The Human Immunodeficiency Virus Type 1 Accessory Protein Vpu Induces Apoptosis by Suppressing the Nuclear Factor κB–dependent Expression of Antiapoptotic Factors

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Abstract

Human immunodeficiency virus (HIV) type 1 Vpu is an integral membrane protein with a unique affinity for βTrCP (TrCP), a key member of the SkpI-Cullin-F-box E3 ubiquitin ligase complex that is involved in the regulated degradation of cellular proteins, including IκB. Remarkably, Vpu is resistant to TrCP-mediated degradation and competitively inhibits TrCP-dependent degradation of IκB, resulting in the suppression of nuclear factor (NF)-κB activity in Vpu-expressing cells. We now report that Vpu, through its interaction with TrCP, potently contributes to the induction of apoptosis in HIV-infected T cells. Vpu-induced apoptosis is specific and independent of other viral proteins. Mutation of a TrCP-binding motif in Vpu abolishes its apoptogenic property, demonstrating a close correlation between this property of Vpu and its ability to inhibit NF-κB activity. The involvement of NF-κB in Vpu-induced apoptosis is further supported by the finding that the levels of antiapoptotic factors Bcl-xL, A1/Bfl-1, and TNF receptor-associated factor (TRAF)1, all of which are expressed in an NF-κB–dependent manner, are reduced and, at the same time, levels of active caspase-3 are elevated. Thus, Vpu induces apoptosis through activation of the caspase pathway by way of inhibiting the NF-κB–dependent expression of antiapoptotic genes.

Key words: TrCP • caspase • TNF-α • Bcl-xL • TRAF1

Introduction

HIV-1 infection is generally associated with a progressive decrease in the number of CD4+ T lymphocytes. This phenomenon is presumably one of the key factors contributing to the virus-induced impairment of the host immune response and is generally considered to be caused by programmed cell death (apoptosis; reference 1). In vitro, two distinct mechanisms for the induction of apoptosis in CD4+ T cells have been observed: one is the induction of apoptosis in uninfected bystander cells; the second mechanism involves the direct killing of infected cells by HIV-1 (1). Apoptosis of uninfected bystander cells can be caused by gp120, which may induce aberrant T cell signaling through binding to CD4 molecules on uninfected cells. In addition, the secretion of HIV-encoded factors such as Tat, Nef, or Vpr or the release from HIV-infected cells of cellular apoptosis-inducing factors such as Fas ligand, TNF-α, or TNF-related apoptosis-inducing ligand were all found to trigger apoptosis in uninfected bystander cells (1). One of the viral factors known to induce direct killing of infected cells in vitro is Vpr, which was found to induce cell cycle arrest in the G2/M phase, followed by induction of apoptosis (2–4). However, CD4+ T cells infected with *vpr*-defective HIV-1 still undergo apoptosis, suggesting that viral product(s) other than Vpr contribute to the induction of apoptosis in infected cells (2, 3).

Vpu is a viral membrane protein that regulates the release of virions from infected cells and induces degradation of CD4 (5–7). These two functions of Vpu are mechanistically distinct (8, 9). In HIV-2, virus release is regulated by the viral Env product thus compensating for the lack of a *vpu* gene (10–12). In contrast, the ability of Vpu to induce CD4 degradation has no functional complement in HIV-2 or simian IV viruses and thus constitutes one of the distinguishing characteristics of HIV-1. CD4 degradation re-

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quires the formation of ternary complexes between Vpu, CD4, and βTrCP (13, 14). βTrCP (TrCP) is a component of E3 ubiquitin ligase complexes (14) and regulates degradation of various cellular substrates including β-catenin or IκB-α, the latter being a potent inhibitor of nuclear factor (NF)*-κB (15). Unlike normal cellular substrates of TrCP, which are directly targeted for degradation, Vpu is insensitive to degradation and can form stable complexes with TrCP (14). As a result, we found that Vpu is able to competitively inhibit the cellular function of TrCP, including the virus- or cytokine-induced degradation of IκB-α (16). Vpu did not inhibit the cytokine-mediated activation of the IκB kinase, but instead interfered with the subsequent TrCP-dependent degradation of phosphorylated IκB-α and resulted in a pronounced reduction of NF-κB activity (16). NF-κB has a central role in the regulation of genes involved in cell proliferation, cytokine production, as well as in the regulation of apoptosis (17, 18). Therefore, Vpu expression in HIV-1–infected cells could have a profound impact on NF-κB regulated gene expression and thus could contribute to the virus-induced cytopathic effects.

Based on these observations, we have explored in this study the possible involvement of Vpu in HIV-1–induced apoptosis. We found that in HIV-1–infected CD4+ T cells Vpu contributed significantly to the induction of apoptosis. Using an inducible expression system we found that the effect of Vpu on apoptosis was direct and did not require the coexpression of other viral proteins. Analysis of cellular factors involved in the induction of apoptosis demonstrated that Vpu downmodulated the NF-κB–dependent expression of antiapoptotic genes such as Bcl-xL and A1/Bfl-1. Concomitantly, Vpu expression resulted in increased levels of active caspase-3. These effects of Vpu involved an interaction with TrCP as evidenced by the fact that mutation of the TrCP binding motif in Vpu abolished its apoptogenic potential. These results suggest that Vpu promotes apoptosis through its inhibition of NF-κB.

Materials and Methods

Plasmids. The full-length HIV-1 molecular clone pNL4–3 was used for the production of wild-type infectious virus. Construction of the Env- and Vpu-defective variants pNL43-K1 (10) and pNL4–3/Udel (6), respectively, was described previously. Plasmid pNL4–3/$U_{2/6}$ encodes a TrCP-binding deficient variant of Vpu and carries two serine to alanine mutations in its cytoplasmic domain ($S_{52,56}A$). Construction of this plasmid has been described previously (8). To inactivate the *env* and/or *vpr* genes in pNL4–3, pNL4–3/Udel, or pNL4–3/$U_{2/6}$, frame-shift mutations were introduced at a *Kpn*I site (NL4–3 pos. 6343) in the *env* gene or an *EcoR*I site (NL4–3 pos. 5743) in the *vpr* gene (or both), resulting in pNL43-K1/Udel (Env$^-$, Vpu$^-$), pNL43-K1/$U_{2/6}$ (Env-, Vpu-TrCP binding mutant), pNL43-EcK1/Udel (Vpr$^-$, Vpu$^-$, Env$^-$), or pNL43-EcK1/$U_{2/6}$ (Vpr-, Env-, Vpu-TrCP binding mutant). The plasmid pHCMV-G contains the vesicular stomatitis virus glycoprotein G (VSV-G) gene under the transcriptional regulation of the human cytomegalovirus immediate early promoter and was used for the production of VSV-G pseudotyped viruses.

Cells. 293T cells were maintained in DMEM containing 10% FBS. Jurkat cells were cultured in RPMI 1640 medium supplemented with 10% FBS. HeLa cell lines for the inducible expression of the CD4-Vpu chimeric proteins CD4U or CD4$U_{2/6}$ under the control of a tetracycline/doxycycline (Dox) repressed promoter have been described previously (16). These cells were maintained in complete DMEM medium supplemented with G418 (1 mg/ml), Dox (20 ng/ml), and hygromycin (200 μg/ml). PBLs were isolated from leukapheresed blood of HIV-seronegative donors by countercurrent centrifugal elutriation as described previously (19). CD4+ T lymphocytes were purified using a magnetic bead system (Miltenyi Biotec) according to the manufacturer's instructions. The purity of the preparation was >90% as determined by flow cytometry. The CD4+ cells were then stimulated with phytohemagglutinin-P (Bacto) at 1 μg/ml in RPMI 1640 medium supplemented with 10% FBS and 10 U/ml recombinant human IL-2 (Boehringer Mannheim) for 2 d before infection.

