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Bcl-2 and Bax regulate the channel activity of the mitochondrial adenine nucleotide translocator

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Bcl-2 family protein including anti-apoptotic (Bcl-2) or pro-apoptotic (Bax) members can form ion channels when incorporated into synthetic lipid bilayers. This contrasts with the observation that Bcl-2 stabilizes the mitochondrial membrane barrier function and inhibits the permeability transition pore complex (PTPC). Here we provide experimental data which may explain this apparent paradox. Bax and adenine nucleotide translocator (ANT), the most abundant inner mitochondrial membrane protein, can interact in artificial lipid bilayers to yield an efficient composite channel whose electrophysiological properties differ quantitatively and qualitatively from the channels formed by Bax or ANT alone. The formation of this composite channel can be observed in conditions in which Bax protein alone has no detectable channel activity. Cooperative channel formation by Bax and ANT is stimulated by the ANT ligand atractyloside (Atr) but inhibited by ATP, indicating that it depends on the conformation of ANT. In contrast to the combination of Bax and ANT, ANT does not form active channels when incorporated into membranes with Bcl-2. Rather, ANT and Bcl-2 exhibit mutual inhibition of channel formation. Bcl-2 prevents channel formation by Atr-treated ANT and neutralizes the cooperation between Bax and ANT. Our data are compatible with a *ménage à trois* model of mitochondrial apoptosis regulation in which ANT, the likely pore forming protein within the PTPC, interacts with Bax or Bcl-2 which influence its pore forming potential in opposing manners. *Oncogene* (2000) **19,** 329–336.

Keywords: anti-oncogene; mitochondrial transmembrane potential; oncogene; permeability transition; planar lipid bilayer

Introduction

It has recently been recognized that a critical event leading to apoptosis induced in many cell death stimuli involves a change in mitochondrial membrane permeability, culminating in the release of apoptogenic factors such as cytochrome *c*, mitochondrial procaspases, and apoptosis-inducing factor (AIF) from the mitochondrial intermembrane space (Liu *et al*., 1996; Mancini *et al*., 1998; Susin *et al*., 1999b,c; Zamzami *et al*., 1996). Changes in the flux of ions and water across the inner mitochondrial membrane characterize the early phase of apoptosis, during which an increase in matrix volume may precede a collapse of the inner transmembrane potential ($\Delta\Psi_m$) (Green and Reed, 1998; Kroemer *et al*., 1998; vander Heiden *et al*., 1997). These changes are suppressed by Bcl-2/Bcl-X_L, facilitated by Bax, and mediated at least in part by the so-called permeability transition pore complex (PTPC). The PTPC is a two-membrane spanning polyprotein complex containing one of the most abundant inner transmembrane proteins, the adenine nucleotide translocator (ANT, also called ATP/ADP carrier), the most abundant outer membrane protein, voltage-dependent anion channel (VDAC, also called porin), cyclophilin D (a soluble matrix protein), and members of the Bcl-2 family including Bax, Bcl-2 and Bcl-X_L (Crompton *et al*., 1998; Marzo *et al*., 1998a,b; Narita *et al*., 1998; Woodfield *et al*., 1998). Thus, Bcl-2-like oncoproteins and Bax-like tumour suppressors can interact with sessile mitochondrial proteins involved in the trafficking of intermediate metabolites, in particular in the exchange of ATP and ADP between the matrix and the cytosol (Marzo *et al*., 1998a,b; Narita *et al*., 1998).

Bax and Bcl-2 influence the molecular device determining apoptosis resistance or susceptibility (the 'apostat'), at least in part by direct effects on the PTPC (Kroemer, 1997; Marzo *et al*., 1998a,b; Narita *et al*., 1998; Shimizu *et al*., 1998; Zamzami *et al*., 1996). In isolated mitochondria as well as in purified PTPC reconstituted in liposomes, Bcl-2 can completely prevent the membrane permeabilizing effect of atractyloside (Atr) (Marzo *et al*., 1998b; Shimizu *et al*., 1998; Zamzami *et al*., 1996), a specific ANT ligand whose conformational effects may mimic those mediated by perturbed ADP/ATP gradients (vander Heiden *et al*., 1997). In contrast, Bax is required for Atr to permeabilize the membranes of purified mitochondria, and recombinant Bax protein can collaborate with purified ANT to create an Atr-responsive channel in artificial membranes (Marzo *et*

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al., 1998a). Although proteins from the Bcl-2/Bax family have been originally thought to be confined to the outer membrane, recent data indicate that they are enriched in the inner/outer membrane contact site (where the PTPC is expected to form) (Krajewski *et al*., 1994) and that they can insert into the inner mitochondrial membrane, in a reaction which depends on the conformation of the ANT/cyclophilin D complex (Marzo *et al*., 1998a).

Bcl-2, Bcl-X_L, and Bax have been reported to form ion channels when incorporated into artificial membranes (Antonsson *et al*., 1997; Minn *et al*., 1997; Schendel *et al*., 1997; Schlesinger *et al*., 1997). The channels formed by Bcl-X_L and Bcl-2 have several levels of conductance (80, 130, 180, 280 pS for Bcl-X_L; 20, 40, 90 pS and up to 1.9 nS for Bcl-2) and exhibit mild K^+ selectivity (Minn *et al*., 1997; Schendel *et al*., 1997; Schlesinger *et al*., 1997). In contrast, Bax demonstrates mild Cl^- specificity. Its conductance may raise from values of 26, 80, 180, and 250 pS to maximum levels of 1.5-2 nS (Antonsson *et al*., 1997; Schlesinger *et al*., 1997). Purified ANT, which normally is a strict ADP/ATP antiporter, forms channels in response to Ca^{2+} in the range of 300–600 pS (Brustovetsky and Klingenberg, 1996).

