Control of Mitochondrial Membrane Permeabilization by Adenine Nucleotide Translocator Interacting with HIV-1 Viral Protein R and Bcl-2

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Abstract

Viral protein R (Vpr), an apoptogenic accessory protein encoded by HIV-1, induces mitochondrial membrane permeabilization (MMP) via a specific interaction with the permeability transition pore complex, which comprises the voltage-dependent anion channel (VDAC) in the outer membrane (OM) and the adenine nucleotide translocator (ANT) in the inner membrane. Here, we demonstrate that a synthetic Vpr-derived peptide (Vpr52-96) specifically binds to the intermembrane face of the ANT with an affinity in the nanomolar range. Taking advantage of this specific interaction, we determined the role of ANT in the control of MMP. In planar lipid bilayers, Vpr52-96 and purified ANT cooperatively form large conductance channels. This cooperative channel formation relies on a direct protein–protein interaction since it is abolished by the addition of a peptide corresponding to the Vpr binding site of ANT. When added to isolated mitochondria, Vpr52-96 uncouples the respiratory chain and induces a rapid inner MMP to protons and NADH. This inner MMP precedes outer MMP to cytochrome c. Vpr52-96–induced matrix swelling and inner MMP both are prevented by preincubation of purified mitochondria with recombinant Bcl-2 protein. In contrast to König’s polyanion (PA10), a specific inhibitor of the VDAC, Bcl-2 fails to prevent Vpr52-96 from crossing the mitochondrial OM. Rather, Bcl-2 reduces the ANT–Vpr interaction, as determined by affinity purification and plasmon resonance studies. Concomitantly, Bcl-2 suppresses channel formation by the ANT–Vpr complex in synthetic membranes. In conclusion, both Vpr and Bcl-2 modulate MMP through a direct interaction with ANT.

Key words: ADP/ATP translocase • HIV • Vpr • mitochondria • Bcl-2
Introduction

Mitochondrial membrane permeabilization (MMP) is a key event of apoptotic cell death associated with the release of caspase activators and caspase-independent death effectors from the intermembrane space, dissipation of the inner mitochondrial transmembrane potential ($\Delta \Psi_m$), and a perturbation of oxidative phosphorylation (1–7). Pro- and antiapoptotic members of the Bcl-2 family regulate inner and outer MMP through interactions with the adenine nucleotide translocator (ANT) in the inner membrane (IM), the voltage–dependent anion channel (VDAC) in the outer membrane (OM), and/or through autonomous channel-forming activities (5–10). ANT and VDAC are major components of the permeability transition pore complex (PTPC), a polypeptide structure organized at sites at which the two mitochondrial membranes are apposed (7, 11). It is a matter of debate whether Bcl-2 primarily regulates MMP through its interaction with VDAC or ANT.

The HIV-1 viral protein of regulation, viral protein R (Vpr), is abundant in virions (12–14) and is detectable in the serum of HIV-1 carriers, correlating with the viral load (15). Vpr has pleiotropic effects on viral replication and cellular proliferation, differentiation, cytokine production, nuclear and membrane fractions as well as the consequence of the viral life cycle, but also independently from the infection process (i.e., via a “bystander effect”). In addition, Vpr can localize to mitochondria to kill cells by apoptosis (20, 22–25). Full-length (Vpr52-96) or truncated synthetic forms of Vpr act on the PTPC to induce all mitochondrial hallmarks of apoptosis, including $\Delta \Psi_m$ loss and the release of cytochrome c and apoptosis-inducing factor (AIF) (22). The MMP-inducing activity of Vpr resides in its COOH-terminal moiety (Vpr52-96), within an $\alpha$-helical motif of 12 amino acids (Vpr71-82) containing several critical arginine (R) residues (R73, R77, R80) which are strongly conserved among different pathogenic HIV-1 isolates (22–24).

In this study, we used Vpr and Vpr-derived peptides as a tool to dissect the molecular events of MMP and its control by Bcl-2. Here we demonstrate that Vpr52-96 physically and functionally interacts with ANT to form large conductive channels, closure of VDAC abrogates Vpr52-96 binding to mitochondria, Vpr triggers inner MMP through its interaction with ANT before outer MMP, and Bcl-2 prevents the Vpr52-96–induced MMP through a direct interaction with ANT.

