpr_v treatment or hVpr transfection of the mutants (Fig. 1C and D). However, cultures infected with the mutant Vpr virus strains failed to proliferate (Fig. 5D), even though the G₂/M block was reduced. Thus, L67E and I74E Vpr mutants caused less G₂/M accumulation in an HIV-1 infection but still inhibited cell proliferation as well as WT Vpr. This apparently occurs through a partial G₁ block, since approximately half of the culture contained 2N DNA content (Fig. 5C). A small population of WT Vpr-arrested cells also stops in G₁ with diploid DNA content (Fig. 5C, for exam-

FIG. 3. Exposed hydrophobic third-helix residues are important for Vpr nuclear localization. (A) Confocal microscopic images of Jurkat T cells transfected with pcDNA3.1 containing WT (wt) or mutant hVpr as indicated. At 48 h after transfection, cells were fixed and immunostained for Vpr (red, left) and imaged by confocal microscopy ($\times 80$). Insets depict higher-magnification images of representative cells for each sample ($\times 100$). The nucleus was stained with Hoechst (green), and an overlay image with Vpr is shown (right). (B) Quantitation of cytoplasmic versus nuclear localization for WT and mutant Vpr. Immunofluorescent staining of Vpr localization in panel A was visually assessed per cell and designated nuclear, mostly nuclear (nuc > cyto), mostly cytoplasmic (cyto > nuc), or cytoplasmic. The percentage of cells with each staining pattern is plotted for the indicated version of Vpr ($n \geq 120$). (C) Jurkat cells transfected with WT (1), I70S (2), or R80A (3) hVpr were harvested 4 days posttransfection for fractionation (top) into cytoplasmic (left) and nuclear (right) protein or DNA content analysis (bottom). Lysates were blotted for Vpr, PARP (nuclear marker), and β -actin (cytoplasmic loading control).

ple), which is consistent with the G₁ block Vpr causes in rodent cells (3). To confirm the G₁ cell cycle arrest in these mutants, we pulsed infected cultures with BrdU to measure DNA synthesis. Although 42% of mock-infected and 30% of Vpr-deficient NL4-3_{e-n-GFP}-infected cells incorporated BrdU, only 5.2 and 12% of the L67E and I74E mutant-infected cells, respectively, were BrdU positive (Fig. 5E). The low level of BrdU incorporation among these mutants was similar to that caused by WT Vpr (8.6%). Thus, G₁ exit is markedly reduced by L67E and I74E Vpr.

To test HIV-1 cytopathicity with a Vpr mutant even more deficient in G₂/M arrest activity, we performed the same comparison using NL4-3_{e-n-GFP} encoding R80A Vpr. Matching infections based on the percentage of infected (GFP⁺) cells

(Fig. 5B), R80A was also as lethal as WT Vpr, killing approximately half of the culture by 100 h postinfection (Fig. 5A). Cell cycle analysis confirmed that infection with R80A Vpr virus (Vif⁻) failed to block cells in G₂/M (Fig. 5C). However, these cells did not proliferate as rapidly as NL4-3_{e-n-GFP}-infected cultures by day 2 (before the onset of viral cytopathic effects; Fig. 5D), and they had a similar defect for BrdU incorporation as cells expressing WT Vpr (11 and 8.6%, respectively, compared to 30% with deleted Vpr [Fig. 5E]). Hence, the R80A mutant demonstrates that G₂/M blockade is not required for Vpr to facilitate HIV-1 cell killing, supporting our observations of the hydrophobic patch mutants L67E and I74E. Rather, inhibition of cell proliferation, by blocking the cell cycle predominantly at the G₁ phase with some

FIG. 4. Vpr self-associates in live cells, but oligomerization is not required for G_2/M arrest activity. 293T cells were transfected with pECFP-C1 and pEYFP-C1-derived constructs encoding either an ECFP-EYFP fusion protein (intramolecular FRET-positive control), ECFP-Vpr and EYFP (negative control), or ECFP-Vpr and EYFP-Vpr fusions containing WT (wt) or mutant Vpr as indicated. FRET analysis was performed 24 h after transfection by flow cytometry on double-positive cells expressing both ECFP and EYFP indicated by the gate in the contour plot on the left. The ECFP-Vpr and EYFP cotransfected cells serve as the baseline FRET signal (thin line) for Vpr cotransfected samples (bold line) in each histogram. The units for the x and y axes are shown for the plots in the bottom row and are identical for all plots. A shift in the FRET MFI indicates protein-protein interaction in real time in living cells.

residual G_2/M arrest, correlated with cell death in R80A-infected cells.

Chemical blockade of the cell cycle in G_1 does not protect against HIV-1 cytopathicity. Since previous reports investigating HIV-induced cell death have implicated the G_2/M arrest in viral cytopathicity, we next tested chemical inhibitors of the cell cycle. Caffeine, a cyclic AMP phosphodiesterase inhibitor that prevents DNA damage checkpoint activation in irradiated cells, reverses Vpr-induced G_2/M arrest (49, 56). As expected, HIV-infected cells treated with caffeine failed to accumulate in G_2/M (Fig. 6A). We found that shifting the cell cycle status did not, however, protect the cells from viral cytopathic effects, since caffeine-treated infected cells died with similar kinetics and magnitude as untreated cells (Fig. 6B). Interpretation of these results was complicated by two factors, however. First, caffeine, like many of the compounds that perturb cell cycle regulation, exhibited moderate intrinsic toxicity on Jurkat T cells (Fig. 6B). Second, caffeine interfered with viral infection at early time points since the percentage of GFP⁺ cells was reduced at 12 and 38 h postinfection (Fig. 6B, right), although complete infection of the culture was obtained by day 3. Overall, however, infected cell death proceeded unabated, and only a minority of the cells was in G_2/M . Thus, accumulation in G_2/M is apparently unnecessary for viral cell killing.

