

Human Immunodeficiency Virus Type 1 Vpr Induces Apoptosis through Caspase Activation

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Human immunodeficiency virus type 1 (HIV-1) Vpr is a 96-amino-acid protein that is found associated with the HIV-1 virion. Vpr induces cell cycle arrest at the G₂/M phase of the cell cycle, and this arrest is followed by apoptosis. We examined the mechanism of Vpr-induced apoptosis and found that HIV-1 Vpr-induced apoptosis requires the activation of a number of cellular cysteinyl aspartate-specific proteases (caspases). We demonstrate that ectopic expression of anti-apoptotic viral proteins, which inhibit caspase activity, and addition of synthetic peptides, which represent caspase cleavage sites, can inhibit Vpr-induced apoptosis. Finally, inhibition of caspase activity and subsequent inhibition of apoptosis results in increased viral expression, suggesting that therapeutic strategies aimed at reducing Vpr-induced apoptosis in vivo require careful consideration.

Human immunodeficiency virus type 1 (HIV-1) is a member of the lentivirus family and is the etiologic agent of AIDS. The HIV-1 genome encodes the *gag*, *pol*, and *env* genes, which are common to all members of the retrovirus family, and two regulatory genes, *tat* and *rev*. In addition to these genes, there are a number of accessory genes encoded in the HIV-1 genome that are dispensable for in vitro replication but necessary, albeit to differing degrees, for pathogenesis (for a review, see reference 7).

One HIV-1 accessory gene encodes a 96-amino-acid protein termed Vpr. Analysis of the HIV-1 virion has demonstrated that Vpr is found associated with the viral particle through an association with the Gag p6 molecule (31, 33, 58, 60). The presence of Vpr in the virion has suggested that Vpr has a role early in infection, prior to viral gene expression. One possible role early in infection may be nuclear translocation of the preintegration complex. Studies of Vpr mutants have demonstrated that Vpr is capable of translocating the preintegration complex to the nucleus, and studies in terminally differentiated macrophages have shown that this function is required for productive infection (12, 15, 16, 21, 33, 54). In addition, Vpr in conjunction with the related *vpx* gene is necessary for disease progression in simian immunodeficiency virus-infected macaques (17).

In addition to Vpr's role in disease and nuclear translocation, a number of in vitro functions have been ascribed to Vpr over the past few years. Vpr was initially shown to induce differentiation of rhabdomyosarcoma cells and was later shown to induce cell cycle arrest at the G₂ phase of the cell cycle (3, 20, 25, 29, 45, 46). Vpr has been shown to increase viral transcription (8, 18, 40, 50), and it was recently suggested that this occurs through manipulation of the cell cycle (50). Vpr has also

been shown to induce apoptosis following G₂ arrest and to retard tumor cell growth in mice (32, 48, 57).

The mechanism by which Vpr induces G₂ arrest and apoptosis is unclear. A number of Vpr-interacting proteins have been defined, but their role in G₂ arrest, if any, remains to be determined. One protein of interest is the human homolog of Rad23A (HHR23A) which was shown to interact with Vpr in vivo and to alleviate Vpr-induced G₂ arrest in transient transfection assays (56). However, what function Vpr-HHR23A interaction plays in viral replication is unknown. One noteworthy aspect of Vpr-induced G₂ arrest is that the susceptibility to G₂ arrest is conserved from budding yeast to humans (61), indicating that Vpr perturbs a highly conserved signal transduction pathway(s).

HIV-1 infection results in elimination of the CD4 cell population within the infected individual. Some studies have suggested that direct viral infection of T cells may be responsible for much of the CD4 cell loss in infected individuals (24, 41, 55). Precisely how HIV-1 kills cells in vivo is still an area of debate; however, apoptosis appears to be one mechanism for HIV-1-induced cell death. Comparisons between normal donors and HIV-1-infected donors indicated that in vitro stimulation of cells from AIDS patients results in increased apoptosis (19, 36). In addition, comparison of cells from acutely infected patients versus asymptomatic patients revealed an increase in apoptotic cell death in cells from the acutely infected patients, presumably due to higher viral titers (36). In addition, in vitro studies demonstrated that HIV-1 infection of T cells results in apoptosis (27, 52).

Apoptosis is an ordered suicide mechanism that is characterized by cell shrinkage, loss of membrane integrity, chromosome condensation, and internucleosomal cleavage of DNA (reviewed in reference 35). Studies of the mechanisms responsible for apoptosis have revealed that the cysteinyl aspartate-specific protease (caspase) family constitutes the effector arm of apoptosis. The caspases are characterized by a cysteine located within their active site. Following activation of the caspases, they recognize specific sequences within their various target proteins and characteristically cleave these proteins 3' of an aspartic acid residue within the recognition sequence (reference 37 and references therein). Over 10 caspases have been

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identified thus far and have been shown to differ with regard to the stimuli that activate them and to their substrate specificity and sensitivity to various inhibitors.

A number of apoptotic stimuli, including viral infection, have been identified which result in the activation of various caspase cascades (51; reference 53 and references therein). Therefore, apoptosis is an important cellular defense against viral infection. Because of this, a number of viruses have evolved genes encoding proteins which inhibit caspase activation, presumably because this is where most apoptotic pathways converge. Therefore, by inhibiting caspase activation and the subsequent abrogation of apoptosis viral production is preserved.