How ANT, Bcl-2 and Bax differentially regulate mitochondrial membrane permeabilization in intact organelles (ANT and Bax as facilitators, Bcl-2 as an inhibitor) is unclear, given that they all can act on artificial membranes to increase their permeability. Intrigued by this paradox, we decided to evaluate possible cooperative effects between ANT, Bax and Bcl-2 in artificial membranes, comparing the electrophysiological properties of these proteins in pairwise combinations (ANT-Bax or ANT-Bcl-2) with each of the proteins alone. Our data indicate that Bax increases the capacity of ANT to form active channels with Atr, whereas Bcl-2 reduces channel formation by ANT. These observations may explain the mode of action of Bcl-2 which, in its physiological context, is likely to act as a channel inhibitor rather than as an autonomous ion channel.

Results and discussion

Functional interaction between ANT and mitochondrion-targeted Bcl-2 in intact cells

Bcl-2 prevents the Atr-induced mitochondrial swelling, $\Delta\Psi_m$ dissipation, and release of soluble intermembrane proteins in isolated mitochondria (Susin *et al*., 1996, 1999b; Zamzami *et al*., 1996). We have validated this result in intact cells, in which the mitochondrion-targeted expression of Bcl-2 (but not the expression of Bcl-2 in the endoplasmic reticulum) prevents the $\Delta\Psi_m$ collapse and nuclear apoptosis induced by microinjection of Atr (Figure 1a,b). As a control, Bcl-2, however, fails to inhibit manifestations of apoptosis induced by microinjected apoptogenic proteins such as cytochrome *c* (Figure 1b). Collectively, these data confirm and extend our earlier observations suggesting a close functional interaction between the target of Atr, ANT, and proteins from the Bcl-2/Bax family within the PTPC (Marzo *et al*., 1998a,b).

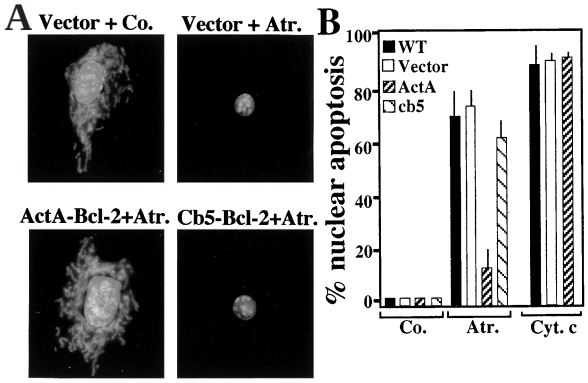

Figure 1 Differential effect of mitochondrial or extramitochondrial Bcl-2 overexpression on apoptosis induced by the ANT ligand Atr. Rat-1 cell transfected with a control vector or cells expressing Bcl-2 either in mitochondria (actA-Bcl-2 fusion protein) or in the endoplasmic reticulum plus nucleus (cb5-Bcl-2 fusion protein) were microinjected with buffer only (control) or Atr. Two hours after microinjection of Atr, cells were stained with the $\Delta\Psi_m$-sensitive dye CMXRos and the DNA-intercalating dye Hoechst 33342. Representative cells are shown on the left and the frequency of cells having undergone nuclear apoptosis after injection of buffer only, Atr, or cytochrome *c* is shown on the right. At least 200 cells were injected for each data point (mean ± s.d., $n=3$)

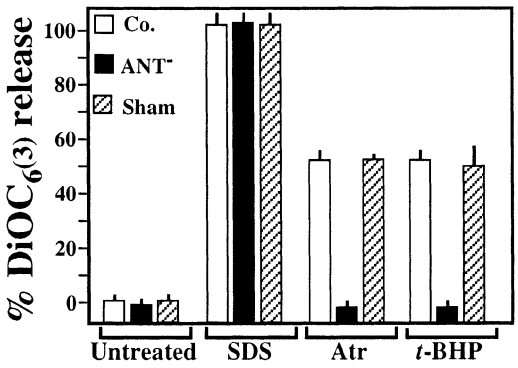

Figure 2 Identification of ANT as a functionally important PTPC constituent. PTPC were either ANT depleted or sham-immunodepleted and reconstituted into liposomes, treated with Atr or *tert*-butylhydroperoxide, and determination of $DiOC_6$ (3) retention within liposomes in the flow cytometer, as described in the Materials and methods section ($n=2$). Incubation of liposomes with SDS marks the 100% value of liposomal membrane permeabilization

ANT is the major Atr target within the PTPC

The semi-purified PTPC can be reconstituted into liposomes and responds to a variety of inducers including Atr or the pro-oxidant *t*-BHP by increasing membrane permeability to small (<1500 Da) solutes such as $DiOC_6$(3). Upon immunodepletion of ANT, the capacity of PTPC to confer Atr- or *t*-BHP-induced membrane permeabilization is abolished, suggesting that ANT is the likely Atr target within the PTPC (Figure 2). Purified ANT, which lacks any immunodetectable contamination by ANT-interacting proteins such as VDAC, cyclophilin D, Bax, or Bcl-2 (Figure 3a), can be reconstituted into planar lipid bilayers. In

