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Human Immunodeficiency Virus Type 1 Vpr Induces Apoptosis following Cell Cycle Arrest

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The human immunodeficiency virus type 1 (HIV-1) *vpr* gene encodes a protein which induces arrest of cells in the G_2 phase of the cell cycle. Here, we demonstrate that following the arrest of cells in G_2, Vpr induces apoptosis in human fibroblasts, T cells, and primary peripheral blood lymphocytes. Analysis of various mutations in the *vpr* gene revealed that the extent of Vpr-induced G_2 arrest correlated with the levels of apoptosis. However, the alleviation of Vpr-induced G_2 arrest by treatment with the drug pentoxifylline did not abrogate apoptosis. Together these studies indicate that induction of G_2 arrest, but not necessarily continued arrest in G_2, was required for Vpr-induced apoptosis to occur. Finally, Vpr-induced G_2 arrest has previously been correlated with inactivation of the Cdc2 kinase. Some models of apoptosis have demonstrated a requirement for active Cdc2 kinase for apoptosis to occur. Here we show that accumulation of the hypophosphorylated or active form of the Cdc2 kinase is not required for Vpr-induced apoptosis. These studies indicate that Vpr is capable of inducing apoptosis, and we propose that both the initial arrest of cells and subsequent apoptosis may contribute to CD4 cell depletion in HIV-1 disease.

Human immunodeficiency virus type 1 (HIV-1) is a member of the lentivirus family. In addition to the *gag*, *pol*, and *env* genes, which are found in all simple retroviruses, the lentiviruses also contain a number of accessory genes. Some of these genes are required for HIV-1 replication, while others have been implicated in pathogenesis. One such gene, *vpr*, encodes a 14-kDa, 96-amino-acid nuclear protein that is highly conserved between HIV-1, HIV-2, and simian immunodeficiency virus type 1 (SIV-1) isolates (28, 68). In addition to containing *vpr*, HIV-2 and SIV-1 also contain *vpx*, which is closely related to *vpr* and may have arisen through either a gene duplication or homologous recombination event (57, 63). Deletion of both *vpr* and *vpx* from SIV-1 resulted in an acute infection, but no disease was observed in rhesus monkeys, indicating that both genes are required for pathogenesis (20). HIV-1 carries *vpr* alone, which is thought to encompass the functions of both the *vpr* and *vpx* genes found within HIV-2 and SIV-1.

A number of functions that are necessary for viral replication and may be important for pathogenesis have been defined in vitro for HIV-1 Vpr. Vpr was shown to possess weak transcriptional activity (8, 9, 45) and was required for productive infection of nondividing cells such as macrophages (1, 13, 22, 66). Analysis of the mature virions has shown that through an interaction with Gag p6, Vpr is incorporated into virions (36, 38, 70). Following infection, it is likely that virion-associated Vpr, in conjunction with the matrix (MA) protein, localizes the preintegration complex to the nucleus (25).

More recently, Vpr has been shown to induce arrest of cells in the G_2 phase of the cell cycle (23, 29, 54, 55). We and others have shown that Cdc2 kinase is inactive in Vpr-arrested cells, suggesting that Vpr function disrupts regulation of Cdc2 kinase, resulting in G_2 arrest (4, 23, 29, 54). In addition to causing G_2 arrest in mammalian cells, Vpr also causes growth arrest in the fission yeast *Schizosaccharomyces pombe* (72). This observation suggests that Vpr interacts with highly conserved components of the cell cycle regulation pathway. Interestingly, BouHamdan et al. (5) reported that Vpr specifically binds to the highly conserved DNA repair enzyme uracil DNA glycoslylase (UNG), which is involved in removing uracil from DNA. The relationship between UNG-Vpr interactions and cell cycle arrest is unclear.

Mutational analysis of the Vpr protein has mapped the above-described functions to different regions of the protein (15, 68). The amino terminus is predicted to form an amphipathic alpha helix, and it has been proposed that the hydrophobic face of the helix determines virion incorporation and that the hydrophilic face is required for nuclear localization (68). The carboxy terminus of the protein, which contains predominantly basic amino acids, is required to induce G_2 arrest. The carboxy terminus alone, however, is not sufficient for G_2 arrest. A recent report has shown that specific point mutations throughout the protein can result in loss of the G_2 arrest phenotype (15).

It has not yet been established how HIV-1 infection results in CD4-positive-T-cell depletion and, ultimately, leads to the development of AIDS. Many reports have described a number of mechanisms for HIV-1-induced T-cell death in vitro. Apoptosis is an ordered self-destruction process which is characterized by cell shrinkage, loss of membrane integrity, chromosome condensation, and internucleosomal cleavage of DNA (reviewed in reference 40). In vitro stimulation of CD4-positive T cells from HIV-1-infected individuals resulted in apoptosis (47). In addition, higher levels of apoptosis were observed in cells from patients with acute infection than in patients in the asymptomatic phase of the disease (41).

In addition to induction of apoptosis in patient peripheral blood lymphocytes (PBL), it has also been demonstrated that in vitro infection of lymphoid cell lines and primary PBL with HIV-1 resulted in apoptosis (33, 62). Studies examining the roles of individual HIV-1 genes have revealed that HIV-1 Env and Tat can sensitize cells to apoptosis in the presence of a second apoptotic signal, such as Fas (CD95) signaling (35, 37, 65). These studies further suggested that apoptosis could contribute to CD4 cell depletion in vivo. Apoptosis, however, is

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not the only form of cell death observed after HIV-1 infection in vitro. Cellular trauma can also lead to necrosis, which, unlike apoptosis, usually results in swelling and lysis of a cell (12). A number of groups have reported that in vitro infection of CD4-positive T cells can result in single-cell lysis or syncytium formation, which ultimately results in necrosis (6, 30, 60, 61). The majority of these reports suggested that these forms of death are dependent on the presence of the HIV-1 *env* gene (6, 32). Together these studies suggest that multiple forms of cell death could contribute to CD4 cell depletion in vivo.

In this report, we demonstrate that expression of HIV-1 Vpr, either alone or in the context of a viral infection, induces apoptosis. Analysis of *vpr* point mutants suggests a correlation between the extent of G_2 arrest and the levels of apoptosis observed. Mutants which cause more cells to accumulate in the G_2 phase of the cell cycle show increased levels of apoptosis. We also demonstrate that Vpr-induced apoptosis does not require maintenance of G_2 arrest. Specifically, treatment of Vpr-arrested cells with the methylxanthine pentoxifylline alleviated G_2 arrest but did not reduce the levels of apoptosis observed. Finally, we show that the Cdc2 kinase remained hyperphosphorylated during apoptosis, indicating that Cdc2 kinase was not required for activation of the apoptotic pathway induced by Vpr.

MATERIALS AND METHODS

Preparation of viral stocks. COS cells were maintained in Dulbecco modified Eagle medium plus 10% bovine calf serum (Gibco-BRL, Grand Island, N.Y.) at 37°C and 5% CO_2. COS cells were transfected (230 V) with 10 μg of NL4-3ThyΔBgl or NL4-3ThyΔBglVprX, containing a wild-type *vpr* or mutant *vpr* gene (51), respectively, and 10 μg of pCMVVSV-G, which encodes the vesicular stomatitis virus envelope. The medium was changed 24 h posttransfection, and virus was concentrated as previously described (17, 69). Briefly, supernatants were removed from cells at 48 and 72 h posttransfection. Supernatants were centrifuged for 5 min at 1,900 × *g*, passed through a 0.45-μm-pore-size filter, and ultracentrifuged at 50,000 × *g* for 90 min. Supernatants were removed, and the viral pellet was resuspended in 0.1× Hanks balanced salt solution overnight at 4°C. Viral stocks were stored in the presence of 10% fetal calf serum (FCS) at −70°C. Viral stock titers were determined on HeLa cells, and viral stocks were analyzed by Thy 1.2 staining and flow cytometric analysis.

DNA constructs. All DNA constructs used for transfection contained the untranslated intron of the cytomegalovirus immediate-early promoter and were previously described (29). Briefly, *vpr* or the mutant, *vprX*, was expressed from the same expression cassette as *thy 1.2* (BSVprThy and BSVprXThy, respectively), allowing identification of individually transfected cells by flow cytometry. The mutant, *vprX*, was created by introducing a frameshift in the carboxy terminus of the *vpr* reading frame. The frameshift mutation within *vpr* results in an unstable protein which does not induce cell cycle arrest and is not visible by immunofluorescence microscopy (50) (see Fig. 1C). The *vpr* point mutants were cloned in place of the wild-type *vpr* gene, creating the plasmids BSA30LThy, BSA30PThy, and BSR80AThy. Each plasmid was modified from the original by the addition of sequence coding for the nanopeptide from the influenza virus hemagglutinin protein, which was placed at the 5′ end of the wild-type *vpr* gene and the *vpr* mutants.

Synchronization of HeLa cells. HeLa cells were synchronized by using a double thymidine block, as previously described (10). Briefly, HeLa cells were treated with 2 mM thymidine (Sigma, St. Louis, Mo.) for 19 to 21 h. Thymidine was removed, and after 9 h, it was added back and left for another 17 to 19 h. Synchrony was examined by DNA analysis, as described below.

Infection of HeLa cells, SupT1 cells, and PBL. HeLa cells were maintained in Dulbecco modified Eagle medium plus 10% bovine calf serum, and SupT1 cells were maintained in RPMI (BioWhittaker, Walkersville, Md.) plus 10% FCS. PBL were isolated from normal, uninfected donors and stimulated for 48 h in RPMI plus 20% FCS and 5 mg of phytohemagglutinin per ml. PBL were maintained in RPMI plus 10% FCS and 20 U of recombinant interleukin-2 per ml. Cells were mock infected or infected with either HIV-$1_{NL4\text{-}3Thy}$env(−)/VSV-G or HIV-$1_{NL4\text{-}3Thy}$env(−)VprX/VSV-G for 4 h in the presence of 1 μg of Polybrene per ml. For mock infections, cells were exposed to 1 μg of Polybrene per ml for 4 h in parallel with cells infected with our various viral constructs. Infectious units were determined by measuring the titers of concentrated virus stocks in HeLa cells and analyzing infection efficiencies by Thy 1.2 staining and flow cytometry. Following infection, the virus was removed and fresh medium was added to the cells.

