The HIV-1 Viral Protein R Induces Apoptosis via a Direct Effect on the Mitochondrial Permeability Transition Pore

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Abstract

Viral protein R (Vpr) encoded by HIV-1 is a facultative inducer of apoptosis. When added to intact cells or purified mitochondria, micromolar and submicromolar doses of synthetic Vpr cause a rapid dissipation of the mitochondrial transmembrane potential (ΔV_{m,mt}) as well as the mitochondrial release of apoptogenic proteins such as cytochrome c or apoptosis inducing factor. The same structural motifs relevant for cell killing are responsible for the mitochondrial toxic effects of Vpr. Both mitochondrial and cytotxic Vpr effects are prevented by Bcl-2, an inhibitor of the permeability transition pore complex (PTPC). Coincubation of purified organelles revealed that nuclear apoptosis is only induced by Vpr when mitochondria are present yet can be abolished by PTPC inhibitors. Vpr favors the permeabilization of artificial membranes containing the purified PTPC or defined PTPC components such as the adenine nucleotide translocator (ANT) combined with Bax. Again, this effect is prevented by addition of recombinant Bcl-2. The Vpr COOH terminus binds purified ANT, as well as a molecular complex containing ANT and the voltage-dependent anion channel (VDAC), another PTPC component. Yeast strains lacking ANT or VDAC are less susceptible to Vpr-induced killing than control cells yet recover Vpr sensitivity when retransformed with yeast ANT or human VDAC. Hence, Vpr induces apoptosis via a direct effect on the mitochondrial PTPC.

Key words: apoptosis • Bcl-2 • cell death • mitochondria • Vpr

Introduction

AIDS is associated with an enhanced apoptotic decay of various cell types, in particular lymphocytes, monocytes, and neurons. The mechanisms of this deregulated cellular turnover are complex and involve host factors, direct viral effects, and soluble viral proteins including gp120, Tat, Nef, and viral protein R (Vpr)1 (1–4). Although none of these mechanisms or factors, taken on their own, can explain the AIDS-associated depletion of important cell types, it appears important to understand their function individually. The 14-kD protein Vpr is abundant in virions (3, 5) and is detectable in the sera of HIV-1 carriers, correlating with the viral load (6). Vpr is likely to be important for AIDS pathogenesis, and loss-of-function mutations of Vpr are negatively selected in vivo (7). Vpr interacts with multiple intracellular targets and has pleiotropic effects on viral replication, cell cycle, and differentiation (3, 5). In addition,
Vpr kills lymphocytes (8), monocytes (9), and neurons (10), either upon infection with vpr-positive HIV-1 isolates (8, 9) or upon extracellular addition of the Vpr protein (10, 11). Intrigued by the pleiotropic cytotoxic potential of Vpr, we decided to explore the apoptogenic mode of action of this HIV-1 accessory protein.

Apoptosis research has recently been boosted by the development of cell-free systems in which isolated organelles (nuclei, mitochondria, cytosol, etc.) are coincubated in vitro (12–19). This approach has generated evidence that mitochondrial intermembrane proteins, including cytochrome c, apoptosis inducing factor (AIF), procaspases, and heat shock proteins, are released during apoptosis and are crucial for the activation of caspases and DNases (17–23). The mechanism responsible for mitochondrial membrane permeabilization has been found to involve proapoptotic members of the Bcl-2 family (Bax, Bak, Bid, etc.; reference 24–28) and/or the permeability transition pore complex (PTPC), a polypeptide complex organized around the two most abundant proteins of the inner and outer mitochondrial membranes, the adenine nucleotide translocator (ANT; inner membrane) and the voltage-dependent anion channel (VDAC; outer membrane). ANT, VDAC, Bcl-2, and Bax physically interact within the inner–outer membrane contact site (27–30). Cell-free systems also allow mapping of the site of action of xenobiotic apoptosis inducers. Schematically, two classes of inducers can be distinguished. First, a variety of different inducers act directly on mitochondria and/or purified PTPC. This is true for experimental anticancer agents such as lonidamine (31), betulinic acid (32), arsenite (33), and diamide (34), as well as for toxins such as salicylate (35) and mastoparan (16). In contrast, the majority of apoptosis inducers act indirectly on mitochondria, e.g., via triggering of the ceramide pathway, increased Ca^2+ levels, effects on the subcellular distribution of proteins from the Bcl-2/Bax family, caspase activation, or shifts in redox potentials, which then affect the PTPC (and perhaps alternative permeabilization mechanisms (16, 24–28, 36–38).