Preparation of VSV-G Pseudotyped Viruses. VSV-G pseudotyped viruses were produced in 293 T cells by cotransfection of 20 μg of pNL4–3 DNA, or one of its variants together with 2 μg of pHCMV-G per 2×10^7 cells in 75 cm^2 tissue culture flasks. Virus supernatants were harvested 48 h after transfection. Filtered (0.45 μm Sterivex-HV filter; Millipore) supernatants were ultracentrifuged for 1 h at 25,000 rpm using an SW41 rotor (Beckman Coulter). Concentrated viruses were suspended in RPMI 1640 medium. Virus stocks were quantified by reverse transcriptase assay and infectious titers were determined by MAGI assay (20).

Detection of HIV-1-infected Cells. HIV-1–infected Jurkat cells were fixed in 1% formaldehyde (in PBS) for 15 min at 4°C and then permeabilized using FACS® permeabilizing solution (Becton Dickinson) for 15 min at 4°C. The cells were incubated for 15 min at 4°C with 10 μg/ml of mouse IgG to block nonspecific binding sites. Samples were then labeled with a PE-conjugated anti–HIV-1 p24 mAb (KC57; Beckman Coulter) for 30 min at 4°C. Cells were then suspended in 1% formaldehyde and analyzed for fluorescence intensity by flow cytometry.

Analysis of Apoptosis and Cell Cycle. Apoptotic cells were identified by either annexin V binding (21, 22) or by staining with the vital dye 7-amino-actinomycin D (7-AAD). Binding of annexin V is observed in early and late apoptotic cells while staining with 7-AAD is indicative of late apoptosis. To stain HeLa cells, 2×10^5 cells were detached by treatment with Trypsin/EDTA (0.05% Trypsin, 0.53 mM EDTA; Life Technologies) and suspended in 300 μl of ice-cold annexin V binding buffer containing Hepes-NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$ (BD PharMingen). Cells were then reacted for 15 min on ice with 5 μl each of PE-conjugated annexin V (BD PharMingen) and 7-AAD (BD PharMingen), followed immediately by flow cytometric analysis. For analysis of HIV-1–infected Jurkat cells, the cells were treated and analyzed as described for HeLa cells, except that cells were fixed in 1% formaldehyde (in annexin V binding buffer) before FACS® analysis to inactivate virus.

To detect fragmentation of chromosomal DNA, terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL)

**Abbreviations used in this paper:* 7-AAD, 7-amino-actinomycin D; c-IAP, cellular inhibitor of apoptosis; Dox, doxycycline; MIP, macrophage inflammatory protein; m.o.i., multiplicity of infection; NF, nuclear factor; PI, propidium iodide; TRAF, TNFR-associated factor; TUNEL, transferase dUTP nick end-labeling; RANTES, regulated on activation, normal T cell expressed and secreted; VSV-G, vesicular stomatitis virus glycoprotein G.

assay was performed using an APO-DIRECT kit (BD PharMingen). Reactions were done as per the manufacturer's instructions.

For microscopic analysis of apoptosis-related phenotypic changes of the nuclei, cells were stained with propidium iodide (PI) as follows: HeLa cells grown on coverslips were fixed with 1% formaldehyde (in PBS) for 30 min at 4°C followed by incubation with 70% ethanol for 30 min at 4°C. Cells were then treated with RNase A (1 mg/ml in PBS) for 15 min at 37°C before PI was added to a final concentration of 50 μg/ml. PI staining continued for 15 min at room temperature before samples were mounted on microscope slides for confocal microscopy.

For cell cycle analysis Jurkat cells were fixed in 1% formaldehyde (in PBS) and permeabilized using FACS® permeabilizing solution. The cells were washed and treated with RNaseA (1 mg/ml in PBS) for 15 min at 37°C, followed by staining with PI (50 μg/ml) for more 15 min at room temperature. Cells were then analyzed by flow cytometry and the data were evaluated for cell cycle status using the ModFit LT software (Becton Dickinson).

Detection of Active Caspase-3. The active form of caspase-3 was detected using an active form-specific antibody. Briefly, HeLa cells were detached by treatment with Trypsin/EDTA (0.05% Trypsin, 0.53 mM EDTA; Life Technologies) and fixed in 1% formaldehyde (in PBS) for 15 min at 4°C. Cells were permeabilized using FACS® permeabilizing solution for 15 min at 4°C. The cells were then incubated for 15 min at 4°C with mouse IgG (10 μg/ml) to block nonspecific binding followed by a 30-min incubation at 4°C with a FITC-conjugated rabbit antiactive caspase-3 polyclonal antibody (BD PharMingen). Finally, the stained cells were fixed in 1% formaldehyde (in PBS) and analyzed for fluorescence intensity using a FACSort™ (Becton Dickinson).

Western Blot Analysis. Western blot analyses were performed using the ECL detection system (Amersham Pharmacia Biotech) as described previously (16). The Abs used were as follows: anti–TNFR-associated factor (TRAF1) mAb (H-3; Santa Cruz Biotechnology, Inc.); anticaspase-8 polyclonal antibody (BD PharMingen); anti–Bcl-xL mAb (2H12; BD PharMingen); anti-A1/Bfl-1 polyclonal antibody (Santa Cruz Biotechnology, Inc.); anti–α-tubulin mAb (DM 1A; Sigma-Aldrich); anti-Vpu polyclonal antibody (U2–3; reference 23); and anti-p24 capsid mAb (provided by S. Zolla-Pazner, AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH; reference 24).

Results

Vpu Promotes Apoptosis in HIV-1-infected Jurkat Cells. In the first set of experiments, Jurkat cells were single-cycle infected with VSV-G-pseudotyped HIV-1NL4–3 (NL4–3/G) at a multiplicity of infection (m.o.i.) of 5. Cells were analyzed 48 h after infection for the induction of apoptosis using annexin V and 7-AAD staining as markers (Fig. 1 A). In mock-infected cultures, only 1.7% of the cells were annexin V-positive with half of those cells also scoring positive for 7-AAD (Fig. 1 A, panel a). In contrast, in cultures infected with NL4–3/G, 51.1% of the cells were in the early phase of apoptosis (annexin V^+ 7-AAD^-) and 28.8% of the cells were already in the late phase of apoptosis (annexin V^+ 7-AAD^+; Fig. 1 A, panel b). To assess the relative impact of Env expression on the induction of apoptosis, Jurkat cells were infected with a VSV-G-pseudotyped Env-defective variant, HIV-1NL43-K1 (Fig. 1 A, panel c). The results showed that the proportion of cells found in the early (48.1%) or late phase of apoptosis (24.4%) was comparable to that in NL4–3/G-infected cells (compare panels b and c). This indicates that the expression of HIV-1 Env is not a major factor in the induction of apoptosis in our experimental system. Surprisingly, infection of Jurkat cells by the pseudotyped Vpu-defective variant, NL4–3/Udel/G (Fig. 1 A, panel d) showed a significant reduction of annexin V- and 7-AAD-positive cells. In fact, the total number of annexin V-positive cells was reduced by >40% in the absence of Vpu relative to wild-type virus (compare Fig. 1 panels b and d). These results suggest that Vpu may play a significant role in the induction of apoptosis in HIV-1-infected cells. To ascertain that the results from Fig. 1 A were not biased by different infection efficiencies, we determined the intracellular expression levels of Gag proteins for the cultures shown in Fig. 1 A by staining with a p24 capsid (CA) antibody followed by flow cytometry (Fig. 1 B). The results demonstrate that all cultures (except mock) were infected with similar efficiency and