Transfection of HeLa cells. Synchronized HeLa cells were incubated in 1.2× RPMI–20% FCS plus 10 μg of DNA for 20 min on ice. Following incubation, cells were electroporated at 250 V and incubated on ice for an additional 20 min. Transfected cells were plated in either six-well plates or chamber slides for use in immunofluorescence staining. The medium was changed 24 h posttransfection.

Immunofluorescence staining. Following either infection or transfection, HeLa cells were grown on chamber slides (Fisher, Pittsburgh, Pa.). Growth medium was removed from the slides, and the cells were fixed with either 1 or 4% paraformaldehyde (in phosphate-buffered saline) for 20 min at room temperature. Following fixation, cells were permeabilized with either 0.1 or 0.2% Triton X-100 (Sigma) for 15 min at room temperature. Fixed cells were then stained with anti-Thy 1.2–phycoerythrin (PE) conjugate (Caltag, South San Francisco, Calif.). Stained cells were washed once with fluorescence-activated cell sorter buffer (phosphate-buffered saline, 2% FCS, and 0.2% sodium azide). The DNA structure was examined by staining cells with 1 μg of Hoechst 33342 (Sigma) per ml for 5 min at room temperature. Slides were observed under an Olympus microscope, and photographs were taken with an Olympus 35-mm camera.

Analysis of DNA content within transfected and infected cells. Transfection and infection efficiencies for Fig. 1 to 4A and 5 to 8 were monitored by flow cytometry. In the case of HeLa cells, both attached and floating cells were collected and analyzed. Cells (1×10^5 to 2×10^6) were stained with anti-Thy 1.2–fluorescein isothiocyanate (FITC) conjugate (Caltag) for 20 min on ice. Following staining, cells were washed with fluorescence-activated cell sorter buffer and resuspended in hypotonic propidium iodide (10 μg of propidium iodide [Sigma] per ml and 11.25 kU of RNase [Sigma] per ml). All flow cytometry protocols are a modification of a procedure developed by Schmid et al. (56).

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 in Fig. 4B (18). Briefly, 1×10^5 to 1.5×10^6 cells were stained with anti-Thy 1.2–PE for 15 min on ice to identify individually transfected or infected cells. Following antibody staining, 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 $MgCl_2$, 1.8 mM $CaCl_2$, and 3.6 μg of 7-aminoactinomycin D [7-AAD] per ml) (Biosource, Camarillo, Calif.). Stained cells were acquired on a FACScan instrument and analyzed with the Lysis II software package.

Cell lysis and protein analysis. HeLa cells were lysed at 4°C in lysis buffer (Hanks balanced salt solution with 1% Nonidet P-40, 50 mM β-glycerophosphate, 10 mM NaF, 1% aprotinin, 1 mM sodium orthovanadate, and 1 μg of leupeptin per ml), and protein concentrations were estimated by the Bio-Rad protein assay according to the manufacturer's protocol. Equivalent amounts of protein per sample were subjected to electrophoresis on sodium dodecyl sulfate–15% polyacrylamide gels. Western blotting (immunoblotting) was performed with a monoclonal antibody for Cdc2 kinase (anti-Cdc2 p34, Santa Cruz Biotechnology, Santa Cruz, Calif.) and developed with the enhanced chemiluminescence assay (Amersham, Arlington Heights, Ill.) (29).

RESULTS

HIV-1 Vpr induces apoptosis. (i) Microscopic analysis. We and others have previously shown that transfection of a Vpr expression vector resulted in the arrest of cells in the G_2 phase of the cell cycle within 48 h (4, 23). Here we report on the fate of Vpr-arrested cells. We noted, through initial microscopic analysis of HeLa cells, that transfection with HIV-1 Vpr resulted in cell death, as evidenced by rounding and lifting of cells from the tissue culture flask. To determine how HIV-1 Vpr induced cellular death we utilized both morphologic and immunologic assays. HeLa cells were transfected with expression plasmids carrying the murine *thy 1.2* gene and either wild-type *vpr* or a *vpr* gene which contained a mutation in the carboxy terminus, *vprX*. Cells were stained with anti-Thy 1.2 to identify individually transfected cells and with a DNA dye (Hoescht 33342) that allowed us to examine the nuclear structure of transfected cells. Initial analysis revealed that all Thy 1.2-expressing cells were depleted within 5 days of transfection with BSVprThy (data not shown). When BSVprThy-transfected cultures were analyzed microscopically, we observed the presence of pyknotic nuclei, which are characteristic structures often observed in the later stages of apoptosis (Fig. 1F). Similar but smaller structures were also observed in HeLa cells induced to undergo apoptosis by treatment with Fas antibodies (Fig. 1H). In contrast, no pyknotic nuclei were observed in HeLa cells transfected with BSVprXThy, which expresses VprX (Fig. 1D). These observations suggested that one consequence of Vpr expression was apoptotic death.

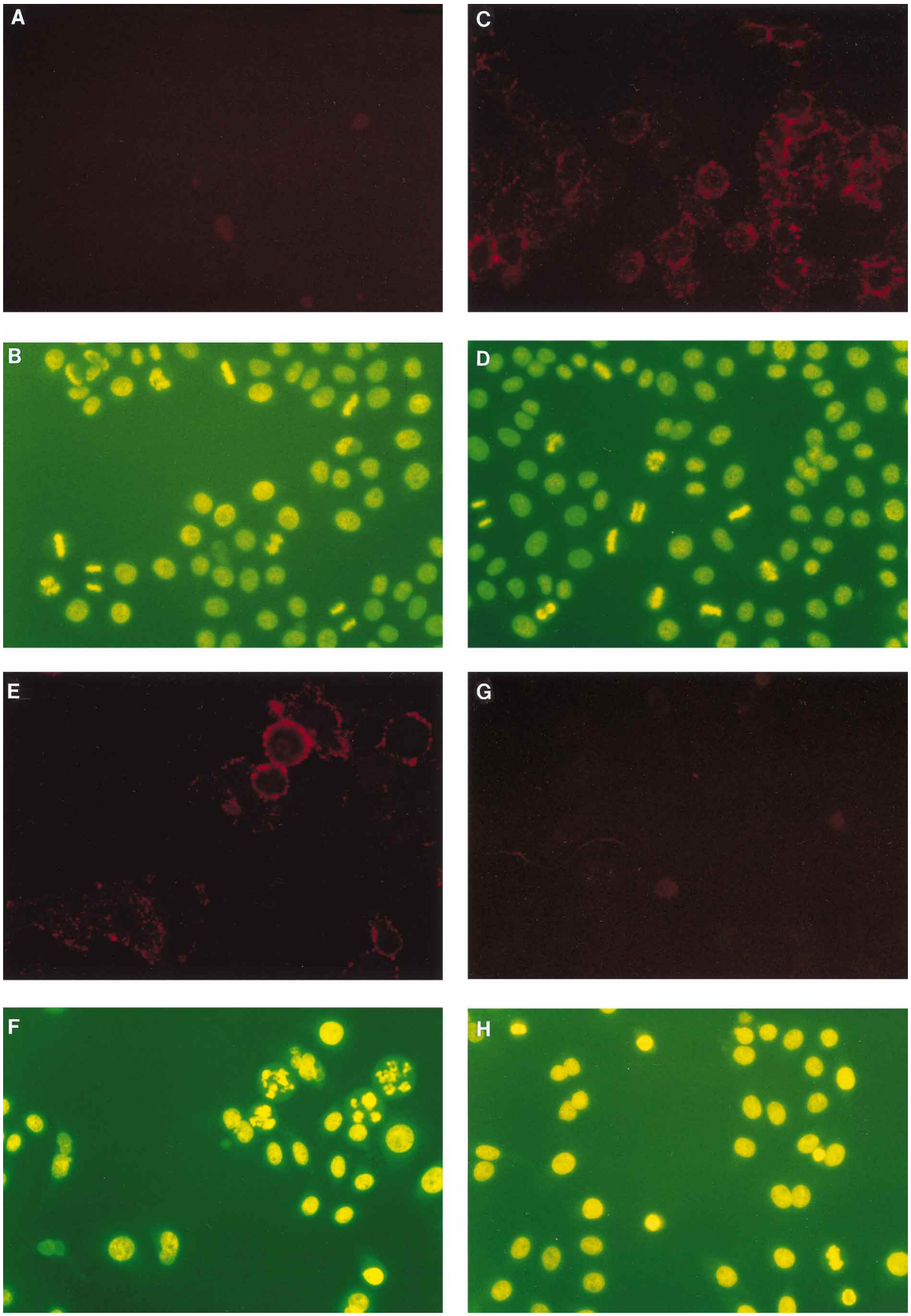

FIG. 1. Microscopic analysis of synchronized HeLa cells transfected with *vpr* and *thy 1.2* (BSVprThy) demonstrates the presence of pyknotic nuclei characteristic of apoptosis. HeLa cells (10^7) were transfected with 10 μg of DNA. At 72 h posttransfection, HeLa cells were fixed with 1% paraformaldehyde and permeabilized with 0.1% Triton X-100, and the same cells were stained with anti-Thy 1.2–PE (A, C, E, and G) and Hoescht 33342 (B, D, F, and H). (A and B) Mock-transfected HeLa cells; (C and D) BSVprXThy-transfected HeLa cells; (E and F) presence of pyknotic nuclei in BSVprThy-transfected HeLa cells; (G and H) HeLa cells pretreated with 100 U of gamma interferon per ml for 48 h and with 100 ng of anti-Fas per ml for 24 h. Anti-Fas-treated cells were used as a positive control for apoptosis in all assays. Magnification for all panels, ×40.