Based on the above premises, we decided to elucidate the apoptogenic mode of action of Vpr, both in cells and in cell-free systems. Our results indicate that Vpr can directly target mitochondrial PTPC and permeabilize mitochondrial membranes in cell-free systems. Moreover, Vpr can act on purified PTPC or PTPC components reconstituted into synthetic membranes. Cells lacking key proteins from the PTPC become relatively resistant to the cytotoxic effect of Vpr. Thus, Vpr represents a novel type of viral peptide that can interact with the PTPC to permeabilize mitochondrial membranes and trigger the apoptotic program.

**Figure 1.** Proapoptotic effects of Vpr and Vpr-derived peptides (35). (A) Cytocidal effects of Vpr on Jurkat cells. After overnight incubation, plasma membrane permeability was assessed by PI staining. This experiment was reproduced five times. (B) Acute mitochondrial Vpr effects. Jurkat cells were cultured for 2 h with 0.5 μM Vpr52-96, 1 μM Vpr1-96, 1 μM Vpr1-51, 10 μM CsA, and/or 50 μM BA, followed by staining with Δψm-sensitive DiOC6(3) and hydroethidium (HE), which reacts with superoxide anion to the fluorochrome ethidium (Eth). (C) Structural requirements, caspase independence, and Bcl-2-mediated inhibition of Vpr-mediated killing. Jurkat cells transfected with the human bcl-2 gene, the neomycin resistance gene only (Neo), or Neo optionally cultured with 50 μM of Z-VAD.fmk or 50 μM of Z-VAD-07 were treated with 2 μM Vpr1-96, 2 μM Vpr1-51, 1 μM Vpr52-96 (or mutants), 5 μM Vpr52-96 (or mutants), 50 μM Vpr71-96 (or mutants), followed by staining with Δψm-sensitive DiOC6(3) (after 2 h as in B), an annexin V–FITC conjugate (for the determination of hypoploid cells (at 12 h). Data are pooled from five independent experiments, and each data point has been repeated at least three times.
Materials and Methods

\( Vpr \) Peptides and Constructs. \( Vpr1-96, Vpr \)-derived peptides, and NCp7 were synthesized by automated solid phase synthesis using the FMOC strategy and purified by reverse-phase HPLC (39, 40). The peptides were analyzed by electrospray mass spectrometry and found to be >98% pure. The FLAG-\( Vpr \)-expressing vector was constructed by PCR amplification of p90, which contains the whole HIV-1 Lai genome. Both primers, FIVpr (TCCGGATC-CACCATGGACTAACAAGCAGCTGACAAATCGATGGAACAGCCC [coding sequence of the FLAG-derived epitope (MDYKDDDDK) plus sequence 5,141-5,153 of Lai]) and VLC as (ATT T T CC TAT ATTT C AT G TACTGGAC C [5,737-5,707]), resulted in a 638-bp fragment, which was cloned into the blunt EcoR1-BamHI sites of the pcDNA3.1 eukaryotic expression vector (Invitrogen Corp.). This construct was transfected into COS cells using Lipofectamine (Life Technology).

Cells and Apoptosis Modulation. Jurkat-Neo and Jurkat-Bcl-2 clones (reference 41; a gift from Dr. N. Israel, Pasteur Institute, Paris, France), and CEM-C7 cells were cultured in RPMI 1640 Glutamax medium supplemented with 10% FCS, antibiotics, and 0.8 \( \mu \)g/ml G418. 2B4.11 mouse T cell hybridoma cell lines stably transfected with an SFFV.neo vector, containing the human \( bcl-2 \) gene or the neomycin (Neo) resistance gene, and COS cells were cultured in DMEM Glutamax medium supplemented with Hepes, antibiotics, and 10% FCS. PBS-washed cells (1-5 \( \times \) 10^6/ml) were incubated for 30 min with Vpr or \( Vpr \)-derived peptides in isotonic glucose–Hepes buffer (2.4% glucose, 13 mM Hepes, 68 mM NaCl, 1.3 mM KCl, 4 mM Na\(_2\)HPO\(_4\), and 0.7 mM KH\(_2\)PO\(_4\), pH 7.2), followed by culture in complete culture medium supplemented with cyclosporin A (CsA; 1 \( \mu \)M; Novartis), bongkrekic acid (BA; 50 \( \mu \)M; gift of Dr. J.A. Duine, Delft University, The Netherlands), and/or the caspase inhibitor N-benzzyloxy carbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk; 50 \( \mu \)M; Bachem Biosciences, Inc.). During exposure to Vpr or \( Vpr \)-derived peptides, human primary PBLs from healthy donors, purified with Lyphoprep (Pharmacia), were cultured in RPMI 1640 Glutamax medium without any addition of serum. In contrast, PHA blasts (24 h of 1 \( \mu \)g/ml PHA-P [Wellcome Industries]; 48 h with 100 U/ml human recombinant IL-2 [Boehringer Mannheim]) were cultured with 10% FCS.