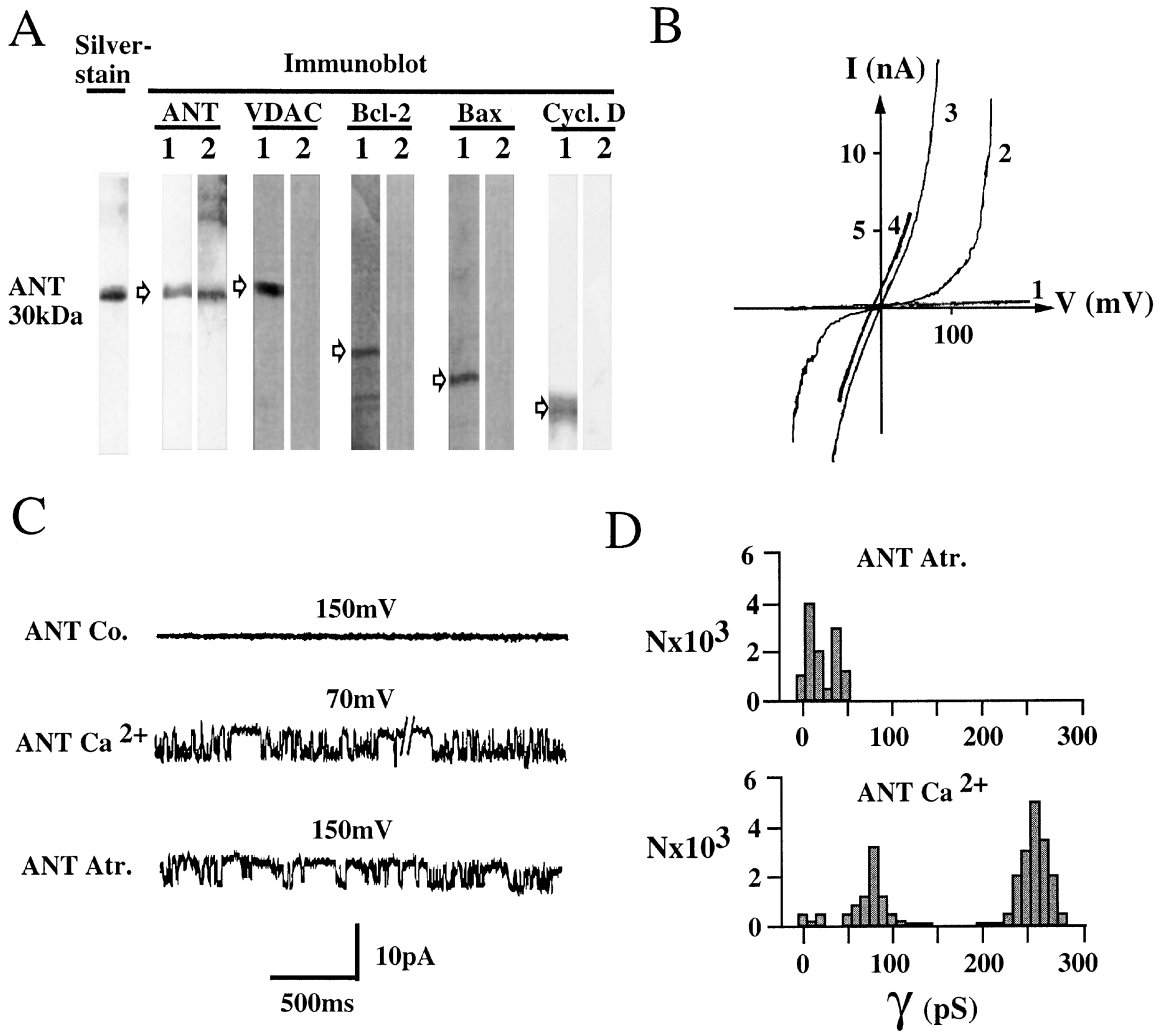

Figure 3 Electrophysiological properties of purified ANT. (**a**) Purification of ANT. Three μg of purified ANT was separated by SDS–PAGE and silver-stained. Immunoblots of 50 μg of proteins from rat heart mitochondria (lane 1) or 3 μg purified ANT were performed for the detection of ANT, Bax, VDAC, and cyclophilin D, as described in Materials and methods (lane 2). As positive control for Bcl-2 immunodetection, mitochondria from the HT 29 cell line were used (lane 1). Arrows indicate the presence of specific bands in positive controls. (**b**) Macroscopic conductance of ANT. ANT was reconstituted into synthetic lipid bilayers, and submitted to slow voltage ramps (1 min/cycle) to determine the current at different voltages in symmetric conditions (100 mM KCl on both sides of the membrane), either in the absence (line 1) or in the presence of 40 μM Atr (line 2) or 1 mM $CaCl_2$ (line 3). The macroscopic conductance of Atr-treated ANT was also recorded in asymmetric conditions (100/400 mM $CaCl_2$ KCl + 1 mM $CaCl_2$, line 4). Results are representative of three independent determinations. (**c**) Single-channel recordings of ANT channels induced by Ca^{2+} or Atr. Recordings were obtained using the dip-tip method (same concentrations of ANT, Atr or $CaCl_2$ as in (**b**) and were representative of the most frequently observed channels. c: closed state; o: open state. (**d**) Statistical analysis of conductances of Atr. and Ca^{2+} induced ANT channels. The conductance levels (γ) observed for single channel openings (obtained as in **c**) were plotted against the frequency (N) of events

accord with previous observations (Brustovetsky and Klingenberg, 1996), ANT has little if any capacity to increase the current on planar lipid bilayers in a voltage-dependent fashion (line 1 in Figure 3b). However, after addition of Atr or Ca^{2+}, ANT mediates significant conductance (lines 2 and 3 in Figure 3b) in macroscopic determinations. Of note, the channel formed by ANT is slightly cation specific under an applied 400/100 mM KCl (cis/trans) gradient, yielding a Vrev of ~ -15 mV (line 4 in Figure 3b) and a P_K:P_{Cl} ratio of 2.8. Single channel recordings obtained at optimal voltages confirm that ANT by itself does not form any channels (Figure 3c), yet forms channels in response to Ca^{2+} with two levels of conductance (70 and 250 pS in Figure 3c,d). In response to Atr, ANT forms only one channel species with a relatively low conductance (30 pS in Figure 3c,d), as compared to Ca^{2+}. In conclusion, ANT is required for the Atr response of PTPC and can, by itself, form an Atr responsive channel.