In this study, we show that Vpr is sufficient to induce caspase activation. Both viral caspase inhibitors and synthetic peptides representing caspase cleavage sites were capable of inhibiting Vpr-induced apoptosis. Finally, we demonstrate that inhibition of Vpr-induced apoptosis results in an increase in viral production.

MATERIALS AND METHODS

Preparation of viral stocks. 293T cells were maintained in Dulbecco modified Eagle medium (DMEM) plus 10% bovine calf serum (CS) (Gibco-BRL, Grand Island, N.Y.) at 37°C and 5% CO₂. For production of HIV-1_{NL4-3}Thyenv(-)/VSV-G and of HIV-1_{NL4-3}Thyenv(-)VprX/VSV-G viruses, 293T cells were transfected with 12.5 µg of NL4-3ThyΔBgl or NL4-3ThyΔBglVprX plasmid DNA and 5 µg of pCMVΔR8.2ΔVpr by a modified calcium phosphate method (46). HR'Thy and HR'Vpr virus stocks were produced by cotransfection of 293T cells with 12.5 µg of pHR'Thy or pHR'Vpr, 12.5 µg of pCMVΔR8.2ΔVpr, and 5 µg of pCMVΔR8.2ΔVpr. Following an 8-h transfection, cells were washed and the media was replaced. At 48 h and 72 h posttransfection, supernatants were collected and pooled. Viral stocks were concentrated as previously described (13, 48, 59). Briefly, supernatants were centrifuged for 5 min at 1,900 × g, were passed through a 0.45-µm-pore-size filter, and were ultracentrifuged at 25,000 × g for 90 min. Supernatants were removed, and the viral pellet was resuspended in DMEM plus 10% CS overnight at 4°C. Viral stocks were stored in the presence of 10% fetal calf serum (FCS) at -70°C.

DNA constructs. The NL4-3ThyΔBgl, NL4-3ThyΔBglVprX, and pCMV VSV-G constructs have been described previously (48). pCMVΔR8.2ΔVpr was derived from pCMVΔR8.2 by PCR mutagenesis, resulting in the introduction of stop codons within the Vpr open reading frame (1, 42). pHR'Thy and pHR'Vpr were derived from pHR'LucZ (39, 48). The baculovirus inhibitor of apoptosis (IAP) and p35 genes were cloned into pXC which was previously described (34). pEFFlagcmApGKpuropA, pCGN-IE1, and pCGN-IE2 were described previously (49, 63).

Infection of HeLa cells. HeLa cells were maintained in DMEM plus 10% CS. Cells were mock infected or infected with HIV-1_{NL4-3}Thyenv(-)/VSV-G, HIV-1_{NL4-3}Thyenv(-)VprX/VSV-G, HR'Thy(Vpr-), or HR'Vpr for 4 h in the presence of 10 µg of polybrene per ml. Infectious units were determined by measuring the titers of concentrated virus stocks in HeLa cells and analyzing infection efficiencies by Thy 1.2 staining for HIV-1_{NL4-3}Thyenv(-)/VSV-G, HIV-1_{NL4-3}Thyenv(-)VprX/VSV-G, or HR'Thy(Vpr-) and measuring hemagglutinin (HA)-staining for HR'Vpr.

Transfection of HeLa cells. HeLa cells were incubated in 1.2× RPMI medium containing 20% FCS and 10 µg of DNA for 20 min on ice. Following incubation, cells were electroporated at 250 V and were incubated on ice for an additional 20 min. Transfected cells were plated in either 6- or 12-well plates. Media was changed 24 h posttransfection.

Peptide inhibitors. The apoptosis peptide inhibitor was resuspended in dimethyl sulfoxide (DMSO). The peptide inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk) was added fresh every 24 h (Enzyme Systems, Livermore, Calif.).

Analysis of DNA content within transfected and infected cells. Transfection and infection efficiencies were monitored by flow cytometry. For analysis of HeLa cells, both attached and floating cells were collected and analyzed. Cells (10⁵ to 2 × 10⁶) infected with HIV-1_{NL4-3}Thyenv(-)/VSV-G, HIV-1_{NL4-3}Thyenv(-)VprX/VSV-G, or HR'Thy(Vpr-) were stained with anti-Thy 1.2-fluorescein isothiocyanate (FITC) conjugate (Caltag, South San Francisco, Calif.) for 20 min on ice. Stained cells were washed once with fluorescence-activated cell sorter (FACS) buffer (phosphate-buffered saline [PBS], 2% FCS, and 0.2% sodium azide), and were resuspended in hypotonic propidium iodide (PI) (2.0 µg of RNAase A per ml, 100 µg of PI per ml, 0.3% Triton X-100, 1 µg of sodium citrate per ml [Sigma, St. Louis, Mo.]). Stained cells were acquired on a FACScan and were analyzed by the Lysis II software package.