Determination of Apoptosis-associated Alterations in Intact Cells. For cytofluorometry, the following fluorochromes were employed: 3,3‘-dihexyloxacarbocyanine iodide (DiOC\(_{15}(3), 40 \)nM) for mitochondrial transmembrane potential (\( \Delta \psi_m \)) quantification, hydroethidine (4 \( \mu \)M; Molecular Probes, Inc.), the \( \psi_m \)-sensitive dye Mitotracker green (1 \( \mu \)M; Molecular Probes, Inc.), and/or Hoechst 33342 (2 \( \mu \)M; Sigma Chemical Co.) (27). Confocal microscopy was performed on a Leica TCS-SP (Leica Microsystems) equipped with an ArKr laser mounted on an inverted Leica DM IFBE microscope with a 63 \( \times \) 1.32 NA oil objective.

Preparation of Organelles, Cell-free Systems of Apoptosis, and Assays of Mitochondrial Parameters. Mitochondria were purified from rat liver (36) and resuspended in 250 mM sucrose plus 0.1 mM EGTA plus 10 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, pH 7.4. Cytosols from control or \( \alpha \)CD95-treated (CH-11; 500 ng/ml; 2 h; Immunotech) cells (10^7 cells/100 \( \mu \)l in cell-free assay buffer [reference 37]) were prepared by five freeze-thaw cycles in liquid nitrogen, followed by centrifugation (1.5 \( \times \) 10^6 g, 4°C, 1 h) as described (37). HeLa cell nuclei (10^7 nuclei per microliter) were incubated (60 min at 37°C) in the presence (or not) of isolated mitochondria, mitochondrial supernatants cytosols from CEM-C7 cells or recombinant AIF (19), and/or Vpr peptides. Then, nuclei were stained with PI (10 \( \mu \)M/ml; Sigma Chemical Co.), followed by cytofluorometric determination of the frequency of hypoploid nuclei (37). To determine large amplitude swelling, mitochondria (0.5 mg protein per milliliter) were resuspended in Swelling buffer (200 mM sucrose, 10 mM Tris-MOPS [3-N-morpholino]-propanesulfonic acid), pH 7.4, 5 mM...
Tris succinate, 1 mM Tris-phosphate, 2 μM rotenone, and 10 μM EGTA-Tris) and monitored in an F5400 fluorescence spectrometer (Hitachi) for 90° light scattering (545 nm) after addition of 1 mM atractyloside (Atr; Sigma Chemical Co.), 1 μM Ca, 50 μM BA, and/or Vpr peptides. For determination of ΔΨ\textsubscript{m}, mitochondria (0.5 mg protein per milliliter) were incubated in swelling buffer supplemented with 1 μM rhodamine 123 (R h123; Molecular Probes, Inc.), and the dequenching of Rh123 fluorescence (excitation 505 nm, emission 525 nm) was measured (44). Supernatants from mitochondria (6,800 g for 15 min, then 20,000 g for 1 h; 4°C) were frozen at −80°C until determination of AIF activity or immunodetection of cytochrome c (mouse mAb clone 7H8.2C12; Pharmingen) and AIF (rabbit polyclonal antiserum; reference 19). Caspase activity in the mitochondrial supernatant was measured using Ac-DEVD-amido-4-trifluoromethylcoumarin (Bachem Bioscience, Inc.) as fluorogenic substrate (18).

Binding Assays and Immunoblots. Isolated rat liver mitochondria (250 μg of protein in 100 μl of swelling buffer) were incubated for 30 min at RT with 5 μM Vpr52-96 or biotin-Vpr52-96. The washed mitochondrial pellet (10^6 g, 10 min, 4°C; two washes) was then lysed with 150 μl of a buffer containing 20 mM Tris/HCl, pH 7.6, 400 mM NaCl, 50 mM KCl, 1 mM EDTA, 0.2 mM PMSF, aprotinin (100 U/ml), 1% Triton X-100, and 20% glycerol. Such extracts were diluted with two volumes of PBS plus 1 mM EDTA before the addition of 150 μl avidin–agarose (ImmunoPure; Pierce Chemical Co.) to capture the biotin-labeled Vpr52-96 complexed with its mitochondrial ligand(s) (2 h at 4°C in a roller drum). The avidin–agarose was washed batchwise with PBS (five times, 5 ml; 4°C) were frozen at −80°C until determination of AIF activity or immunodetection of cytochrome c (mouse mAb clone 7H8.2C12; Pharmingen) and AIF (rabbit polyclonal antiserum; reference 19). Caspase activity in the mitochondrial supernatant was measured using Ac-DEVD-amido-4-trifluoromethylcoumarin (Bachem Bioscience, Inc.) as fluorogenic substrate (18).