Materials and Methods

Plasmon Resonance. NH2-terminally biotinylated peptides (Vpr52-96, Vpr52-96[R73A,80A]), or an irrelevant control peptide, HWWRAEDDEA-RCCYNDPKCCDFVTNR), were immobilized on biosensor chips of a BIAcore™ apparatus (Amersham Pharmacia Biotech), and 140 nM highly purified ANT from rat heart mitochondria (26) was added (time 0, on) in the mobile phase (5 $\mu$L/min) for real time measurement (in resonance units, RU). When required, the surface was regenerated using 1 M NaCl containing 0.05% P20 detergent (BIAcore). The data were analyzed using the v3.0 software and fitted to a 1:1 Langmuir binding model with separate $k_{on}$ and $k_{off}$ determination. The association constant ($K_a$) was determined as $k_{on}/k_{off}$, and the dissociation constant ($K_d$) as $1/K_a$. Control experiments revealed no specific binding of Vpr52-96 to recombinant VDAC (a gift from C. Mannella, Wadsworth Center, Albany, NY; reference 27), without any difference between Vpr52-96 and Vpr52-96[R73A, 80A] and without detectable saturation for VDAC concentrations up to 10 $\mu$M.

ANT Purification and Reconstitution in Liposomes. ANT was purified from rat heart mitochondria as described previously (8). After mechanical shearing, mitochondria were suspended in 200 mM mannitol, 70 mM sucrose, 10 mM Hepes, 200 $\mu$L EDTA, 100 mM dithiothreitol, 0.5 $\mu$g/ml subtilisin, pH 7.4, kept on ice for 8 min, and sedimented twice by differential centrifugations (5 min, 500 g; 10 min, 10,000 g). Mitochondrial proteins were solubilized by 6% (vol/vol) Triton X-100 (Boehringer) in 40 mM K$_2$HPO$_4$, 40 mM KCl, 2 mM EDTA, pH 6.0, for 6 min at room temperature (RT), and solubilized proteins were recovered by ultracentrifugation (30 min, 24,000 g, 4°C). Then, 2 ml of this Triton X-100 extract was applied to a column filled with 1 g of hydroxyapatite (BioGel HTP; Bio-Rad Laboratories), eluted with previous buffer, and diluted (vol/vol) with 20 mM MES, 200 $\mu$L EDTA, 0.5% Triton X-100, pH 6.0. Subsequently, the sample was separated with a Hitrap SP column using an FPLC system (Amersham Pharmacia Biotech) and a linear NaCl gradient (0–1 M). Protein concentration was determined using micro-BCA assay (Pierce Chemical Co.). Purified ANT and/or recombinant Bcl-2 was reconstituted in phosphatidylcholine (PC)/cardiolipin liposomes. In brief, to prepare liposomes, 45 mg PC and 1 mg cardiolipin were mixed in 1 ml of chloroform, and the solvent was evaporated under nitrogen. Dry lipids were resuspended in 1 ml liposome buffer (125 mM sucrose plus 10 mM Hepes, pH 7.4) containing 0.3% n-octyl-β-D-pyranoiside and mixed by continuous vortexing for 40 min at RT. ANT (0.1 mg/ml) or recombinant Bcl-2 (0.1 mg/ml) was then mixed with liposomes (vol/vol) and incubated for 20 min at RT. Proteoliposomes were finally dialyzed overnight at 4°C.

Vpr Binding to Liposomes. Proteoliposomes or plain liposomes were exposed to different concentrations of FITC–labeled Vpr52-96 for 30 min in liposome buffer, washed (290,000 g, 45 min, 10°C), and analyzed in a FACS Vantage™ (Becton Dickinson) cytofluorometer (X ± SD, n = 3).

