The C-terminal moiety of HIV-1 Vpr induces cell death via a caspase-independent mitochondrial pathway

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Abstract

Previous biochemical studies suggested that HIV-1-encoded Vpr may kill cells through an effect on the adenine nucleotide translocase (ANT), thereby causing mitochondrial membrane permeabilization (MMP). Here, we show that Vpr fails to activate caspases in conditions in which it induces cell killing. The knock-out of essential caspase-activators (Apaf-1 or caspase-9) or the knock-out of a mitochondrial caspase-independent death effector (AIF) does not abolish Vpr-mediated killing. In contrast, the cytotoxic effects of Vpr are reduced by transfection-enforced overexpression of two MMP-inhibitors, namely the endogenous protein Bcl-2 or the cytomegalovirus-encoded ANT-targeted protein vMIA. Vpr, which can elicit MMP through a direct effect on mitochondria, and HIV-1-Env, which causes MMP through an indirect pathway, exhibit additive (but not synergic) cytotoxic effects. In conclusion, it appears that Vpr induces apoptosis through a caspase-independent mitochondrial pathway.
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Abbreviations: AIF, apoptosis-inducing factor; ANT, adenine nucleotide translocator; COX, cytochrome *c* oxidase; (Asp)$_2$R110, bis-(L-aspartic acid amide) rhodamine 110; CMV, cytomegalovirus; Cyt *c*, cytochrome *c*; DiOC$_6$(3), 3,3′dihexyloxacarbocyanine iodide; $\Delta\Psi_m$, mitochondral transmembrane potential; ES, embryonic stem; JC-1, 5,5′,6,6′-tetrachloro-1,1′, 3,3′-tetraethylbenzimidazolylcarbocyanine iodide; MMP, mitochondrial membrane permeabilization; PI, propidium iodide; SIMP, soluble intermembrane protein; STS, staurosporin; VDAC, voltage-dependent anion channel; vMIA, viral mitochondrial inhibitor of apoptosis; Z-VAD.fmk, *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone

Introduction

HIV-1 encodes several apoptosis inducing proteins, including the envelope glycoprotein complex (Env) and accessory proteins such as Tat, Nef, Vpu, and Vpr.[1−3] Vpr, which is present in virions as well as in infected cells, has the particularity to be secreted into culture supernatants (*in vitro*) or body fluids such as serum and cerebrospinal liquid (*in vivo*). The Vpr protein can penetrate into non-infected bystander cells. Upon addition to cell cultures, it exerts pleiotropic effects including an arrest in the G2 phase of the cell cycle,[4,5] inhibition of activation-induced T cell death,[6,7] and induction of apoptosis in a wide range of cell types.[5,7−11] When expressed as a transgene in mice, it causes a lymphocyte depletion linked to an excess of apoptosis, suggesting that its pro-apoptotic action prevails *in vivo*.[12] Similarly, co-expression of Vpr with a DNA vaccine reveals its immunosuppressive potential *in vivo*.[13]

The intact Vpr protein of 96 amino acids as well as its C-terminal moiety (Vpr 52-96, but not its N-terminus Vpr 1-51) can trigger apoptosis through a pathway which involves mitochondrial membrane permeabilization (MMP).[14] Experiments conducted on isolated mitochondria or on model membranes indicate that Vpr can trigger MMP through its capacity to interact with the adenine nucleotide translocase (ANT), thereby creating a composite ion channel within the inner mitochondrial membrane whose opening results into the dissipation of the mitochondrial transmembrane potential ($\Delta\Psi_m$), followed by permeabilization of the outer mitochondrial membrane with consequent release of soluble intermembrane proteins (SIMPs).[15] Apoptogenic SIMPs include apoptosis inducing factor (AIF), a flavoprotein which translocates to the nucleus where it activates a sessile DNase,[16,17] and cytochrome *c*, a heme protein which translocates to the cytosol where it interacts with Apaf-1 and pro-caspase-9 to create the apoptosome, the caspase activation complex.[18]

Based on the above premises, we comparatively evaluated the role of MMP, caspase activation and AIF in Vpr-induced cell death. To this end, we tested the effect of Vpr 52-96 on cells in which the AIF, caspase-9 or Apaf-1 genes had been invalidated by homologous recombination. Moreover, we determined the effect of Vpr on cells expressing MMP-inhibitory molecules such as Bcl-2 or the viral mitochondrial inhibitor of apoptosis (vMIA), as well as on cells in which the mitochondrial pathway leading to apoptosis is stimulated by another HIV-1 gene product, namely the envelope (Env) glycoprotein complex. Collectively, our data reveal MMP to be a critical step for Vpr-

induced killing, while caspase activation and AIF appear to be dispensable for the cytotoxic action of Vpr.