Cells (10⁵ to 2 × 10⁶) infected with HR'Vpr were fixed with 4%

paraformaldehyde in PBS for 20 min on ice. Following fixation, cells were permeabilized with 0.1% Triton X-100 (Sigma) for 15 min on ice. Fixed cells were stained with an anti-HA antibody (12CA5) followed by staining with a secondary goat anti-mouse-FITC antibody (Molecular Probes, Eugene, Oreg.). After the antibody stain, cells were resuspended in DNA stain buffer (10 µg of PI per ml and 11.25 kunits of RNAase in FACS buffer). Stained cells were acquired on a FACScan and were analyzed by the Lysis II software package.

Cells transfected with pXC-IAP, pCGN-IE1, and pCGN-IE2 were treated in the same manner as HR'Vpr-infected cells. IAP was detected by staining with the anti-HA antibody as described above. pCGN-IE1- and pCGN-IE2-transfected cells were also treated in the same manner, but both proteins were detected with MAB810, a monoclonal antibody that recognizes both proteins (Chemicon, Temecula, Calif.).

Flow cytometric analysis of apoptotic cells. The Annexin V assay was carried out as recommended by the manufacturer, and Thy 1.2 staining was used to monitor infection efficiencies (14, 48). Briefly, 10⁵ to 1.5 × 10⁶ cells were resuspended in Annexin V buffer (2.5 µg of Annexin V-FITC per ml, 10 mM HEPES-NaOH [pH 7.4], 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, and 3.6 µg of actinomycin D, 7-amino [7-AAD] per ml) (Biosource, Camarillo, Calif.). The percentages of apoptotic cells reported in each figure were defined as cells that were both Annexin V positive and 7-AAD negative. Stained cells were acquired on a FACScan and were analyzed by the Lysis II software package.

RESULTS

HIV-1 Vpr is sufficient to induce apoptosis. We previously demonstrated that both transient transfection of an HIV-1 Vpr-expressing plasmid and infection with a pseudotyped HIV-1 virus, HIV-1_{NL4-3}Thyenv(-)/VSV-G, resulted in apoptosis (48). Furthermore, infection with a virus carrying a frameshift mutation in *vpr*, HIV-1_{NL4-3}Thyenv(-)VprX/VSV-G, also induced apoptosis, albeit at a lower percentage than that observed with HIV-1_{NL4-3}Thyenv(-)/VSV-G, suggesting that other viral gene products influenced the induction of apoptosis (48). We determined whether Vpr expressed during a viral infection, in the absence of any other viral components, was capable of inducing apoptosis. Two retroviral vectors, HR'Vpr and HR'Thy(Vpr-), were designed, expressing Vpr and the murine Thy 1.2 cell surface marker, respectively. HeLa cells were infected with HIV-1_{NL4-3}Thyenv(-)/VSV-G, HIV-1_{NL4-3}ThyenvVprX/VSV-G, HR'Vpr, or HR'Thy(Vpr-) and were analyzed for apoptosis by Annexin V staining. Annexin V specifically binds to phosphatidylserine, which is normally located in the plasma membrane. During the early stages of apoptosis, phosphatidylserine becomes exposed and is bound by Annexin V. Subsequent staining with a dead cell exclusion dye such as 7-AAD allows live, dead, and apoptotic cells to be distinguished by flow cytometry (14) (Fig. 1A).

Annexin V analysis of HIV-1_{NL4-3}Thyenv(-)/VSV-G-infected cells from five independent experiments consistently had significantly increased levels of Annexin V-positive cells compared to HIV-1_{NL4-3}Thyenv(-)VprX/VSV-G-infected cells, which in turn had increased levels relative to mock-infected cells (Fig. 1B). The variation in the levels of apoptosis observed was due to the percentage of cells infected in each independent experiment. This observation confirmed previous studies that indicated that the presence of Vpr increased the levels of apoptosis induced by infection with HIV-1 (48). We have previously reported that HIV-1_{NL4-3}Thyenv(-)VprX/VSV-G induces some apoptosis but significantly less than HIV-1_{NL4-3}Thyenv(-)/VSV-G (48), presumably due to the presence of other viral gene products, such as Tat, which have also been reported to induce apoptosis. Therefore, to determine whether Vpr alone was sufficient to induce apoptosis during a viral infection, we also analyzed cells infected with HR'Thy(Vpr-) and HR'Vpr. Infection with HR'Vpr resulted in arrest in the G₂/M phase of the cell cycle while HR'Thy(Vpr-) infected cells had a similar cell cycle profile as mock-infected cells (data not shown). Infection with HR'Vpr resulted in a significant increase in the levels of apoptosis com-