Pore-opening Assay. ANT proteoliposomes were sonicated in the presence of 1 mM 4-methylumbelliferylphosphate (4-MUP) and 10 mM KCl (50 W, 22 s, sonifier model 250; Branson) on ice as described previously (28). Then, liposomes were separated on Sephadex G-25 columns (PD-10; Amersham Pharmacia Biotech) from unencapsulated products. 25-$\mu$L aliquots of liposomes were diluted to 3 ml in 10 mM Hepes, 125 mM saccharose, pH 7.4, mixed with various concentrations of the proapoptotic inducers, and incubated for 1 h at RT. Potential inhibitors of MMP such as BA, ATP, and ADP were added to the liposomes 30 min before the addition of Vpr or Vpr-derived peptides.
Chondria were isolated as described (22). For the determination of monoclonal antibody clone 7H8.2C12; BD PharMingen). For potentials for electrical silence. Before addition to the bilayer, protein addition, bare membranes were checked under applied mation was monitored by the capacitance response and, before introduction, with Bcl-2 or Bax and added to one compartment, then. Rad Laboratories) to eliminate Triton X-100. In some experi- ments, Vpr52-96 was added directly to the aqueous subphase af- ter bilayer formation. Single channel recordings are representative of the most frequently observed events. ANT was preincubated or not with Bcl-2 or Bax and added to one compartment, then Vpr52-96 was added to the same compartment.

**Binding Assays and Western Blot Analysis.** Mouse liver mito- chondria were isolated as described (22). For the determination of cytochrome c release, supernatants from Vpr52-96–treated mito- chondria (6,800 g for 15 min; 20,000 g for 1 h, both at 4°C) were frozen at −80°C until immunodetection of cytochrome c (mouse monoclonal antibody clone 7H8.2C12; BD PharMingen). For binding assays, purified mitochondria were incubated (250 μg of protein in 100 μl swelling buffer) for 30 min at RT 5 μM (binding assay) Vpr52-96 or biotin–Vpr52-96. Mitochondria were lysed ei- ther after incubation with biotinylated Vpr52-96 or before 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, 100 U/ml aprotinin, 1% Triton X-100, and 20% glycerol. Such extracts were diluted with 2 vol 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 mitochon- drial ligand(s) (2 h at 4°C in a roller drum). The avidin-agarose was washed batchwise with PBS (five times with 5 ml; 1,000 g, 5 min, 4°C), resuspended in 100 μl of twofold concentrated Laemmli buffer containing 4% SDS and 5 mM β-mercaptoetha- nol, incubated for 10 min at RT, and centrifuged (1,000 g, 10 min, 4°C). Finally, the supernatants were heated at 95°C for 5 min and analyzed by SDS-PAGE (12%), followed by Western blot analysis and immunodetection with a rabbit polyclonal antiserum against human ANT (provided by Dr. Heide H. Schmid, The Hormel Institute, University of Minnesota, Austin, MN; reference 31).

**Cell Culture and Microinjection Experiments.** COS cells (cultured in DMEM Glutamax medium supplemented with Hepes, antibiotics, and 10% FCS) that were growing on a premarked V-shaped area of a coverslip (>200 per experiment) were microinjected into the cytoplasm using a computer-controlled microinjection equipment (Eppendorf; pressure 200 hPa, 0.5 s) with PBS only, pH 7.2, recombinant human Bcl-2 (a gift of Dr. Heide H. Schmid, Konstanz, Germany). After microinjection, cells were cultured in the absence (Co.) or presence of 1 μM Vpr52-96 for 3 h and stained for 30 min with 2 μM ΔΨm-sensitive dye 5,5′,6,6′-tetraclororo-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) (red fluorescence shows mitochondria with a high ΔΨm; green fluorescence shows mitochondria with a low ΔΨm; reference 33).

**Flow Cytometric Analysis of Purified Mitochondria.** Purified mitochondria were resuspended in PT buffer (200 mM sucrose, 10 mM Tris-MOPS, pH 7.4, 5 mM Tris-succinate, 1 mM Tris- phosphate, 2 μM rotenone, and 10 μM EGTa). Cytolipofluorometric (FACS Vantage™; Becton Dickinson) detection was re- stricted to mitochondria by gating on the forward scatter (FSC)/ side scatter (SSC) parameters and on the main peak of the FSC-W parameter. Confirmation a posteriori of the validity of this double gating was obtained by the labeling of mitochondria with m-insensitive mitochondrial dye MitoTracker® Green (75 nM, green fluorescence; Molecular Probes). To determine the percentage of mitochondria having a low ΔΨm, the ΔΨm-sensitive fluorescent probe JC-1 (200 nM, 570–595 nm) was added 10 min before carboxylamide m-chlorophenylhydrazone (CCCP) or Vpr-derived peptides. The percentage of mitochondria having a low ΔΨm was determined in dot plot FSC/FL-2 (red fluorescence) windows.