Given the complexity of interpreting viral cytopathicity curves in the presence of a slightly toxic drug such as caffeine, we evaluated less toxic alternatives. Indirubin, a potent cyclin-dependent kinase (Cdk) inhibitor that arrests Jurkat cells in G_1 (28), and piceatannol, a tyrosine kinase inhibitor that prevents Stat3 activation and centrosome duplication (40, 64), were

tested in HIV-infected Jurkat cells. We observed that treatment of Jurkat cells with these compounds alone did not significantly alter the DNA content profile in uninfected cells (Fig. 6C, left column), but HIV-1 infection-induced G_2/M accumulation was dramatically reduced (middle column). This was due primarily to blocking cell division at the G_1 phase, since both piceatannol and indirubin prevented cell proliferation while DNA analysis showed cells to be mostly in G_1 (Fig. 6C and D, lower right panel). Unlike caffeine, indirubin and piceatannol were not toxic at concentrations that effectively prevented the G_2/M blockade (10 μ M), although mild toxicity was observed at higher concentrations of piceatannol (30 μ M, Fig. 6D). As with caffeine, HIV-1-induced cell death proceeded unabated in the presence of these drugs. The viral infection process was not disrupted by piceatannol or indirubin, except at the higher concentration of piceatannol (30 μ M), since we found that the percentage of cells expressing the provirus was comparable between treated and untreated infected cultures (Fig. 6D, upper right panel). Together, these data provide strong evidence that G_2/M -phase arrest is not required for HIV-1 cytopathicity. Furthermore, the G_2/M -independent death is not observed when Vif and Vpr are removed from the virus (NL4-3_{ΔVif-ΔVpr}; Fig. 6D), indicating that this killing requires Vif, Vpr, or a combination of the two proteins.

Cytopathic effects of a G_2/M arrest deficient HIV-1 strain at a high MOI. The observation that HIV-1-induced cell death occurs independently of G_2/M blockade is at odds with the observation that a *vif-vpr*- derivative of NL4-3_{ΔVif-ΔVpr} (NL4-3_{ΔVif-ΔVpr}) lacking G_2/M arrest activity is largely noncytopathic (55). To test the possibility that increased proviral

FIG. 5. Vpr mutants attenuated for G₂/M arrest activity retain cytotoxicity function within HIV-1 infection. Jurkat T cells were mock infected or infected with VSV-G-pseudotyped NL4-3_{e-n-GFPf} (Vif-) derivatives expressing WT Vpr (wt), deleted Vpr (Δ), or Vpr point mutants as indicated (MOI = 3). (A) The viability of infected cultures was monitored over time by flow cytometry (PI negative, high forward scatter). (B) Infected cell percentage was measured by flow cytometric quantitation of GFP⁺ live cells for the cultures shown in panel A. (C) DNA content analysis of mock-infected or NL4-3_{e-n-GFPf} infected cells (gated on GFP⁺) 46 h postinfection for the samples in panels A and B. The ratio of cells in G₂/M to G₁ (R) is indicated within the DNA content histogram in boldface type. (D) Total viable cell counts at 26, 45, and 72 h postinfection for the cultures shown in panels A to C measured by constant time flow cytometric acquisition. (E) Jurkat T cells were infected with WT or mutant Vpr derivatives of NL4-3_{e-n-GFPf} as in panel A, and BrdU incorporation was measured after a 60-min pulse with 5 mM BrdU at 42 h postinfection. DNA content (7-AAD) is shown on the x axis, and BrdU staining is shown on the y axis. The percentage of BrdU-positive cells in the gate is indicated in the upper right corner. Analysis was performed on GFP⁺ cells (gate drawn within inset histogram) for infected cultures.

expression during a G₁ or G₂/M cell cycle blockade is responsible for cell killing, we examined the cytopathicity of NL4-3_{e-n-GFPf-r} in Jurkat cells at MOIs ranging up to 18 with elevated viral protein production. Infected cells remained predominantly in G₁ regardless of the MOI (Fig. 7A), confirming the inability of *vif-vpr*- NL4-3 to arrest in G₂/M. At MOIs of 1 to 1.5, cells supported viral infection with minimal effects on viability, even though the majority (60 to 75%) of the culture was actively expressing the provirus (Fig. 7B, top panels). At greater MOIs, viability fell to 50 and 30% by day 5 postinfection for cultures infected at MOIs of 7 and 18, respectively (Fig. 7B, top left plot). Although cell killing could not be attributed to G₂/M arrest, cell proliferation kinetics indicated that normal cell expansion was impaired in cultures infected at the higher MOIs (Fig. 7B, bottom right plot). Another viral protein, such as Tat, which can cause G₁ arrest (32), may be responsible for the residual cell cycle inhibition.

From these observations, we hypothesized that infected cell

death occurs when a viral component, such as one of the remaining viral proteins (Gag, Pol, Tat, Rev, and Vpu) or nucleic acid, accumulates in nondividing cells to concentrations greater than that in cells permitted to divide. Similarly, cells infected at high MOIs with multiple copies of an integrated provirus would be more likely to contain high concentrations of viral components due to more viral gene expression. We tested this by flow cytometric detection of the GFP MFI to measure quantitative differences in relative protein expression at the single-cell level. The GFP MFI was notably enhanced with increasing MOIs throughout the course of the infection (Fig. 7B, bottom left plot). Thus, elevated viral expression correlated with the MOI, as well as with decreased proliferation. To verify that the proliferation block and cell death caused by NL4-3_{e-n-GFPf-r} infection at high MOIs was specific to HIV-1 infection and not an artifact of elevated GFP expression, accumulation of unintegrated proviral DNA, or VSV-G exposure, we tested a VSV-G-pseudotyped NL4-3-derived lentivirus, NL-EGFP, that lacks all HIV-1 genes (42). This virus,