Results and Discussion

Caspase activation and AIF are dispensable for Vpr-induced cell killing

To determine to which degree caspases and AIF contribute to Vpr-mediated killing, synthetic Vpr 52-96 peptide[19] was added to mouse embryonic stem (ES) cells with a normal genotype (Wt) or cells in which either of two essential apoptosome components (Apaf-1 or caspase-9)[20,21] or, alternatively, AIF had been invalidated by homologous recombination.[17] Vpr 52-96 caused a $\Delta\Psi_m$ loss (measured by means of the $\Delta\Psi_m$-sensitive fluorochrome 3,3'dihexyloxacarbocyanine iodide, DiOC$_6$(3), followed by cytofluorometric analysis) as well as a loss of viability (determined by measuring the incorporation of the vital dye propidium iodide, PI)[22] (Figure 1A). The loss of $\Delta\Psi_m$ and viability were indistinguishable in Wt, Apaf-1$^{-/-}$, caspase-9$^{-/-}$; or AIF$^{-/y}$ ES cells (Figure 1A), both as far as the dose response (Figure 1B) and the kinetics (not shown) of the cell death process were concerned. Moreover, the combined inhibition of

caspase-activation (by addition of the pan-caspase inhibitor Z-VAD.fmk) and the knock-out of AIF did not affect the cytotoxic effect of Vpr (Figure 1A,B), indicating that post-mitochondrial effectors such as caspases and AIF are dispensable for Vpr-mediated killing.

Failure of Vpr to elicit caspase activation in different cell lines

When caspase activation was assessed in a direct fashion, using the fluorogenic, cell-permeable substrate bis-(L-aspartic acid amide) rhodamine 110 ((Asp)$_2$R110), we found that ES cells failed to activate caspase-3 in response to Vpr 52-96, although they did cleave (Asp)$_2$R110 when incubated with the commonly used apoptosis inducer staurosporin (STS)[23] (Figure 1C). Similarly, HeLa cells incubated with Vpr 52-96 failed to activate caspase-3 in conditions in which Vpr did provoke significant cell kiling (Figure 2A,B). In the same experiment, the caspase inhibitor Z-VAD.fmk did provoke a significant inhibition of caspase-3 activation induced by STS. Z-VAD.fmk, however, did not prevent the loss of viability induced by Vpr 52-96. Thus, in contrast with another study in which HeLa cells were transfected with the Vpr cDNA,[24] exogenous addition of Vpr 52-96 kills without caspase
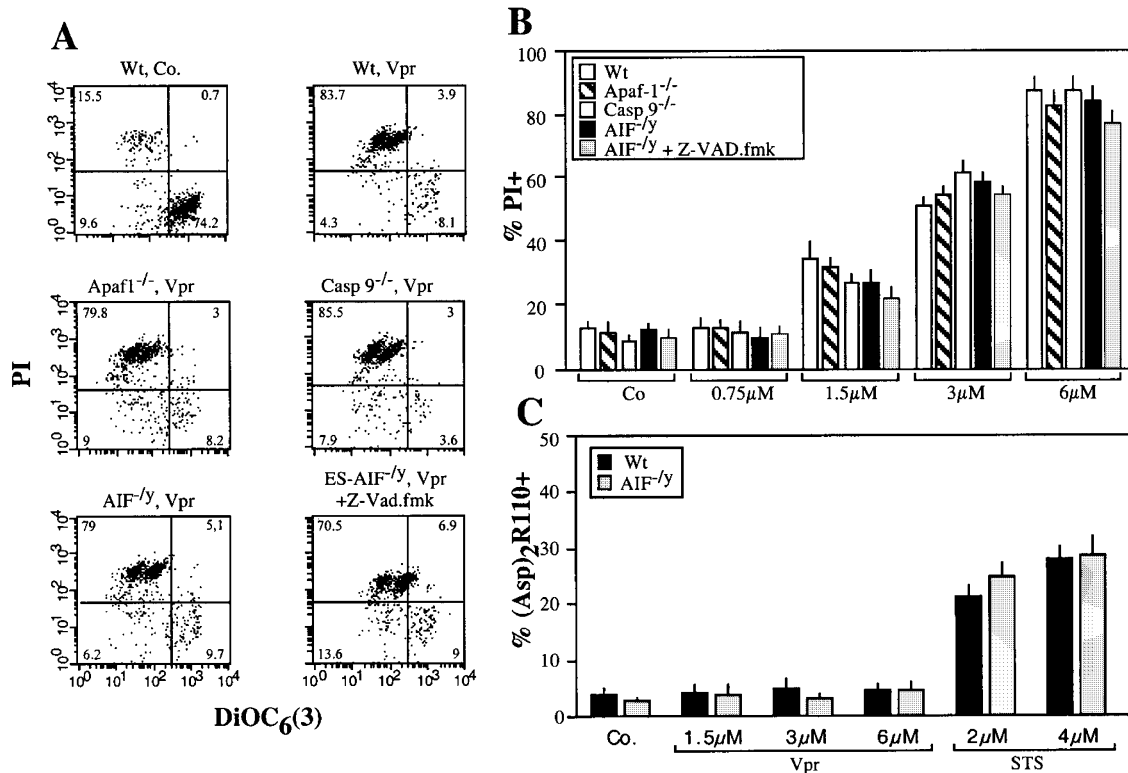