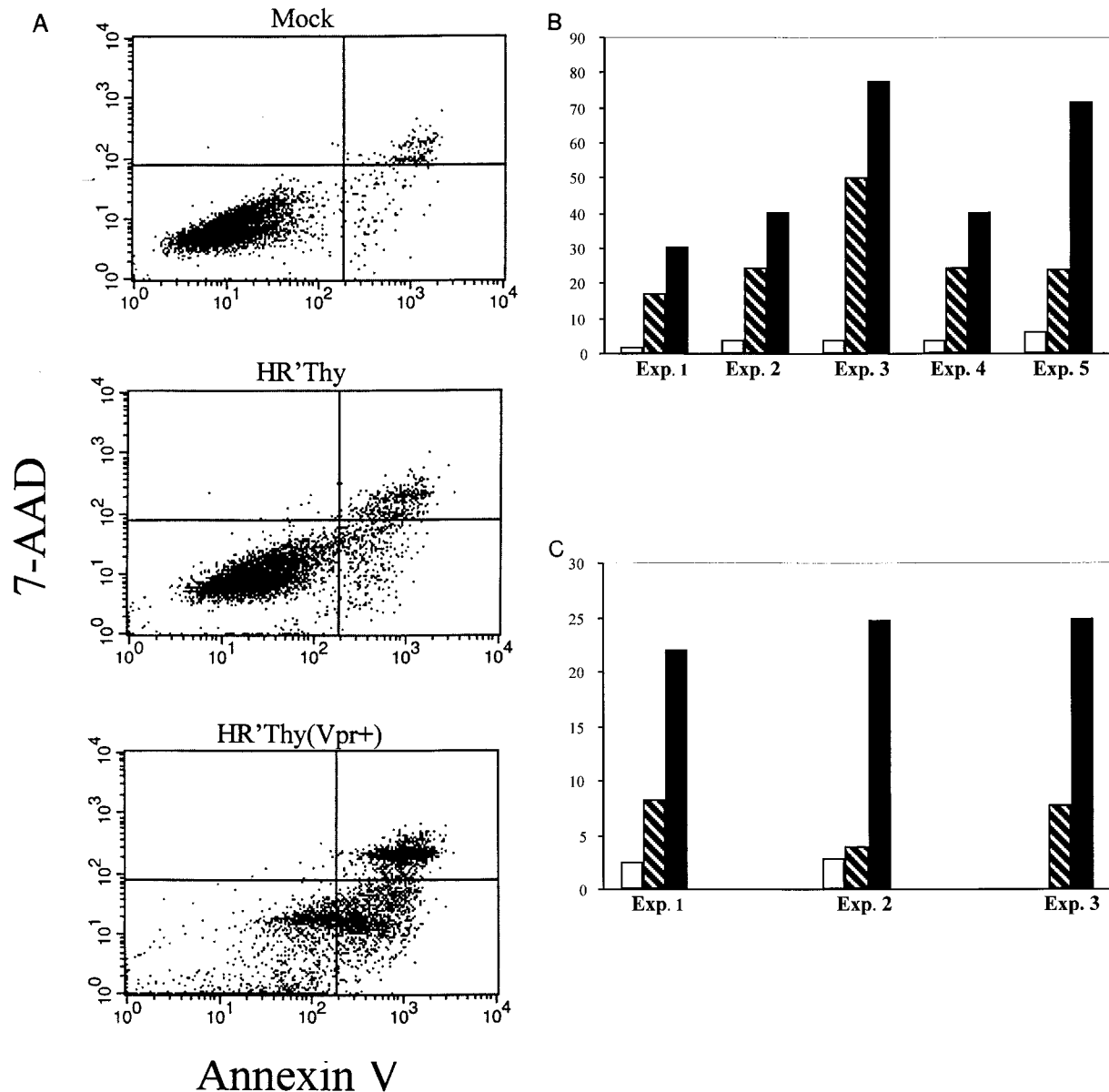


FIG. 1. Vpr expressed from the retroviral vector HR'Vpr is sufficient to induce apoptosis. (A) Representative diagram of Annexin V staining of mock-, HR'Thy-, and HR'Thy (Vpr⁺)-infected HeLa cells. Fluorescence intensity of Annexin-V-FITC is represented on the x axis, and fluorescence intensity of 7-AAD is represented on the y axis. The population in the lower left quadrant represents the live cells, the cells in the upper right quadrant represent the dead cell population, and the lower right quadrant represents the apoptotic cell population. (B) Bar graph represents five independent experiments showing the percentages of mock- (open bars), HIV-1_{NL4-3}Thyenv(-)/VprX/VSV-G- (hatched bars), and HIV-1_{NL4-3}Thyenv(-)/VSV-G-infected cells (filled bars) staining Annexin V positive 48 h postinfection. Values obtained with HIV-1_{NL4-3}Thyenv(-)/VSV-G were significantly different from those obtained with HIV-1_{NL4-3}Thyenv(-)/VprX/VSV-G, which in turn were higher than those obtained from media alone ($P < 0.0001$ by Page's test for ordered alternatives in a two-way layout) (22). (C) Bar graph represents three independent experiments showing the percentages of mock- (open bars), HR'Thy(Vpr-) (hatched bars), and HR'Vpr-infected cells (filled bars) staining Annexin V positive 72 h postinfection. Values obtained with HR'Vpr were significantly higher than those obtained with HR'Thy(Vpr-), which in turn were higher than those obtained with media alone ($P < 0.0001$ by Skillings-Wolf nonparametric test for ordered alternatives in a two-way unbalanced layout) (22).

pared to infection with HR'Thy(Vpr-), which in turn had increased levels relative to mock-infected cells (Fig. 1C). Similar to the results shown in Fig. 1B, the levels of apoptosis observed were dependent on the percentage of cells infected. These data indicate that Vpr produced from a retroviral vector is sufficient to induce apoptosis.