ANT and Bax cooperate to form an Atr-responsive channel

Single channel recordings reveal that a mixture of Bax and ANT (molar ratio 1 : 4) has a higher probability of pore opening and possesses at 150 mV conductance levels (30 and 80 pS) different from what might be expected from the simple addition of Bax-mediated channels ($\sim$200 pS) to Atr-treated ANT (30 pS, see above, Figure 3d) (Figure 4a,b). Moreover, when employed at relatively low doses (1 nM), Bax does not yield any major macroscopic conductance (line 1 in

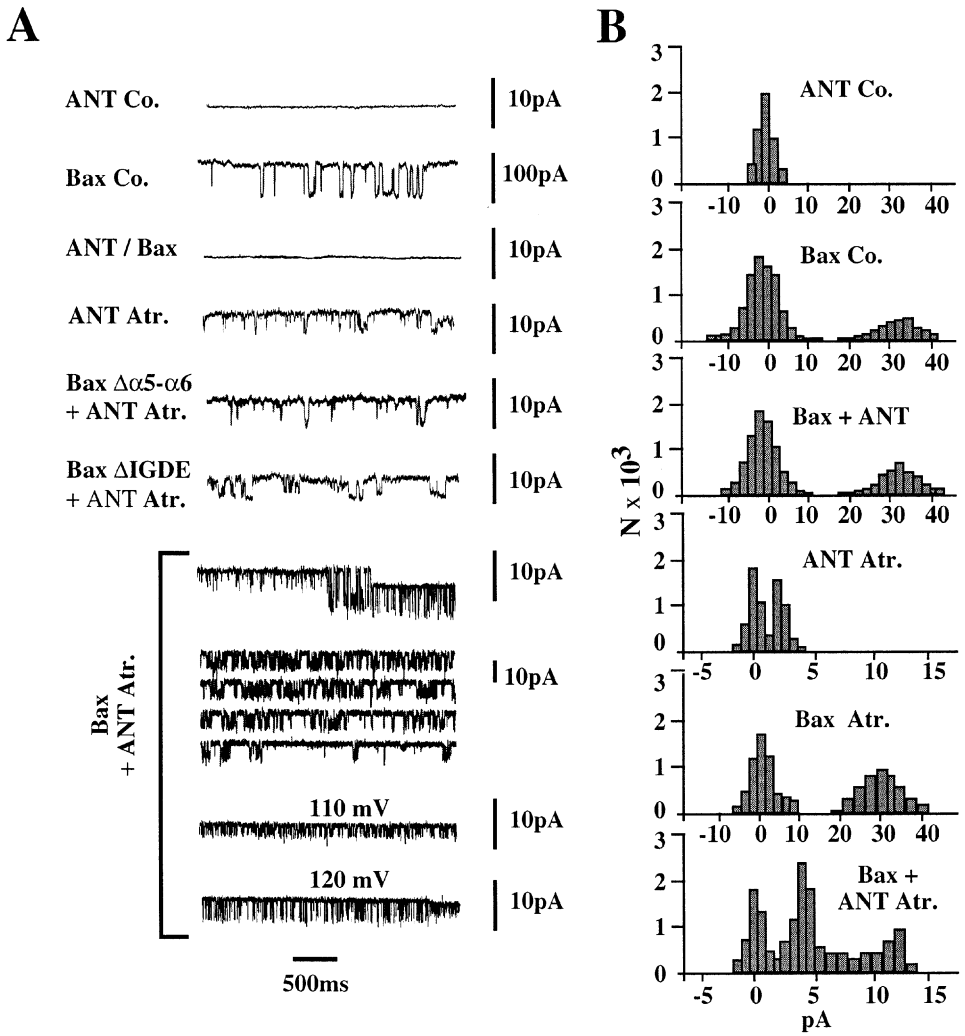

Figure 4 Cooperation between ANT and Bax in tip-dip experiments. (**a**) Cooperative effect at the single channel level. ANT (treated with 40 μM Atr or not) and/or recombinant Bax protein (molar ratio ANT : Bax = 4 : 1) or Bax mutant proteins (Bax $\Delta\alpha$5/6 or Bax ΔIGDE, used at the same ratio as Bax wild-type protein) were incorporated into synthetic membranes and single channel recordings were performed using the dip-tip technique. The applied voltage was 150 mV unless specified differently and the recordings shown are representative for at least three independent determinations. Note that the scale of the ordinate is indicated for each recording. (**b**) Statistical analysis of conductances obtained in (**a**). Results were expressed as current distributions at different voltages. Conductances may be calculated by division of current by voltage

Figure 5a), unless combined with ANT treated with Atr (line 4 in Figure 5a). Thus, the combination of ANT + Atr + Bax (line 4) forms a much more efficient membrane permeabilizing channel than do the combinations ANT + Atr (line 2), ANT + Bax (line 3 in Figure 5a), or Atr + Bax (not shown). The ANT/Bax cooperation is not observed when wild-type Bax is replaced by two mutant proteins which have lost their apoptogenic potential (Matsuyama *et al*., 1998; Ying *et al*., 1994): Bax ΔIGDE, which lacks a homodimerization domain in BH3, and Bax $\Delta\alpha$5/6, which lacks a putative pore forming domain (Figure 5b). Preincubation of ANT with its physiological ligand ATP completely inhibits the channel activity mediated by ANT + Atr + Bax (Figure 5b). The channel formed by ANT + Bax is K^+ specific (line 5 in Figure 5a). Thus the combined ANT/Bax channel resembles that formed by ANT (which is also cation-specific, line 4 in Figure 3b), yet differs from that formed by Bax alone (which is anion-specific) (Schendel *et al*., 1997).