Figure 1 Cytotoxic effects of Vpr on ES cells lacking Apaf-1, caspase-9, or AIF. ES cells exhibiting different genotypes were cultured for 4 h in the presence of different doses of synthetic Vpr 52-96 peptide, followed by staining of cells with the $\Delta\Psi_m$-sensitive dye DiOC$_6$(3) (green fluorescence) and with the vital dye PI (red fluorescence) and cytofluorometric analysis. Numbers refer to the percentage of cells found in each quadrant. (**A**) Representative FACS pictograms obtained by culturing cells in the presence or absence of 6 μM Vpr 52-96 and 100 μM Z-VAD.fmk. (**B**) Dose response of the loss of viability (PI positivity) induced by Vpr 52-96 in different ES cell lines (X$\pm$SEM, *n*=6). (**C**) Failure of Vpr to induce caspase activation in ES cells. Wild type ES cells or AIF knock-out ES cells were cultured in the presence of the indicated dose of Vpr or STS, and the percentage of cells activating caspase-3 was measured cytofluorometrically using the fluorogenic substrate (Asp)$_2$-R110. Note that STS but not Vpr induced caspase activation
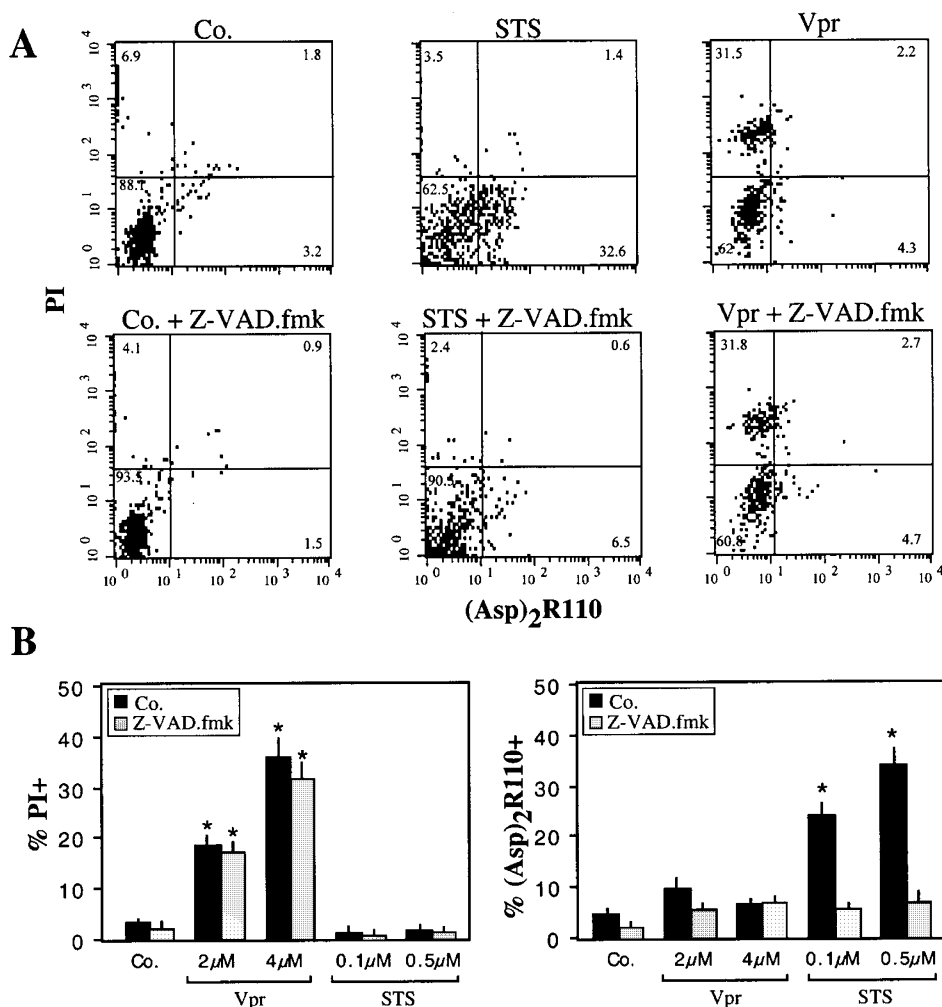
Figure 2 Failure of Vpr to induce caspase activation in HeLa cells. (**A**) Representative cytofluorometric results obtained by double staining with propidium iodide (PI) and the caspase-3 substrate (Asp)$_2$-R110. Cells were cultured for 6 h with STS (500 nM) or Vpr (6 μM), in the presence or absence of 100 μM Z-VAD.fmk, followed by staining and FACS analysis. (**B**) Quantitation of cell death and caspase-3 activation. The percentage of dying cells (PI-positive, left panel) or of cells exhibiting caspase-3 activation (which are (Asp)$_2$-R110-positive) is shown. Asterisks denote significant ($P < 0.01$, paired Student t-test) effects of Vpr or STS, as compared to untreated control (Co.) cells