Viral caspase inhibitors modulate Vpr-induced apoptosis. Induction of apoptosis by HIV-1 Vpr occurs through an unknown mechanism. Therefore, to define which pathways may be involved in Vpr-induced apoptosis, we tested the ability of

a number of viral genes to inhibit Vpr-induced apoptosis. HeLa cells were transfected with expression constructs expressing baculovirus p35, cowpox virus CrmA, or human cytomegalovirus IE1 or IE2. All of these gene products negatively effect the induction of apoptosis at a variety of levels. CrmA and p35 are known to function as substrates for many of the caspases. IE1 and IE2 inhibit tumor necrosis factor alpha-induced apoptosis (6, 10, 26, 34, 43, 62, 63).

Following transfection with the various anti-apoptotic viral genes, HeLa cells were infected with HIV-1_{NL4-3}Thyenv(-)/

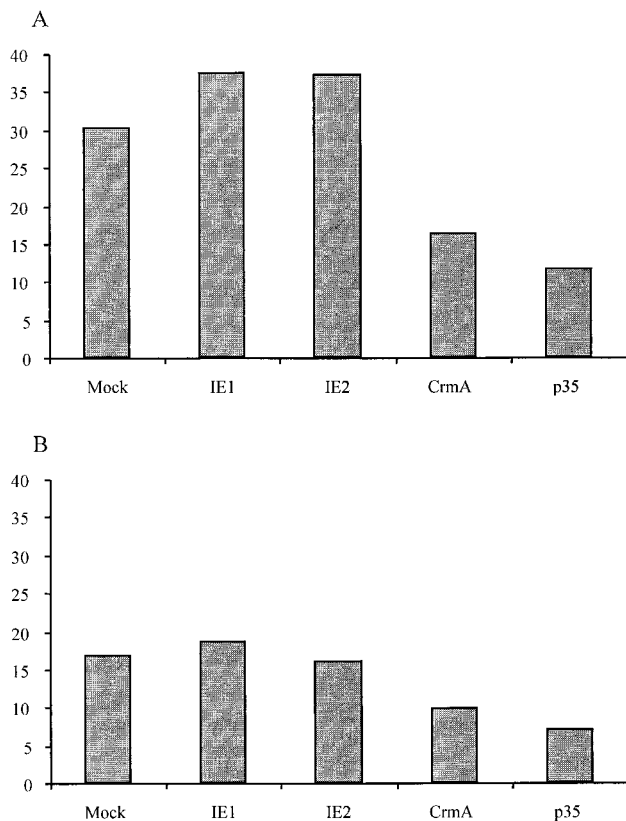


FIG. 2. CrmA and p35 reduce Vpr-induced apoptosis. Transfection efficiencies were analyzed by flow cytometry at 48 h posttransfection. The data shown is representative of three independent experiments. (A) Bar graph representing Annexin V staining of cells transfected with the indicated viral anti-apoptosis gene and infected with HIV-1_{NL4-3}Thyenv(-)/VSV-G. The percentage of Annexin V staining is represented on the y axis. The data indicated are results from 72 h posttransfection and 48 h postinfection. (B) Bar graph representing Annexin V staining of cells transfected with the indicated viral anti-apoptosis gene and infected with HIV-1_{NL4-3}Thyenv(-)VprX/VSV-G. The percentage of Annexin V staining is represented on the y axis. The data indicated are results from 72 h posttransfection and 48 h postinfection.

VSV-G encoding wild-type Vpr or HIV-1_{NL4-3}Thyenv(-)VprX/VSV-G encoding a frameshift mutant of Vpr. Thy 1.2 staining of HIV-1_{NL4-3}Thyenv(-)/VSV-G- and HIV-1_{NL4-3}Thyenv(-)VprX/VSV-G-infected cells at 24 h postinfection indicated that 90 to 96% of the cells were productively infected regardless of the presence of the anti-apoptotic genes (data not shown). Transfected and infected HeLa cells were analyzed for DNA content and Annexin V expression 72 h posttransfection and 48 h postinfection. DNA analysis of transfected cells indicated that the anti-apoptotic genes alone did not significantly alter the cell cycle profile (data not shown). As previously reported, infection with HIV-1_{NL4-3}Thyenv(-)VprX/VSV-G did not result in significant alteration of the cell cycle (data not shown). In contrast, infection with HIV-1_{NL4-3}Thyenv(-)/VSV-G resulted in G₂ arrest, regardless of the presence of the anti-apoptotic genes (data not shown). Annexin V analysis at 48 h postinfection revealed that infection with HIV-1_{NL4-3}Thyenv(-)/VSV-G resulted in increased Annexin V-positive cells versus mock-infected cells. Transfection with IE1 or IE2 did not have a significant effect on Vpr-induced apoptosis (Fig. 2A). In contrast, transfection of HeLa cells with p35 and *crmA* resulted in a reduction in apoptosis (in the representative experiment shown in Fig. 2A, 12.0 and

16.5%, respectively, compared with 30.4% in mock-treated HIV-1_{NL4-3}Thyenv(-)/VSV-G-infected cells) while having little effect on HIV-1_{NL4-3}Thyenv(-)VprX/VSV-G-infected cells (Fig. 2B). This experiment suggested that inhibition of cellular caspases could protect cells from Vpr-induced apoptosis while other apoptosis effector proteins, such as IE1 and IE2, had no effect on Vpr-induced apoptosis. In addition, since these studies show that p35 and CrmA can inhibit apoptosis without altering G₂ arrest (data not shown), the mechanism of action is independent of the cell cycle.