In summary, Bax and ANT can cooperate by forming a new class of channels which acquire distinct electrophysiological properties as well as a far higher probability of opening than either of the two compounds on its own. This cooperative effect depends on the conformation of the ANT, as manifested by the effects of actractyloside and ATP.

Bcl-2 inhibits the formation of an Atr-responsive, ANT-dependent channel

As previously reported (Minn *et al*., 1997; Schendel *et al*., 1997; Schlesinger *et al*., 1997), Bcl-2 can form a channel when incorporated into artificial membranes (Figure 6a). This effect was obtained at neutral pH, provided that Bcl-2 was mixed with dried lipids, prior to generation of the lipid bilayer (Figure 6a,b); it was not observed for a Bcl-2 mutant protein lacking the putative α5/6 membrane insertion domain (Schendel *et al*., 1997) and was modified by a single amino acid exchange in the BH1

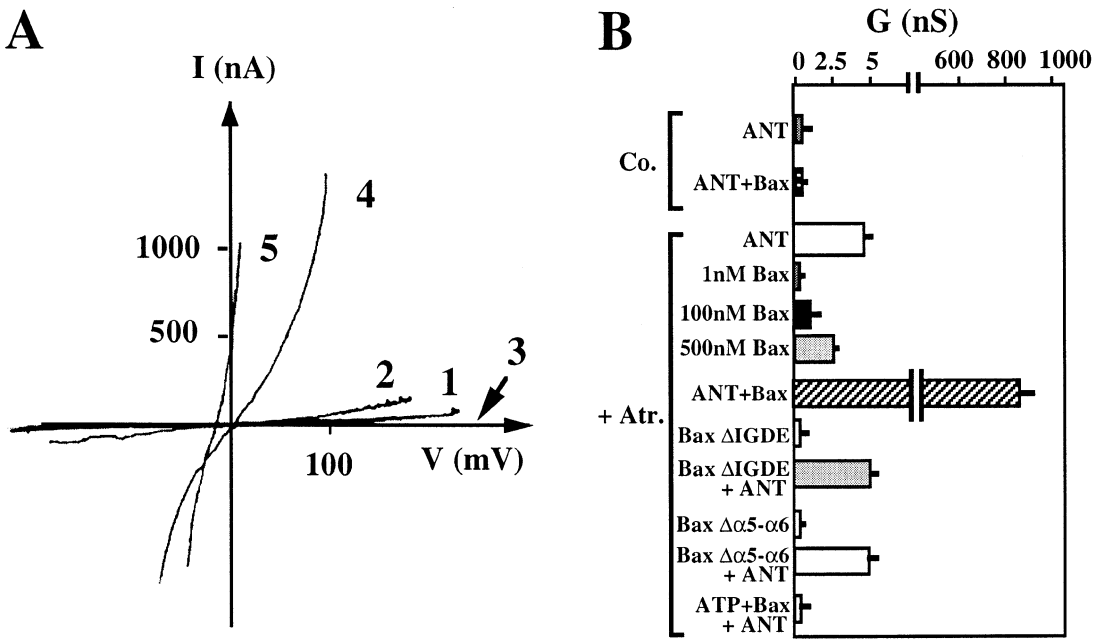

Figure 5 Macroscopic determination of the ANT-Bax cooperation. (**a**) Effects of Bax addition on macroscopic conductance of ANT. Current/voltage (I–V) curves were determined for preparations containing Bax only (1), Atr-treated ANT (2), ANT + Bax (3) or Atr-treated ANT + Bax (4, 5), in symmetric (1–4) or asymmetric (5) conditions. (**b**) Conductances determined from I–V curves in symmetric conditions. The conductance (corresponding to the slope of the I–V curve obtained in **c**) was calculated for different preparations of ANT (4 nM), in the presence of Bax (1 nM unless specified), mutated Bax proteins (1 nM BaxΔIGDE or BaxΔα5/6), ATP (1 mM) and/or Atr (40 μM), as indicated. These experiments have been repeated two to five times, yielding similar results

domain (G145A), which yields a channel with a reduced conductance (Figure 6b). Intriguingly, we found that, in combination with ANT, Bcl-2 does not form channels in artificial membranes (nor does Bcl-2 G145A), suggesting that the physical interaction between ANT and Bcl-2 (Marzo *et al*., 1998a) can change the ion channel properties of Bcl-2. This effect is optimal in conditions of molar equivalence of ANT and Bcl-2. It becomes particularly evident when macroscopic measurements are performed (Figure 6c). The combination of ANT plus Bcl-2 results in a virtual cessation of ion movement indicative of the closure of both ANT and Bcl-2 channels. Though not shown, the channel activity of Bcl-2 was not influenced by Atr. If Bcl-2 is replaced by a mutant protein (Bcl-2 Δα5/6) lacking a putative pore formation domain (Matsuyama *et al*., 1998; Schendel *et al*., 1997), the inhibition of ANT-mediated conductance is suboptimal, both at the macroscopic (Figure 6c) and single channel levels (Figure 6a). In conclusion, Bcl-2 and ANT exhibit mutual inhibition of ion channel formation *in vitro*.

As expected (Antonsson *et al*., 1997; Oltvai and Korsmeyer, 1994), an excess of Bcl-2 prevented the formation of channels induced by the combination of Bax and ANT (Figure 6d). Thus, in addition to its Bax-independent effect on the ANT (Figure 6a,b), Bcl-2 can neutralize Bax (Antonsson *et al*., 1997; Oltvai and Korsmeyer, 1994), including the cooperative effect of Bax on ANT.

