Bcl-2 and Bax Modulate Adenine Nucleotide Translocase Activity

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ABSTRACT

Bcl-2 is a prosurvival factor that reportedly prevents the nonspecific permeabilization of mitochondrial membranes, yet enhances specific ADP/ATP exchange by these organelles. Here, we show that Bcl-2 enhances the ADP/ATP exchange in proteoliposomes containing the purified adenine nucleotide translocase (ANT) in isolated mitochondria and mitoplasts, as well as in intact cells in which mitochondrial matrix ATP was monitored continuously using a specific luciferase-based assay system. Conversely, Bax, which displaces Bcl-2 from ANT in apoptotic cells, inhibits ADP/ATP exchange through a direct action on ANT. The Bax-mediated inhibition of ADP/ATP exchange can be separated from Bax-stimulated formation of nonspecific pores by ANT. Chemotherapy-induced apoptosis caused an inhibition of ANT activity, which preceded the loss of the mitochondrial transmembrane potential and could be prevented by overexpression of Bcl-2. These data are compatible with a model of mitochondrial apoptosis regulation in which ANT interacts with either Bax or Bcl-2, which both influence ANT function in opposing manners. Bcl-2 would maintain the translocase activity at high levels, whereas Bax would inhibit the translocase function of ANT.

INTRODUCTION

Bcl-2-like proteins are assumed to exert most of their apoptotic-regulatory function at the level of mitochondrial membranes (1–5). Bcl-2-like antiapoptotic proteins are constitutively present in mitochondria and would favor the maintenance of normal mitochondrial function, namely by preventing nonspecific permeabilization of mitochondrial membranes while simultaneously maintaining the exchange of small molecules (e.g., ADP, ATP, NADH) on mitochondrial membranes, which is essential for oxidative phosphorylation. Conversely, Bax-like proapoptotic proteins, which translocate to mitochondria only in conditions of apoptosis induction, would favor MMP4 and irreversible loss of mitochondrial function. Although Bcl-2 and Bax can form heterodimers and neutralize each other through direct physical interactions, it appears that both proteins can modulate mitochondrial function and apoptosis independently from each other (1–5). The exact molecular mechanisms through which these effects are achieved are a matter of intense debate exacerbated by contradictory findings. For instance, one group reported that Bcl-2 (or its close homologue Bcl-XL) would prevent the VDAC to form nonspecific, large channels leading to the MMP-associated release of proteins from mitochondria (6–8). In strict contrast, another group reported that Bcl-2 would prevent VDAC to close and that this effect would maintain specific nutrient exchange on mitochondrial membranes (9, 10).

Intrigued by these contradictions, we decided to reevaluate the effect of Bcl-2 and Bax on another mitochondrial protein, namely the ANT, which is known to interact with VDAC within the so-called PTPC (5, 11, 12). ANT is a bifunctional protein that, in physiological conditions, exchanges ATP and ADP on the inner mitochondrial membrane and, in apoptotic conditions, can form a nonspecific pore. According to our published observations (13–15), pore formation by ANT would require a physical interaction with Bax as well as specific interactions with proapoptotic ANT ligands such as Atr and the protein Vpr from human immunodeficiency virus-1. In contrast, Bcl-2 would suppress pore formation by ANT (14). Here, we report that Bcl-2 and Bax do not only modulate pore formation by ANT but, importantly, also influence the enzymatic function of ANT as an ADP/ATP antiporter. These results have been obtained in a variety of different systems (proteoliposomes containing purified ANT, Bcl-2, and/or Bax; isolated mitochondria and mitoplasts; intact cells) and shed new lights on the apoptosis-regulatory functions of Bcl-2/Bax-like proteins.

MATERIALS AND METHODS

Immunogold and Coinmunoprecipitation Assays. HeLa-Bcl-2 cells (16) were cryofixed and subjected to immunoelectron microscopy for the detection of ANT (16) and Bcl-2 (anti-Bcl-2 2C 21 mAb; Santa Cruz Biotechnology, Santa Cruz, CA). HT29 cells (5 × 10⁴ cells/75 cm² flask) were treated with 100 μM etoposide for 20 h to induce apoptosis, and mitochondria were isolated (17) and resuspended in 10 mM Tris·HCl, 0.15 mM MgCl₂, 10 mM KCl (pH 7.6) containing 0.5% Triton X-100 and 0.4% phenylmethylsulfonyl fluoride at a concentration of 0.4 mg protein/ml. The immunoprecipitation was performed by adding 20 μl of a rabbit polyclonal anti-rat heart ANT serum (18) to 100 μl of mitochondrial suspension (90 min, 37°C). Then, 40 μl of protein A/protein G agarose beads (Santa Cruz Biotechnology) were added (30 min, 37°C). The beads were washed three times with 1 ml of PBS and resuspended in electrophoresis sample buffer. Proteins were analyzed by SDS-PAGE (12.5%, 25 μg of protein/lane) and immunoblotting with anti-rat heart ANT polyclonal serum (17) or mAbs specific for Bax (P-19; Santa Cruz Biotechnology), cytochrome c (7H8.2C12; Pharmingen), or Bcl-2 (anti-Bcl-2 ΔC 21 mAb; Santa Cruz Biotechnology).