(ii) Flow cytometric analysis by Annexin V staining. A quantitative flow cytometric analysis for apoptosis was used to confirm and extend our initial microscopic analysis. Annexin V is a protein which specifically binds phospholipidserine, an integral part of the inner plasma membrane. During the initial steps of apoptosis, membrane integrity is lost and phospholipidserine is exposed, allowing Annexin V to bind to cells (18). Therefore, we were able to differentiate between live, apoptotic, and dead cells by staining cells with Annexin V and a dead-cell exclusion dye and analyzing these cells by flow cytometry. In addition, staining cells with the Thy 1.2 antibody allowed us to analyze only those cells transfected with BSVpr-Thy and thus expressing Vpr. Therefore, this assay allowed us to specifically examine the percentage of apoptosis within only the Thy 1.2-positive population. Representative flow cytometric analyses of Annexin V staining of mock-, BSVprXThy-, and BSVprThy-transfected cells are shown in Fig. 2A.

Annexin V analysis of BSVprThy-transfected HeLa cells revealed that at 21 h posttransfection, 11.6% of cells were Annexin V positive. By 69 h posttransfection, Annexin V staining increased to 34.2% (Fig. 2B). Analysis of BSVprThy-transfected cells revealed that only 8.5% Thy 1.2-positive cells remained at 123 h posttransfection, indicating that virtually all Vpr-expressing cells die by apoptosis. In contrast, only 4.8% of BSVprXThy-transfected cells were Annexin V positive at 69 h posttransfection (Fig. 2B). Concurrent analysis of Thy 1.2 in both Vpr- and VprX-expressing populations revealed similar transfection efficiencies, ranging from 62.2% in BSVprXThy-transfected cultures to 59.2% in BSVprThy-transfected cultures (data not shown). At each time point, cell numbers were quantified. As seen in Fig. 2C, reduced cell numbers correlated with increased levels of apoptosis following transfection with the Vpr-expressing plasmid, BSVprThy. The increase in cell number at 91 h posttransfection in the BSVprThy-transfected culture is likely due to the expansion of nontransfected cells within the culture.

Vpr expressed during HIV-1 infection increases apoptosis. We next examined the effects of Vpr in the context of a viral infection to establish its effects at biologically relevant concentrations. Synchronized HeLa cells were infected with HIV-1 bearing either a wild-type *vpr* gene [HIV-$1_{NL4\text{-}3Thy}$env(−)/VSV-G] or the *vprX* mutant [HIV-$1_{NL4\text{-}3Thy}$env(−)VprX/VSV-G], and the murine *thy 1.2* gene expressed in place of *nef*, which allowed us to analyze individually infected cells by flow cytometry (50). In addition, these constructs contained a deletion in the *env* gene which allowed us to examine the effects of Vpr in the absence of viral spread and without the complication of envelope-mediated cell death. Flow cytometric analysis of Thy 1.2 expression indicated that approximately 80% of cells were infected in both the HIV-$1_{NL4\text{-}3Thy}$env(−)/VSV-G- and HIV-$1_{NL4\text{-}3Thy}$env(−)/VprX/VSV-G-infected populations. Analysis of HeLa cells infected with HIV-$1_{NL4\text{-}3Thy}$env(−)/VSV-G showed that apoptosis increased over time. At 30 h postinfection, 11.6% of cells infected with HIV-$1_{NL4\text{-}3Thy}$env(−)/VSV-G were Annexin V positive, and this increased to 78.9% at 90 h postinfection (Fig. 3A). In contrast, only 6.4% of cells infected with the *vpr* mutant virus [HIV-$1_{NL4\text{-}3Thy}$env(−)VprX/VSV-G] were Annexin V positive, and this increased to 25% at 90 h. Annexin V staining of mock-infected cells remained low throughout the time course of the experiment (Fig. 3A). Increases in apoptosis coincided with decreases in the number of viable cells present in our cultures (Fig. 3B). It is noteworthy that we observed an induction of apoptosis by our *vpr* mutant virus, HIV-$1_{NL4\text{-}3Thy}$env(−)VprX/VSV-G, suggesting that other viral factors in addition to Vpr may also influence the induction of apoptosis; however, this induction was consistently less than in the wild type.

HIV-1 Vpr increases apoptosis in human T cells. Human T cells are the natural host cells for HIV-1 infection. Therefore, we examined the effects of HIV-1 Vpr on the human T-cell line SupT1 and on stimulated PBL. SupT1 cells were infected with either HIV-$1_{NL4\text{-}3Thy}$env(−)/VSV-G or HIV-$1_{NL4\text{-}3Thy}$env(−)VprX/VSV-G, and the bulk cultures were analyzed by Annexin V staining. Infection of SupT1 cells with HIV-$1_{NL4\text{-}3Thy}$env(−)/VSV-G resulted in an increase in Annexin V staining over time (Fig. 4A). At 24 h postinfection, 7.5% of cells infected with HIV-$1_{NL4\text{-}3Thy}$env(−)/VSV-G were Annexin V positive, and this increased to 42.9% by 72 h postinfection (Fig. 4A). Increases in Annexin V staining of HIV-$1_{NL4\text{-}3Thy}$env(−)/VSV-G-infected cells correlated with a loss of Thy 1.2-positive cells (data not shown). Analysis of cells infected with the *vpr* mutant virus [HIV-$1_{NL4\text{-}3Thy}$env(−)VprX/VSV-G] revealed a more modest increase in Annexin V staining over time (Fig. 4A). At 24 h postinfection, Annexin V staining was 6.7%, and it increased to 27.9% at 72 h postinfection, again indicating that viral factors other than Vpr may influence apoptosis in the context of infection.

As described for SupT1 cells, PBL were infected with HIV-$1_{NL4\text{-}3Thy}$env(−)/VSV-G or HIV-$1_{NL4\text{-}3Thy}$env(−)VprX/VSV-G. Thy 1.2 staining revealed that infection of PBL with HIV-$1_{NL4\text{-}3Thy}$env(−)/VSV-G or HIV-$1_{NL4\text{-}3Thy}$env(−)VprX/VSV-G resulted in maximums of 12.0 and 6.3% Thy 1.2-positive cells, respectively (data not shown). At 24 h postinfection, 19.4% of cells infected with HIV-$1_{NL4\text{-}3Thy}$env(−)/VSV-G were Annexin V positive. Over the following 96 h, Annexin V staining remained constant, ranging from 19.4 to 25.7%, and concurrent Thy 1.2 analysis revealed depletion of Thy 1.2-positive cells over the same time period (Fig. 4B). In contrast, Annexin V staining of cells infected with the mutant virus [HIV-$1_{NL4\text{-}3Thy}$env(−)VprX/VSV-G] remained low. Over 96 h, Annexin V staining of mock-infected cells ranged from 4.2 to 6.2%, while Annexin V staining of cells infected with the *vpr* mutant virus, HIV-$1_{NL4\text{-}3Thy}$env(−)VprX/VSV-G, ranged from 8.6 to 12.8% (Fig. 4B). Concurrent analysis of Thy 1.2-expressing cells infected with the *vpr* mutant virus revealed that the percentage of these cells remained relatively constant, suggesting that they persisted, while cells infected with HIV-$1_{NL4\text{-}3Thy}$env(−)/VSV-G died. Furthermore, Annexin V analysis of the Thy 1.2-positive and Thy 1.2-negative cells revealed that Annexin V staining was limited to the Thy 1.2-positive cells (Fig. 4C). Our results demonstrate that the natural target cells for HIV-1, human T cells, are susceptible to the apoptotic effects of Vpr and that they appear to be more sensitive to the effects of Vpr than HeLa cells.

Analysis of HIV-1 Vpr mutants. We examined the effects of *vpr* point mutants on cellular survival. Mutation of amino acids 30 and 80 resulted in stable proteins which have been reported to affect G_2 arrest (15). Residue 30 is located within the predicted alpha-helical region of the protein and is thought to affect nuclear localization of the protein. Mutation of alanine 30 to leucine was predicted to have little effect on the protein structure and did not alter the cellular localization of Vpr. Mutation of residue 30 to proline changed the cellular localization of Vpr from the nucleus to the cytoplasm (15). Residue 80 lies within the basic carboxyl terminus and has been shown to affect G_2 arrest. Synchronized HeLa cells were transiently transfected with plasmids expressing either *vpr*, the *vpr* mutant *vprX*, or one of the *vpr* point mutants (BSA30LThy, BSA30PThy, or BSR80AThy) and the *thy 1.2* gene. The cell cycle profiles of Thy 1.2-positive cells were analyzed at various time points throughout the experiment. As seen in Fig. 5A, the mutants