A synthetic caspase inhibitor can reduce Vpr-induced apoptosis. The experiments described above indicated the involvement of caspase-dependent apoptosis following HIV-1 infection. There are a number of cellular caspases involved in the induction of apoptosis (reference 37 and references therein). To more directly determine whether caspases were activated during Vpr-induced apoptosis, we treated infected HeLa cells with the synthetic caspase inhibitor, z-VAD-fmk. The studies described above (Fig. 1) indicated that Vpr expressed from a retroviral vector was sufficient to induce apoptosis in infected HeLa cells. Therefore, we examined the effect of z-VAD-fmk specifically upon Vpr-mediated apoptosis in the absence of other viral components by infecting HeLa cells with HR'Vpr which expresses wild-type Vpr. DNA profiles of cells infected with the control vector without Vpr, HR'Thy(Vpr-), were similar to the profiles of mock-infected cells. In contrast, cells infected with HR'Vpr were arrested in the G₂/M phase of the cell cycle, and treatment with DMSO or the peptide inhibitor z-VAD-fmk did not significantly affect this induction of G₂/M arrest by Vpr (data not shown).

In contrast to G₂ arrest, Annexin V analysis of infected cells demonstrated that the peptide inhibitor did affect Vpr-induced apoptosis. Peptide treatment of both HR'Thy(Vpr-)-infected and mock-infected cells had little effect on the percentage of Annexin V-positive cells (data not shown). Control treated cells showed no effect on Annexin V staining at 72 h postinfection. In contrast, treatment of HR'Vpr-infected cells with z-VAD-fmk resulted in a decrease of Annexin V-positive cells (Fig. 3), indicating that z-VAD-fmk can reduce Vpr-induced apoptosis.

Caspase inhibitors inhibit Vpr-induced apoptosis in SupT1 cells. Our previous results demonstrate that Vpr-induced apoptosis could be inhibited in HeLa cells by the addition of the peptide inhibitor z-VAD-fmk. To extend these studies to human T cells, we either mock infected SupT1 cells or infected them with HR'Vpr in the presence or absence of z-VAD-fmk and analyzed the cells for apoptosis. SupT1 cells infected with HR'Vpr arrested in G₂ (data not shown) and underwent apoptosis (Fig. 4). Treatment of HR'Vpr-infected SupT1 cells with z-VAD-fmk resulted in a significant reduction in the levels of apoptosis (Fig. 4) without affecting the percentage of cells arrested in G₂ (data not shown). This experiment shows that Vpr-induced apoptosis in human T cells occurs through the activation of caspases.

Reduction of Vpr-induced apoptosis by caspase inhibitors increases viral production. Previous studies by Chinnaiyan et al. examined viral production from a spreading HIV-1 infection and indicated that the inhibition of HIV-1-induced apoptosis leads to higher viral production over time (5). Since many additional factors such as viral envelope, processing, and packaging may be affected in a spreading infection, we examined whether inhibition of Vpr-induced apoptosis, in the context of a nonspreading viral infection, similarly increased viral production.

Infection of HeLa cells with HIV-1_{NL4-3}Thyenv(-)/VSV-G

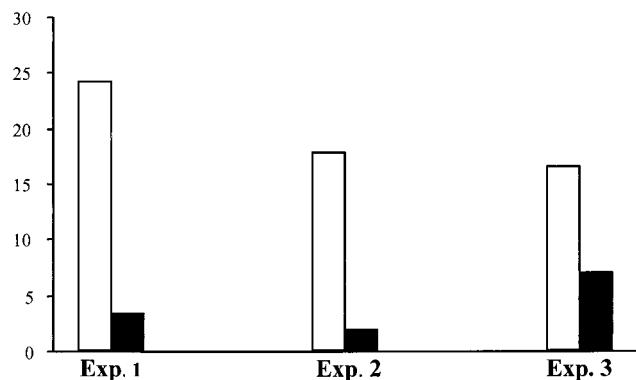


FIG. 3. Synthetic peptide inhibitor of apoptosis has no significant effect on cell cycle arrest but does reduce the amount of Vpr-induced apoptosis. HeLa cells (10^6) were mock infected or infected with HR'Vpr. Following infection, cells were washed and 200 μ M z-VAD-fmk was added (filled bars). In addition, one set of infected cultures (open bars) received DMSO alone, which was used to dissolve the inhibitors. Fresh inhibitor was added every 24 h throughout the experiment. Infected cells were analyzed 72 h postinfection. Data shown is representative of three independent experiments.

resulted in the infection of 59.7% of the cells (data not shown). At 72 h postinfection, HIV-1_{NL4-3}Thyenv(-)/VSV-G-infected cells treated with either DMSO diluent as a control or z-VAD-fmk were analyzed for apoptosis by Annexin V staining. In agreement with the above studies, treatment of infected cells with synthetic peptides reduced the amount of apoptosis as measured by Annexin V staining (Fig. 5A). Analysis of Gag p24 production at 84 h postinfection showed that p24 production was inversely related to the percentage of Annexin V-positive cells (compare 72.5 ng/ml in DMSO-treated cells to 147.7 ng/ml in the z-VAD-fmk-treated cells [Fig. 5B]). These data indicate that p24 production is closely correlated with survival of the cells and that induction of apoptosis by Vpr results in reduced p24 production.