activation. These differences can probably be attributed to the distinct experimental design (transfection *versus* addition of the peptide). Since Vpr fails to induce caspase activation, it may be argued that it induces a non-apoptotic cell death. However, we found that Vpr did induce some hallmarks of apoptosis (as opposed to necrosis), in particular pyknosis and chromatin condensation (see below). It thus induces caspase-independent cell death with some features of apoptosis.

vMIA from cytomegalovirus (CMV) and Bcl-2 inhibit Vpr-induced MMP and cell killing

Bcl-2 and vMIA represent two proteins with MMP-inhibitory properties. Bcl-2 is found inserted in intracellular membranes (in particular the endoplasmic reticulum and the outer mitochondrial membrane) and stabilizes the mitochondrial membrane barrier function by local interactions with pore forming proteins contained in the permeability

transition pore complex, namely ANT, voltage-dependent anion channel (VDAC), and pro-apoptotic Bcl-2 family members.[25-28] Viral mitochondrial inhibitor of apoptosis (vMIA or UL37) is encoded by CMV and is exclusively found in mitochondrial membranes, where it specifically interacts with ANT but not with VDAC.[29-32] Both vMIA and Bcl-2 inhibited the $\Delta\Psi_m$ dissipation induced by Vpr 52-96, as determined by two different $\Delta\Psi_m$-sensitive fluorochromes, DiOC$_6$(3) (Figure 3A,C) and 5,5',6,6'-tetrachloro-1,1', 3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1, which undergoes a red–green shift in the emission spectrum when the $\Delta\Psi_m$ drops) (Figure 3B). vMIA also largely reduced the Vpr 52-96-stimulated increase in the production of rective oxygen species (ROS, measured by means of the ROS-induced conversion of non-fluorescent hydroethidine into fluorescent ethidium[33]) (Figure 3A), the translocation of cytochrome *c* to the cytosol (Figure 4A), as well as the translocation of AIF from mitochondria to the
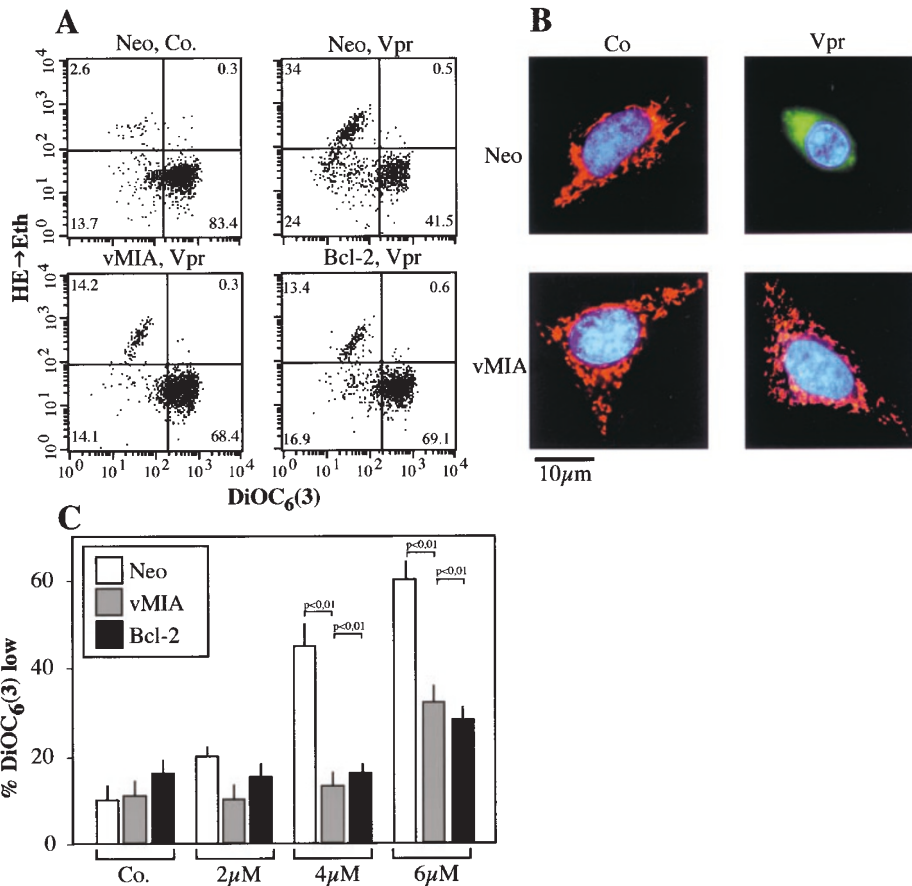
Figure 3 vMIA and Bcl-2 prevent the $\Delta\Psi_m$ dissipation induced by Vpr. HeLa cells stably transfected with the neomycin resistance gene only (Neo), vMIA or Bcl-2 were cultured for 6 h in the presence or absence of 6 μM Vpr 52-96, followed by (**A**) staining with DiOC$_6$(3) and hydroethidine (HE), to determine the $\Delta\Psi_m$ loss (detectable as a reduction of DiOC$_6$(3) staining) and ROS production (resulting in the conversion of HE into Ethidium, Eth) and FACS analysis, or (**B**) staining with JC-1 (which stains mitochondria with a high $\Delta\Psi_m$ in red and those with a low $\Delta\Psi_m$ in green) and Hoechst 33342 (which stains chromatin, blue fluorescence), followed by fluorescence microscopy. Cells representing the dominant phenotype ($>60\%$ of total) are shown. (**C**) Dose response of the $\Delta\Psi_m$ dissipation (quantitated by DiOC$_6$(3) staining as in (**A**)) induced by Vpr 52-96. This experiment has been repeated three times, and concordant results were obtained for DiOC$_6$(3) and JC-1 stainings. Results are X$\pm$S.E.M. (*n*=3), and *P* values have been calculated using the Student *t*-test