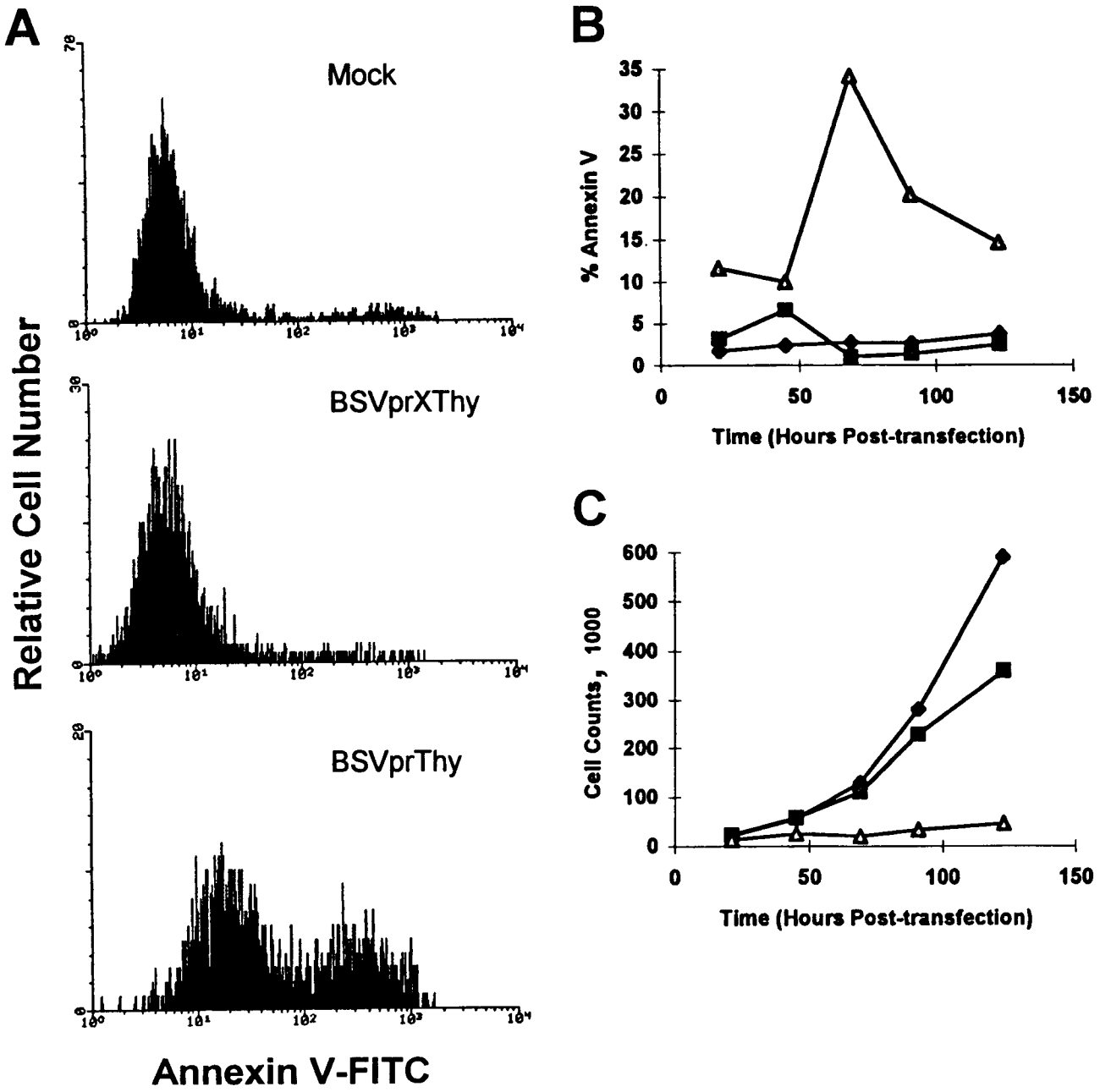

FIG. 2. Flow cytometric analysis of Annexin V staining demonstrates apoptosis in Vpr-expressing cells. The data shown are representative of five independent experiments. (A) Representative histograms of Annexin V-FITC staining of Thy 1.2-positive cells. Plots represent transfected populations at 69 h posttransfection. Results for mock-, BSVprXThy-, and BSVprThy-transfected cells are shown. (B) Annexin V analysis of transfected HeLa cells. HeLa cells (10^7) were transfected with either a wild-type Vpr- or mutant VprX-expressing plasmid. At 21, 45, 69, 91, and 123 h posttransfection, both attached and floating cells were stained with Annexin V, 7-AAD, and anti-Thy 1.2–PE, and 5×10^3 cells were analyzed by flow cytometry. Thy 1.2 staining indicated that transfection efficiencies were 59.2% for BSVprThy-transfected cells and 62.2% for BSVprXThy-transfected cells (data not shown). The graph represents apoptosis in the Thy 1.2-positive population only. Mock-transfected cells were also added for comparison. Results for mock (diamonds)-, BSVprXThy (squares)-, and BSVprThy (triangles)-transfected cells are shown. (C) Cell counts. At each time point analyzed by Annexin V staining, transfected cells were also counted by trypan blue exclusion in duplicate to correlate apoptosis with cell number. In all experiments, cell counts were carried out on the same transfected population of cells as that used in the Annexin V analysis in panel B. Symbols are as described for panel B.

affected the cell cycle profile to various degrees compared to Vpr. At 46 h posttransfection, 62.4, 47.2, and 34.7% of BSA30LThy-, BSA30PThy-, and BSR80AThy-expressing cells, respectively, were in the G_2 phase of the cell cycle, compared to 84.1% of Vpr-expressing cells and 37.0% of VprX-expressing cells (Fig. 5A and B). Given that the Vpr mutants varied in their abilities to induce cell cycle arrest, we examined whether this correlated with the ability to induce apoptosis.

Annexin V analysis of transfected HeLa cells revealed that 45.6% of cells transfected with BSVprThy underwent apoptosis at 75 h posttransfection, and this increased to 73.5% at 92 h (Fig. 5C). In contrast, only 7.3, 11.9, and 16.4% of BSVprXThy-,

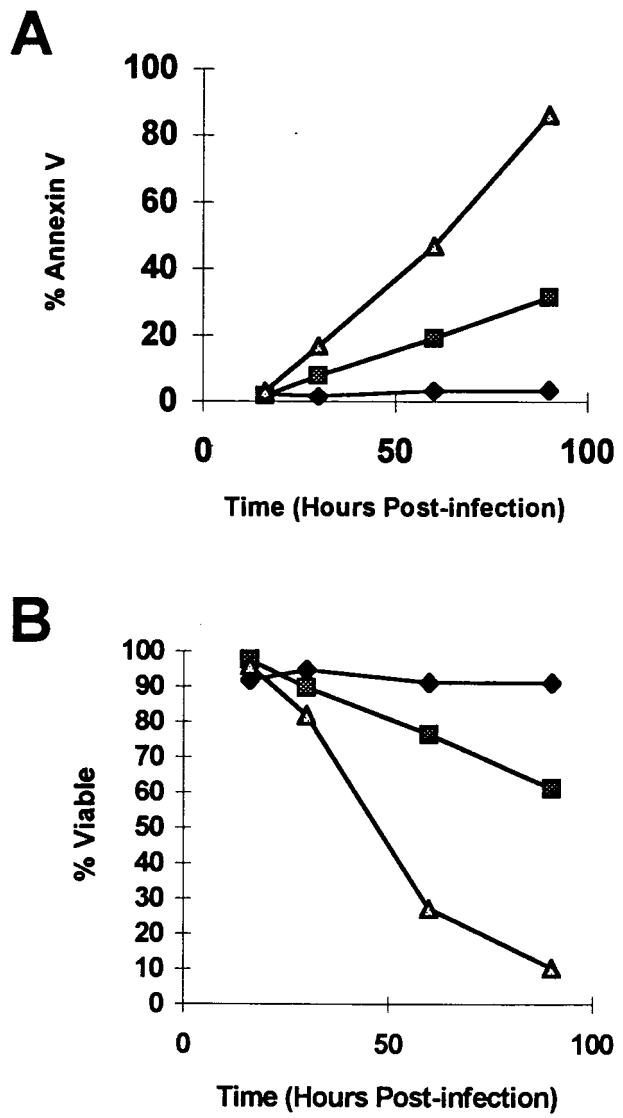

FIG. 3. Analysis of HIV-$1_{NL4\text{-}3Thy}$env(−)/VSV-G- and HIV-$1_{NL4\text{-}3Thy}$env(−)VprX/VSV-G-infected HeLa cells reveals that Vpr increases apoptosis following viral infection. HeLa cells (5×10^5) were infected, and multiplicities of infection were determined as described in Materials and Methods. The data shown are representative of three independent experiments. (A) HIV-1-infected HeLa cells (5×10^3) were analyzed by Annexin V staining at 16, 30, 60, and 90 h postinfection. Concurrent Thy 1.2 analysis of infected cells indicated that 80% of cells in both the HIV-$1_{NL4\text{-}3Thy}$env(−)/VSV-G and HIV-$1_{NL4\text{-}3Thy}$env(−)VprX/VSV-G populations were infected at 30 h postinfection (data not shown). The Annexin V analysis is for Thy 1.2-positive cells. Results for mock (diamonds)-, HIV-$1_{NL4\text{-}3Thy}$env(−)/VSV-G (triangles)-, and HIV-$1_{NL4\text{-}3Thy}$env(−)VprX/VSV-G (squares)-infected cells are shown. (B) Cell viabilities were determined at the same time cells were analyzed for Annexin V staining. Symbols are as described for panel A.

BSR80AThy-, and BSA30PThy-expressing cells, respectively, were Annexin V positive at 92 h posttransfection (Fig. 5C). The ability of point mutant BSA30LThy to induce apoptosis was intermediate to those of the other mutants and Vpr. At 75 h posttransfection, 14.3% of BSA30LThy-expressing cells were Annexin V positive, and this increased to 24.1% at 92 h posttransfection. As expected, cell counts revealed the presence of fewer cells in the cultures which showed a higher incidence of apoptosis, such as those cells transfected with the Vpr-expressing construct, BSVprThy (Fig. 5D). Morphologic analysis of the point mutants indicated that increases in apoptosis corresponded with the increased presence of pyknotic nuclei, which were similar in appearance to pyknotic nuclei in Vpr-expressing cells (Fig. 6). In general, there was a correlation between the extent of cell cycle arrest and the extent of apoptosis induced by the mutants.

Alleviation of Vpr-induced G_2 arrest does not abrogate apoptosis. We determined whether maintenance of G_2 arrest was required to induce apoptosis by examining whether treatment with agents which alleviate Vpr-induced arrest would also affect Vpr's ability to induce apoptosis. Pentoxifylline is a drug used in many clinical settings, but its mechanisms of action are not clear. In vitro, pentoxifylline has been shown to alleviate G_2 arrest induced by a number of DNA-damaging agents, such as nitrogen mustard (44). Experiments in our laboratory have recently shown that pentoxifylline treatment also alleviated Vpr-induced G_2 arrest (52). Therefore, we tested whether apoptosis would still occur in cells treated with pentoxifylline.