**Polycromatic Studies.** Purified mitochondria were incubated at 37°C in a thermostated cell fitted with a Clark electrode (Hanse- tach) and containing 250 ml of 0.3 M mannitol, 10 mM KCl, 5 mM MgCl2, 1 mg/ml BSA, and 10 mM KH2PO4, pH 7.4, as described (34). Cytochrome c oxidase activities were spectrophoto- metrically measured (34).

**Results**

**Physical Interaction between Vpr and ANT.** Surface plas- mon resonance measurements indicate that purified deter- gent-solubilized ANT protein binds to the immobilized Vpr COOH-terminal moiety biotin–Vpr52-96 (but to a far lesser extent to mutated biotin–Vpr52-96[K73,80A]) with an affinity in the nanomolar range (Fig. 1, A and B). This interaction was modulated by two ANT ligands which differentially affect ANT conformation (35), namely the
PTPC activator (opener) Atr, which favored Vpr binding, and the PTPC inhibitor (closer) bongkrekic acid (BA), which reduced Vpr binding (Fig. 1 C). Bcl-2-like proteins bind to a motif of ANT (amino acids 105–155; reference 8) whose implication in apoptosis control has been confirmed by deletion mapping (36). This motif partially overlaps with the second ANT loop (amino acids 92–116), a regulatory domain exposed to the intermembrane space (37, 38). A peptide corresponding to the overlap between the Bcl-2 binding motif and this loop (ANT104-116) inhibited the ANT–Vpr interaction (Fig. 1 C), presumably via direct association with Vpr52-96 (Fig. 1 D). Neither a topologically related peptide motif derived from the human phosphate carrier nor mutated and scrambled versions of ANT104-116 (control peptides in Fig. 1 C) had such inhibitory effects. Vpr also interacted with ANT incorporated into liposomal membranes. Indeed, Vpr52-96 binding to membranes was greatly facilitated in liposomes in which ANT has been reconstituted compared with protein-free liposomes (Fig. 2 A). The ANT-facilitated incorporation of Vpr into membranes was inhibited by BA (Fig. 2 B). In conclusion, Vpr binds to ANT, at least in part via an interaction with a domain of ANT (ANT104-116) that is exposed to the intermembrane side of this protein, coinciding with the apoptogenic portion of ANT.

Cooperative Channel Formation by Vpr and ANT in Synthetic Membranes. Vpr52-96 (but not the NH2-terminal moiety of Vpr [Vpr1-51] or mutated Vpr52-96, in which arginine 77 is replaced by alanine, Vpr52-96[R77A]) caused permeabilization of ANT proteoliposomes (Fig. 2 C), yet had no effect on plain liposomes (data not shown). ANT104-116 (but not the control peptides) prevented the Vpr52-96–induced membrane permeabilization of ANT proteoliposomes (Fig. 2 C), indicating that in the context of the lipid bilayer, the effect of Vpr involved a direct interaction with ANT. In planar lipid bilayers, low doses of Vpr52-96 (<1 nM) were incapable of forming channels, unless ANT was present. ANT and Vpr52-96 cooperated to form discrete channels whose conductance (190 ± 2 pS; reference 39), yet was in the range of those formed by Ca2+-treated ANT (26, 40). These biophysical experiments demonstrate that ANT and Vpr directly interact in membranes to form functionally competent channel-forming hetero(poly)mers.

Vpr-induced IM Permeabilization Studied in Isolated Mitochondria. Compared with untreated organelles (Fig. 4 A, trace a), purified mitochondria preincubated with Vpr52-96 (Fig. 4 A, trace b) exhibited a gross deficiency in respiratory control (RC). Vpr increased succinate oxidation preceding ADP addition and abolished both the inhibitory effect of oligomycin (a specific ATPase inhibitor) and the stimulatory effect of uncoupling by the protonophore CCCP. Thus, Vpr52-96 (but not Vpr1-51) reduced the RC (ratio of oxygen consumption with oligomycin versus CCCP) to a value of 1.1, compared with 5.3 in control mitochondria (Table I). The entire Vpr protein (Vpr1-96), and a short peptide corresponding to the minimum “mitochondriotoxic” motif of Vpr (Vpr71-82) (22, 23), also reduced the RC values (Table I). Noticeably, the Vpr-induced loss of RC was not associated with a significant decrease of the oxidation rate (Fig. 4 A), suggesting that no major loss of membrane-bound cytochrome c occurred on short-term incubation with Vpr52-96. Accordingly, adding cytochrome c to Vpr52-96-treated mitochondria oxidizing succinate did not stimulate the rate of oxygen uptake (Fig. 4 A, trace b). The observation of Vpr-mediated uncoupling of the respiratory chain prompted us to test its capacity to in-
duce IM permeabilization. The IM being essentially impermeable to NADH (41), no significant oxygen uptake could be measured when NADH was added to control mitochondria (Fig. 5 A, trace a). However, addition of Vpr52-96 prompted a significant, NADH-stimulated oxygen consumption (Fig. 5 A, trace b). This indicates that Vpr permeabilized the IM both to protons (leading to uncoupling; Fig. 4 A, trace b) and NADH (Fig. 5 A, trace b). Moreover, addition of ADP (the physiological substrate of ANT) strongly inhibited the Vpr-dependent, NADH-stimulated oxygen consumption (Fig. 5 A, trace c), suggesting that the ANT–Vpr interaction is essential for inner MMP by Vpr.