FIG. 6. Accumulation in G_2/M is not required for HIV-1 cytopathicity. (A) Jurkat cells were mock infected or infected with NL4-3_{c-n-GFP} (MOI = 3) in the presence or absence of 3 mM caffeine (caf). DNA content analysis was performed 37 h postinfection and plotted against GFP (x axis). Lower and upper quadrants depict approximate G_1 and G_2/M populations, respectively, and the percentage within each gate is indicated. Inset histograms depict the DNA content for each sample, pregated on GFP⁺ cells for NL4-3_{c-n-GFP}-infected samples. (B) Viable cell (PI-negative, high forward scatter) percentages of cultures are shown in panel A over time as measured by flow cytometry. The percentage of GFP⁺ cells is shown on the right. (C) Jurkat cells were mock infected or infected with NL4-3_{c-n-GFP} (NL4-3) or NL4-3_{c-n-GFP}vif-vpr- (NL4-3_{f-r}) (MOI = 2) in the presence or absence of indirubin (ind) or piceatannol (pic) at the μ M concentration indicated in parentheses. DNA content analysis at 24 h postinfection is shown in dot plot format with GFP on the x axis as in panel A. (D) Viable cell percentages (left panel), GFP-expressing cell percentages (upper right panel), and live cell counts (bottom right panel) of samples in panel C over time for mock-infected (open symbols), NL4-3-infected (filled symbols), and NL4-3_{f-r}-infected (half-filled symbols) cultures.

which encodes EGFP under the cytomegalovirus promoter, failed to have any deleterious effects on cell viability and division (Fig. 7C, left plots), despite a threefold increase in the GFP MFI compared to NL4-3_{c-n-GFPf-r}-infected cells (Fig. 7C, right plot inset). Therefore, HIV-1 cytopathicity appears to be a direct result of elevated HIV-1 provirus expression in non-dividing cells and can occur independently of Vpr expression and G_2/M arrest under extreme infection conditions.

DISCUSSION

AIDS is due to the massive depletion of CD4⁺ T cells that occurs during the acute and chronic stages of HIV-1 infection. Direct viral killing of infected CD4⁺ T-lymphocytes likely plays a role in this process. Given the established involvement of Vpr in HIV-1 cytopathicity (31, 60), we investigated how Vpr mediates T-cell death. The recent NMR structure for Vpr re-

vealed a series of exposed hydrophobic residues in the third alpha-helix that we envision could function as a protein-protein binding domain (43). Characterization of Vpr mutants in this region has led us to conclude that residues I63, L67, I70, and I74 are important for cell cycle arrest, cytopathicity, nuclear localization, and self-association. In particular, replacement of I63, L67, and I74 with glutamic acid severely disrupted Vpr cell cycle blockade activity and cytopathicity. These substitutions did not lead to decreased protein stability or diminished virion packaging or delivery, indicating that they did not cause global Vpr dysfunction. However, impaired nuclear localization and self-association of the cell cycle active mutant, I70S, suggests that Vpr does not execute its G_2/M block function from within the nucleus and that Vpr oligomerization is likely not necessary for cell cycle blockade. Regardless, our data represent the first confirmation that Vpr-Vpr binding

FIG. 7. NL4-3 vif-vpr- expression and cytopathicity increases with the MOI in the absence of G₂/M accumulation. Jurkat cells were mock infected (open symbol) or infected with increasing concentrations of NL4-3_{c-n-GFP} vif-vpr- (NL4-3_{f-r-}, filled symbols), and the DNA content and viability were measured by flow cytometry. (A) DNA content FACS histograms show cell cycle profiles at 48 h postinfection. The MOI is indicated for each sample below the corresponding symbol. (B) The percentage of viable (high forward scatter, PI-negative) cells is plotted at the indicated hours after infection (upper left panel) for the samples in panel A. Concurrent measurements of the percentage of cells expressing GFP were made (upper right panel). The GFP MFI of infected GFP⁺ cells determined by flow cytometry is also plotted over time (lower left panel). Live cell counts at 24 and 48 h postinfection were measured flow cytometrically by constant time acquisition (lower right panel). (C) Jurkat cells were infected with increasing concentrations of NL-EGFP (filled symbols) and viability, GFP, and cell counts were measured as in panel A. The MOI for each sample is indicated adjacent to the plot symbol on the far right.

occurs in live, unaltered human cells expressing endogenous Vpr. Vpr oligomerization may actually diminish G₂/M arrest, however, or the arrest may involve a release from self-binding. Finally, G₂/M arrest-impaired Vpr mutants were cytopathic when expressed in the context of an HIV-1 infection, which may be due to cell cycle dysfunction in the G₁ phase rather than in G₂/M. HIV-1 toxicity independent of G₂/M cell cycle blockade was also observed by chemical inhibition of the cell cycle in G₁. Combined, these data suggest that an overall arrest of cell proliferation, and not accumulation in G₂/M, is required for viral cytotoxicity.

Analysis of these mutants has also provided important insights into the significance of Vpr nuclear localization and self-association for cell cycle blockade activity. Neither of these properties was required for Vpr cell cycle arrest, since the active mutant, I70S, was expressed predominantly in the cytoplasm and did not oligomerize. Although the low-level nuclear

expression observed for this mutant may be sufficient for cell cycle blockade, other cytoplasmic Vpr mutants, such as L68S, that retain G₂/M block activity have been reported (14, 36). Thus, our data are consistent with a model in which Vpr inhibits cell cycle progression from an extranuclear site, such as through interaction with the cytoplasmic scaffolding protein 14-3-3 (30). Since the hydrophobic patch mutants were generally deficient in nuclear localization and self-binding, this region may mediate Vpr binding to nuclear transport factors (50, 67) or other Vpr molecules. Moreover, this correlation may indicate a role for Vpr self-association in nuclear import. Although NMR studies of Vpr C-terminal fragments implicated the neighboring hydrophobic residues—I61, L64, and L68—in mediating Vpr dimerization (6), altered protein folding in the absence of the N terminus or the *in vitro* conditions used in these studies may account for this discrepancy. Interestingly, the enhanced self-association of mutants in the C-terminal

basic domain relative to WT Vpr suggests that this region may govern the extent of Vpr oligomerization. For example, the highly charged domain might function as a mild Vpr repellent. In summary, Vpr oligomerization does not play a direct role in G₂/M arrest induction but may serve to facilitate nuclear entry and is likely mediated by hydrophobic residues in the third alpha-helix. It is also possible that Vpr self-association negatively regulates the G₂/M arrest activity, since many of the mutants showed an inverse relationship between self-binding and G₂/M blockade. Although interpretation of these experiments is limited by their reliance on Vpr overexpression, Vpr is highly expressed in HIV-1-infected cells and thus may behave similarly under physiologic conditions.