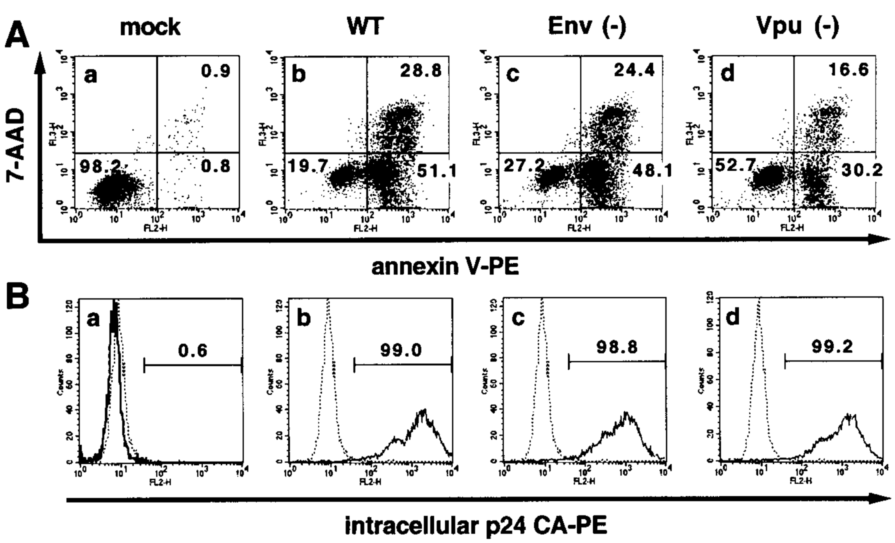


Figure 1. Vpu induces apoptosis in HIV-1–infected Jurkat cells. Uninfected Jurkat cells (a) or Jurkat cells infected with an m.o.i. of 5 with VSV-G-pseudotyped virus stocks of (b) wild-type (WT) NL4–3, (c) NL43-K1 (Env^-), or (d) NL4–3/Udel (Vpu^-) variants were used for this analysis. (A) Cultures were analyzed 48 h after infection for the presence of apoptotic cells by staining with 7-AAD and PE-conjugated annexin V, followed by flow cytometric analysis. Numbers represent the percentages of cells in the respective quadrants. (B) The same cultures were evaluated 24 h after infection for HIV-1 infection by intracellular p24 staining using PE-conjugated mouse mAb to HIV-1 p24 followed by flow cytometry. Numbers represent the percentages of p24-positive cells. The dotted lines in panels a–d represent p24-staining of mock-infected cells. The solid lines in panels a–d represent p24-staining of infected cells. The results shown are representative of three independent experiments.

produced comparable levels of p24 CA antigen, as determined by fluorescence intensity.

Vpu-induced Apoptosis Is Independent of HIV-1 Env or Vpr and Requires a TrCP Binding Motif. We have previously shown that Vpu can act as a competitive inhibitor of TrCP and, as a consequence, suppress the activation of NF-κB (16). This effect is dependent on a motif in the cytoplasmic domain of Vpu that includes two phosphoserine residues and constitutes a binding domain for TrCP (14). To examine whether the inhibition of TrCP could be functionally related to the Vpu-induced apoptosis observed in Fig. 1, we compared the apoptotic potential of wild-type Vpu and a TrCP binding mutant, $Vpu_{2/6}$. At the same time, we wanted to compare the relative effects of Vpu with those of Vpr, a known inducer of apoptosis (2, 3). Also, since Vpu can affect surface transport of Env in CD4-expressing cells (25) and to rule out second cycles of infection, we employed Env-defective viruses for the following experiment.

Env-defective virus preparations, pseudotyped with VSV-G, lacking Vpu and/or Vpr expression or expressing $Vpu_{2/6}$ were prepared as described for Fig. 1 and used for single-cycle infection of Jurkat cells. Intracellular p24 staining followed by FACS® analysis, as performed in Fig. 1, confirmed that infection efficiency and protein expression levels were comparable for all samples (data not shown). As shown in Fig. 2 A, uninfected cells exhibited only low levels (<2%) of spontaneous apoptosis (Fig. 2 A, panel a). In contrast, infection of Jurkat cells by Vpu- and Vpr-expressing virus (panel b) induced severe apoptosis as evidenced by the fact that 72% of the cells were positive for annexin V within 48 h after infection. Deletion of Vpu (Vpr^+, Vpu^-, panel c) or mutation of the TrCP binding motif (Vpr^+, $Vpu_{2/6}$, panel d) reduced the proportion of annexin V-positive cells in the cultures to 39 and 45%, respectively. Similarly, deletion of Vpr (Vpr^-, Vpu^+, panel e) resulted in a marked reduction of annexin V^+ cells (41%) relative to cultures expressing both Vpr and Vpu (compare panels a and e). However, cultures infected with viruses lacking both Vpr and Vpu (Vpr^-, Vpu^-, panel f) or expressing $Vpu_{2/6}$ (Vpr^-, $Vpu_{2/6}$, panel g) exhibited even lower levels of apoptotic cells (15 and 20%, respectively). The residual apoptogenic property of HIV-1 lacking Env, Vpu, and Vpr (panels f and g) presumably is due to the expression of other viral proteins such as Tat (1). Results of Fig. 2 A indicate that Vpu and Vpr have the ability to induce apoptosis with similar efficiency. Moreover, the data show that Vpu-mediated apoptosis is not dependent on the presence of Vpr, raising the question as to whether Vpu mimics the activity of Vpr or induces apoptosis through a novel mechanism. The results of this experiment are summarized in Table I.

While the precise mechanism of Vpr-induced apoptosis is currently unclear, it appears to be correlated with the protein's ability to induce cell cycle arrest in the G2 phase