Protein-Protein Interactions by Surface Plasmon Resonance. To study the interaction of Vpr with proteins of known structure, recombinant human Bcl-2 (1-218) or mouse Bax (1-171), both lacking the hydrophobic transmembrane domain and produced and purified as described (27, 29), were added during the dialysis step. Liposomes reconstituted with PTPC or ANT, respec-

Figure 3. Mitochondrial Vpr effects in intact cells. COS cells were treated for 3 h with 1 μM Vpr1-51 (negative control) or Vpr52-96, fixed, permeabilized, and immunostained with antibodies specific for AIF or cytochrome C (Cyt.c) (both normally in the mitochondrial intermembrane space revealed PE, red fluorescence) and the mitochondrial matrix protein Hsp60 or the inner mitochondrial membrane protein COX (both revealed by FITC, green fluorescence). In addition, cells were stained with the ΔΨ\textsubscript{m}-sensitive dye CMXRos (red fluorescence) and the DNA intercalating agent Hoechst 33342 (blue fluorescence). The histograms indicate the percentage of cells manifesting mitochondrionuclear AIF translocation, mitochondriocytoplasmic cytochrome C translocation, or a low ΔΨ\textsubscript{m} after treatment with different Vpr peptides (1 μM) in the presence or absence of Z-VAD-fmk (50 μM).
Results and Discussion

Structural M oltifs R equired for the Cytotoxic Effects of V pr on Intact Cells. Synthetic Vpr protein (96 amino acids) kills Jurkat lymphoma cells (Fig. 1A) as well as a variety of other cell lines (references 10 and 11; data not shown). This effect was mimicked by the COOH-terminal moiety of the molecule Vpr52-96 but not by its NH2-terminal moiety (Vpr1-51; Fig. 1A). As described for other models of apoptosis (38), Vpr (or Vpr52-96, not Vpr1-51) induced an early loss of ΔΨm, as detected by the potential-sensitive fluorochrome DiOC6(3) (Fig. 1B). Abolition of Vpr52-96 homodimerization by replacement of two leucine residues by alanines (L60A L67A; reference 40) did not affect its apoptogenic function. In contrast,

![Diagram](image_url)

**Figure 4.** Effects of Vpr on a cell-free system of apoptosis. (A) Determination of the subcellular target of Vpr in a cell-free system of nuclear apoptosis. Nuclei purified from HeLa cells were incubated for 60 min with the indicated combinations of Vpr alone (1 μM), cytosol (Cyt.; obtained from CEM cells/10^6 g protein per milliliter), mitochondria (Mit.; purified from mouse liver; 0.5 mg mitochondrial protein per milliliter), recombinant mouse AIF1-612 (100 μg/ml), cytosol from Fas-treated CEM cells (10 μg protein per milliliter), or Atr (1 mM), followed by PI staining and DNA content analysis of nuclei (16). (B) Structural requirements and CsA-mediated inhibition of Vpr-induced release of mitochondrial apoptogenic factors. Mitochondria were treated for 30 min with 1 μM of Vpr or Vpr-derived peptides, 1 μM CsA, and/or 1 mM Atr. The supernatants of these mitochondria were added to purified nuclei for a period of 60 min, followed by PI staining and determination of the frequency of hypoploid nuclei. Alternatively, Vpr-derived peptides were added directly to nuclei. (C) Vpr-induced mitochondrial release of potentially apoptogenic proteins. Mitochondrial supernatants treated as in B were subjected to immunoblot detection of cytochrome (Cyt.;) or AIF. Alternatively, the capacity of supernatants to cleave the fluorogenic caspase substrate DEVD-AFC was assessed. (D) Bcl-2-mediated inhibition of nuclear apoptosis induced in the cell-free system. Mitochondria (Mit.) were purified from 2B4.11 T cell hybridoma cells expressing a Neo control vector or human Bcl-2. These organelles were left untreated (Co.), subjected to lysis or treated with 1 μM of the indicated Vpr-derived peptide, followed by recovery of the supernatant and determination of its apoptogenic effects on isolated HeLa nuclei as in A.