Our site-directed mutagenesis studies suggest that the third alpha-helix of Vpr mediates diverse Vpr functions. We speculate that this is most likely due to disruption of a crucial protein-protein interaction function served by the hydrophobic residues on the outer surface of this helix. Vpr is a 14-kDa protein without known enzymatic activity or domain motifs, so it seems likely that it works by binding other, presumably cellular, proteins. The unique ability of glutamic acid substitutions in this region to cripple Vpr activity, compared to alanine or serine, suggests that the negative charge or larger side chain is particularly damaging to Vpr function. It is also possible that the remaining unaltered hydrophobic residues in the alanine and serine substitution mutants may provide sufficient surface area for a protein partner to bind. One possibility is that the basic C-terminal domain is drawn in toward the negatively charged mutant third helix by electrostatic forces, competing for binding of a cellular partner to the hydrophobic patch. This could also prevent proper functioning or modification of the C terminus, which is known to be critical for Vpr cell cycle arrest activity (74). It is also possible that this region is important for proper folding of Vpr, although the external orientation of the targeted residues may not affect intrinsic folding. Thus, we favor the conclusion that the third helix is directly responsible for Vpr functions, perhaps by associating with a cellular protein.

Our results uncovered a hitherto-unrecognized distinction between direct Vpr cytotoxicity and cell death mediated by HIV-1 infection. For death induced by Vpr alone (i.e., virion-associated Vpr), cell cycle blockade in G₂/M was important. Cell death was proportional to the dose of Vpr_v added to cells and invariably preceded by cell cycle blockade for at least 24 h (data not shown). In addition, Vpr point mutants attenuated in G₂/M arrest activity showed a direct correlation between cell death and cell cycle blockade. This is consistent with previous correlations using other Vpr mutants (60, 71) and abrogation of Vpr-induced cell death by chemically blocking cells in G₁ (2). It is important to note that these studies, like our Vpr_v experiments, were conducted in systems in which Vpr was expressed in the absence of other viral proteins and bone fide HIV-1 infection. However, examples of Vpr mutants, such as I81P, which kill independent of cell cycle activity, are at odds with our findings (11, 47). One possible explanation for this discrepancy is that, in the case of I81P, introduction of a proline in the middle of a potential fourth alpha-helix might significantly change the overall structure and function of the protein, in spite of the fact that the protein was intact (43, 47). Other studies demonstrating independent G₂/M arrest and cell

death activity by Vpr were conducted in fission yeast (11). Effects in this simple eukaryote may not recapitulate the interplay between cell cycle regulation and cell death in human cells. Thus, our data strengthen the argument for a correlative, and likely causal, relationship between G₂/M cell cycle arrest and cell death.

In contrast to direct Vpr death induced upon virion delivery, HIV-1 cytopathicity can occur independently of G₂/M cell cycle blockade. That is, although Vpr toxicity may rely on a G₂/M block, death due to actual HIV-1 infection did not. Vpr mutants that cannot arrest in G₂/M retained lethal properties when expressed in the context of viral infection but not during virion-mediated delivery. This appears to be due to a previously unknown ability of Vpr to cause cell cycle arrest in the G₁ phase, as well as in G₂/M, in human T cells. It is not clear whether Vpr blockade in G₁ occurs in contexts other than during viral infection, although two lines of evidence suggest that this may be an inherent property of the protein: (i) cell cultures arrested by WT Vpr_v contain a minor population (~10%) of cells in G₁ with diploid DNA that fail to proliferate and (ii) Vpr arrests murine cells in G₁ (3, 46). One explanation for this phenomenon is that Vpr G₁ arrest is most apparent in mammalian cells only if the G₂/M blockade activity is attenuated. Given the emerging role of Cdk1 in regulating multiple phases of the mammalian cell cycle (37), it seems possible that Vpr could inactivate aspects of Cdk1 function besides entry into mitosis. Alternatively, the classical G₁ kinases, Cdk4 and Cdk6, which are essential for hematopoietic cell proliferation in mice (37), could also be targeted by Vpr. This could lead to an arrest in G₁ even if the Cdk1 inactivating function of Vpr is not intact. Regardless of the mechanism, the cytopathicity of our Vpr mutant HIV-1 strains is consistent with *ex vivo* studies of human lymphoid tissue infected with R80A mutant CXCR4-tropic HIV-1 (51). Rajan et al. observed significant CD4⁺ T-cell depletion using either the R80A mutant or the WT Vpr virus. In contrast, *vpr*-deleted HIV-1 was impaired for CD4⁺ T-cell depletion. Although it has also been reported that *in vitro* T-cell infection with HIV-1 harboring non-G₂/M-arresting mutants such as R80A fail to cause cell killing (71), these studies did not control for the level of infection at the single-cell level.