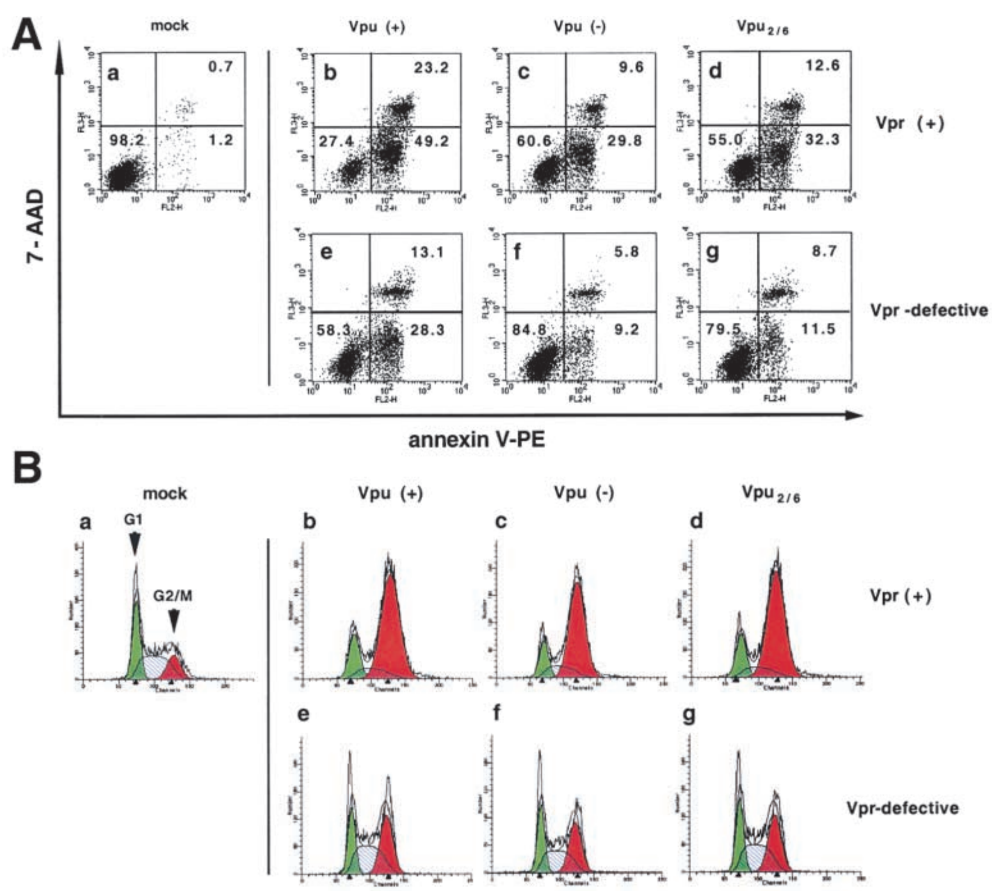


Figure 2. Vpu-induced apoptosis is independent of HIV-1 Env or Vpr and requires a TrCP-binding motif. Jurkat cells were (a) mock-infected or infected with an m.o.i. of 5 with VSV-G-pseudotyped virus stocks of (b) NL43-K1 (Env^-), (c) NL43-K1/Udel (Env^-, Vpu^-), (d) NL43-K1/$U_{2/6}$ (Env^-, $Vpu_{2/6}$), (e) NL43-EcK1 (Env^-, Vpr^-), (f) NL43-EcK1/Udel (Env^-, Vpr^-, Vpu^-), or (g) NL43-EcK1/$U_{2/6}$ (Env^-, Vpr^-, $Vpu_{2/6}$). (A) Cultures were analyzed 48 h after infection for apoptotic cells as in Fig. 1 A. (B) The same cultures were examined 24 h after infection for their cell cycle status by propidium iodide staining followed by flow cytometry. Similar results were obtained from three independent experiments.

Table I. *Effect of Vpu and Vpr on Induction of Apoptosis in HIV-1–infected Jurkat Cells*

	HIV-1 phenotype			% annexin V^+ cells	
Clones	*vpr*	*vpu*	*env*	Day 1	Day 2
Mock				1.9	1.9
NL43-K1	+	+	−	4.4	72.4
NL43-K1/Udel	+	−	−	3.0	39.4
NL43-K1/$U_{2/6}$	+	m[a]	−	4.1	44.9
NL43-EcK1	m[b]	+	−	2.3	41.4
NL43-Eck1/Udel	m[b]	−	−	2.1	15.0
NL43-Eck1/$U_{2/6}$	m[b]	m[a]	−	2.3	20.2

[a]$Vpu_{2/6}$.
[b]*vpr*-defective mutant.

Table II. *Effect of Vpu on Induction of Apoptosis in HIV-1–infected Primary CD4*$^+$ *T Lymphocytes*

	% annexin V^+ cells		
Clones	Day 1	Day 2	Day 3
Exp. 1			
mock	5.9		3.4
NL43-K1	8.4		30.1 (100)
NL43-K1/Udel	6.2		21.7 (69)
NL43-K1/$U_{2/6}$	5.8		23.9 (77)
Exp. 2			
mock	6.6	5.9	6.3
NL43-K1	7.1	12.8	28.9 (100)
NL43-K1/Udel	6.7	10.6	17.2 (48)
NL43-K1/$U_{2/6}$	6.9	11.2	18.4 (54)

The numbers in parenthesis indicate the relative percentages of annexin V-positive cells.

(2, 3). Numerous steps in cell cycle control are regulated by proteasome-dependent degradation of cell cycle regulators (26). Because of the transdominant negative effect of Vpu on the function of TrCP, a known regulator of proteasome-dependent protein degradation (14), it is possible that Vpu competitively suppresses the proteasome degradation of cell cycle-related factor(s), which in turn could promote apoptosis by a mechanism similar to that of Vpr. Such a mechanism would be in agreement with our finding that the TrCP-binding mutant of Vpu ($Vpu_{2/6}$) did not promote apoptosis (Fig. 2 A).

To assess the impact of Vpu on cell cycle control in HIV-infected Jurkat cells, we performed a cell cycle analysis on the cultures shown in Fig. 2 A. Aliquots of cells from each culture were removed 24 h after infection and processed for staining with propidium iodide as described in the Materials and Methods section. The results of this experiment are shown in Fig. 2 B. Infection of cells with virus expressing Vpr and Vpu (panel b) resulted in a significant accumulation of cells in G2. However, the absence of Vpu (panel c) or expression of $Vpu_{2/6}$ (panel d) had no significant effect on the HIV-induced cell cycle arrest. In contrast, the absence of Vpr largely reversed the HIV-induced G2 arrest irrespective of the presence or absence of Vpu (panels e–g). Thus, Vpu has no obvious impact on cell cycle control in infected Jurkat cells, suggesting that Vpu-induced apoptosis is due to an unrelated mechanism.

Vpu Promotes Apoptosis in HIV-1-infected Primary CD4$^+$ *T Lymphocytes.* We next wanted to ascertain that the apoptotic effect of Vpu noted above was not a phenomenon restricted to transformed cell lines but could be observed in primary cell types as well. To address this issue, activated primary $CD4^+$ T lymphocytes were infected for single-cycle analysis with the VSV-G pseudotyped, env-defective variants NL43-K1 (wild-type Vpu), NL43-K1/Udel (Vpu-), or NL43-K1/$U_{2/6}$ ($Vpu_{2/6}$). Cells were analyzed for annexin V staining as described for Fig. 2. The results from two different donors are summarized in Table II. Consistent with the results in Jurkat cells, expression of wild-type Vpu resulted in significantly increased annexin V staining when compared with cultures infected with the Vpu-defective variant or with a variant expressing $Vpu_{2/6}$. Infection of cells was comparable based on intracellular p24 staining (data not shown).

*Expression of CD4U but not CD4*$U_{2/6}$ *Causes Spontaneous Apoptosis in HeLa Cells.* The experiments presented in the previous sections demonstrate that Vpu significantly contributes to the induction of apoptosis in HIV-1–infected T cells independent of Vpr. However, it is nevertheless possible that Vpu alone is insufficient for induction of apoptosis and requires other viral protein(s). We have previously reported on the inducible expression of CD4-Vpu or CD4-$Vpu_{2/6}$ chimeras using a tetracycline/Dox-inducible vector system in stable HeLa cell lines (16). The CD4U and CD4$U_{2/6}$ chimeric molecules were found to have biological activities indistinguishable to those of wild-type Vpu and $Vpu_{2/6}$ (16, 27, 28). To assess the effect of Vpu on apoptosis in the absence of other HIV-1–specific proteins, we made use of these inducible cell lines. In a first set of experiments we compared the induction of apoptosis over time in the CD4U and CD4$U_{2/6}$ lines after removal of Dox. Cells were analyzed at various times after induction by annexin V staining (Fig. 3). The results of this experiment demonstrate that induction of CD4U but not CD4$U_{2/6}$ caused a dramatic increase in the number of annexin V-positive cells. Therefore, Vpu alone is sufficient for the induction of apoptosis. Furthermore, the fact that induction of CD4$U_{2/6}$ did not increase the number of apoptotic cells with time indicates that the observed effect of CD4U is specific and not the result of a nonspecific toxicity caused by the overexpression of a heterologous protein in these cells.