nucleus (Figure 4B), as determined by *in situ* immuno-fluorescence.[34] Concomitantly, vMIA reduced the frequency of cells exhibiting an apoptotic chromatin condensation, as determined by staining with the chromatin dye Hoechst 33342 (Figure 4C). These results confirm that MMP induction, presumably mediated by a direct effect of Vpr on ANT (which is antagonized by vMIA) is a decisive step in Vpr-induced cytotoxicity.

Additive cytotoxic effects of Vpr and Env

When cells expressing lymphotropic HIV-1 Env are co-cultured with cells expressing a suitable combination of the Env receptor CD4 and the co-receptor CXCR4, syncytia are formed, as this can be detected by pre-staining individual cells with non-toxic dyes (CellTracker® green CMFDA for HeLa Env cells and CellTracker® blue CMAC for HeLa CD4 cells), followed by FACS detection of double-stained fusion products (Figure 5A). We have previously found that Env-elicited syncytia undergo apoptosis in an MMP-dependent fashion, through a pathway which involves the transcriptional upregulation of p53 target genes including that of the MMP-inducing Bcl-2 antagonist Bax.[35–37] This pathway is also activated in primary lymphoblasts infected by HIV-1.[37–39] Based on the observation, that a ternary ANT/Bax/Vpr complex permeabilizes membranes more efficiently than ANT/Vpr,[15] we suspected that Env-elicited syncytia (which upregulate Bax, as mentioned above) might be more susceptible to Vpr 52-96-induced MMP and killing than control cells. However, Vpr caused a loss of viability (positive PI staining) in freshly formed syncytia (24 h of coculture) and individual cells with a similar efficacy (Figure 5A). Moreover, careful dose response studies revealed no difference in the Vpr 52-96-induced $\Delta\Psi_m$ dissipation (determined by means of the $\Delta\Psi_m$-sensitive dye JC-1, as described in reference 36) and chromatin condensation (determined by staining with Hoechst 33342) between individual cells and syncytia, even in an aging (48 h) syncytial population in which a significant portion of cells is undergoing apoptosis (Figure 5B). In conclusion, Vpr and Env exert additive rather than synergic cytotoxic effects.
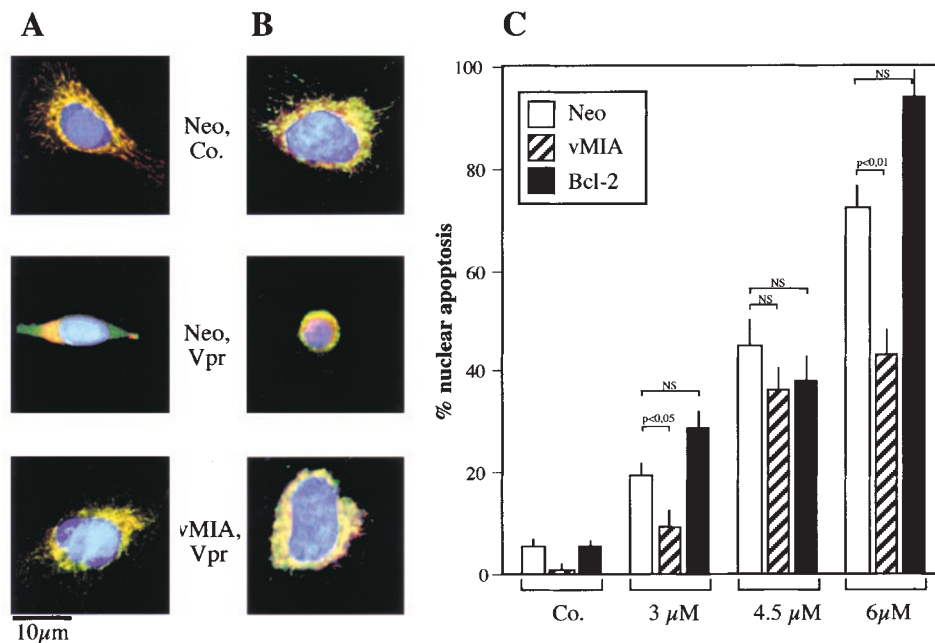
Figure 4 vMIA prevents the mitochondrio-cytosolic translocation of cytochrome *c*, the mitochondrio-nuclear translocation of AIF and nuclear apoptosis triggered by Vpr 52-96. HeLa cells expressing the Neo resistance gene only or vMIA were cultured for 6 h in the presence of 4 μM Vpr, fixed and permeabilized and then subjected to immunofluorescence staining with antibodies specific for cytochrome *c* (revealed by phycroerythrin, red fluorescence) and COX (revealed by fluorescein isothiocyanate, green fluorescence) (**A**) or specific for AIF (red) and Hsp60 (green) (**B**) and counterstained with Hoechst 33342 (blue). Representative photomicrographs are shown. A yellow (red+green) punctate staining indicates co-localization, within mitochondria, of cytochrome *c* and the inner mitochondrial membrane protein COX (**A**) or AIF and the matrix protein Hsp60 (**B**). The diffuse red fluorescence indicates the presence of cytochrome *c* in the cytosol (**A**). Purple nuclear fluorescence (blend of red+blue) is indicative of the AIF translocation to the nucleus (**B**). Note that the extra-mitochondrial localization of cytochrome *c* or AIF was detectable in 12$\pm$3 and 16$\pm$4% of untreated Neo control cells, 10$\pm$4 and 14$\pm$4% of untreated vMIA cells, 44$\pm$4 and 48$\pm$6% of Vpr 52-96-treated Neo cells, and 15$\pm$3 and 21$\pm$5% of Vpr 52-96-treated vMIA cells, respectively. (**C**) Frequency of cells exhibiting an apoptotic chromatin condensation detectable with Hoechst 33342 (X$\pm$S.E.M., *n*=5). At least 200 cells were scored for each point