DISCUSSION

We demonstrate that HIV-1 Vpr-induced apoptosis occurs through the activation of cellular caspases. Furthermore, inhibition of caspase activation by either viral caspase inhibitors or synthetic peptide inhibitors inhibits Vpr-induced apoptosis. Finally, inhibition of Vpr-induced apoptosis by synthetic peptides results in an increase in viral gene production.

Chinnaiyan et al. had previously shown that treatment of HIV-1-infected peripheral blood mononuclear cells with z-VAD-fmk enhanced viral replication (5). Similarly, we have shown that treatment of infected cells with z-VAD-fmk increased the amount of viral p24 detected within the supernatants. The relative increase in the amount of p24 directly correlated with the ability of the synthetic peptides to inhibit apoptosis. These studies indicate that the level of p24 production during an HIV-1 infection increases with inhibition of apoptosis.

The cellular caspases are the effector arm of apoptosis. Cells receive an apoptotic stimulus which transduces a signal that converges on the caspase pathway. Once activated, these proteases cleave a variety of critical cellular proteins, such as poly-ADP-ribose polymerase and laminin, which results in apoptosis (reviewed in reference 35). We have examined Vpr-induced apoptosis and found that Vpr stimulates caspase activation. Both viral protein inhibitors and synthetic peptide inhibitors were capable of inhibiting Vpr-induced apoptosis.

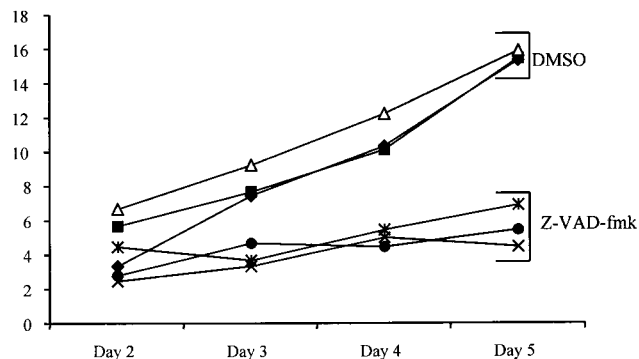


FIG. 4. Synthetic caspase inhibitor protects human T cells from Vpr-induced apoptosis. SupT1 cells (4×10^5) were mock infected or infected with HR'Vpr for 4 h in the presence of 10 μ g of polybrene per ml. Following infection, cells were washed and DMSO or 100 μ M z-VAD-fmk, dissolved in DMSO, was added. Fresh DMSO or z-VAD-fmk was added every 24 h for the duration of the experiment. The experiment was carried out in triplicate, and percentage of apoptosis is plotted against time for HR'Vpr-infected cells treated with either DMSO or z-VAD-fmk as indicated. The difference between estimated mean response at day 3 is significantly lower for z-VAD treatment than for treatment with DMSO ($P < 0.005$ by testing contrasts for fixed effects in a mixed linear model for repeated measurements) (30).

This observation suggests that Vpr interacts with a component(s) within the cell that results in activation of the caspase cascade either directly or indirectly. It was interesting to note that the more general synthetic inhibitor, z-VAD-fmk, resulted in greater reduction of apoptosis than other synthetic inhibitors with more-restricted action (data not shown). Similarly, baculovirus p35 demonstrates the most widespread activity against the caspase family members (51), and, in our assays, it is also the most active against Vpr-induced apoptosis (51). The activity of CrmA, on the other hand, was shown to be more restricted to inhibition of caspases one and eight (62), and analysis of Vpr-induced apoptosis demonstrated that the inhibition of Vpr-induced apoptosis by CrmA was weaker than inhibition by p35. This observation suggests that the action of Vpr results in the activation of a number of cellular caspases.

Our studies demonstrating Vpr-induced apoptosis are in contrast to those by Ayyavoo et al. and Conti et al. who showed that Vpr protects cells from apoptosis under some circumstances (2, 9). Ayyavoo et al. reported that soluble Vpr induced apoptosis in A1.1 T cells. However, upon pretreatment with anti-CD3, which normally results in induction of apoptosis, Vpr was protective. Studies by Conti et al. demonstrated that Jurkat cells stably transfected and expressing Vpr were protected from the induction of apoptosis by serum starvation, anti-Fas, or cycloheximide/tumor necrosis factor. Analysis of Vpr-expressing cells revealed that their cell cycle profiles were normal. It is possible in this case that selection for Vpr-expressing clones resulted in cells which were refractory to the effects of Vpr (9) and, therefore, do not reflect the action of Vpr on normal cells.