Liposome Technology. ANT was purified from rat heart mitochondria (19), routinely checked to be VDAC-free (14) and reconstituted into proteoliposomes (phosphatidylcholin/cardiolipin [45:1; w:w]) either alone or in the presence of recombinant Bax, BaxΔGDE, Bcl-2, or Bcl-2Δ145 (20) at the indicated molar ratios. Proteoliposomes were loaded either with 4-MUP (17) or with ATP (1 mM) in 10 mM KCl, 10 mM HEPES, 125 saccharose (pH 7.4), by sonication (25% of 250 W, 22 s on ice, Branson sonifier 250), washed on Sephadex PD-10 columns (Pharmacia, Uppsala, Sweden), dispended in 96-well microtiter plates, and incubated with the indicated agents (AMP, ADP, Atr) at room temperature for 30 min, as described previously (13, 21, 22). The dose of ANT (quantified by the Bradford method) in liposomes was 0.1 mg/ml. To quantify ATP release from liposomes, a luciferase/luciferin-based luminescence method (kit HS II; Boehringer Mannheim, Germany) was used. The
release of 4-MUP was quantified by addition of alkaline phosphatase (which converts 4-MUP into the fluorochrome 4-methylumbelliferone; Ref. 17). The maximum 4-MUP release was determined by adding 5% Triton X-100 to proteoliposomes. The percentage of 4-MUP release induced by treatment of liposomes by Atr was determined as [(Atr – treated liposomes fluorescence – untreated liposomes fluorescence)/(TX-100-treated liposomes fluorescence)] × 100. The maximal fluorescence induced by 200 μM Atr (which induces a specific, fully ADP-inhibitable 4-MUP release from ANT proteoliposomes) was then identified as 100% 4-MUP release, and the fluorescence induced by the treatment of liposomes by another product was calculated as a percentage of Atr-induced 4-MUP release.

ADP/ATP Translocase Activity in Isolated Mitochondria and Mitoplasts. Liver mitochondria (from female, 8–12-month-old C57Bl6 mice and age- and sex-matched congenic mice expressing a Bcl-2 transgene under the t-type pyruvate kinase gene promoter) (23) were purified (18) and suspended (7 mg protein/500 μl) in 0.6 M mannitol, 0.2% BSA, 10 mM MOPS, and 0.1 mM EDTA (pH 6.8). The respiratory rate, respiratory control, and membrane potential of mitochondria purified from control livers and Bcl-2-overexpressing livers were indistinguishable (data not shown). For kinetic analyses of ADP/ATP exchange, we modified a method developed by Klingenberget al. (24). Isolated mitochondria were incubated with 15 μl of [2,8-3H] ATP (40 Ci/mmol) for 45 min at 4°C in the presence or absence of recombinant Bax protein (2 μg/ml), and washed twice to eliminate free [2,8-3H] ATP. The exchange was initiated by addition of cold ADP (standard dose: 400 μM) and stopped by addition of 100 μM Atr after 10 s and centrifugation (6800 × g, 10 min, 4°C). Mitoplasts were generated by incubation of fresh mitochondria (7 mg protein/500 μl) [mitochondria: hypotonic buffer, 1:10, v:v] in 20 mM HEPES, 1 mg/ml BSA (pH 7.4) for 20 min at 4°C. KPA and NADH were used at 2 and 300 μM, respectively.

**RESULTS AND DISCUSSION**

**ANT Physically Interacts with Bcl-2 in Normal Cells and with Bax during Apoptosis.** Bcl-2 reportedly is confined to the outer mitochondrial membrane, which would be in conflict with a possible Bcl-2 effect on ANT (see Refs. 1–4, 6–10, 29, 30 but Refs., 31, 32). Immune electron microscopic examination of mitochondria in intact cells revealed, however, that Bcl-2 is also found within the inner mitochondrial membrane, mainly organized in clusters (Fig. 1A). Coimmunoprecipitation assays confirmed an interaction between Bcl-2 and ANT (Fig. 1B, IP) previously suggested by yeast-two-hybrid studies (13). Upon induction of apoptosis, the interaction between ANT and Bax increased while that with Bcl-2 decreased (Fig. 1B), concomitantly with the translocation of Bax to mitochondria (Fig. 1B, Mito), and the release of cytochrome c (Fig. 1B, Mito). These observations indicate dynamic changes in the intramolecular interactions operating within the PTPC. Moreover, they underscore the possibility that ANT switches from a preponderant Bcl-2-associate...
ANT (Fig. 2E). Bcl-2 (but not its inactive truncation mutant Bcl-2Δ145) counteracted the Atr-stimulated ANT-mediated 4-MUP release (Fig. 2F). In addition, full-length Bcl-2 did cause a dose-dependent increase in ANT-mediated ADP/ATP antiport (Fig. 2G). These results indicate that, in addition to regulating pore formation by ANT, Bcl-2 and Bax influence the ADP/ATP translocase activity of ANT.