Yeast Strains and Clonogenic Assays. Synthetic Vpr protein (96 amino acids) kills Jurkat lymphoma cells (Fig. 1A) as well as a variety of other cell lines (references 10 and 11; data not shown). This effect was mimicked by the COOH-terminal moiety of the molecule Vpr52-96 but not by its NH2-terminal moiety (Vpr1-51; Fig. 1A). As described for other models of apoptosis (38), Vpr (or Vpr52-96, not Vpr1-51) induced an early loss of ΔΨm, as detected by the potential-sensitive fluorochrome DiOC6(3) (Fig. 1B). Abolition of Vpr52-96 homodimerization by replacement of two leucine residues by alanines (L60A L67A; reference 40) did not affect its apoptogenic function. In contrast,
replacement of arginine (R) residues situated within or between the two functionally important H(S/F)R1G motifs (52) (R73A or R77A or R80A) greatly reduced the apoptogenic effect of Vpr52-96. A peptide containing this motif (Vpr71-96, but not Vpr71-96 R73A R80A) was sufficient to induce apoptosis (Fig. 1 C). Systematic dose-response studies revealed a significant difference in the ED_{50} of these R-mutated peptides and their wild-type equivalents (see Fig. 6 A). These observations correlate with the fact that R80 mutations reduce cell killing by vesicular stomatitis virus (VSV)-G–pseudotyped HIV-1 in vitro (8) and that R73 and R80 are extremely conserved among pathogenic HIV-1 isolates. Agents that interact with the H(S/F)R1G motifs such as RNA or DNA (53) neutralized the cytocidal effect of Vpr (not shown). A strict correlation was found between the ΔΨ_{m} collapse induced by different Vpr-derived peptides and apoptosis induction at the plasma membrane and nuclear levels (Fig. 1 C). Very similar data have been obtained with several human cell lines (U937, CEM, HELa), COS cells, and mouse thymocytes (not shown), as well as human primary PBLs (Fig. 2).

Thus, Vpr or Vpr52-96 (but not Vpr1-51) causes a ΔΨ_{m} dissipation that precedes the loss of viability in human PBLs, and this effect is reduced when the mutant Vpr52-96 R73A is employed (Fig. 2).

Mitochondrial Effects of Vpr Added to Intact Cells. In Jurkat cells, Vpr caused a loss of ΔΨ_{m}, which was followed by an increase in the production of superoxide anion (Fig. 1 B) and nuclear apoptosis (Fig. 1 C). This early effect on the ΔΨ_{m} (1–2 h after addition of Vpr or Vpr52-96) was transiently inhibited by CsA and BA, two inhibitors of the PTPC (Fig. 1 B). Similar results were obtained with primary cells such as mouse thymocytes (not shown) and human primary PBLs, in which the ΔΨ_{m} reducing effect of Vpr or Vpr52-96 is counteracted by the ANT ligand BA (Fig. 2 A). The ΔΨ_{m} loss was also inhibited by overexpression of Bcl-2 (Fig. 1 C), an endogenous cytoprotective protein acting on the PTPC (27, 29). Bcl-2 concomitantly prevented other Vpr-induced features of apoptosis, such as phosphatidylserine exposure on the plasma membrane and nuclear DNA loss (Fig. 1 C). In contrast, the pancaspase inhibitor Z-Val-Ado.fmk

**Figure 5.** Vpr-induced swelling and ΔΨ_{m} dissipation in isolated mitochondria. Rat liver mitochondria were exposed to Vpr (2 μM) or the indicated Vpr derivative (1 μM), CsA (5 μM; added 1 min before Vpr to mitochondria), BA (50 μM; added 1 min before Vpr to mitochondria), a random eicosadisoxynucleotide (DNA; 1 μM), total cell RNA (1 μM), or Ncp7 (10 μM; added to Vpr 1 min before joint addition to mitochondria). Mitochondrial swelling (measured as 90° light scattering at 545 nm) or the ΔΨ_{m} (measured as Rh123 dequenching) were monitored continuously. ΔΨ_{m} and swelling determinations yielded concordant results. Representative curves obtained with either of the two methods are shown.
were obtained in six independent experiments in which each peptide was added to purified mitochondria as in Fig. 5, and the dose yielding an ED$_{50}$ to mitochondria. Different concentrations of Vpr or Vpr-derived peptides were used in six independent experiments in which each peptide was tested at least three times. (B) ED$_{50}$ of Vpr-derived peptides on purified mitochondria. Different concentrations of Vpr or Vpr-derived peptides were added to purified mitochondria as in Fig. 5, and the dose yielding an ED$_{50}$ on mitochondrial swelling (measured as 90° light scattering at 545 nm, 300 s after addition of the peptide) was extrapolated from the dose-response curve (log 2 dilutions). Results were obtained in 10 independent experiments, and each peptide was used at least three times.