Why a failure of HIV-1-infected cells to proliferate leads to cell death remains unclear. Proviral proteins and nucleic acid may accumulate to higher concentrations in cells prevented from dividing and diluting their contents into daughter cells. Elevations of one or more viral components could ultimately be lethal. Increased viral protein expression in noncycling infected cells may also result from augmented transcription. Transcription from the HIV-1 promoter is upregulated specifically in G₂/M, but a G₁/S blockade also transactivates the long terminal repeat, albeit to a smaller extent (19). Alternatively, protein translation or stability may be enhanced in nonproliferating cells. This model is supported by *in vivo* studies demonstrating a correlation between viral protein levels and cell death, since cells expressing HIV-1 mRNA at high levels have a shorter life span than those with less mRNA (9). It is also a distinct possibility that conflicting cell cycle regulatory signals, such as from Vpr and Tat, which can arrest cells in the G₁ phase (32), may trigger cells to die through a process related to mitotic catastrophe (54). In summary, blocking cell prolifera-

tion, regardless of the cell cycle phase, increases the cytopathic nature of HIV-1 infection, although a specific death mechanism remains to be elucidated.

ACKNOWLEDGMENTS

We thank Nathaniel Landau, Eric Freed, and Hirofumi Akari for plasmids; Klaus Strebel for Vpr antibody; Kevin Holmes, Calvin Eigsti, and David Stephany for assistance performing flow cytometric FRET and BrdU detection; and Anthony Fauci and members of his laboratory for use of the BSL-2 research facilities. DNA sequencing was performed at the NIAID Rocky Mountain Laboratories. We are also grateful to Tony Barnitz, Keiko Sakai, Lixin Zheng, and other members of the Lenardo laboratory for helpful insights and inspiring discussions and to John Coffin, Malcolm Martin, Andrew Snow, and Helen Su for critical reading of the manuscript.

D.L.B. was a participant in the FAES (NIH)-Johns Hopkins University Cooperative Graduate Program in Biomedical Sciences. This study was supported by the intramural program of the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