Induction of apoptosis following viral infection is a common cellular defense against infection. A number of viruses have devised strategies to inhibit induction of apoptosis and thus provide more time to replicate. The cowpox virus protein CrmA is similar to the serpin family of proteins and is able to inhibit caspase activation (26, 62). Baculovirus encodes p35, which is also able to inhibit caspase activation (4, 6). Other viruses also inhibit apoptosis by intersecting apoptosis pathways prior to caspase activation. The adenovirus encodes a protein, E1B 19K, which is a functional homolog of Bcl-2 and

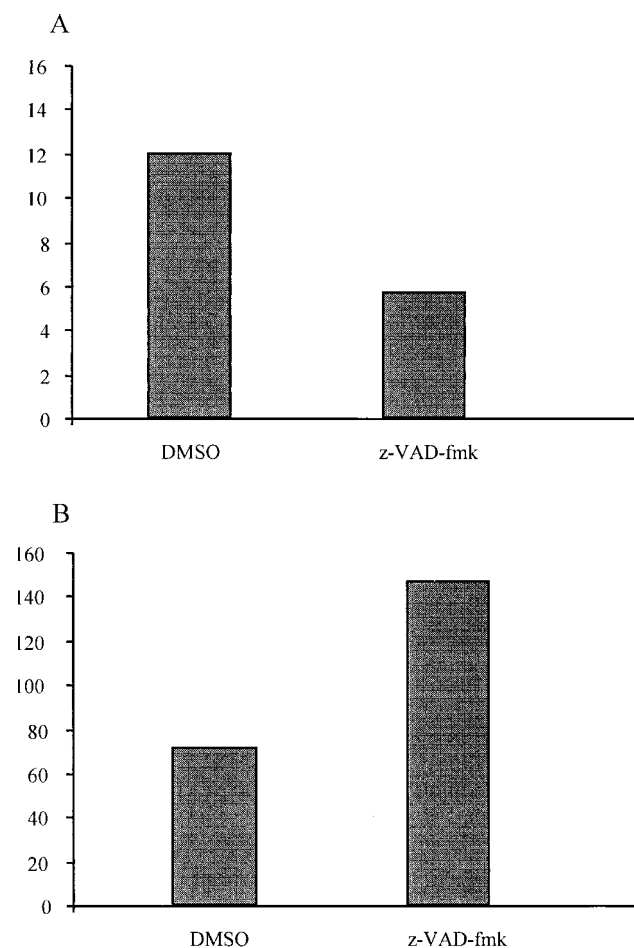


FIG. 5. Synthetic peptide inhibitor of apoptosis reduces the levels of apoptosis while increasing the amount of p24 produced during an HIV-1 infection. HeLa cells (10^5) were infected with HIV-1_{NL4-3}Thyenv(-)/VSV-G for 4 h in the presence of 10 μ g of polybrene per ml. Following infection, the cells were washed twice with PBS and fresh media containing DMSO or 100 μ M z-VAD-fmk, dissolved in DMSO, was added. Every 12 h, fresh DMSO or z-VAD-fmk was added to the media. The data shown is representative of four independent experiments. (A) Bar graph representing the percentage of Annexin V-staining cells within the cultures infected with HIV-1_{NL4-3}Thyenv(-)/VSV-G 72 h postinfection. Thy 1.2 analysis revealed that 47.1% of the cells were infected at 48 h postinfection. (B) Bar graph representing p24 (ng/ml) at 84 h postinfection. In each of four independent experiments, we observed a similar negative association: DMSO-treated cultures had higher levels of Annexin V-positive cells and lower levels of p24 than z-VAD-fmk-treated cultures ($P = 0.06$ by testing the signs of independent Spearman rank correlations for evidence of no association versus negative association) (22).

is thought to interact with positive regulators of apoptosis such as Bax, thereby inhibiting initiation of apoptosis. In addition to these, there are other viral gene products, such as the human cytomegalovirus IE1 and IE2 proteins, that negatively regulate apoptosis in an unknown manner (63).

While many viruses actively inhibit apoptosis, there are also those which activate apoptosis. Evidence suggests that some viruses may induce apoptosis as a means for dissemination, and therefore, apoptosis may be an important step in the viral life cycle (51). The nonstructural protein from the B19 parvovirus induces apoptosis in various cell lines (38). Infection by Sindbis virus results in apoptosis that can be blocked by the expression of Bcl-2 (28). Interestingly, inhibition of apoptosis by Bcl-2 shifts the viral infection from lytic to persistent. Other viruses, including influenza virus, Dengue virus, and various herpesvi-

rus, have also been reported to induce apoptosis as a result of cellular infections, although the mechanism behind induction of apoptosis remains to be elucidated (11, 23; reference 51 and references therein). HIV-1, therefore, is not unique with regard to its ability to induce apoptosis. The adaptive role of apoptosis for HIV-1 may differ from that of the above-mentioned viruses. HIV-1, like all retroviruses, integrates into the genome as part of its life cycle. Integrated viruses that constitutively express viral gene products would serve as good targets for the cellular immune response. Indeed, inhibition of apoptosis leads to increased viral p24 production. HIV-1-induced apoptosis may, therefore, play an important role in the viral life cycle through limiting the host immune responses to the virus and thus facilitating viral persistence.

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