Concluding remarks

The data shown here provide genetic evidence that Vpr kills cells through a pathway that does not rely on caspases or AIF, yet involves MMP as a critical step. That MMP is involved in cell killing by Vpr is suggested by the fact that it is inhibited by Bcl-2, a protein thought to mediate apoptosis inhibition via local effects on mitochondria,[40–42] although local effects on the endoplasmic reticulum have also been reported.[43–46] More convincingly, vMIA, an apoptosis inhibitor exclusively targeted to mitochondria,[29] antagonizes Vpr-induced cell killing. In contrast, it appears that the absence of AIF or that of essential ingredients of the post-mitochondrial caspase activation machinery (Apaf-1, caspase-9) has no incidence on Vpr-mediated cytotoxicity. Speculatively, the MMP-associated bioenergetic catastrophe could suffice to cause cell death,[47] without that catabolic hydrolases (apoptosome-elicited caspases and AIF-elicited nucleases) would be required for the execution of the cell death program. It appears particularly intriguing that vMIA from CMV, which targets ANT to *inhibit* apoptosis,[29] can antagonize the cytotoxicity of HIV-1 Vpr, which targets ANT to *induce* apoptosis.[15] Whether these effects involve a competition for ANT binding between the two antagonistic viral proteins (vMIA and Vpr) and whether they are relevant for the pathogenesis of simultaneous HIV-1/CMV infections remains to be

elucidated. Nonetheless, the fact that several viruses have developed independent strategies to modulate apoptosis by targeting ANT[30] underlines the probable importance of ANT (or ANT-associated proteins) for the regulation of cell death.

Although Env and Vpr both induce apoptosis through induction of MMP, Vpr in a direct fashion[14,15] and Env in an indirect (p53-dependent) pathway,[36,37,48,49] the two proteins do not exhibit a synergistic action of apoptosis induction, at least in the model that we studied. As a possibility, Env and Vpr may cooperate in the pathophysiology of HIV-1 infection by inducing apoptosis in different cellular populations rather than by cooperating within the same cell. Irrespective of these possibilities, our data underline the general importance of MMP for the cytotoxic effects mediated by HIV-1-encoded proteins.