At 24 h posttransfection, cells were treated with 0.5 mM pentoxifylline for the course of the experiment. Examination of Annexin V staining revealed that alleviation of Vpr-induced G_2 arrest by pentoxifylline did not reduce the percentage of Annexin V-positive cells observed in BSVprThy-transfected cultures. At 68 h posttransfection, 82.4% ± 1.5% of Vpr-transfected cells were in the G_2 phase of the cell cycle, and Annexin V analysis indicated that 29.9% ± 0.5% of these cells were Annexin V positive (Fig. 7A and B, respectively). Treatment of cells with pentoxifylline resulted in 59.6% ± 3.9% of Vpr-transfected cells in G_2. Concurrent Annexin V analysis revealed that 28.0% ± 0.6% of Vpr-transfected cells were Annexin V positive, indicating that a reduction in G_2-arrested cells did not result in a corresponding reduction in the levels of apoptosis (Fig. 7B). Pentoxifylline treatment of VprX-transfected cells showed that drug treatment alone had no substantial effect on apoptosis (Fig. 7B). Specifically, 28.8% ± 2.7% of BSVprXThy-transfected cells were found in the G_2 phase of the cell cycle at 68 h posttransfection. Pentoxifylline treatment of BSVprXThy- and mock-transfected cells had no effect on the number of cells found in G_2, indicating that drug treatment alone did not perturb normal cell cycling. In addition, we found that addition of pentoxifylline at the time of transfection (0 h) had an effect on cell cycle progression and apoptosis similar to that when the drug was added at 24 h posttransfection (Fig. 7C and D, respectively). While these studies cannot establish whether pentoxifylline actually blocked initiation of G_2 arrest or alleviated arrest, they do demonstrate that Vpr-induced apoptosis does not require the continued maintenance of G_2 arrest.

Phosphorylation state of Cdc2 kinase during Vpr-induced apoptosis. We and others have previously shown that HIV-1 Vpr induces accumulation of hyperphosphorylated, or inactive, Cdc2 kinase (4, 23, 29, 54). Since it was reported that some apoptotic pathways require the activation of Cdc2 kinase (58), we examined whether Cdc2 kinase was activated prior to the onset of Vpr-induced apoptosis. HeLa cells were infected with the wild-type virus [HIV-$1_{NL4\text{-}3Thy}$env(−)/VSV-G] or the *vpr* mutant virus [HIV-$1_{NL4\text{-}3Thy}$env(−)VprX/VSV-G], and the phosphorylation state of Cdc2 kinase was monitored by Western blot analysis. At 19 and 66 h postinfection, 1.1 and 7.0%, respectively, of cells infected with *vpr* mutant virus were Annexin V positive, and no shift in the Cdc2 kinase phosphorylation state was observed (Fig. 8). Compared to cells infected with the *vpr* mutant virus, analysis of cells infected with the wild-type virus [HIV-$1_{NL4\text{-}3Thy}$env(−)/VSV-G] showed an accumulation of hyperphosphorylated Cdc2 kinase that correlated with G_2 arrest (Fig. 8). The proportion of Annexin V-

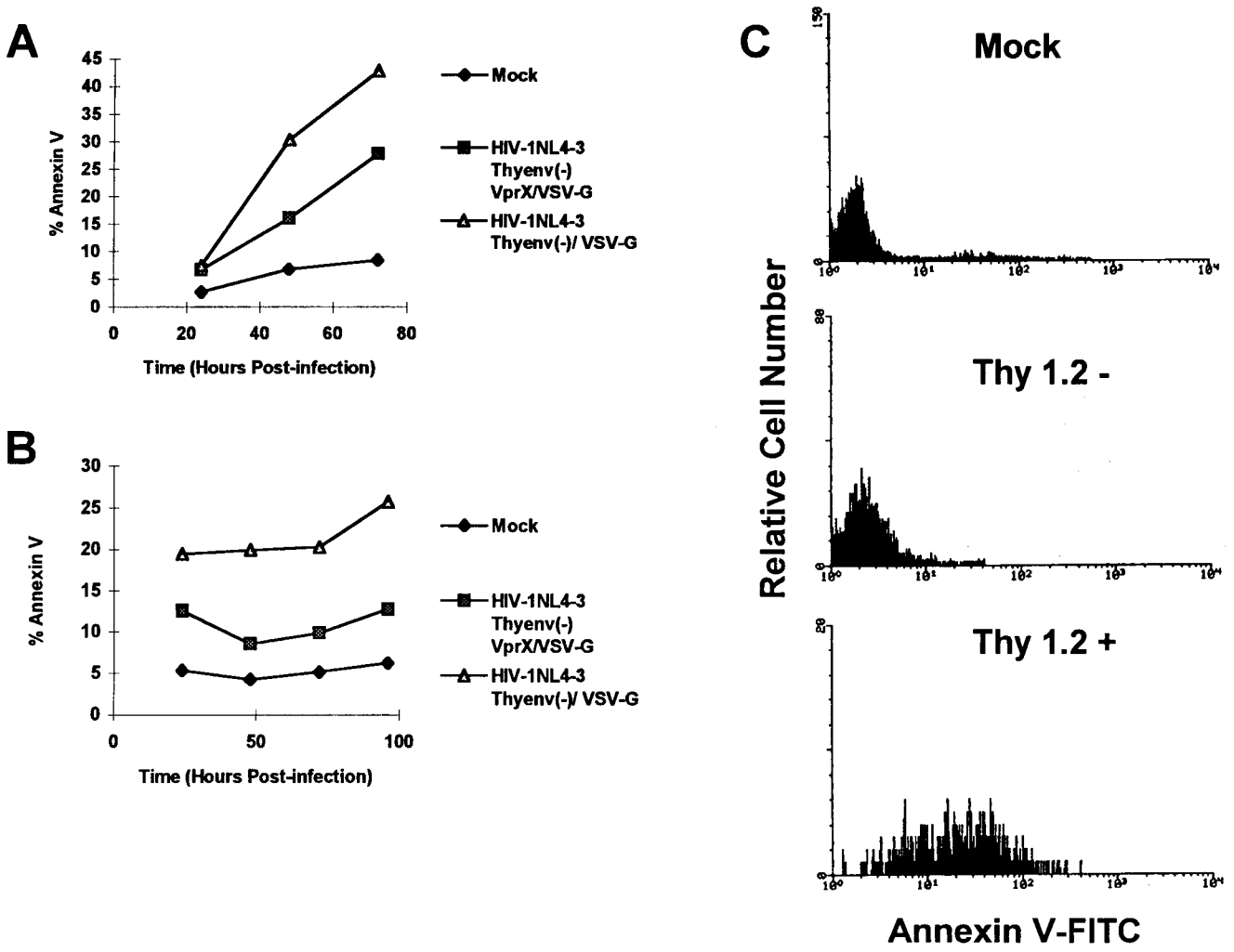

FIG. 4. HIV-1 Vpr induces apoptosis in both SupT1 cells and PBL. (A) Annexin V staining of infected SupT1 cells. SupT1 cells (5×10^5) were infected, and multiplicities of infection were determined as described in Materials and Methods. Infection efficiencies were determined by DNA and Thy 1.2 staining and were 58.9% for HIV-$1_{NL4\text{-}3Thy}$env(−)/VSV-G- and 50.5% for HIV-$1_{NL4\text{-}3Thy}$env(−)VprX/VSV-G-infected cells at 48 h postinfection (data not shown). At 24, 48, and 72 h postinfection, cells were stained with Annexin V, and 5×10^3 cells were analyzed by flow cytometry. The graph represents bulk populations and is representative of two independent experiments. (B) PBL (5×10^5) were stimulated for 48 h and infected with either HIV-$1_{NL4\text{-}3Thy}$env(−)/VSV-G or HIV-$1_{NL4\text{-}3Thy}$env(−)VprX/VSV-G. Thy 1.2 analysis of cells stained with Annexin V, Thy 1.2, and 7-AAD showed infection efficiencies ranging from 12.0% in HIV-$1_{NL4\text{-}3Thy}$env(−)/VSV-G-infected cultures to 6.3% in HIV-$1_{NL4\text{-}3Thy}$env(−)VprX/VSV-G-infected cultures (data not shown). Annexin V analysis was carried out on 5×10^3 cells at 24, 48, 72, and 96 h postinfection. The graph represents bulk populations and is representative of three independent experiments. (C) Annexin V staining of HIV-$1_{NL4\text{-}3Thy}$env(−)/VSV-G-infected (Thy1.2+) and -uninfected (Thy1.2−) PBL.

positive cells in these cultures increased from 1.7 to 33.8% at 19 and 66 h postinfection, respectively. Since a similar proportion of Cdc2 kinase remained hyperphosphorylated while Annexin V staining increased considerably over time, we conclude that the apparent state of Cdc2 kinase phosphorylation does not change during Vpr-induced apoptosis (Fig. 8).

DISCUSSION

We examined the fate of cells expressing HIV-1 Vpr. As previously reported, HIV-1 Vpr induced G_2 arrest within 24 h of expression (29). Transient-transfection assays indicated that cells expressing HIV-1 Vpr displayed typical morphological changes associated with apoptosis. Analysis of *vpr* point mutants suggested that the ability to arrest cells in G_2 correlated with induction of apoptosis. To determine whether alleviation of Vpr-induced G_2 arrest could inhibit apoptosis, we treated Vpr-arrested cells with pentoxifylline. Pentoxifylline treatment of Vpr-transfected cells reduced the proportion of cells in the G_2 phase of the cell cycle. However, alleviation of Vpr-induced arrest by pentoxifylline did not result in a corresponding reduction in the levels of apoptosis observed, indicating that maintenance of G_2 arrest was not required for the induction of apoptosis. Finally, we showed that the Cdc2 kinase remained hyperphosphorylated during Vpr-induced apoptosis. These experiments describe a new phenotype for HIV-1 Vpr.