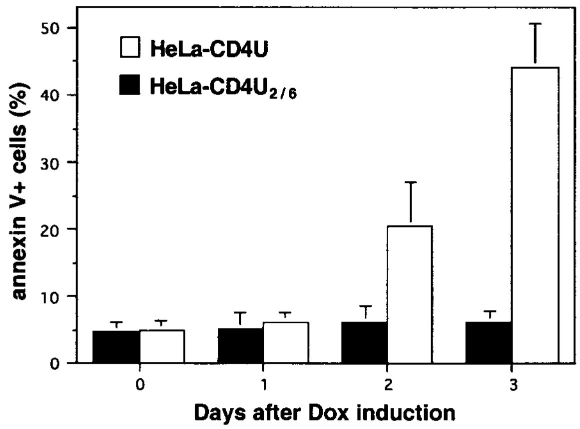
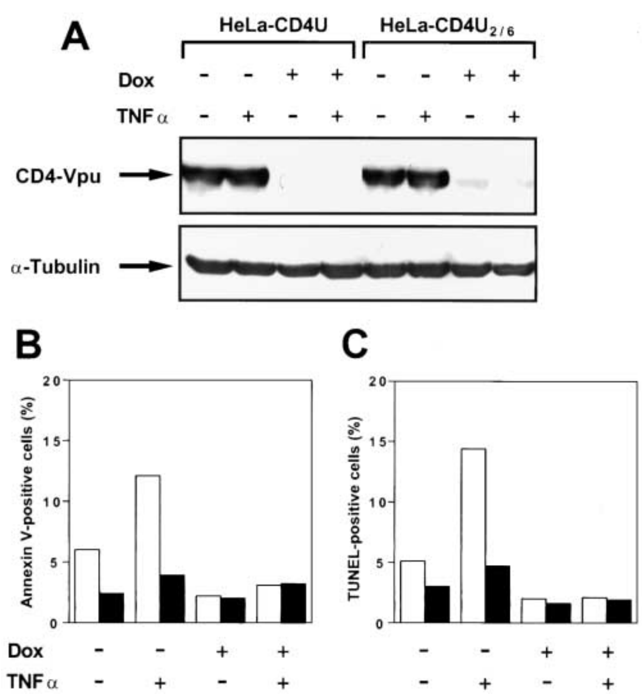


Figure 3. Expression of CD4U but not $CD4U_{2/6}$ causes spontaneous apoptosis in HeLa cells. Inducible HeLa-CD4U and $CD4U_{2/6}$ cell lines were cultured after the removal of Dox for the times indicated. The cells were then evaluated for induction of apoptosis by staining with PE-conjugated annexin V, followed by flow cytometry. Error bars reflect SDs from three independent experiments.

TNF-α Treatment Amplifies the Effect of Vpu on Apoptosis. We have recently shown that Vpu can inhibit cellular NF-κB activity by blocking the TrCP-dependent degradation of its inhibitor IκB (16). Therefore, we examined whether the induction of apoptosis in CD4U-expressing cells could be due to the inhibition of NF-κB activity, which controls the expression of antiapoptotic genes (1). We made use of our inducible cell lines to assess the impact of TNF-α treatment on apoptosis either in uninduced cells or after induction of CD4U. It is well documented that TNF-α can stimulate the activation of both NF-κB and caspase cascades, even though TNF-α stimulation alone does not cause apoptosis in most cells (17). The resistance to TNF-α requires the activation of NF-κB (29–31), which in turn induces the expression of various antiapoptotic factors such as TRAF1, TRAF2, cellular inhibitor of apoptosis (c-IAP)1, c-IAP2, Mcl-1, IEX-1L, Bcl-xL, and A1/Bfl-1, all of which are able to inhibit activation of caspases at various steps in the caspase pathway (32–37).

HeLa-CD4U and as a control HeLa-$CD4U_{2/6}$ cells were grown in the presence or absence of Dox for 24 h to inhibit or to induce Vpu expression, respectively. Cells were then treated with or without TNF-α for 16 h in the presence or absence of Dox. Induction of CD4U or $CD4U_{2/6}$ was confirmed by immunoblotting using a Vpu-specific antibody (Fig. 4 A, top). The same blot was subsequently reblotted with an antibody to α-tubulin as a loading control (Fig. 4 A, bottom). The expression levels of CD4U and $CD4U_{2/6}$ were comparable and were not affected by treatment of the cells with TNF-α. Induction of apoptosis was measured either by annexin V staining (Fig. 4 B), TUNEL assay (Fig. 4 C) or confocal microscopic analysis of nuclear staining with PI (Fig. 4 D). Expression of CD4U (white bars in Fig. 4 B and C) but not $CD4U_{2/6}$ (black bars in Fig. 4 B and C) in the absence of TNF-α led to a small



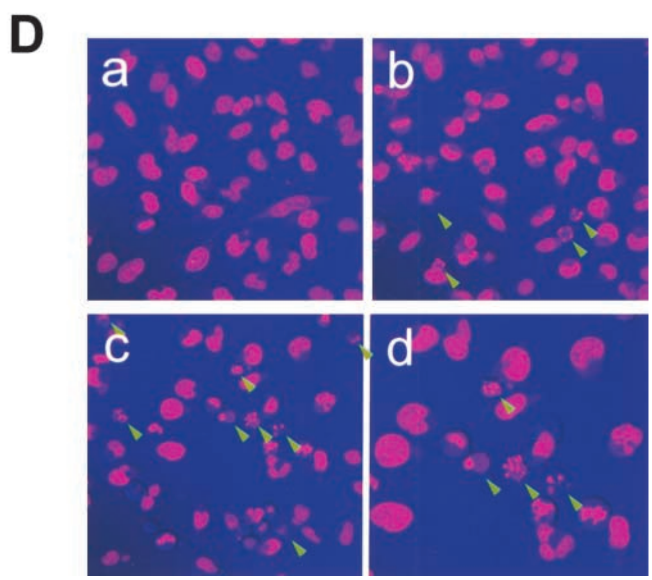


Figure 4. TNF-α treatment amplifies CD4U-induced apoptosis. CD4U and $CD4U_{2/6}$ cell lines were cultured in complete DMEM medium in the presence or absence of Dox for 24 h TNF-α (20 ng/ml) was then added to the samples as indicated and cultures were incubated for an additional 16 h before analysis. (A) The expression of CD4U and $CD4U_{2/6}$ (top) and α-tubulin (bottom) was determined by immunoblot analysis using a rabbit anti-Vpu polyclonal antibody (U2–3) and a mouse anti–α-tubulin mAb, respectively. (B and C) The cells were evaluated for induction of apoptosis by annexin V assay (B) or TUNEL assay (C), followed by flow cytometry. (D) Cultures were examined for apoptosis-related morphological changes of the nuclei by staining with PI, followed by confocal microscopic analysis. Panel a: Dox^{+}/TNF-α^{-}; panel b: Dox^{-}/TNF-α^{-}; panels c and d: Dox^{-}/TNF-α^{+} by low and high power magnification, respectively. Arrowheads mark cells containing pyknotic apoptotic bodies.

but detectable increase in the number of apoptotic cells noticeable in all three assay systems (compare TNF-α^- and Dox$^{+/-}$ in Fig. 4 B and C, also compare Fig. 4 D panels a and b). However, the effect of CD4U on apoptosis was significantly more pronounced in cultures treated with TNF-α where 12–15% of the cells were found to be apoptotic (TNF-α^+ and Dox$^{+/-}$ in Fig. 4 B and C, and Fig. 4 D panels c and d). PI staining revealed pyknotic nuclear apoptotic bodies, which are typical morphological characteristics of apoptosis, in Dox-depleted CD4U cell lines (indicated by arrowheads in Fig. 4 D) but not in $CD4U_{2/6}$ cells (data not shown). These results suggest that TNF-α promotes CD4U-induced apoptosis in HeLa cells. The fact that TNF-α alone, i.e., in the absence of CD4U expression, did not cause apoptosis under these experimental conditions suggests that TNF-α–mediated induction of apoptosis is facilitated by the Vpu-dependent suppression of NF-κB–dependent expression of antiapoptotic genes.