Concluding remarks

The electrophysiological data contained in this work reveal a close functional interaction between ANT and Bax, as well as between ANT and Bcl-2. Although each of these molecules can form ion channels, their combination has new, unexpected properties. Bax potentiates the capacity of ANT to form an Atr-responsive channel, whereas the combination of Bcl-2 and ANT is inactive. These observations may resolve the long-standing contradiction between Bcl-2 effects on synthetic membranes and on mitochondria. In artificial lipid bilayers, Bcl-2/Bcl-X_L forms a mostly cation-specific ion channel (Minn *et al*., 1997; Schendel *et al*., 1997; Schlesinger *et al*., 1997). However, in isolated mitochondria or intact cells, Bcl-2/Bcl-X_L prevents the influx of water into the mitochondrial matrix and the dissipation of the $\Delta\Psi_m$ (Kroemer, 1997; Marzo *et al*., 1998a,b; Narita *et al*., 1998; Shimizu *et al*., 1998; vander Heiden *et al*., 1997; Zamzami *et al*., 1996). As shown here, it becomes plausible that, within its physiological context (that is in the presence of other proteins including ANT), Bcl-2 acquires electrophysiological properties that have not been revealed in a more artificial setting. As a word of caution, it has to be stated, however, that the electrophysiological experiments in which ANT was reconstituted into the same synthetic lipid bilayer as Bax and Bcl-2 might inaccurately reflect the *in vivo* physiology of mitochondria, in which ANT is confined to the inner membrane whereas Bax and Bcl-2 are enriched in the outer membrane, within the contact site between inner and outer membranes (Krajewski *et al*., 1993). At present, it remains unclear to which extent the interactions *in situ* involve close interactions between ANT and Bcl-2-like proteins (as they are suggested by co-immunoprecipitation studies