Materials and Methods

Cell lines, culture conditions, and Vpr treatment

Embryonic stem (ES) stells (wild type, Apaf-1$^{-/-}$, caspase-9$^{-/-}$ or AIF$^{-/y}$) were maintained in the presence of leukemia-inhibitory factor, as described.[17,20,21] HeLa cells transfected with the cytomegalovirus UL37 exon 1 gene coding for vMIA and with the human Bcl-2 and Bcl-X$_L$ gene (kindly provided by Dr. V Goldmacher),[29] as well as control
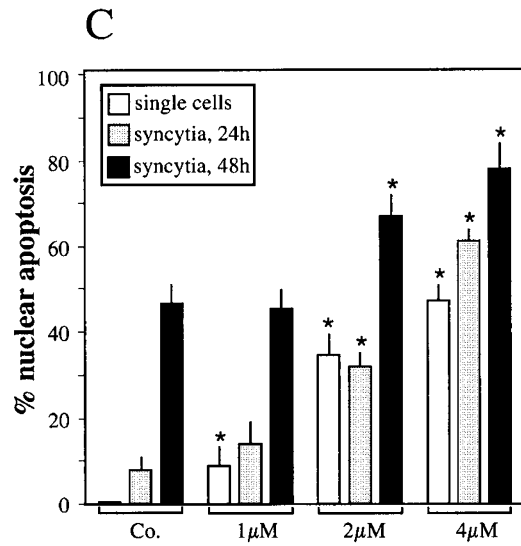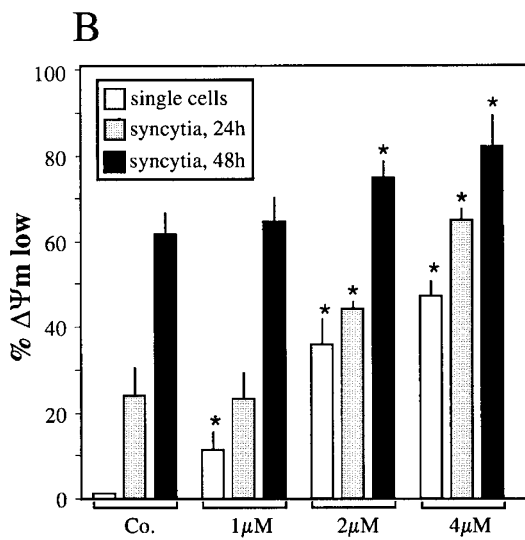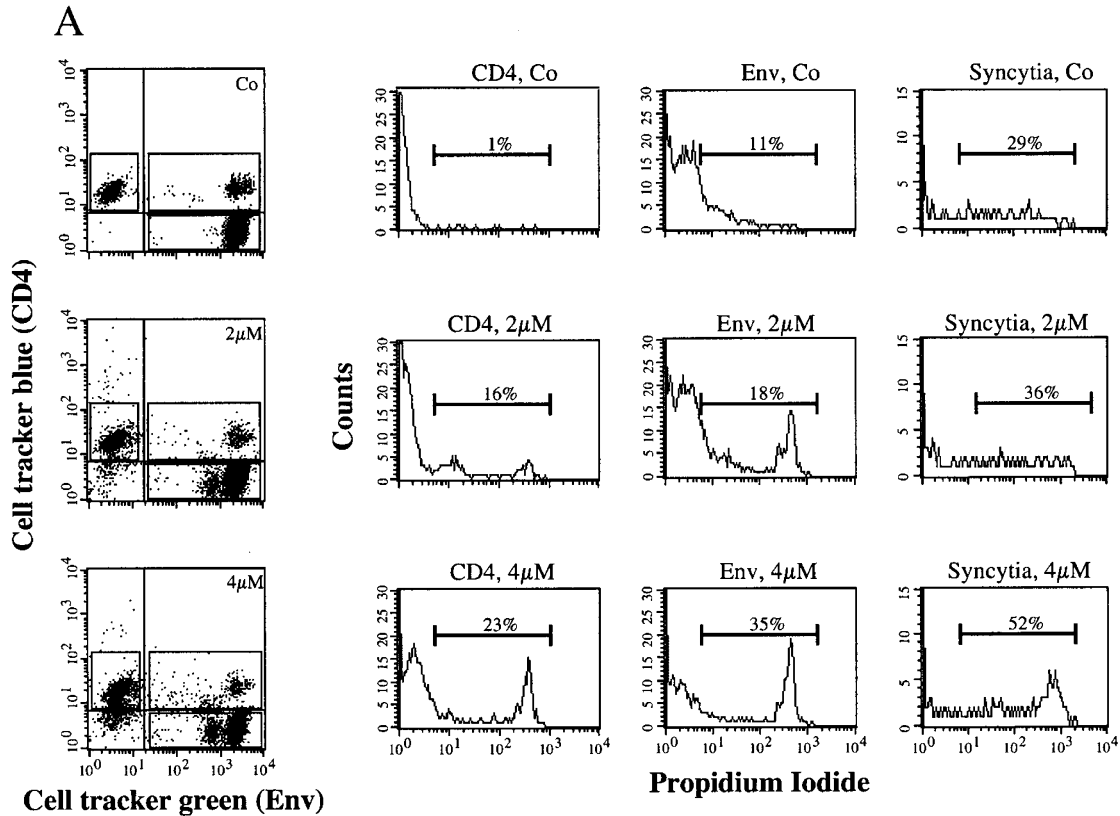
Figure 5 Additive cytotoxic effects of Vpr and Env. (**A**) Vpr kills Env-elicited syncytia and individual control cells with a similar efficacy. Env-expressing HeLa cells, pre-stained with CellTracker® green CMFDA, were cultured together with CD4/CXCR4-expressing HeLa cells, pre-stained with CellTracker® blue CMAC (7-amino-4-chloromethylcoumarin) for 24 h, and then in the absence (Co.) or in the presence of 2 or 4 μM Vpr 52-96. Cells were incubated for 15 min in the presence of PI, followed by three colour fluorescence analysis. Two-colour blots (right panels) allowed for gating on single Env HeLa cells (green), single CD4/CXCR4 HeLa cells (blue) and syncytia (green+blue). The PI staining profiles (red) of each of the gates are shown, revealing that Vpr 52-96 killed a similar percentage of single cells and syncytia. (**B** and **C**) Quantitation of the $\Delta\Psi_m$ loss (determined by JC-1 staining as in Figure 2B) and nuclear apoptosis (determined with Hoechst 33342) in single cells and syncytia of different ages (24 and 48 h after beginning of coculture). Results are representative of nine independent experiments. Asterisks indicate signicant ($P < 0.01$) Vpr effects as compared to untreated controls