Vpu-induced Apoptosis Involves Activation of the Caspase Pathway. In view of our observation that Vpu has the ability to suppress spontaneous and TNF-α-induced NF-κB activation (16), it seems likely that Vpu-induced apoptosis is the result of an indirect activation of the caspase pathways by downmodulating the expression levels of antiapoptotic factor(s). To address this issue, we initially tested whether Vpu-induced apoptosis is dependent on the caspase pathway. For that purpose, we determined the effect of a broad-range inhibitor of caspases, Z-VAD-fmk, on CD4U-induced and TNF-α–enhanced apoptosis. HeLa-CD4U cells were cultured in the absence of Dox for 24 h and then treated for 16 h with TNF-α (10 ng/ml) either in the presence or absence of Z-VAD-fmk as indicated in Fig. 5. Cells were then reacted with PE-conjugated annexin V and analyzed by flow cytometry (Fig. 5 A). The results of this experiment show that treatment of cells with the caspase inhibitor reduced the level of annexin V-positive cells to background levels despite the presence of CD4U and TNF-α. Of note, treatment with Z-VAD-fmk did not affect the level of CD4U expression by Dox deprivation (data not shown).

Caspase-3 is a critical downstream protease in the caspase cascade, which is involved in the killing of cells in response to a number of apoptotic stimuli including TNF-α ligation with the TNF-receptor (38, 39). We evaluated the levels of the active form of caspase-3 in uninduced or induced HeLa-CD4U cells either in the presence or absence of TNF-α and/or Z-VAD-fmk as indicated in Fig. 5. Caspase-3 activity was determined by direct staining of cells with an FITC-conjugated rabbit antiactive caspase-3 polyclonal antibody followed by FACS® analysis (Fig. 5 B). The results of this experiment show that the percentage of cells expressing the active form of caspase-3 was proportional to the percentage of annexin V-positive cells. Moreover, Z-VAD-fmk treatment, which reduced the proportion of annexin V-positive cells to background levels (Fig. 5 A), simultaneously reduced the fraction of active caspase-3-positive cells to background levels (Fig. 5 B). These results indicate that Vpu-induced apoptosis and its enhancement by TNF-α are dependent on the activation of the caspase pathway, which eventually leads to the activation of the downstream effector caspase-3.

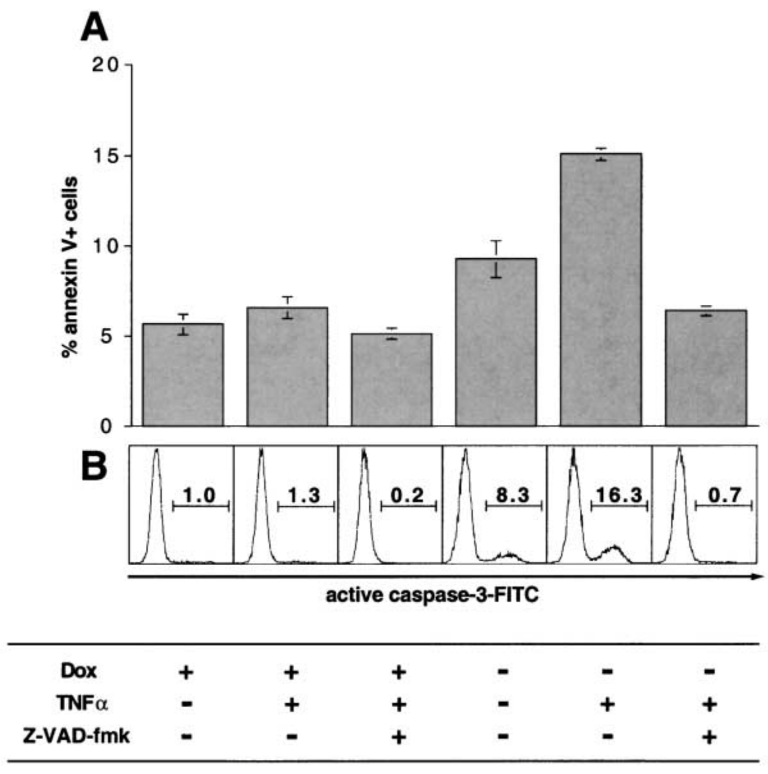


Figure 5. Vpu-induced apoptosis involves activation of the caspase pathway. CD4U and $CD4U_{2/6}$ cell lines were cultured in complete DMEM medium in the presence or absence of Dox for 24 h. TNF-α (20 ng/ml) and z-VAD-fmk (50 μM) were then added where indicated and the cultures were incubated for an additional 16 h before analysis. (A) Cultures were evaluated for induction of apoptosis by annexin V staining. Error bars reflect SDs from three independent experiments. (B) The same cultures were analyzed by flow cytometry for the expression of the active form of caspase-3 using a FITC-conjugated rabbit antiactive caspase-3 polyclonal antibody. The numbers indicate percentages of FITC-positive cells.

Vpu Inhibits the NF-κB–dependent Expression of the Antiapoptotic Factors Bcl-xL, A1/Bfl-1, and TRAF1. One of the mechanisms leading to apoptosis is mitochondrial dysfunction, which leads to the release of cytochrome c into the cytoplasm. This is followed by activation of caspase-9 through the formation of a ternary complex with Apaf-1 and procaspase-9, and results in the activation of caspase-3 (40). Members of the Bcl-2 family, which includes Bcl-xL and A1/Bfl-1, can inhibit this process by blocking the release of cytochrome c from mitochondria (34, 41).

There is a possibility that the expression levels of Bcl-xL and A1/Bfl-1, which are transcriptionally regulated by NF-κB, might be reduced in Vpu-expressing cells due to its inhibitory effect on NF-κB activity (16). To examine this possibility, we determined the levels of Bcl-xL and A1/Bfl-1 expression in the CD4U-HeLa cells by immunoblot analyses (Fig. 6 A). In uninduced CD4U cells (Fig. 6 A, lanes 3 and 4), both Bcl-xL and A1/Bfl-1 were expressed at considerable levels, reflecting the relatively high basal level of NF-κB activity in HeLa cells, and stimulation with TNF-α did not significantly augment their expression levels. After induction of CD4U expression, however, the steady-state levels of both factors were reduced to ~60% of their levels in uninduced cultures (Fig. 6 A, lane 1 versus lane 3). Moreover, TNF-α treatment of the CD4U-induced cells further reduced the levels of Bcl-xL and A1/Bfl-1 to <20% of those in TNF-α–treated uninduced cells (Fig. 6 A, lane 2 versus lane 4). These results are significant considering that <10% of the cells shown in lane 1 and <20% of the cells shown in lane 2 were annexin V-positive at the time of the analysis (data not shown). Thus, Vpu indeed downregulated the steady-state levels of Bcl-xL and A1/Bfl-1. The further reduction of Bcl-xL and A1/Bfl-1 levels after TNF-α treatment is presumably a consequence of the concomitant activation of caspase-3 by TNF-α, which is known to proteolytically cleave Bcl-xL (42, 43).

It has been shown that TRAF1 is a component of the TNFR complex (44) and recruits the c-IAPs to the complex. Recruitment of c-IAPs is required to inhibit activation of caspase-8 and thus to prevent the initiation of the caspase pathway (33, 45). Like Bcl-xL and A1/Bfl-1, expression of TRAF1 is regulated by NF-κB. In contrast, the steady-state levels of TRAF1 in unstimulated HeLa cells are low (Fig. 6 B, lane 3) but are efficiently induced after TNF-α stimulation (Fig. 6 B, lane 4). As for Bcl-xL and A1/Bfl-1, Vpu expression significantly inhibited the TNF-mediated induction of TRAF1 (Fig. 6 B, lane 2). To assess the physiological relevance of this phenomenon, we analyzed the effect of Vpu on the activation of caspase-8, which is regulated by TRAF1 (33). As seen in Fig. 6 B, TNF-α stimulation alone was not sufficient to induce caspase-8 activation (Fig. 6 B, lane 4). Similarly, Vpu expression alone was insufficient to activate caspase-8 (Fig. 6 B, lane 1). However, TNF-α treatment of cells expressing Vpu (Fig. 6 B, lane 2) resulted in the activation of caspase-8. These results confirm that the reduced expression of TRAF1 in Vpu-expressing cells can disturb the equilibrium between pro and antiapoptotic regulators and promote proapoptotic signaling in response to cytokine stimulation. The fact that Vpu expression alone did not induce caspase-8 activation further highlights the significance of the reduced expression of Bcl-xL and A1/Bfl-1 for the apoptogenic properties of Vpu.