failed to prevent the ΔΨ$_{m}$ dissipation, although it did reduce the (caspase-dependent) DNA loss resulting in hypoploidy (Fig. 1 C). Vpr52-96 induced, in intact cells, the mitochondrionuclear translocation of AIF and the mitochondrial translocation of cytochrome c as detected by confocal immunofluorescence microscopy (Fig. 3). Vpr also caused nuclear chromatin condensation (measured with Hoechst 33342), as well as a dissipation of the ΔΨ$_{m}$, as measured with the ΔΨ$_{m}$-sensitive dye CMX Rsos (Fig. 3). Again, Z-VAD.fmk (which did prevent end-stage nuclear chromatin condensation) had no mitochondrioprotective effects (Fig. 2). Altogether, these findings indicate that the mitochondrial effects of Vpr are caspase independent yet suppressed by PT pore inhibitors such as CsA, BA, or Bcl-2.

Determination of the Subcellular Target Responsible for the Apoptogenic Vpr Effect in a Cell-free System. Vpr has been suggested to act on different subcellular targets including the nucleus (5), the plasma membrane (10, 54), and mitochondria (55). To map the subcellular site of its apoptogenic action, we added Vpr to purified HeLa nuclei and determined the minimum requirements for the induction of chromatin degradation. Vpr alone had no effects on nuclei, nor did it activate any cytosolic activity resulting in nuclear apoptosis (Fig. 4 A). In contrast, Vpr did become apoptogenic in the presence of mitochondria (Fig. 4 A). This suggests that Vpr acts primarily on mitochondria (rather than on nuclei or cytosolic proteins) to trigger the induction of apoptosis. Supernatants of mitochondria treated with Vpr contain a factor that provokes nuclear apoptosis in the cell-free system (Fig. 4 B), immunodetectable AIF (which accounts for this bioactivity; reference 19), immunodetectable cytochrome c and a caspase activity cleaving DEVD-afce (Fig. 4 C) (18, 56). The release of these mitochondrial intermembrane proteins was induced by the entire Vpr molecule, its COOH-terminal moiety (Vpr52-96 or Vpr52-71), or a short peptide fragment containing the two H(S/F)RGIG motifs (Vpr71-82) (Fig. 4, B and C), but not by Vpr-derived peptides in which R 73 and R 80 were mutated (Fig. 4 B). Altogether, the data obtained in the cell-free system suggest that Vpr can exert most if not all of its apoptogenic potential by directly compromising the barrier function of mitochondrial membranes.

Mechanisms of Vpr Effects on Isolated Mitochondria: Structural Analysis and Evidence for the Involvement of the PT Pore. The release of mitochondrial proteins induced by Vpr in vitro was blocked by the PT pore inhibitor CsA (Fig. 4, B and C). Moreover, mitochondria isolated from Bcl-2-overexpressing cells were refractory to the Vpr-induced release of AIF activity (Fig. 4 D). The fact that some of the Vpr effects were inhibited by PT pore inhibitors (CsA, BA, or Bcl-2) suggested that Vpr can act on the mitochondrial PT pore, the opening of which can be a rate-limiting step of the apoptotic process. Accordingly, Vpr induced two hallmark PTPC opening when added to purified mitochondria, namely mitochondrial volume increase and ΔΨ$_{m}$ dissipation (Fig. 5), and both of these effects were inhibited by CsA and BA. The effect of free holo Vpr on isolated mitochondria is fully mimicked by Vpr52-96 but not by Vpr52-96 R73A, Vpr52-96 R77A, or Vpr52-96 R80A (Fig. 5). Preincubation of Vpr with a molar excess of RNA or DNA (which bind to the Vpr71-82 motif; reference 53) abolished its effects on isolated mitochondria (Fig. 5), correlating with the data obtained in cells (not shown). In contrast, synthetic HIV-1 nucleocapsid protein NCp7 (which binds to the extreme COOH terminus of Vpr; reference 39) does not inhibit Vpr effects on mitochondria (Fig. 5). Thus, the structural motifs of Vpr responsible for direct, presumably PT pore-mediated mitochondrial effects in vitro (Fig. 5) and apoptosis induction in intact cells (Fig. 1 C) are the same. This fact is also underscored by the comparison of the ED$_{50}$ of different Vpr peptides determined on intact cells (Fig. 6 A) and purified mitochondria (Fig. 6 B).