Bcl-2 and Bax Modulate ADP/ATP Exchange in Mitochondria and Mitoplasts. When added to purified mouse liver mitochondria, recombinant Bax incorporated into mitochondrial membranes (35, 36). We used nonoligomeric Bax at a dose that does not induce mitochondrial swelling (35, 36). Preincubation with Bax caused a significant reduction in ADP/ATP exchange, as compared with sham-preincubated control mitochondria, with a decrease in the $K_m$ and $V_{max}$ of ADP/ATP exchange (determined after 10 s of incubation with ADP), suggesting (but by no mean proving) that Bax competes for ADP binding (Fig. 3A), a notion that would be in accord with the vicinity of the ADP and Bax-binding domains of ANT (13, 37). Alternatively, a conformational transition could explain the Bax effect. In addition, we found that purified mitochondria from mice overexpressing a liver-targeted Bcl-2 transgene (23) exhibited an increased ADP/ATP exchange, with a doubling of $V_{max}$, yet no major change in the $K_m$ (Fig. 3B). Similar results were found when mitoplasts (i.e., mitochondria with disrupted outer membrane) were used to assess the Bax and Bcl-2 effects on ADP/ATP antiport. Bax inhibited and Bcl-2 stimulated the ADP-induced ATP release (Fig. 3C). In addition, König’s polyanion and NADH, two VDAC channel blockers, failed to interfere with ADP/ATP exchange in mitoplasts, whereas Bax and Bcl-2 continued to affect the ADP/ATP antiport. This suggests that VDAC does not mediate the Bax and Bcl-2 effects.

Fig. 2. Effects of Bax and Bcl-2 on ANT proteoliposomes. A, cryoelectronic microscopy analysis of ANT proteoliposomes. Proteoliposomes population consisted mainly in small unilamellar vesicles with a 50 nm-mean diameter. B, assay for ANT pore opening. ANT proteoliposomes loaded with 4-MUP were exposed to the indicated doses of Atr, a specific ligand of ANT, AMP (as a negative control) or Atr + ADP (ADP added 30 min before Atr) for 30 min. Then, alkaline phosphatase (which normally cannot access the liposomal lumen) is added and the phosphatase-catalyzed conversion of 4-MUP into a fluorescent product is measured. C, assay for ADP/ATP exchange. ANT proteoliposomes loaded with ATP were exposed to the indicated dose of ADP or AMP (as a negative control) for 30 min, followed by addition of luciferase and luciferin. D, influence of Bax on the Atr-induced permeabilization of ANT proteoliposomes. ANT and Bax (or the inactive Bax mutant ΔGDE) were inserted into liposomal membranes at the indicated molar ratios. Then, liposomes were loaded with 4-MUP and the Atr-induced 4-MUP release was quantified as in B. E, effect of Bax on the translocase activity. The same liposomes as in D were loaded with ATP, exposed to ADP, and the release of ATP was assessed. Untreated liposomes, liposomes treated with 400 μM ADP. F, Bcl-2-mediated inhibition of ANT-dependent pore formation. ANT and Bcl-2 (or as a negative control the Bcl-2Δ145 mutant) were inserted into liposomes, followed by the determination of Atr-induced 4-MUP release. G, effect of Bcl-2 on ADP/ATP exchange. The same liposomes as in F were loaded with ATP instead of 4-MUP, and the ADP-driven ATP release was measured. Results are means of three independent experiments (each done in triplicate) ± SD. * indicates significant Bcl-2 or Bax effects as compared with controls ($P < 0.01$; paired Student t test).
ADP/ATP translocation inhibitor Atr, underlining a new functional interaction between Bcl-2 and ANT (Fig. 4E), not mediated by a pore opening effect of Atr (Fig. 4F). When Neo cells were treated with STS to induce apoptosis, the ADP/ATP exchange was reduced after as little as 1 h of incubation (Fig. 5A), which is well before the loss of the mitochondrial transmembrane potential (Fig. 5B). In contrast, STS failed to cause a reduction in mitochondrial ADP/ATP exchange in Bcl-2-overexpressing cells (Fig. 5A). Altogether, these data confirm that Bcl-2 can modulate the ADP/ATP translocase activity in vivo during chemotherapy-induced apoptosis.