Our results are contrary to a report by Bartz et al. (4), who suggested that Vpr is cytostatic but not cytotoxic. That group reported that infection of Jurkat T cells with a *vpr* wild-type virus resulted in G_2 arrest but not apoptosis. In their hands, cells infected with the *vpr* wild-type virus continued to accumulate in G_2 over a 72-h period, but no significant cell death was detected compared to cells infected with a *vpr* mutant virus. Our infection data indicated that all infected cells accu-

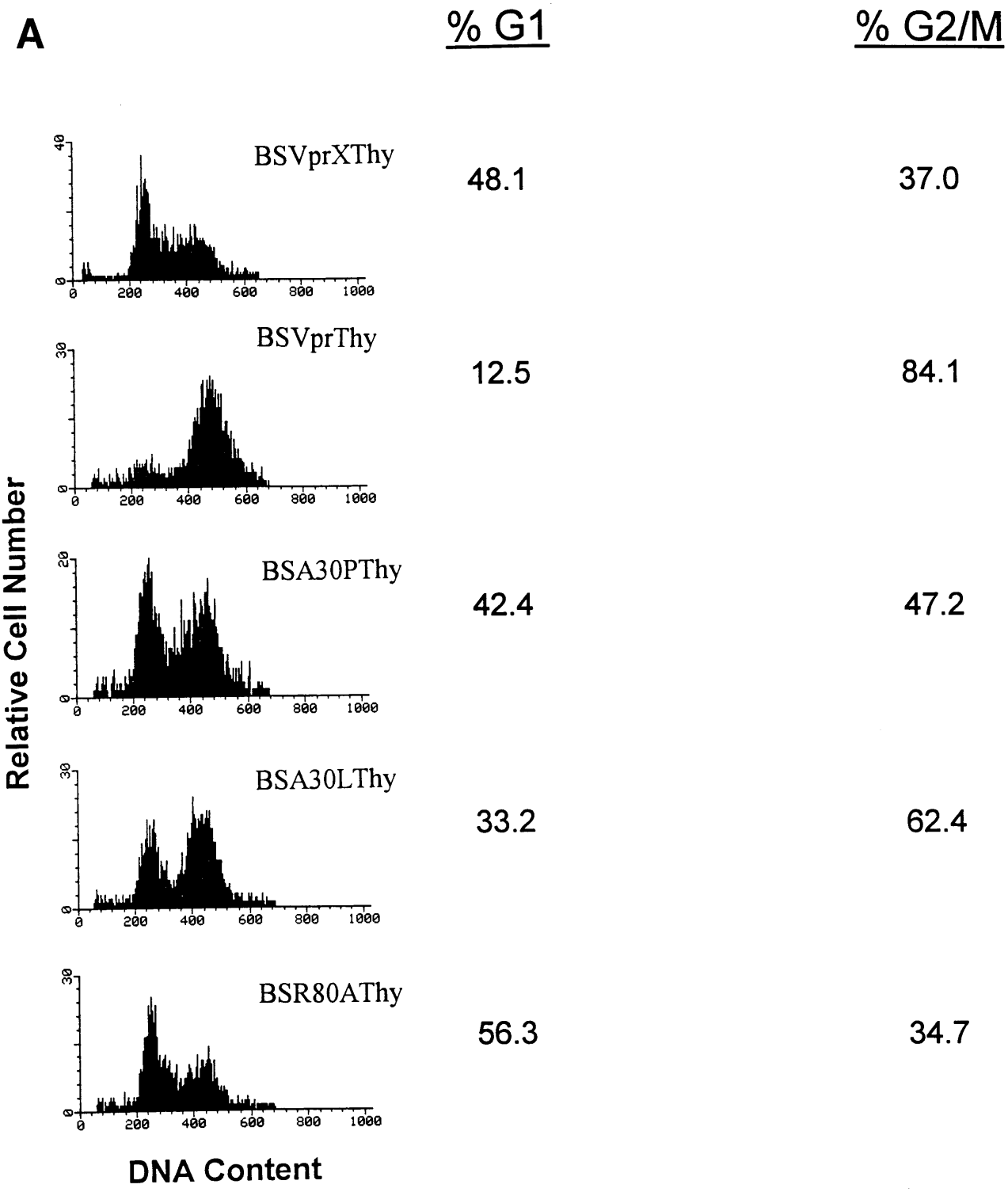

FIG. 5. Analysis of HeLa cells transfected with *vpr* point mutants indicates that the extent of cell cycle arrest correlates with the extent of apoptosis. Data are representative of five independent experiments. (A) DNA profiles of transfected HeLa cells stained with propidium iodide. HeLa cells (10^7) were transfected with 10 μg of the indicated plasmids, as described in Materials and Methods. Single-parameter histograms show DNA profiles of 5×10^3 cells analyzed at 46 h posttransfection. The percent Thy 1.2-positive cells found in the G_1 and the G_2/M phases of the cell cycle is presented on the right. The analyses presented in panels A to D were carried out on the same transfected populations. (B) Graphic representation of the percentage of Thy 1.2-positive cells in the G_2/M phase of the cell cycle. At 22, 46, 75, 92, and 116 h posttransfection, 5×10^3 cells were analyzed by flow cytometry. The graph shows mock-transfected cells (diamonds) and cells expressing BSVprXThy (squares), BSVprThy (triangles), BSA30PThy (crosses), BSA30LThy (asterisks), and BSR80AThy (circles). The analysis was carried out on the same population of cells as examined in panel A. (C) Annexin V staining of Thy 1.2-positive cells. Transfected HeLa cells were stained with Annexin V, 7-AAD, and anti-Thy 1.2–PE. At 22, 46, 75, 92, and 116 h posttransfection, 5×10^3 cells were analyzed by flow cytometry. Symbols are as in panel B. The analysis was carried out on the same population of cells as that examined in panel A. (D) Cell counts. Transfected HeLa cells were counted in duplicate at each time point by trypan blue exclusion to correlate cell survival with Annexin V staining. Symbols are as described for panel B. The analysis was carried out on the same population of cells as that examined in panel A.

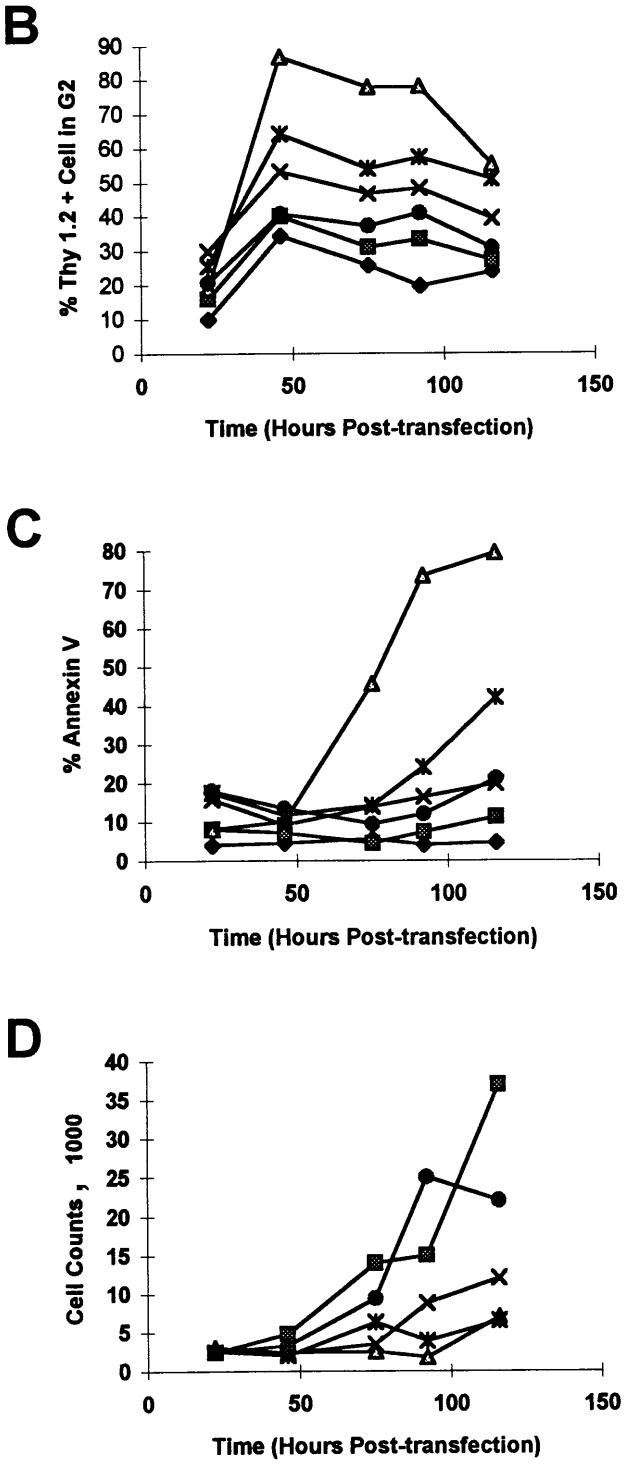

FIG. 5—*Continued.*

mulated in G_2 within 16 to 24 h of infection. Following arrest, we began to detect increased cell death at 30 h, which peaked at 60 to 90 h postinfection. It is likely that the discrepancy between our data and those of Bartz et al. is due to the postinfection time point at which the cells were monitored. Specifically, they monitored infected cells for only 3 days, whereas we have observed that apoptosis increased over time and was usually maximal between 3 and 4 days postinfection.

Numerous studies have examined the effects of various HIV-1 gene products on cellular survival. Depending on the model system used, seemingly discordant reports regarding the role of viral genes on cellular survival have appeared in the literature. Tat, a viral transcription factor, has been shown to affect transcription of genes involved in cell survival. Tat was found to up-regulate Bcl-2 expression, protecting cells from apoptosis (71). In contrast to protection of cells from apoptosis, establishment of stable Tat-expressing cell lines or addition of exogenous Tat has also been reported to sensitize cells to anti-T-cell receptor- and anti-CD4-induced apoptosis (35, 48). In those studies, Tat appeared to sensitize cells to apoptosis, and only upon a second signal, such as CD4 cross-linking, did death occur. These studies demonstrated that Tat alone was insufficient to induce apoptosis. In our system employing HIV-1 and natural host cells, the presence of Vpr increased apoptosis without addition of a second stimulus. Therefore, Tat was not protective when produced at naturally occurring concentrations. In addition, we observed that the HIV-$1_{NL4\text{-}3Thy}$env(−)VprX/VSV-G virus, which contains a *vpr* mutant, could also induce apoptosis, albeit at lower levels than the HIV-$1_{NL4\text{-}3Thy}$env(−)/VSV-G virus, indicating that other viral factors influence induction of apoptosis.