Mitochondrial Localization of Vpr. If Vpr acted on mitochondria to induce apoptosis, then at least some Vpr protein should be found in mitochondria from intact cells. To determine the subcellular localization of Vpr, epitope-tagged (FLAG) Vpr was transfected into COS cells and was revealed by a PE-labeled anti-FLAG antibody (green fluorescence). Simultaneously, mitochondria were stained with an FITC-conjugated anti-Hsp60 antibody (green fluorescence). In accord with previous observations of a punctuate cytoplasmic localization of Vpr (57, 58), we found that ~30% of Vpr-expressing cells exhibited an exclusively cytoplasmic Vpr staining pattern (Fig. 7 A). These cells appear to be programmed to die (not shown), which may explain why they represent only a fraction of the entire population. In such cells, most of the Vpr-dependent red fluorescence colo-
calizes with the Hsp60 protein, giving rise to a yellow (red plus green) staining pattern. Very little Vpr is localized in the nonmitochondrial compartment (red fluorescence; Fig. 7 A). To confirm this observation in another experimental system, we added biotinylated Vpr52-96 to human primary PBLs or to PHA lymphoblasts. Vpr52-96 was then detected by means of a streptavidin–PE conjugate. Cells were counterstained with Mitotracker green (which labels mitochondria independently from their ΔΨm) and Hoechst 33342 (which labels nuclei) to determine the subcellular distribution of Vpr. After an initial enrichment in the plasma membrane (not shown), biotinylated Vpr52-96 was specifically recruited to mitochondria (Fig. 7 B).

Direct Interaction of Vpr with the PTPC via ANT. To identify the putative mitochondrial receptor of Vpr, purified mitochondria were incubated with biotinylated Vpr52-96 (which is as efficient as nonmodified Vpr52-96 in inducing mitochondrial swelling; not shown), followed by purification of biotin–Vpr52-96 binding proteins on avidin–agarose. This led to the selective recovery of very few proteins, among which we identified VDAC and ANT (but not COX) by immunoblotting (Fig. 8 A). Neither VDAC nor ANT was recovered if mitochondria were pretreated with BA (Fig. 8 B), indicating that BA can compete with Vpr52-96 for ANT binding and/or that a BA-induced conformational change abolishes the Vpr–ANT interaction. Surface plasmon resonance (see Materials and Methods) measurements confirmed that biotinylated Vpr52-96 immobilized to a streptavidin matrix binds to purified (>95%) ANT with an affinity constant of Kd = 7.4 × 10⁶ M⁻¹ (koff = 1.61 × 10⁶ M⁻¹s⁻¹; kon = 2.16 × 10⁻³ s⁻¹). These results suggest that Vpr is recruited to the PTPC via a direct, specific interaction with ANT.

To confirm the hypothesis that Vpr might permeabilize mitochondrial membranes by a direct effect on the PTPC, we purified this molecular complex from brain (27, 29), reconstituted it into liposomes, and measured the capacity of Vpr to permeabilize the liposomal membrane (Fig. 9 A). Vpr or Vpr52-96 increases the permeability of liposomes containing the PTPC, and this effect is inhibited by CsA or BA (Fig. 9 A). In addition, Vpr52-96 acts on a combination of two proteins from the PTPC, ANT plus Bax, and this effect is suppressed by recombinant Bcl-2 (Fig. 9 B). Thus, Vpr acts on the PTPC to perturb the barrier function of mitochondrial membranes.

Genetic Evidence for PTPC-mediated Vpr Cytotoxicity. The essential components of the PTPC include the two most abundant proteins of the outer and inner mitochondrial membranes, VDAC and ANT, respectively (27, 29, 30). We therefore examined the cytotoxic effect of Vpr52-96 on a series
of S. cerevisiae (yeast) strains in which VDAC or ANT had been invalidated by homologous recombination. Yeast cells rendered deficient for one or two of the principal VDAC isoforms (VDAC\textsubscript{D1} or VDAC\textsubscript{D1D2}) or the two principal ANT isoforms (ANT\textsubscript{D1D2}) are more resistant to Vpr\textsubscript{52-96} than their respective wild-type control cells (Fig. 10, A and B). This relative resistance is abolished by genetic interventions known to correct the metabolic deficiencies caused by VDAC\textsubscript{1} knock-out (overexpression of VDAC\textsubscript{2} or transfection with human VDAC\textsubscript{1}; references 46 and 47; Fig. 10 B) or ANT\textsubscript{1/2} knock-out (retransfection with yeast ANT\textsubscript{2}; references 48 and 49; Fig. 10 A). Thus, genetic manipulations confirm that PTPC components are rate limiting for the cytotoxic effect of Vpr.