To validate the results from our inducible CD4U cell lines we examined the effect of Vpu on the expression of antiapoptotic factors such as Bcl-xL, A1/Bfl-1, and TRAF1 in HIV-infected T cells. For that purpose, Jurkat cells were single-cycle infected with VSV-G pseudotyped NL43-K1, NL43-K1/Udel, or NL43-K1/$U_{2/6}$ as described for Table

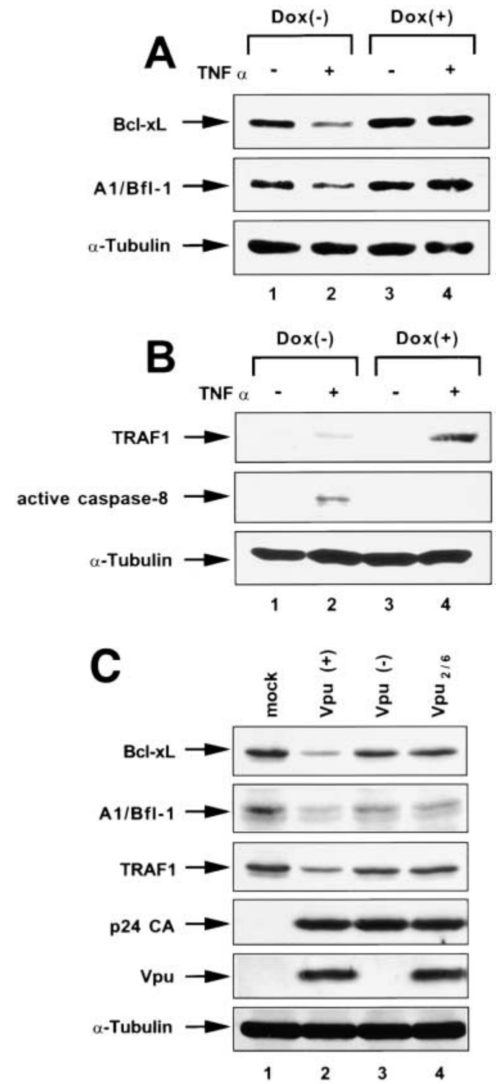


Figure 6. Vpu affects the expression of antiapoptotic factors and induces caspase-8 activation. (A and B) HeLa-CD4U cells were cultured in complete DMEM medium in the presence or absence of Dox for 24 h. TNF-α (20 ng/ml) was then added to the cultures as indicated and incubation was continued for an additional 16 h. Cell lysates were analyzed by immunoblotting for the expression of Bcl-xL and A1/Bfl-1 (A) as well as TRAF1 and the active form of caspase-8 (B). Lysates were normalized for tubulin using an α-tubulin antibody. (C) Jurkat cells were single-cycle infected with VSV-G-pseudotyped NL43-K1, NL43-K1/Udel, or NL43-K1/$U_{2/6}$. Cell lysates were analyzed 40 h after infection by immunoblotting to detect expression of Bcl-xL, A1/Bfl-1, TRAF1, p24 CA, Vpu, or α-tubulin.

II. 40 h after infection, cells were analyzed by immunoblotting with antibodies to Bcl-xL, A1/Bfl-1, or TRAF1 as described for Fig. 6 A and B. To control for comparable infection efficiency and the expression levels of wild-type Vpu and $Vpu_{2/6}$, blots were stained with antibodies to p24 CA and Vpu, respectively. As a loading control, blots were reacted with an antibody to α-tubulin. Consistent with the results from HeLa CD4U cells (Fig. 6 A and B), the steady-state levels of the antiapoptotic proteins were reduced in cultures expressing wild-type Vpu (lanes 2) when compared with mock-infected cultures (lanes 1) or cultures infected with a Vpu-defective variant (lanes 3) or a variant expressing $Vpu_{2/6}$ (lanes 4). Taken together, these results demonstrate that Vpu suppresses the expression of NF-κB–dependent antiapoptotic genes.

Discussion

One of the hallmarks of HIV infection is the gradual elimination of the host's $CD4^+$ T cells due to apoptosis. However, the mechanisms of HIV-induced apoptosis are complex and still controversial (1, 46). Several HIV-1 proteins have been attributed with apoptogenic properties, including Vpr (2, 3), Env, and Tat (1). More recently, Vpu was reported to increase the sensitivity of HIV-infected cells to Fas killing (47). However, the underlying mechanism remained unclear. In this study, we investigated in detail the apoptogenic properties of Vpu and we performed an in-depth analysis of the molecular mechanism. Our data suggest that Vpu, aside from Vpr, is one of the main inducers of apoptosis in HIV-infected cells and functions by inhibiting the NF-κB–dependent expression of antiapoptotic genes.

Both Vpr- and Vpu-induced apoptosis involve the activation of the caspase pathway (references 48 and 49, and this study). Although the precise mechanism for Vpr-induced apoptosis is still unclear, recent observations suggest that it might be caused by a Vpr-induced permeabilization of mitochondrial membranes resulting in the release of apoptogenic proteins such as cytochrome c or apoptosis inducing factor and the subsequent activation of caspase (50). While it was suggested that Vpu itself might have poreforming properties (51, 52) making a mechanism for induction of apoptosis similar to that of Vpr conceivable, our data suggest that Vpu instead functions by inhibiting the NF-κB–dependent expression of antiapoptotic genes. This is supported by the observation that mutation of the TrCP-binding motif ($Ser_{52, 56}Asn$), which in fact stabilized the pore-forming property of Vpu (52), abolished its apoptogenic potential (Table I). Based on the available experimental evidence, we therefore propose the following model for Vpu-induced apoptosis (Fig. 7): in unstimulated cells, NF-κB resides in the cytoplasm in an inactive complex with its inhibitor IκB (15). Upon stimulation of cells by cytokines such as TNF-α (Fig. 7 no. 1), IκB is rapidly phosphorylated by an IκB-specific kinase (Fig. 7 no. 2), which results in the rapid degradation of IκB via a TrCP-dependent pathway (Fig. 7 no. 3). Infection of cells by HIV-1 results in the gradual intracellular accumulation of