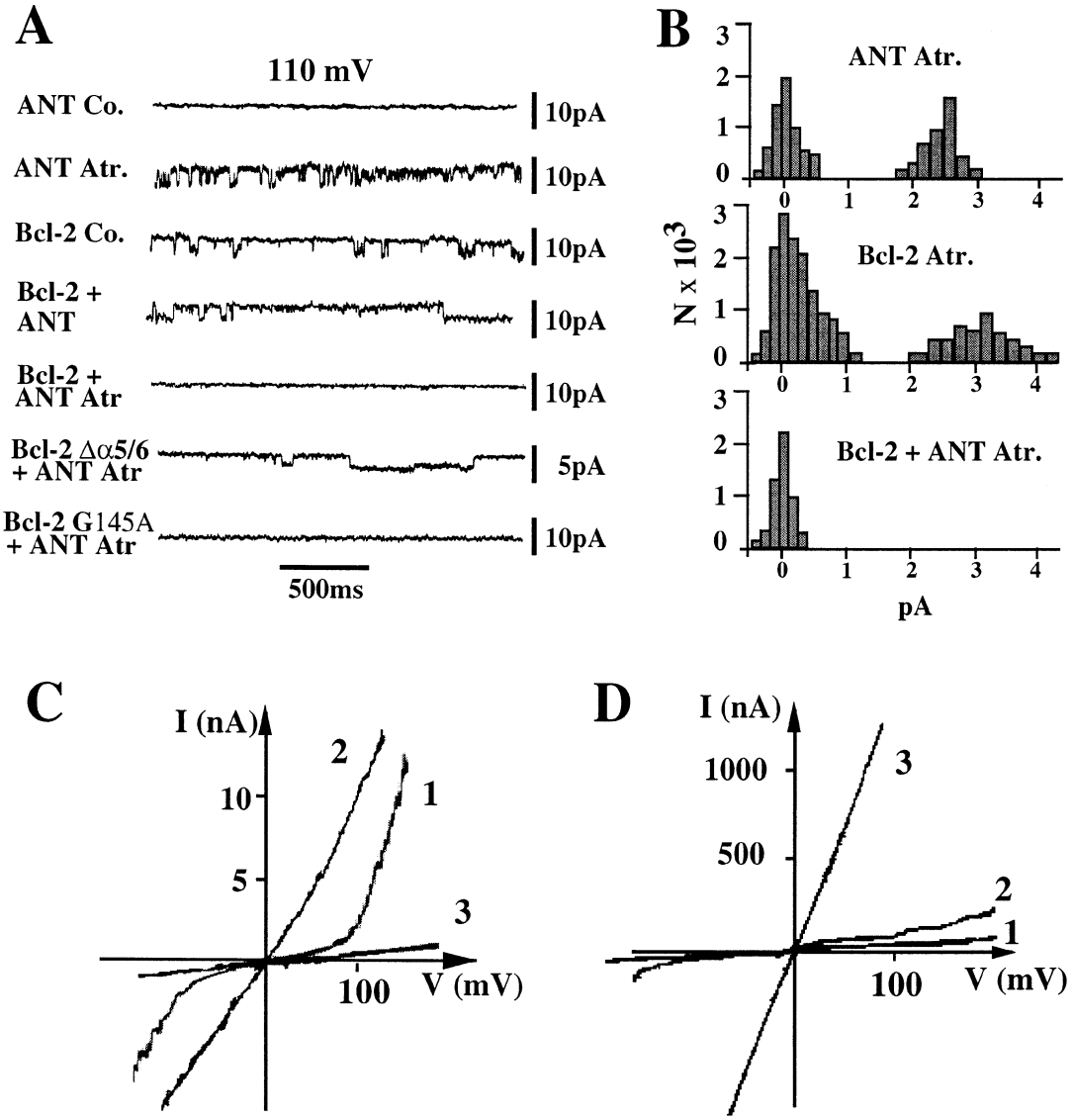

Figure 6 Electrophysiology of the ANT/Bcl-2 interaction. (**a**) Bcl-2 inhibits opening of the Atr/ANT channel at the single channel level. Recordings of single-channels of different combinations of ANT, Bcl-2 (molar ratio ANT : Bcl-2 = 1 : 1), Bcl-2 Δα5-α6, Bcl2 G145A and/or Atr obtained at a voltage of 110 mV. (**b**) Statistical analysis of channel opening and conductances obtained for Bcl-2 and Bcl-2 G145A. (**c**) Effects of Bcl-2 addition on macroscopic conductance of ANT. I–V curves were obtained in different conditions, namely in the presence of (Atr-treated) ANT (line 1), Bcl-2 alone (line 2) or an equimolar combination of both (line 3). All recordings were done in equimolar conditions (1 : 1; 20 nM). Note that the scale of the ordinate is different from Figure 2c. (**d**) Effect of Bcl-2 on the Bax/ANT cooperative effect. I–V curves were obtained with (Atr-treated) ANT (line 1), or ANT + BAX + Bcl-2 (10 : 1 : 10 molar ratio; line 2), or ANT + Bax (10 : 1; line 3), the amount of ANT being constant in each preparation. Experiments are representative of two to four independent data sets

(Marzo *et al*., 1998a)) and/or a translocation of Bcl-2/Bax from the outer to the inner membrane.

Although the exact stoichiometry of the ANT/Bax or ANT/Bcl-2 reaction (e.g. heterodimers, heterotetramers or higher-order structures) remains elusive, the present data, as well as the earlier observations of direct physical Bcl-2/Bax (Oltvai *et al*., 1993), ANT/Bax and ANT/Bcl-2 interactions (Marzo *et al*., 1998a) may be integrated into a *ménage à trois* model of promiscuous interactions between ANT, Bax, and Bcl-2 within the PTPC. Hypothetically, one of the functions of Bcl-2 would be to withhold Bax from its functionally relevant site of action, which is the inner mitochondrial membrane, to which some Bax molecules may translocate in a colicin-like fashion (Marzo *et al*., 1998a). An additional, Bax-independent function of Bcl-2 would be to interact with ANT, the core protein of the PTPC, and to prevent it from forming a pore. This Bax-independent function of Bcl-2 might be restricted to those ANT molecules interacting with the outer mitochondrial membrane within the contact site. In contrast, the major function of Bax, apart from neutralizing Bcl-2, would be to interact with ANT to facilitate pore formation in an ANT conformation-dependent fashion, thus allowing for ion and subsequent water influx into the matrix and final $\Delta\Psi_m$ dissipation. This scenario of 'apostat' regulation would be compatible with the reported autonomous, Bax-independent function of Bcl-2 and vice versa (Knudson and Korsmeyer, 1997). If correct, the *ménage à trois* model of ANT/Bcl-2/Bax interactions could have far-

reaching consequences for the development of new classes of apoptosis-inhibitory and apoptosis-inducing agents which selectively inhibit ANT/Bax and ANT/Bcl-2 interactions, respectively.

Materials and methods

Cell culture and microinjection

Rat-1 fibroblast cells transfected with a control vector only, Bcl-2 wild-type or Bcl-2 fusion proteins selectively directed to the mitochondrion (actA-Bcl-2) or the endoplasmic reticulum and nuclear envelope (cb5-Bcl-2) (Zhu *et al*., 1996), were microinjected (pressure 150 hPa; 0.2 s; (Marzo *et al*., 1998a)) with buffer only, atractyloside (50 μM) or horse cytochrome *c* (10 μM, Sigma). After microinjection, cells were cultured for 120 min and stained for 10 min with the $\Delta\Psi_m$-sensitive dye CMXRos and the DNA-intercalating dye Hoechst 33342, as described (Marzo *et al*., 1998a). Microinjected viable cells (100–200 per session, two to three independent sessions of injection) were identified by inclusion of 0.25% (w:v) FITC-dextran (green fluorescence) in the injectate. Only the blue or red fluorescence was recorded.

Functional assays on purified and reconstituted PTPC in liposomes

PTPC from Wistar rat brains were purified and reconstituted in liposomes following published protocols (Marzo *et al*., 1998b). Briefly, membrane-associated, Triton X-100-soluble proteins co-purifying with hexokinase 1 were enriched by anion exchange chromatography. Immunodepletion of ANT was performed at this stage using a polyclonal rabbit antibody (gift from Dr Heide H Schmid; (Giron-Calle and Schmid, 1996)). One volume of PTPC-containing preparation was incubated with one volume of polyclonal anti-ANT or an equivalent concentration of pre-immune rabbit antiserum for 2 h at 4°C. 0.1 volume of protein A and protein G agarose beads was added and the mix was incubated for 30 min at room temperature. The beads were collected by centrifugation (10 min, 2000 *g*), and the supernatant was used for further experiments. The PTPC (approx. 1 μg protein/mg lipid) were reconstituted into phosphatidylcholine/cholesterol (5 : 1 = w:w) vesicles by overnight dialysis. Liposomes recovered from dialysis were ultrasonicated (120 W, Ultrasonic Processor, Bioblock) for 7 s in 5 mM malate and 10 mM KCl, charged on a Sephadex G50 column and eluted with 125 mM sucrose + 10 mM HEPES (pH 7.4). Proteoliposomes containing maximum hexokinase activity were recovered, as described (Marzo *et al*., 1998b) and were incubated at room temperature for 30 min with different PT pore-opening agents in 125 mM sucrose + 10 mM HEPES (pH 7.4). For determination of 3,3′dihexylocarbocyanine iodide ($DiOC_6(3)$) retention, liposomes were equilibrated for 30 min with 80 nM ($DiOC_6(3)$) and then analysed in a cytofluorometer (Marzo *et al*., 1998b).