REFERENCES

- Agostini, I., J. M. Navarro, F. Rey, M. Bouhamdan, B. Spire, R. Vigne, and J. Sire. 1996. The human immunodeficiency virus type 1 Vpr transactivator: cooperation with promoter-bound activator domains and binding to TFIIB. *J. Mol. Biol.* **261**:599–606.
- Andersen, J. L., J. L. Dehart, E. S. Zimmerman, O. Ardon, B. Kim, G. Jacquot, S. Benichou, and V. Planelles. 2006. HIV-1 Vpr-induced apoptosis is cell cycle dependent and requires Bax but not ANT. *PLoS Pathog.* **2**:e127.
- Azuma, A., A. Matsuo, T. Suzuki, T. Kurosawa, X. Zhang, and Y. Aida. 2006. Human immunodeficiency virus type 1 Vpr induces cell cycle arrest at the G₁ phase and apoptosis via disruption of mitochondrial function in rodent cells. *Microbes Infect.* **8**:670–679.
- Bartz, S. R., M. E. Rogel, and M. Emerman. 1996. Human immunodeficiency virus type 1 cell cycle control: Vpr is cytostatic and mediates G₂ accumulation by a mechanism which differs from DNA damage checkpoint control. *J. Virol.* **70**:2324–2331.
- Bolton, D. L., B. I. Hahn, E. A. Park, L. L. Lehnhoff, F. Hornung, and M. J. Lenardo. 2002. Death of CD4⁺ T-cell lines caused by human immunodeficiency virus type 1 does not depend on caspases or apoptosis. *J. Virol.* **76**:5094–5107.
- Bourbigot, S., H. Beltz, J. Denis, N. Morellet, B. P. Roques, Y. Mely, and S. Bouaziz. 2005. The C-terminal domain of the HIV-1 regulatory protein Vpr adopts an antiparallel dimeric structure in solution via its leucine-zipper-like domain. *Biochem. J.* **387**:333–341.
- Brenchley, J. M., T. W. Schacker, L. E. Ruff, D. A. Price, J. H. Taylor, G. J. Beilman, P. L. Nguyen, A. Khoruts, M. Larson, A. T. Haase, and D. C. Douek. 2004. CD4⁺ T-cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. *J. Exp. Med.* **200**:749–759.
- Cao, J., I. W. Park, A. Cooper, and J. Sodroski. 1996. Molecular determinants of acute single-cell lysis by human immunodeficiency virus type 1. *J. Virol.* **70**:1340–1354.
- Cavert, W., D. W. Notermans, K. Staskus, S. W. Wietgreffe, M. Zupancic, K. Gebhard, K. Henry, Z. Q. Zhang, R. Mills, H. McDade, C. M. Schuirth, J. Goudsmit, S. A. Danner, and A. T. Haase. 1997. Kinetics of response in lymphoid tissues to antiretroviral therapy of HIV-1 infection. *Science* **276**:960–964.
- Chang, F., F. Re, S. Sebastian, S. Sazer, and J. Luban. 2004. HIV-1 Vpr induces defects in mitosis, cytokinesis, nuclear structure, and centrosomes. *Mol. Biol. Cell* **15**:1793–1801.
- Chen, M., R. T. Elder, M. Yu, M. G. O’Gorman, L. Selig, R. Benarous, A. Yamamoto, and Y. Zhao. 1999. Mutational analysis of Vpr-induced G₂ arrest, nuclear localization, and cell death in fission yeast. *J. Virol.* **73**:3236–3245.
- Conti, L., G. Rainaldi, P. Matarrese, B. Varano, R. Rivabene, S. Columba, A. Sato, F. Belardelli, W. Malorni, and S. Gessani. 1998. The HIV-1 vpr protein acts as a negative regulator of apoptosis in a human lymphoblastoid T-cell line: possible implications for the pathogenesis of AIDS. *J. Exp. Med.* **187**:403–413.
- de Noronha, C. M., M. P. Sherman, H. W. Lin, M. V. Cavois, R. D. Moir, R. D. Goldman, and W. C. Greene. 2001. Dynamic disruptions in nuclear envelope architecture and integrity induced by HIV-1 Vpr. *Science* **294**:1105–1108.
- Di Marzio, P., S. Choe, M. Ebricht, R. Knoblauch, and N. R. Landau. 1995. Mutational analysis of cell cycle arrest, nuclear localization and virion packaging of human immunodeficiency virus type 1 Vpr. *J. Virol.* **69**:7909–7916.
- Felzien, L. K., C. Woffendin, M. O. Hottiger, R. A. Subbramanian, E. A. Cohen, and G. J. Nabel. 1998. HIV transcriptional activation by the accessory protein, VPR, is mediated by the p300 coactivator. *Proc. Natl. Acad. Sci. USA* **95**:5281–5286.
- Fukumori, T., H. Akari, S. Iida, S. Hata, S. Kagawa, Y. Aida, A. H. Koyama, and A. Adachi. 1998. The HIV-1 Vpr displays strong antiapoptotic activity. *FEBS Lett.* **432**:17–20.
- Fukumori, T., H. Akari, A. Yoshida, M. Fujita, A. H. Koyama, S. Kagawa, and A. Adachi. 2000. Regulation of cell cycle and apoptosis by human immunodeficiency virus type 1 Vpr. *Microbes Infect.* **2**:1011–1017.
- Gibbs, J. S., A. A. Lackner, S. M. Lang, M. A. Simon, P. K. Sehgal, M. D. Daniel, and R. C. Desrosiers. 1995. Progression to AIDS in the absence of a gene for vpr or vpx. *J. Virol.* **69**:2378–2383.