Conclusion. Based on the evidence obtained with cells (Figs. 1–3, 6 A, and 7), cell-free systems of apoptosis (Fig. 4), isolated mitochondria (Fig. 5 and Fig. 6 B), purified PTPC (Fig. 9 A), purified ANT and Bax (Fig. 9 B) and VDAC/ANT-deficient yeast cells (Fig. 10), it appears that the acute apoptogenic effect of Vpr involves a direct effect on the PTPC. This conclusion is corroborated by the interaction of Vpr with mitochondria (Fig. 7), with proteins from the PTPC (Fig. 8 and Fig. 9 A), and in particular with the ANT (Fig. 9 B and surface plasmon resonance data). Additional mechanisms of Vpr-mediated apoptosis induction have been suggested, in particular a glucocorticoid-like effect on T cells (11), plasma membrane permeabilization in neurons (10, 54) (which would, however, involve the NH\textsubscript{2} terminus of Vpr, not the COOH terminus), and cell cycle arrest in proliferating cells (5, 7, 9). Thus, the mechanisms of cell killing by Vpr may be redundant, at least in some systems. However, data supporting mitochondrial Vpr effects have been obtained with different cells (Jurkat, CEM, U 937, COS, R at-1, thymocytes and human primary PBLs Figs. 1–3, Fig. 7, and data not shown), purified mitochondria from distinct organs (lymphocytes and liver; Figs. 4 and 5), PTPC from brain (Fig. 9 A), and a xenogenic yeast system (Fig. 10), underlining the relative importance of this pathway for Vpr-mediated cell killing.

Vpr and its COOH-terminal moiety have acute cytotoxic (2 h) and mitochondriotoxic (5 min) effects at an ED\textsubscript{50} of \(~1\) \(\mu\)M, which is higher than the Vpr serum concentration. This might be used as an argument against the pathophysiological relevance of our studies. Nevertheless, several considerations have to be taken into account. First, the mitochondrial receptor for Vpr, the ANT, possesses a \(K_D\) of \(7.4 \times 10^{-8}\) M\textsuperscript{2-1}, meaning that chronic exposure to Vpr may well have biological effects at lower doses than those required in short-term assays (in which Vpr must cross several diffusion barriers to reach its target). Accordingly, the
Viruses employ several strategies for the inhibition of apoptosis. Thus, viruses may encode homologues of mammalian IAPs, or caspase inhibitors to prevent apoptosis. Thus, viruses may encode homologues of mammalian IAPs, or caspase inhibitors to prevent apoptosis. Thus, viruses may encode homologues of mammalian IAPs, or caspase inhibitors to prevent apoptosis. Thus, viruses may encode homologues of mammalian IAPs, or caspase inhibitors to prevent apoptosis. Thus, viruses may encode homologues of mammalian IAPs, or caspase inhibitors to prevent apoptosis.

ED_{50} of Vpr52-96 was found to be ~120 nM if cytotoxic effects were assessed after 24 h (not shown); that is at least three times lower than the ED_{50} measured after 2 h (Fig. 6 A). Second, compartmentalization effects might give rise to locally elevated concentrations, which suffice to exert biological effects in situ. Third, when cooperating with other cytotoxic mechanisms, in the context of viral infection, Vpr might exert its effects at lower doses. Two other HIV-1 proteins, Tat and PR, may indirectly affect mitochondrial function, Tat via downregulating mitochondrial superoxide dismutase (59) and PR by cleaving Bcl-2 (60). This hints at the possibility that several apoptogenic HIV-1 proteins—Vpr, Tat, and PR—cooperate at the mitochondrial level, thereby explaining that a fraction of circulating and sessile lymphocytes from HIV-1 carriers have a low ΔΨ_{m} (61, 62).

At the time of this writing, the extent to which Vpr contributes to HIV-1–induced apoptosis in infected or bystander cells is elusive. Early during replication, most if not all Vpr (which is of viral origin) is found in the preintegration complex, where it interacts with nucleic acids (which inhibit the mitochondrial effects of Vpr; Fig. 5) and NCP7, as well as other proteins (63). Moreover, during the later stage of the viral life cycle, Vpr synthesized de novo by the host cell may be sequestered into viral particles before it interacts with its mitochondrial receptor. Alternatively, Vpr may be released and then act on noninfected bystander cells. In vitro, HIV-1 strains in which endogenous Env has been replaced by the general fusogene VSV-G can infect most mammalian cell types yet induce apoptosis in a largely Vpr-independent fashion (9). Thus, at least in some particular settings, Vpr is rate limiting for HIV-1–mediated killing. It is not known, however, whether this effect is mediated by Vpr produced by the infected cells or rather involves paracrine effects. However, the fact that such Vpr-dependent killing can be obtained in the absence of HIV-1 replication (64) underlines the possibility that virion-associated Vpr (as opposed to free soluble Vpr) may well have a cytotoxic potential at the beginning of the viral life cycle.

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