Concluding Remarks. Apoptosis induced by growth factor withdrawal reportedly reduces ADP/ATP exchange on mitochondrial membranes. This effect has been attributed to cytosolic alkalization (39), down-regulation of mitochondrion-associated hexokinase II, and other glycolytic enzymes (40–42), as well as a closure of the VDAC (42), perhaps as a result of a hyperpolarization of the inner mitochondrial membrane with charge transfer to the outer membrane (9, 43). Bcl-2 would maintain the flux of metabolites on mitochondrial membranes, either through its ability to maintain a normal proton flow on the inner mitochondrial membrane (44) or through a functional interaction with VDAC preventing it from closing and from shutting down metabolite transport on the outer membrane (42).

Fig. 4. Assessment of ADP/ATP exchange in mitochondrial membranes in vivo. HeLa cells stably expressing the neomycin resistance cassette alone (Neo) or Bcl-2 were transiently transfected with mtLuc. After transfection (efficiency 40–50%), cells were permeabilized with digitonin in conditions that leave the inner mitochondrial membrane intact. A and B, evidence for Bcl-2-mediated increase of ADP/ATP exchange. Oligomycin-preincubated cells (before digitonin permeabilization) were sequentially exposed to luciferin (first arrow) and ATP plus ADP (second and third arrows), and luminescence was monitored. Low residual ATP levels yield a minor signal upon luciferase addition, whereas exogenous ADP/ATP yields a rapid increase in ATP-dependent luminescence which then declines, reflecting the influx and efflux of ATP in and from the mitochondrial matrix, respectively. The experiment shown in B has been performed in the continuous presence of CsA. C–F, control experiments addressing the specificity of the system, performed in the absence of oligomycin (C and D), the presence of Atr (E and F), or the presence of CsA (B, D, and F). Note the comparatively low amplitude of the ADP/ATP-elicted luciferase-dependent signal in the presence of Atr. This experiment has been repeated six times, yielding similar results.

on ANT (Fig. 3C). Altogether, these data confirm the results obtained with ANT proteoliposomes in a more physiological setting and underscore that the effect of Bcl-2 and Bax are not mediated via VDAC (which is irrelevant for ADP/ATP exchange in mitoplasts).

Bcl-2 Enhances the ANT-dependent ADP/ATP Exchange in Intact Cells. To investigate mitochondrial ADP/ATP translocation in intact cells, we engineered HeLa cells to express an ATP sensor in the mitochondrial matrix. This was achieved by transfecting cells with a luciferase cdna construct that directs luciferase expression to the mitochondrial matrix (mtLuc) because of fusion with the NH2-terminal mitochondrial import sequence of cytochrome c oxidase subunit VIII (25, 38). In the presence of luciferin, the luciferase emits a luminescence signal, the amplitude of which depends on the local ATP concentration. When HeLa control cells (Neo) were permeabilized with digitonin (which does not affect the inner mitochondrial membrane) and the mitochondrial F1F0-ATPase was inhibited by oligomycin, addition of exogenous ATP plus ADP led to a sudden increase in the luminescence signal, followed by its decrease, the slope of which reflects ADP/ATP exchange on mitochondrial membrane (Fig. 4A). HeLa cells overexpressing Bcl-2 reproducibly exhibited an accelerated matrix ATP decline, indicative of an increased ATP efflux from mitochondria (Fig. 4B). The experiment reduced the contribution of the F1F0-ATPase and allowed to reveal a specific effect of Bcl-2 on ANT translocase activity (Fig. 4, A versus C). This difference between NEO and Bcl-2-overexpressing cells persisted in the presence of CsA (Fig. 4B), excluding the contribution of CsA-inhibitable PTPC pore opening. However, the difference disappeared in the presence of the
been shown to maintain the ATP/ADP exchange on mitochondrial membranes, and this effect has been explained by an action on the outer membrane, presumably on VDAC (47). Although our data certainly do not exclude a functional interaction between Bcl-2 and VDAC, they suggest that part of the Bcl-2 effect can be attributed to an interaction with ANT. Thus, Bcl-2 enhances the translocase activity of purified (VDAC-free) ANT in proteoliposomes (Fig. 2), and it does stimulate ADP/ATP exchange on mitochondria on which the outer membrane has been permeabilized (Fig. 3). Moreover, our scenario of Bax and Bcl-2-mediated metabolic and apoptotic regulation is compatible with the previously reported finding that both proteins can function independently of each other (48–50).

In summary, the reported effects of Bax and Bcl-2 on ANT activity are of heuristic value for the comprehension of apoptosis regulation. Future studies will have to elucidate the complex interplay between apoptosis-sensitive components of the PTPC (e.g., ANT, VDAC, Bcl-2, Bax, hexokinase II . . . ). Such studies appear particularly important in view of the fact that intra-PTPC protein-protein interactions strongly change during the switch from normal physiology to incipient cellular demise.

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