- Goh, W. C., M. E. Rogel, C. M. Kinsey, S. F. Michael, P. N. Fultz, M. A. Nowak, B. H. Hahn, and M. Emerman. 1998. HIV-1 Vpr increases viral expression by manipulation of the cell cycle: a mechanism for selection of Vpr in vivo. *Nat. Med.* **4**:65–71.
- Gummuru, S., and M. Emerman. 1999. Cell cycle- and Vpr-mediated regulation of human immunodeficiency virus type 1 expression in primary and transformed T-cell lines. *J. Virol.* **73**:5422–5430.
- Haase, A. T. 1999. Population biology of HIV-1 infection: viral and CD4⁺ T-cell demographics and dynamics in lymphatic tissues. *Annu. Rev. Immunol.* **17**:625–656.
- Hazenberg, M. D., D. Hamann, H. Schuitemaker, and F. Miedema. 2000. T-cell depletion in HIV-1 infection: how CD4⁺ T cells go out of stock. *Nat. Immunol.* **1**:285–289.
- He, J., S. Choe, R. Walker, P. Di Marzio, D. O. Morgan, and N. R. Landau. 1995. Human immunodeficiency virus type 1 viral protein R (Vpr) arrests cells in the G₂ phase of the cell cycle by inhibiting p34cdc2 activity. *J. Virol.* **69**:6705–6711.
- Heinzinger, N. K., M. I. Bukinsky, S. A. Haggerty, A. M. Ragland, V. Kewalramani, M. A. Lee, H. E. Gendelman, L. Ratner, M. Stevenson, and M. Emerman. 1994. The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in nondividing host cells. *Proc. Natl. Acad. Sci. USA* **91**:7311–7315.
- Henklein, P., K. Bruns, M. P. Sherman, U. Tessmer, K. Licha, J. Kopp, C. M. de Noronha, W. C. Greene, V. Wray, and U. Schubert. 2000. Functional and structural characterization of synthetic HIV-1 Vpr that transduces cells, localizes to the nucleus, and induces G₂ cell cycle arrest. *J. Biol. Chem.* **275**:32016–32026.
- Herbein, G., C. Van Lint, J. L. Lovett, and E. Verdin. 1998. Distinct mechanisms trigger apoptosis in human immunodeficiency virus type 1-infected and in uninfected bystander T lymphocytes. *J. Virol.* **72**:660–670.
- Ho, D. D., A. U. Neumann, A. S. Perelson, W. Chen, J. M. Leonard, and M. Markowitz. 1995. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. *Nature* **373**:123–126.
- Hoessel, R., S. Leclerc, J. A. Endicott, M. E. Nobel, A. Lawrie, P. Tunnah, M. Leost, E. Damiens, D. Marie, D. Marko, E. Niederberger, W. Tang, G. Eisenbrand, and L. Meijer. 1999. Indirubin, the active constituent of a Chinese antileukaemia medicine, inhibits cyclin-dependent kinases. *Nat. Cell Biol.* **1**:60–67.
- Jowett, J. B., V. Planelles, B. Poon, N. P. Shah, M. L. Chen, and I. S. Chen. 1995. The human immunodeficiency virus type 1 vpr gene arrests infected T cells in the G₂+M phase of the cell cycle. *J. Virol.* **69**:6304–6313.
- Kino, T., A. Gragerov, A. Valentin, M. Tsopanmihalou, G. Ilyina-Gragerova, R. Erwin-Cohen, G. P. Chrousos, and G. N. Pavlakis. 2005. Vpr protein of human immunodeficiency virus type 1 binds to 14-3-3 proteins and facilitates complex formation with Cdc25C: implications for cell cycle arrest. *J. Virol.* **79**:2780–2787.
- Kishi, M., Y. H. Zheng, M. K. Bahmani, K. Tokunaga, H. Takahashi, M. Kakinuma, P. K. Lai, M. Nonoyama, R. B. Luftig, and K. Ikuta. 1995. Naturally occurring accessory gene mutations lead to persistent human immunodeficiency virus type 1 infection of CD4-positive T cells. *J. Virol.* **69**:7507–7518.
- Kundu, M., S. Sharma, A. De Luca, A. Giordano, J. Rappaport, K. Khalili, and S. Amini. 1998. HIV-1 Tat elongates the G₁ phase and indirectly promotes HIV-1 gene expression in cells of glial origin. *J. Biol. Chem.* **273**:8130–8136.
- Laurent-Crawford, A. G., B. Krust, S. Muller, Y. Riviere, M. A. Rey-Cuille, J. M. Bechet, L. Montagnier, and A. G. Hovanessian. 1991. The cytopathic effect of HIV is associated with apoptosis. *Virology* **185**:829–839.
- Lenardo, M. J., S. B. Angleman, V. Bounkeua, J. Dimas, M. G. Duval, M. B. Graubard, F. Hornung, M. C. Selkirk, C. K. Speirs, C. Trageser, J. O. Orenstein, and D. L. Bolton. 2002. Cytopathic killing of peripheral blood CD4⁺ T lymphocytes by human immunodeficiency virus type 1 appears necrotic rather than apoptotic and does not require env. *J. Virol.* **76**:5082–5093.
- Lu, Y. L., P. Spearman, and L. Ratner. 1993. Human immunodeficiency virus type 1 viral protein R localization in infected cells and virions. *J. Virol.* **67**:6542–6550.
- Mahalingam, S., V. Ayyavoo, M. Patel, T. Kieber-Emmons, and D. B. Weiner. 1997. Nuclear import, virion incorporation, and cell cycle arrest/differentiation are mediated by distinct functional domains of human immunodeficiency virus type 1 Vpr. *J. Virol.* **71**:6339–6347.