Purification of ANT and recombinant Bax/Bcl-2 proteins

ANT was purified (Rück *et al*., 1998) from rat heart mitochondria isolated as described (Susin *et al*., 1999a). Mitochondria were subjected to mechanical shearing in a Emvejhem potter; solubilized (8 min, 4°C) in buffer A (220 mM mannitol, 70 mM sucrose, 10 mM HEPES (pH 7.4), 200 μM EDTA, 1 mM β-mercaptoethanol) supplemented with 0.5 mg/ml subtilisin; and subjected to differential centrifugation (5 min 500 *g*, discard pellet; then 10 min 10 000 *g*, discard supernatant). Soluble (30 min, 24 000 *g*, 4°C) mitochondrial proteins (10 mg/ml) were resuspended in buffer B (40 mM KH_2PO_4 (pH 6.0), 40 mM KCl, 2 mM EDTA, and 6% [vol : vol] Triton X100; Boehringer Mannheim, Mannheim, Germany). 2 ml of this solution were applied to a column containing 1 g hydroxyapatite (BioGel HTP, Biorad) and eluted with buffer B, then diluted 1 : 1 with buffer C (20 mM MES, 200 μM EDTA, pH 6.0; 0.5% Triton X-100) supplemented with 1 M NaCl, applied to a HiTrap SP column (Pharmacia Biotech, Uppsala, Sweden) pre-equilibrated with buffer C, washed with buffer C, and eluted at a flow of 1 ml/min by gradually increasing the salt concentration (0 to 500 mM NaCl within 10 min). ANT containing fractions were checked for purity using silver staining and immunoblot (12.5% SDS–PAGE, 3 μg protein/lane) using a monoclonal antibody recognizing VDAC (31HL, Calbiochem), a polyclonal rabbit antisera specific for ANT (see above), an antiserum raised against the N-terminus of cyclophilin D (gift from Paolo Bernardi, University of Padova, Padova, Italy; (Nicolli *et al*., 1996)), Bcl-2 (specific for residues 20-34; Calbiochem, La Jolla, CA, USA), or Bax (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Positive controls were total mitochondrial proteins (50 μg/lane) from the rat heart. Recombinant human Bcl-2 (1-218), mutant Bcl-2(Gly145Ala) and Bcl-2$\Delta\alpha$5/6 (Bcl-2 (Δ143-184), Bcl-2$\Delta\alpha$5/6 (Bcl-2 Δ143-184), murine Bax (1-171) or mutant Bax ΔIGDE (Δ66-69) and a Bax $\Delta\alpha$5/6 (Δ106-153) lacking the hydrophobic transmembrane domain (Δ219-239 in the case of Bcl-2; Δ172-192 for Bax) were purified as described (Jürgensmeier *et al*., 1998; Schendel *et al*., 1998; Xie *et al*., 1998). Circular dichroism spectra revealed correct folding of all recombinant proteins.

Electrophysiological methods

The pore-forming activities of ANT, Bax, Bax ΔIGDE, Bax $\Delta\alpha$5/6, Bcl-2 and/or Bcl-2 $\Delta\alpha$5/6 were investigated at both the macroscopic (black membrane) and single-channel (patch clamp) levels, following published protocols (Brullemans *et al*., 1994). The lipid mixture palmitoyloleoylphosphocholine/dioleoylphosphocholine (7 : 3; w:w) supplemented with 3% cardiolipin (Avanti Polar Lipids Inc., Alabaster, AL, USA) was dissolved in 1 or 0.1%. For the macroscopic conductance configuration, solvent free bilayers were formed by apposition of two lipid monolayers onto a 175–200 μm diameter hole in a 25-μm thick PTFE film sandwiched between two glass half cells pretreated with hexane/hexadecane (40 : 1 v/v) (Montal and Mueller, 1972). Unless specified, recordings were performed in symmetric conditions (100 mM KCl, 2 mM $MgCl_2$, 10 mM HEPES, pH 7.4). To determine anion/cation specificity, asymmetric conditions (400/100 mM KCl, cis/trans) were imposed on the bilayer and the reversal potential (V_{rev}) was calculated by means of the Goldmann-Hodgkin-Katz equation (Hille, 1984). For recording single-channel fluctuations, lipid bilayers were preformed at the tip of patch-clamp glass micropipettes by the 'tip-dip' method (Hanke *et al*., 1984). Bilayer formation was monitored by the capacitance response and, prior to protein addition, bare membranes were checked under applied potentials for electrical silence. Before addition to the bilayer, proteins were incubated with 1 mM $CaCl_2$, 40 μM Atr or control electrolyte (10 mM HEPES, 100 mM KCl, 2 mM $MgCl_2$, pH 7.4) for 30 min at room temperature, then mixed with evaporated lipid mixture (60 μg protein per mg lipid) and treated with Biobeads SM-2 (BioRad) to eliminate Triton X-100. The lipid protein mixture was added to both sides of the bilayer in the macroscopic configuration and to the bath in the tip-dip configuration (2.5 μg protein/ml). In some experiments, Bax was added directly to the aqueous subphase after bilayer formation. Macroscopic current-voltage curves were repeated at least three times, and the single channel recordings (digitally stored and subsequently analysed using Satori 3.01 software from Intracel Ltd, Royston, UK) are representative of the most frequently observed events.

Abbreviations
ANT, adenine nucleotide translocator; Atr, atractyloside; $\Delta\Psi_m$, mitochondrial transmembrane potential; PT, permeability transition; PTPC, permeability transition pore complex.

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