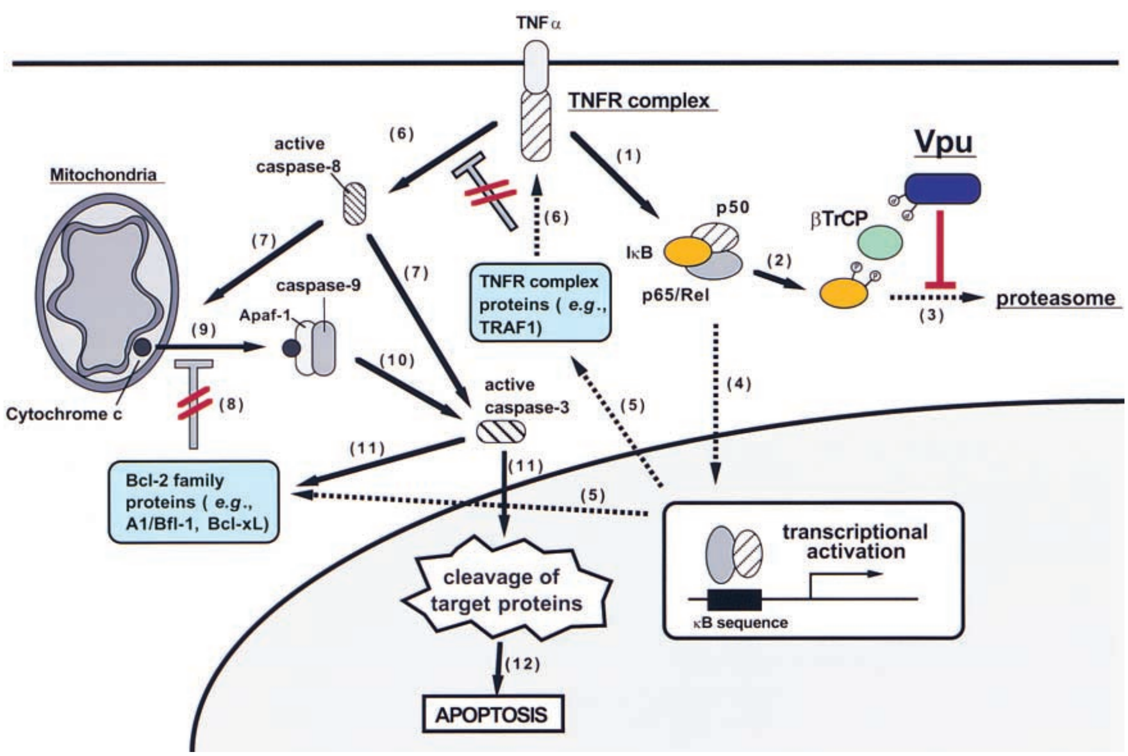


Figure 7. Model for Vpu-induced apoptosis through activation of the caspase pathway. Details of the model are explained in the Discussion. Broken arrows symbolize inhibitory effects. Steps inhibited by Vpu are marked in red.

Vpu. Because of its constitutively active TrCP-binding motif and the fact that it is not sensitive to TrCP-mediated proteolysis, Vpu functions as a competitive inhibitor of TrCP. This results in the gradual accumulation of IκB and the progressive impairment of the cell's ability to activate NF-κB (Fig. 7 no. 4). The inhibition of NF-κB blocks the synthesis of antiapoptotic proteins such as the Bcl-2 family proteins (e.g., Bcl-xL and A1/Bfl-1) or TNFR complex proteins (e.g., TRAF1; Fig. 7 no. 5). TRAF1 is induced by TNF-α treatment and normally inhibits activation of caspase-8 (Fig. 7 no. 6). In Vpu-expressing cells, the levels of TRAF1, in response to TNF stimulation, are reduced and no longer sufficient to inhibit the cytokine-induced activation of caspase-8 (Fig. 7 no. 6). Activated caspase-8 in turn induces the release of cytochrome c from the mitochondria (Fig. 7 no. 7). Release of cytochrome c is normally inhibited by the Bcl-2 family of proteins. However, in Vpu-expressing cells the levels of Bcl-2 proteins are limiting and no longer sufficient to block cytochrome c release (Fig. 7 no. 8). After its release from the mitochondria, cytochrome c forms ternary complexes with Apaf-1 and caspase-9 (Fig. 7 no. 9), resulting in the activation of caspase-3 (Fig. 7 no. 10). Active caspase-3 finally triggers a reaction that results in the cleavage of a number of target proteins including Bcl-2 family proteins (Fig. 7 no. 11) and leads to cell death (Fig. 7 no. 12).

While our data clearly demonstrate the ability of Vpu to induce apoptosis in HIV-infected cells, its role in promoting apoptosis of uninfected bystander cells, which has been observed for $CD4^+$ as well as $CD8^+$ cells (53, 54), remains to be addressed. The latter phenomenon is presumably a consequence of a continuous immune activation and could be due to exposure of these cells to secreted HIV proteins or to the disturbance of cytokine regulatory networks (55–57). Most cytokines important for cellular and humoral immune response, including IL-2, IL-4, IL-10, IL-12, as well as TNF-α are transcriptionally regulated by NF-κB (18, 58) and it is therefore possible that Vpu expression during the course of HIV infection could affect their expression. Thus, even though Vpu is not a secretory protein and is unlikely to directly promote apoptosis of bystander cells, its expression in HIV-infected cells could nevertheless indirectly affect uninfected bystander cells through its possible effect on cytokine production. While it is tempting to speculate on a possible role of Vpu in restricting the cellular immune response to HIV infection through its ability to inhibit NF-κB–dependent gene expression, regulation of cytokine production in vivo is complex and influenced by a multitude of factors, which will make it difficult to assess the contribution of individual viral factors such as Vpu in vivo. Nevertheless, the noted reversion of a Vpu mutant in a monkey model and its correlation with disease progression (59) attests to the importance of Vpu for virus replication in vivo.

Chemokines are another family of cellular proteins that is regulated by NF-κB and whose expression could thus be affected by Vpu. These include: regulated on activation, normal T cell expressed and secreted (RANTES), macrophage inflammatory protein (MIP)-1α, and MIP-1β (18), which are secreted from $CD4^+$ as well as $CD8^+$ cells and act through their specific surface receptor CCR5 (60). Endogenous expression of these chemokines was found to suppress HIV-1 replication in vitro (61) and inhibited HIV replication, presumably through competition for the HIV coreceptor (62). In fact, there appears to be a correlation between increased production of RANTES and resistance to HIV infection (63) and, conversely, decreased production of RANTES and MIP-1α with disease progression (64) in vivo. Thus, suppression of chemokine production by Vpu could provide a selective advantage to the virus and thus have a severe impact on disease progression.

HIV-2 infection is generally associated with a reduced rate of disease development as compared with HIV-1 (65) and is characterized by an extended asymptomatic phase. Interestingly, lymphocytes from HIV-2–infected patients were found to be less susceptible to apoptosis than those derived from HIV-1–infected cells during the asymptomatic phase (66, 67). Therefore, it is tempting to speculate that the apoptogenic property of Vpu, for which there is no functional complement in HIV-2, contributes to the increased pathogenicity of HIV-1. In fact, there is some evidence from the macaque monkey model supporting the importance of *vpu* in vivo. For example, when monkeys were infected with a *vpu*-defective chimeric SHIV variant carrying an ATG to ACG mutation in the *vpu* initiation codon, the *vpu* gene was found to revert back to a functional open reading frame during the course of infection (59), demonstrating the in vivo selective pressure for maintaining a functional *vpu* gene. In addition, reversion of the *vpu* open reading frame was correlated with disease progression in infected animals (59) and expression of Vpu was associated with increased viremia (68), demonstrating the importance of Vpu for viral replication and/or persistence in vivo and suggesting a role for Vpu in viral pathogenesis.

Despite the fact that HIV-1 encodes at least four proteins that promote apoptosis, it is difficult to envision a scenario in which the induction of apoptosis per se could provide a selective advantage for HIV-1. It appears that, in this respect, other primate lentiviruses have much better adapted to their hosts. In particular, simian IVs, which are endemic in their natural hosts, do not generally induce disease (69). It seems therefore more plausible that the apoptogenic properties of HIV-1 proteins are unfortunate side effects of other important functions of these viral proteins. In the case of Vpu, it could be argued that its ability to induce rapid degradation of CD4 provides a selective advantage to HIV-1 by preventing the intracellular retention of Env in CD4/Env complexes (25). Such complexes can form between de novo synthesized Env and CD4 proteins in the endoplasmic reticulum (70–72). They are highly stable and unable to traffic to the cell surface (70–72). The benefits of Vpu-mediated degradation of CD4 for HIV-1 are therefore twofold: (i) it releases Env from its intracellular trap and ensures its expression at the cell surface, and (ii) at the same time, Vpu prevents surface expression of CD4, which would interfere both with virus release (73) as well as with the infectivity of the particles produced (74, 75). These functions of Vpu are particularly important for HIV-1 due to the affinity of its Env protein to CD4, which is significantly higher than HIV-2 Env (76, 77). The evolution of Vpu thus provides an intriguing example of how viruses redirect existing cellular mechanisms to their own advantage even if it is at the expense of their host.

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