37. Malumbres, M., R. Sotillo, D. Santamaria, J. Galan, A. Cerezo, S. Ortega, P. Dubus, and M. Barbacid. 2004. Mammalian cells cycle without the D-Type cyclin-dependent kinases Cdk4 and Cdk6. *Cell* **118**:493–504.
38. Mattapallil, J. J., D. C. Douek, B. Hill, Y. Nishimura, M. Martin, and M. Roederer. 2005. Massive infection and loss of memory CD4⁺ T cells in multiple tissues during acute SIV infection. *Nature* **434**:1093–1097.
39. McCune, J. M. 2001. The dynamics of CD4⁺ T-cell depletion in HIV disease. *Nature* **410**:974–979.
40. Metge, B., S. Ofori-Acquah, T. Stevens, and R. Balczon. 2004. Stat3 activity is required for centrosome duplication in Chinese hamster ovary cells. *J. Biol. Chem.* **279**:41801–41806.
41. Minemoto, Y., M. Shimura, Y. Ishizaka, Y. Masamune, and K. Yamashita. 1999. Multiple centrosome formation induced by the expression of Vpr gene of human immunodeficiency virus. *Biochem. Biophys. Res. Commun.* **258**:379–384.
42. Mochizuki, H., J. P. Schwartz, K. Tanaka, R. O. Brady, and J. Reiser. 1998. High-titer human immunodeficiency virus type 1-based vector systems for gene delivery into nondividing cells. *J. Virol.* **72**:8873–8883.
43. Morellet, N., S. Bouaziz, P. Petitjean, and B. P. Roques. 2003. NMR structure of the HIV-1 regulatory protein VPR. *J. Mol. Biol.* **327**:215–227.
44. Naldini, L., U. Blomer, F. H. Gage, D. Trono, and I. M. Verma. 1996. Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. *Proc. Natl. Acad. Sci. USA* **93**:11382–11388.
45. Nishino, Y., M. Kishi, M. Sumiya, K. Ogawa, A. Adachi, K. Maotani-Imai, S. Kato, K. Hirai, and K. Ikuta. 1991. Human immunodeficiency virus type 1 *vif*, *vpr*, and *vpu* mutants can produce persistently infected cells. *Arch. Virol.* **120**:181–192.
46. Nishino, Y., T. Myojin, M. Kamata, and Y. Aida. 1997. Human immunodeficiency virus type 1 Vpr gene product prevents cell proliferation on mouse NIH 3T3 cells without the G₂ arrest of the cell cycle. *Biochem. Biophys. Res. Commun.* **232**:550–554.
47. Nishizawa, M., M. Kamata, T. Mojin, Y. Nakai, and Y. Aida. 2000. Induction of apoptosis by the Vpr protein of human immunodeficiency virus type 1 occurs independently of G₂ arrest of the cell cycle. *Virology* **276**:16–26.
48. Paxton, W., R. I. Connor, and N. R. Landau. 1993. Incorporation of Vpr into human immunodeficiency virus type 1 virions: requirement for the p6 region of gag and mutational analysis. *J. Virol.* **67**:7229–7237.
49. Poon, B., J. B. Jowett, S. A. Stewart, R. W. Armstrong, G. M. Rishton, and I. S. Chen. 1997. Human immunodeficiency virus type 1 *vpr* gene induces phenotypic effects similar to those of the DNA alkylating agent, nitrogen mustard. *J. Virol.* **71**:3961–3971.
50. Popov, S., M. Rexach, G. Zybarth, N. Reiling, M. A. Lee, L. Ratner, C. M. Lane, M. S. Moore, G. Blobel, and M. Bukrinsky. 1998. Viral protein R regulates nuclear import of the HIV-1 pre-integration complex. *EMBO J.* **17**:909–917.
51. Rajan, D., S. Wildum, E. Rucker, M. Schindler, and F. Kirchhoff. 2006. Effect of R77Q, R77A and R80A changes in Vpr on HIV-1 replication and CD4 T-cell depletion in human lymphoid tissue ex vivo. *AIDS* **20**:831–836.
52. Re, F., D. Braaten, E. K. Franke, and J. Luban. 1995. Human immunodeficiency virus type 1 Vpr arrests the cell cycle in G₂ by inhibiting the activation of p34cdc2-cyclin B. *J. Virol.* **69**:6859–6864.
53. Rogel, M. E., L. I. Wu, and M. Emerman. 1995. The human immunodeficiency virus type 1 *vpr* gene prevents cell proliferation during chronic infection. *J. Virol.* **69**:882–888.
54. Roninson, I. B., E. V. Broude, and B. D. Chang. 2001. If not apoptosis, then what? Treatment-induced senescence and mitotic catastrophe in tumor cells. *Drug Resist. Update* **4**:303–313.
55. Sakai, K., J. Dimas, and M. J. Lenardo. 2006. The Vif and Vpr accessory proteins independently cause HIV-1-induced T-cell cytopathicity and cell cycle arrest. *Proc. Natl. Acad. Sci. USA* **103**:3369–3374.
56. Sarkaria, J. N., E. C. Busby, R. S. Tibbetts, P. Roos, Y. Taya, L. M. Karnitz, and R. T. Abraham. 1999. Inhibition of ATM and ATR kinase activities by the radiosensitizing agent, caffeine. *Cancer Res.* **59**:4375–4382.
57. Savarino, A., C. Martini, G. C. Orofino, C. Cantamessa, L. Castelli, P. G. Pich, A. Sinicco, and A. Pugliese. 1997. Apoptotic DNA fragmentation, and its in vitro prevention by nicotinamide, in lymphocytes from HIV-1-seropositive patients and in HIV-1-infected MT-4 cells. *Cell Biochem. Funct.* **15**:171–179.
58. Schuler, W., K. Wecker, H. de Rocquigny, Y. Baudat, J. Sire, and B. P. Roques. 1999. NMR structure of the (52-96) C-terminal domain of the HIV-1 regulatory protein Vpr: molecular insights into its biological functions. *J. Mol. Biol.* **285**:2105–2117.
59. Siegel, R. M., F. K. Chan, D. A. Zacharias, R. Swofford, K. L. Holmes, R. Y. Tsien, and M. J. Lenardo. 2000. Measurement of molecular interactions in living cells by fluorescence resonance energy transfer between variants of the green fluorescent protein. *Sci. STKE* **2000**:PL1.
60. Stewart, S. A., B. Poon, J. B. Jowett, and I. S. Chen. 1997. Human immunodeficiency virus type 1 Vpr induces apoptosis following cell cycle arrest. *J. Virol.* **71**:5579–5592.
61. Stewart, S. A., B. Poon, J. B. Jowett, Y. Xie, and I. S. Chen. 1999. Lentiviral delivery of HIV-1 Vpr protein induces apoptosis in transformed cells. *Proc. Natl. Acad. Sci. USA* **96**:12039–12043.
62. Stivahtis, G. L., M. A. Soares, M. A. Vodicka, B. H. Hahn, and M. Emerman. 1997. Conservation and host specificity of Vpr-mediated cell cycle arrest suggest a fundamental role in primate lentivirus evolution and biology. *J. Virol.* **71**:4331–4338.
63. Su, H., N. Bidere, L. Zheng, A. Cubre, K. Sakai, J. Dale, L. Salmena, R. Hakem, S. Straus, and M. Lenardo. 2005. Requirement for caspase-8 in NF- κ B activation by antigen receptor. *Science* **307**:1465–1468.
64. Su, L., and M. David. 2000. Distinct mechanisms of STAT phosphorylation via the interferon- α /beta receptor: selective inhibition of STAT3 and STAT5 by piceatannol. *J. Biol. Chem.* **275**:12661–12666.
65. Terai, C., R. S. Kornbluth, C. D. Pauza, D. D. Richman, and D. A. Carson. 1991. Apoptosis as a mechanism of cell death in cultured T lymphoblasts acutely infected with HIV-1. *J. Clin. Invest.* **87**:1710–1715.
66. Tristem, M., A. Purvis, and D. L. Quicke. 1998. Complex evolutionary history of primate lentiviral *vpr* genes. *Virology* **240**:232–237.
67. Vodicka, M. A., D. M. Koepp, P. A. Silver, and M. Emerman. 1998. HIV-1 Vpr interacts with the nuclear transport pathway to promote macrophage infection. *Genes Dev.* **12**:175–185.
68. Wang, L., S. Mukherjee, F. Jia, O. Narayan, and L. J. Zhao. 1995. Interaction of virion protein Vpr of human immunodeficiency virus type 1 with cellular transcription factor Sp1 and transactivation of viral long terminal repeat. *J. Biol. Chem.* **270**:25564–25569.
69. Watanabe, N., T. Yamaguchi, Y. Akimoto, J. B. Rattner, H. Hirano, and H. Nakauchi. 2000. Induction of M-phase arrest and apoptosis after HIV-1 Vpr expression through uncoupling of nuclear and centrosomal cycle in HeLa cells. *Exp. Cell Res.* **258**:261–269.
70. Wei, X., S. K. Ghosh, M. E. Taylor, V. A. Johnson, E. A. Emini, P. Deutsch, J. D. Lifson, S. Bonhoeffer, M. A. Nowak, B. H. Hahn, et al. 1995. Viral dynamics in human immunodeficiency virus type 1 infection. *Nature* **373**:117–122.
71. Yao, X. J., A. J. Moulard, R. A. Subbramanian, J. Forget, N. Rougeau, D. Bergeron, and E. A. Cohen. 1998. Vpr stimulates viral expression and induces cell killing in human immunodeficiency virus type 1-infected dividing Jurkat T cells. *J. Virol.* **72**:4686–4693.
72. Zhang, Y. J., B. Fadeel, V. Hodara, and E. M. Fenyo. 1997. Induction of apoptosis by primary HIV-1 isolates correlates with productive infection in peripheral blood mononuclear cells. *AIDS* **11**:1219–1225.
73. Zhao, L. J., L. Wang, S. Mukherjee, and O. Narayan. 1994. Biochemical mechanism of HIV-1 Vpr function. Oligomerization mediated by the N-terminal domain. *J. Biol. Chem.* **269**:32131–32137.
74. Zhou, Y., and L. Ratner. 2000. Phosphorylation of human immunodeficiency virus type 1 Vpr regulates cell cycle arrest. *J. Virol.* **74**:6520–6527.
75. Zhu, Y., H. A. Gelbard, M. Roshal, S. Pursell, B. D. Jamieson, and V. Planellas. 2001. Comparison of cell cycle arrest, transactivation, and apoptosis induced by the simian immunodeficiency virus SIVagm and human immunodeficiency virus type 1 *vpr* genes. *J. Virol.* **75**:3791–3801.