Vpr-induced IM Permeabilization Precedes OM Permeabilization. To determine the primary site of action of Vpr52-96 on mitochondria, we studied the differential kinetics of inner and outer MMP to NADH and cytochrome c, respectively (Fig. 5 B). NADH oxidation by mitochondria incubated with Vpr52-96 was found maximal 10 min after addition of Vpr52-96 (Fig. 5 B). Under similar conditions, Vpr52-96 only induced a marginal access of cytochrome c to cytochrome c oxidase (Fig. 5 B). Moreover, the ΔΨm loss occurred before cytochrome c release can be detected by immunoblot (Fig. 5 C). Hence, Vpr52-96 causes inner MMP well before OM becomes permeable to cytochrome c. Accordingly, at the ultrastructural level (see also Fig. 6 C), Vpr52-96–treated mitochondria exhibited matrix swelling before OM rupture became apparent.

Bcl-2 and PA10 Both Prevent MMP Induced by Vpr. Extracellular addition of Vpr to intact cells induced a rapid ΔΨm loss before nuclear condensation occurred. This ΔΨm loss could be fully prevented (≥95% of inhibition) by microinjection of recombinant Bcl-2 into the cytoplasm (Fig. 6 A). Less than 5% of Bcl-2–injected cells manifested a ΔΨm reduction, whereas 61 ± 8% (n = 3) of vehicle-microinjected cells exhibited a complete ΔΨm dissipation in response to Vpr.

Preincubation of purified mitochondria with recombinant Bcl-2 also inhibited the Vpr-mediated inner MMP to NADH (Fig. 6 B). Concomitantly, Bcl-2 preincubation inhibited both the Vpr-induced matrix swelling (Fig. 6 C; ~50% inhibition) and ΔΨm loss (Fig. 6, D and E; ~70% inhibition). No such protective effect was observed when Bcl-2 was replaced by Bcl-2ΔΔ5/6, a deletion mutant lacking the putative pore-forming α5 and α6 helices (42). Two pharmacological inhibitors of the PTPC (the ANT ligand BA and the cyclophilin D ligand cyclosporin A [CsA]), as
well as the specific VDAC inhibitor (closer) PA10 (43), also inhibited the manifestations of inner MMP (Fig. 6, B–E). Microinjected PA10 also inhibited the effect of Vpr52-96 on intact cells (Fig. 6 A). In conclusion, both Bcl-2 and PA10 protect mitochondria against Vpr-induced MMP.