HIV-1 Env has also been implicated in induction of apoptosis. Coculture of uninfected H9 cells with HIV-1-infected H9 cells resulted in apoptosis (39). Induction of apoptosis required the presence of the *env* gene and could be blocked by addition of soluble CD4. Furthermore, apoptosis did not require syncytium formation. These studies suggested that CD4-gp120 interaction was required for apoptosis. Banda et al. (3) have shown that cross-linking CD4 with gp120 primed T cells for apoptosis. Following CD4-gp120 cross-linking, a second stimulus, such as cross-linking of the T-cell receptor, resulted in apoptosis. Since a VSV-G pseudotyped virus was utilized, the data presented here do not address the effects of the *env* gene on apoptosis.

Various viruses encode proteins which enhance survival after infection, allowing the virus time to replicate. Both adenovirus and the human papillomavirus have been shown to encode proteins which specifically inhibit p53 (2, 67). p53 is a protein which is critical at the G_1 checkpoint (reviewed in references 7 and 11). Under normal circumstances, p53 allows cells to progress from G_1 to S. However, following DNA damage, p53 arrests cells in G_1. Arrest in G_1 allows repair of the damaged DNA before the cell enters the S phase of the cell cycle. If, however, the damaged DNA cannot be repaired, an apoptotic signal that prevents the cell from duplicating the damaged DNA and transferring it to daughter cells is given. Therefore, inactivation of p53 abrogates the ability of the cell to induce both G_1 arrest and apoptosis, possibly allowing viruses such as human papillomavirus time to replicate. Vaccinia virus encodes a second type of inhibitory protein, CrmA, which specifically inhibits activation of interleukin 1-converting enzyme (31). Interleukin 1-converting enzyme is a cysteine protease involved in the activation of many apoptotic pathways (53). Unlike the case for vaccinia virus, infection with other viruses, such as dengue virus and influenza virus, resulted in increased apoptosis (14, 26). Sindbis virus (SV) is a lytic virus which has been shown to induce apoptosis in nonneuronal cells (34). In contrast, infection of neuronal cells with SV does not lead to apoptosis and results in a persistent infection. It was proposed (34) that apoptosis serves in this model to limit establishment of a chronic infection. Interestingly, induction of

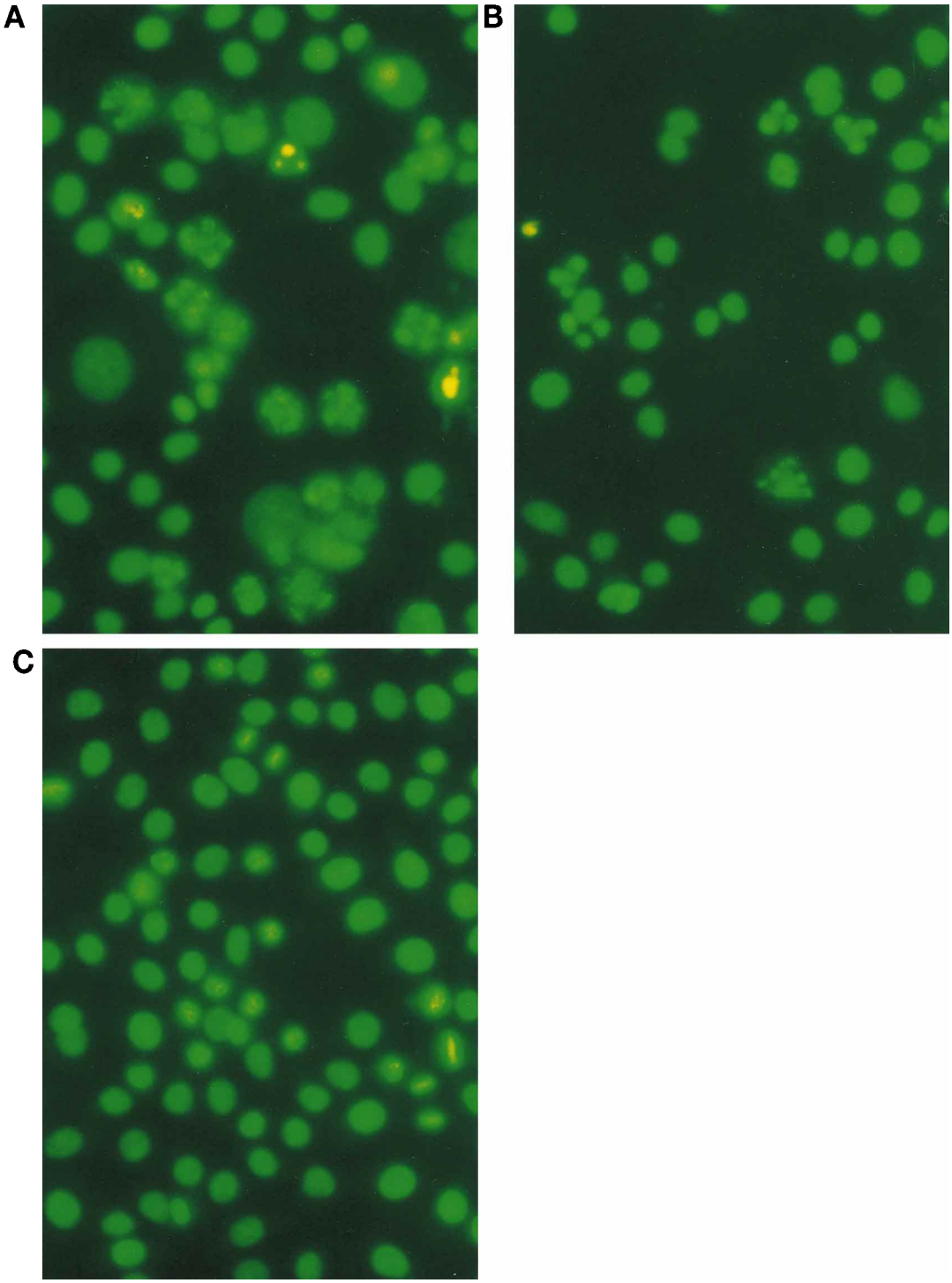

FIG. 6. Microscopic analysis of HeLa cells transfected with BSA30LThy reveals that the pyknotic nuclei observed within these cells were similar to those observed in cells expressing Vpr. Transfected HeLa cells (10^7) were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and stained with Hoescht 33342 to examine DNA content. (A) DNA content of BSVprThy-transfected cells, revealing the presence of pyknotic nuclei. (B) BSA30LThy-transfected cells, similar to the cells in panel A, also contain pyknotic nuclei. (C) HeLa cells treated with gamma interferon and anti-Fas, as described for Fig. 1.

apoptosis by influenza virus and SV can be blocked by overexpression of the cellular *bcl-2* gene.

Apoptosis is a precisely orchestrated sequence of events that allows multicellular organisms to rid themselves of unwanted cells. Interestingly, it has become apparent that different apoptotic stimuli can induce various biochemical pathways which ultimately lead to apoptosis. Granzyme B-induced apoptosis requires activation of the Cdc2 kinase (58). Inhibition of Cdc2 kinase with drug treatment inhibits apoptosis in this system. In contrast, Ongkeko et al. (46) have demonstrated that inactiva-