cells transfected with the pcDNA3.1 vector containing the neomycin resistance gene (Neo), were cultured in DMEM medium supplemented with 2 mM glutamine, 10% FCS, 1 mM pyruvate, 10 mM HEPES and 100 U/ml pencillin/streptomycin at 37°C under 5% CO_2. ES cells

exhibiting different genotypes or HeLa cells were cultured for 4–6 h in the presence of different doses of synthetic Vpr 52-96 peptide,[19,50] as described.[51] In some experiments, the pan-caspase inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (*Z*-VAD.fmk, 100 μM, Enzyme Systems) was added to the cultures.

Determination of apoptosis-associated parameters

The following fluorochromes were employed to assess apoptosis-associated changes by cytofluorometry on a FACS Vantage (Becton Dickonson), while gating the forward and the side scatters on viable cells: 3,3′ dihexyloxacarbocyanine iodide (DiOC$_6$(3), 20 nM) for $\Delta\psi_m$ quantification; propidium iodide (PI, 1 μg/ml) for the determination cell viability; hydroethidine (HE, 1 μM) for the assessment of superoxide anion production.[33] Alternatively, cells cultured on a cover slip were stained with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1, 3 μM, Molecular Probes) and Hoechst 33342 (2 μM, Sigma), followed by fluorescence microscopic assessment of apoptotic parameters.[34,36] Cells were fixed on paraformaldehyde (4% w:v) and picric acid (0.19% v:v) for immunofluorescence assays. Cells were stained for the detection of cytochrome *c* (mAb 6H2.B4 from Pharmingen, detected by a goat anti-mouse IgG conjugated with Alexa® fluor) and cytochrome *c* oxidase (anti-COX subunit IV 20E 8-C12 monoclonal, Molecular Probes detected by a goat antimouse IgG conjugated with Alexa® fluor). For the determination of caspase-3 activation, cells were incubated with 20 μM (Asp)$_2$R110 (20 min, 37°C, Molecular Probes).

Generation and evaluation of Env-dependent syncytia

HeLa cells transfected with the Env gene of HIV-1 LAI (HeLa Env) and HeLa cells transfected with CD4 (HeLa CD4) were cultured together (1:1 ratio) to generate syncytia, as described.[36,52,53] Prior to coculture, HeLa Env cells were pre-stained with 15 μM of CellTracker® green CMFDA and HeLa CD4 cells pre-stained with 15 μM CellTracker® blue CMAC (7-amino-4-chloromethylcoumarin, both from Molecular Probes). Preliminary experiments showed that neither of these dyes had toxic effects on HeLa cells during a 72 h culture period. After 24 or 48 h of co-culture, cells were treated with the indicated dose of Vpr, stained with PI (1 uM, 15 min), and subjected to FACS analysis. Alternatively, syncytia were stained with JC-1 and Hoechst 33324 and subjected to fluorescence microscopic examination, as described.[36,48]

Acknowledgements

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