**Differential Effects of Bcl-2 and PA10 on Vpr Binding to Mitochondria.** When added to purified mitochondria, FITC-labeled Vpr52-96 rapidly bound to intact mitochondria and then provoked ΔΨm loss (Fig. 7 A). Binding of Vpr52-96 to purified mitochondria was completely abolished by preincubation of the organelles with PA10, partially reduced by BA, but not affected by CsA (Fig. 7 B). Thus, Vpr must access mitochondria through VDAC. Bcl-2 may be expected to prevent Vpr from crossing the OM via VDAC (based on the Bcl-2-mediated closure of VDAC; references 8, 26, 45) and/or to inhibit the Vpr effect on ANT (based on its physical and functional interaction with ANT; references 8, 26, 45). Although recombinant Bcl-2 strongly reduced the Vpr52-96-induced matrix swelling (Fig. 6 C) and ΔΨm loss (Fig. 6, D and E), it failed to impair the binding of Vpr52-96 to purified mitochondria (Fig. 7 B). The differential inhibitory effects of PA10 and Bcl-2 on the Vpr-ANT interaction was confirmed in a distinct experimental system. PA10 fully abolished the affinity-mediated purification of ANT using biotinylated Vpr52-96 (Fig. 7 C), provided that its effect was assessed on intact mitochondria (in which Vpr52-96 has to cross the OM to reach ANT). In contrast, PA10 did not affect the Vpr52-96-mediated purification of ANT from Triton-solubilized mitochondria (in which ANT is readily accessible to Vpr52-96). In the same conditions, Bcl-2 reduced the Vpr52-96-mediated recovery of ANT, irrespective of its addition to intact or solubilized mitochondria (Fig. 7 C). Thus, Bcl-2 does not interfere with the PA10-inhibited VDAC-mediated conduit, allowing Vpr52-96 to pass the OM.

**Bcl-2-mediated Inhibition of the Physical and Functional Interaction between Vpr and ANT.** A further series of experiments indicated that Bcl-2 modulated the physical and functional interaction of Vpr with ANT. Recombinant Bcl-2 (but not Bcl-2Δα5/6) reduced Vpr52-96 binding to soluble (Fig. 8 A) or membrane-associated (Fig. 8 B) ANT. In an independent series of control experiments, Vpr53-96 was found not to interact with recombinant Bcl-2, as determined by surface plasmon resonance. Hence, inhibition of the Vpr–ANT binding is likely attributable to a direct Bcl-2–ANT interaction (8, 26). Accordingly, Bcl-2 abolished the formation of Vpr52-96–induced channels in ANT-containing lipid bilayers (Fig. 8 C). In contrast, in the same conditions, Bax exacerbated the conductance of Vpr52-96–ANT channels to a mean value of 245 ± 2 pS (compared with 190 ± 2 pS for Vpr52-96–ANT without any further addition; Fig. 8 C). In conclusion, Bcl-2 specifically prevents cooperative channel formation by Vpr–ANT, presumably by disrupting their interaction.

**Table I. RC Values**

| Peptide   | RC   |
|-----------|------|
| Vpr-51    | 5.1  |
| Vpr52-96  | 1.1  |
| Vpr1-96   | 2.0  |
| Vpr71-82  | 2.6  |
| None      | 5.3  |

RC values were calculated by dividing oxygen consumption in the presence of CCCP by that measured with oligomycin (determined as in the legend to Fig. 4), 10 min after the addition of 1 μM Vpr-derived peptides (mean values of three determinations).
**Discussion**

The mechanism by which endogenous end stage effectors achieve permeabilization of the IM and/or OM is a matter of debate. Depending on the apoptotic stimulus, permeabilization may affect the OM and IM in a variable fashion and may or may not be accompanied by matrix swelling (1, 2, 5–7, 46). In vitro experiments performed on purified mitochondria or proteins reconstituted into artificial membranes suggest at least two competing models of MMP. On the one hand, pore formation by ANT has been proposed to account for IM permeabilization, osmotic matrix swelling, and consequent OM rupture, resulting be-
cause the surface area of the IM with its folded cristae exceeds that of the OM. In support of this hypothesis, proapoptotic molecules such as Bax, Atr, Ca\(^{2+}\), and thiol oxidants cause ANT (which normally is a strictly specific ADP/ATP antiporter) to form a nonspecific pore (8, 26, 28, 40). On the other hand, VDAC has been suggested to account for a primary OM permeabilization not affecting the IM (9, 44). In favor of this hypothesis, the permeabilization of VDAC-containing liposomes to sucrose or cytochrome \(c\) is enhanced by Bax and inhibited by Bcl-2 in vitro (9, 44).