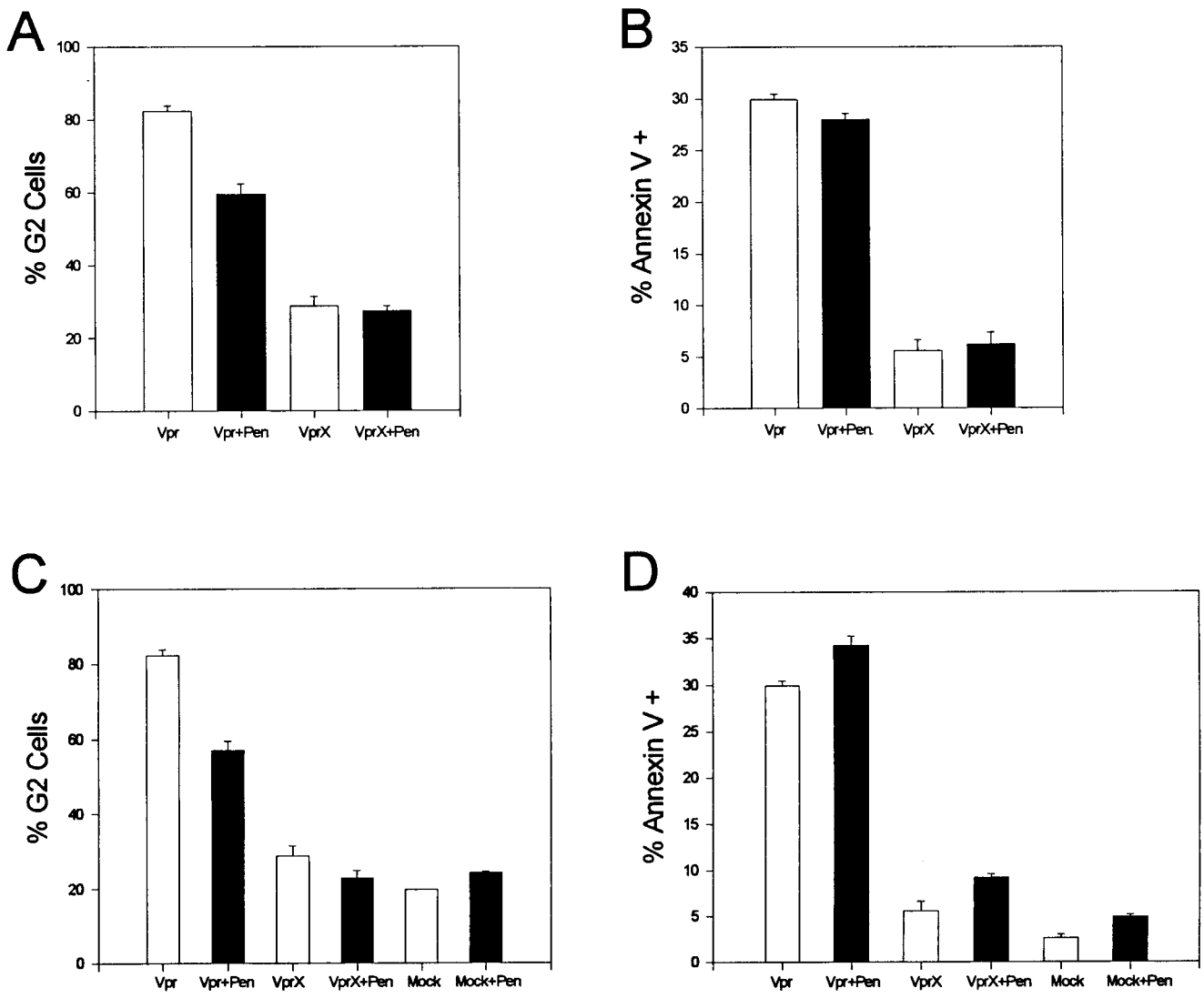

FIG. 7. Pentoxifylline treatment of Vpr-arrested HeLa cells reduces the number of cells in the G_2 phase of the cell cycle but does not reduce the levels of Vpr-induced apoptosis. HeLa cells were transfected with BSVprThy or BSVprXThy, and at either 0 or 24 h posttransfection, 0.5 mM pentoxifylline (Pen) was added to the cultures. Cells were analyzed for DNA content and apoptosis at 68 h posttransfection. Transfected HeLa cells were plated in triplicate to allow statistical analysis of the flow cytometric results. The data are representative of three independent experiments. Standard deviations were determined by analyzing samples in triplicate within the one experiment shown. (A) Effect of pentoxifylline on Vpr-arrested cells. Pentoxifylline treatment at 24 h posttransfection resulted in a decrease in the number of Vpr-arrested cells in the G_2/M phase of the cell cycle. Solid bars represent analysis of BSVprThy- or BSVprXThy-transfected cells (5×10^3) treated with 0.5 μM pentoxifylline at 24 h posttransfection, and open bars represent cells receiving no drug treatment. (B) Annexin V analysis of BSVprThy- or BSVprXThy-transfected HeLa cells (5×10^3) treated either with 0.5 μM pentoxifylline (solid bars) at 24 h posttransfection or with no drug (open bars). The cells were from the same transfected population examined in panel A. (C) Pentoxifylline treatment of transfected cells at the time of transfection (0 h) resulted in a decrease in the number of Vpr-arrested cells in the G_2/M phase of the cell cycle. Solid bars represent analysis of mock-, BSVprThy-, or BSVprXThy-transfected cells (5×10^3) treated with 0.5 μM pentoxifylline, and open bars represent cells receiving no drug. (D) Annexin V analysis of mock-, BSVprThy-, or BSVprXThy-transfected HeLa cells (5×10^3) treated either with 0.5 μM pentoxifylline (solid bars) at 0 h posttransfection or with no drug (open bars). The cells were from the same transfected population examined in panel C.

tion of Cdc2 kinase increased levels of apoptosis induced by DNA strand-breaking drugs. Finally, Norbury et al. (43) have shown that activation of Cdc2 kinase is not required for induction of apoptosis in thymocytes. These discordant reports emphasize that the system used and the type of apoptotic stimuli applied can produce varied results. In light of the various reports on the requirement for Cdc2 kinase activity in apoptosis, we examined the phosphorylation state of Cdc2 kinase in Vpr-induced apoptosis to determine whether activation of Cdc2 kinase was required prior to the onset of apoptosis. Western blot analysis of the Cdc2 kinase indicated that as the levels of HIV-1-induced apoptosis increased over time, the amount of the hyperphosphorylated or inactive Cdc2 kinase appeared to remain constant. We also observed that addition of pentoxifylline to Vpr-arrested cells alleviated G_2 arrest but did not abrogate apoptosis. Together, these studies indicate that Vpr-induced apoptosis occurs independently of Cdc2 kinase activity.

Different *vpr* point mutants had various effects on the induction of apoptosis. Previous studies have demonstrated that mutation of amino acid 30, which lies within a proposed alpha helix, resulted in loss of the G_2 arrest phenotype (15, 68).

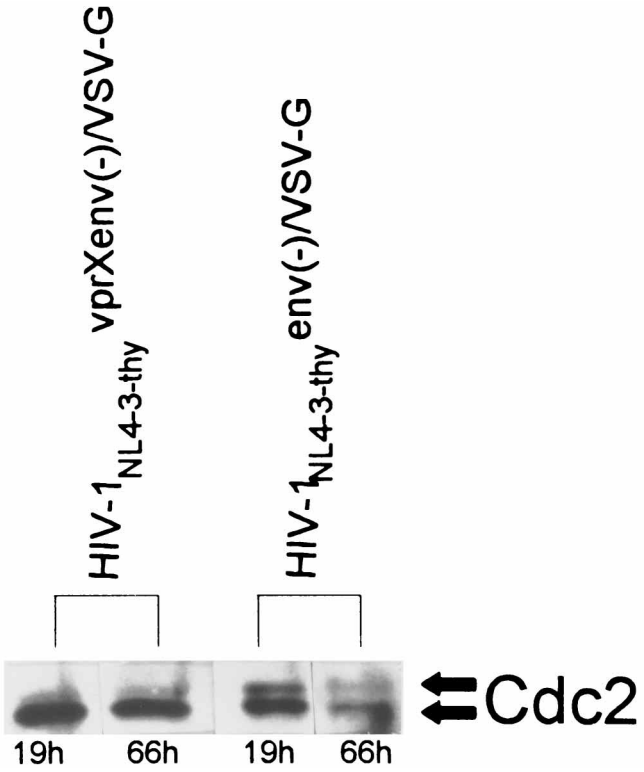

FIG. 8. Vpr-induced apoptosis does not require alteration of the Cdc2 kinase phosphorylation state. Protein was isolated from infected cells, and 5 μg was subjected to Western blot analysis, as described in Materials and Methods. The phosphorylation states of Cdc2 kinase in HeLa cells infected with HIV-$1_{NL4\text{-}3Thy}$env(−)VprX/VSV-G at 19 and 66 h postinfection or infected with HIV-$1_{NL4\text{-}3Thy}$env(−)/VSV-G at 19 and 66 h postinfection are represented. Annexin V analysis of 5×10^3 HIV-$1_{NL4\text{-}3Thy}$env(−)/VSV-G-infected cells revealed that 1.7 and 33.8% were Annexin V positive at 19 and 66 h postinfection, respectively. Analysis of HIV-$1_{NL4\text{-}3Thy}$env(−)VprX/VSV-G-infected cells indicated that 1.1 and 7.0% of cells were Annexin V positive at 19 and 66 h postinfection, respectively. The upper arrow indicates the inactive hyperphosphorylated form of Cdc2 kinase, and the lower arrow indicates the active hypophosphorylated form of Cdc2 kinase.

Similarly, mutation of amino acid 80 also resulted in loss of G_2 arrest. We found that these mutations had different effects on Vpr-induced apoptosis. Mutation of arginine 80 to alanine and alanine 30 to proline resulted in a severe reduction of both G_2 arrest and apoptosis, while mutation of alanine 30 to leucine had a more moderate effect. Specifically, this mutation resulted in an intermediate loss of G_2 arrest and apoptosis compared to that with the wild-type protein. In general, mutants which induced more cell cycle arrest showed higher levels of apoptosis.

Studies examining the *vpr* point mutants revealed that the level of Vpr-induced cell cycle arrest correlated with the level of apoptosis. These studies did not, however, determine whether maintenance of G_2 arrest was required for induction of apoptosis. Therefore, to address this question, we treated Vpr-arrested cells with pentoxifylline, which alleviated Vpr-induced G_2 arrest and allowed cells to continue cycling. We found that alleviation of G_2 arrest did not significantly reduce the levels of apoptosis observed. These studies suggest the possibility that while initial cell cycle perturbation may be required to initiate an apoptotic signal, maintenance of G_2 arrest is not required for Vpr-induced apoptosis to occur. However, we cannot distinguish from these experiments whether the onset of G_2 arrest is required for initiation of Vpr-induced apoptosis.

An important question in HIV-1 pathogenesis is whether virus killing is limited to infected T cells. Gougeon et al. (21) and Meyaard et al. (41) have shown that both CD4 and CD8 cells isolated from AIDS patients die upon culture in vitro. In addition, Finkel et al. (19) have analyzed lymph nodes from HIV-1-infected children and SIV-1-infected macaques and have shown that the majority of T-cell death occurred within uninfected bystander cells. In contrast, studies examining peripheral blood suggested that T-cell death occurs predominately in infected cells, suggesting direct viral killing (25, 49, 59, 64). The differences in these studies may be due to the source of the T-cell population analyzed. In our experiments examining PBL, we noted that Vpr-induced apoptosis occurs predominately in the Thy 1.2-positive population with little or no effect on Thy 1.2-negative bystander cells.

Data presented here suggest that Vpr-induced apoptosis may play a role in the death of infected T cells during HIV-1 disease. It has been shown that during the later stages of disease, T cells in the peripheral blood have an average half-life of 2.2 days. In addition it has been estimated that 1×10^9 to 2×10^9 T cells die daily through direct viral killing (27, 49, 64). This CD4 T-cell death is hypothesized to eventually exhaust the immune system's regenerative properties and lead to CD4 cell depletion and, eventually, AIDS. We have observed that Vpr is capable of arresting cells within 16 h of HIV-1 infection, and Annexin V analysis indicates that in the presence of Vpr, apoptosis can be observed as early as 24 to 30 h postinfection (data not shown). Although the kinetics of infection in vivo and in vitro may be different, our results are consistent with the half-life of HIV-1-infected cells in vivo. While we have not examined the direct effect of HIV-1 Vpr on cell survival in patient tissue, several groups have attempted to study the role of apoptosis in CD4 cell depletion. Some reports have shown that cells isolated from HIV-1-positive patients undergo apoptosis more readily than cells isolated from seronegative donors (41). In addition, apoptosis has been observed in lymph nodes isolated from HIV-1-positive patients (42). Interestingly, chimpanzees can be infected with HIV-1, but they do not develop disease (24). Analysis of chimpanzee T cells revealed that they do not undergo apoptosis following infection, leading to the hypothesis that the ability of HIV-1 to induce apoptosis may be related to disease progression in humans (16, 21). Therefore, we propose that HIV-1 Vpr may contribute to the overall induction of cell death and contribute to CD4 cell decline in disease.

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