Figure 7. Differential effect of Bcl-2 and PA10 on Vpr52-96 binding to mitochondria. (A) Vpr52-96 binds mitochondria before inducing \(\Delta \Psi_m\) loss. Mitochondria were left unstained (inset in control, top left panel) or exposed to the \(\Delta \Psi_m\)-insensitive mitochondrial dye MitoTracker® Green (75 nM), alone (MTG) or with 0.5 \(\mu\)M FITC–Vpr52-96 (green fluorescence) in combination with the \(\Delta \Psi_m\)-sensitive mitochondrial dye MitoTracker® Red (chloromethyl-X-rosamine; red fluorescence) followed by cytofluorometric two-color analysis. Numbers indicate the percentage of mitochondria in each quadrant. (B) PA10, but not Bcl-2, inhibits Vpr52-96 binding to mitochondria. Mitochondria were preincubated for 10 min with the indicated inhibitors, and the percentage of FITC–Vpr52-96–labeled mitochondria was determined as in A. (C) Inhibitory effect of Bcl-2 on affinity purification of ANT by biotinylated Vpr52-96. Mitochondria were lysed either after incubation with biotinylated Vpr52-96 (top) or lysed before (bottom) with Tris-HCl as described in Materials and Methods. Biotinylated Vpr52-96 complexed with its mitochondrial ligands was retained on avidin-agarose and subjected to immunoblot detection of ANT. Co, control.
synthetic, VDAC-free system (Fig. 8). It should be noted that although Bcl-2 is generally considered to be preferentially associated with the mitochondrial OM, some reports also indicate an IM localization (47–49). This suggests that Bcl-2 may adopt different submitochondrial localizations possibly depending on the state of the cell. Although our data do not exclude the possibility that Bcl-2 modulates the permeability of VDAC to relatively large, globular proteins (14.5 kD for cytochrome c, as opposed to the linear, mostly α helical structure of Vpr52-96 resolved by nuclear magnetic resonance; reference 50), they indicate that, at least in this particular model, Bcl-2 exerts its membrane-protective mitochondrial effect via ANT.

The fact that PA10-mediated closure of VDAC completely blocks the binding of Vpr52-96 to intact mitochondria suggests that Vpr52-96 must cross the OM via VDAC to reach its mitochondrial target(s). Bcl-2 inhibits binding of Vpr52-96 to ANT in three experimental systems: plasmon resonance (Fig. 8 A), ANT proteoliposomes (Fig. 8 B), and affinity purification of ANT by biotinylated Vpr added to intact or Triton-solubilized mitochondria (Fig. 8 C). Conversely, Vpr52-96 binding to intact mitochondria was not affected by Bcl-2 (Fig. 7 B). One possible explanation for this result may be that Vpr52-96 has a second mitochondrial target different from ANT. The ANT ligand BA inhibits the ANT–Vpr52-96 binding (Fig. 1 C) and reduces the binding of Vpr to mitochondria to some extent (Fig. 7 B). One explanation to reconcile the differential inhibitory action of Bcl-2 and PA10 is to assume that BA (but not Bcl-2) affects the Vpr target which is different from ANT. Alternatively, BA (but not Bcl-2) might modify the general conformation of the PTPC (including that of VDAC), and thus reduce the (VDAC-mediated) Vpr entry into mitochondria.

Irrespective of this latter possibility, our data are compatible with a scenario in which Vpr affects mitochondrial membrane integrity in four steps (Fig. 9). First, Vpr crosses the OM via VDAC (inhibition by PA10). Second, the COOH-terminal half of Vpr (Vpr52-96) interacts with the ANT104-116 site in ANT (and possibly with other ANT domains). Third, as a consequence of its association with Vpr, ANT is converted into a nonspecific pore, leading to inner MMP. Fourth, inner MMP triggers matrix swelling resulting in OM rupture.

Recent studies have revealed the existence of several viral apoptosis inhibitors acting on mitochondria. For example, adenovirus, Epstein-Barr virus, herpesvirus saimiri, and Kaposi sarcoma–associated human herpesvirus 8 produce apoptosis-suppressive Bcl-2 homologues (51–54). In addition, several viruses encode PTPC-interacting proteins without any obvious homology to the Bcl-2/Bax family. The cytomegalovirus apoptosis inhibitor pUL37X (55) and Vpr, an HIV-1–encoded apoptosis inducer, selectively bind to ANT. The proapoptotic p13 (II) protein derived from the X-II open reading frame of HTLV-1 is also targeted to mitochondria via a peptide motif that bears structural similarities to the mitochondriotoxic domain of Vpr (56). Moreover, the proapoptotic, MMP-inducing hepatitis virus B protein X interacts with VDAC (57). Thus, both VDAC and ANT emerge as major targets of viral apoptosis regulation and, perhaps, as targets for pharmacological intervention on viral pathogenesis.

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