Inhibition of Bax Channel-Forming Activity by Bcl-2

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Proteins of the Bcl-2 family are intracellular membrane-associated proteins that regulate programmed cell death (apoptosis) either positively or negatively by as yet unknown mechanisms. Bax, a pro-apoptotic member of the Bcl-2 family, was shown to form channels in lipid membranes. Bax triggered the release of liposome-encapsulated carboxyfluorescein at both neutral and acidic pH. At physiological pH, release could be blocked by Bcl-2. Bcl-2, in contrast, triggered carboxyfluorescein release at acidic pH only. In planar lipid bilayers, Bax formed pH- and voltage-dependent ion-conducting channels. Thus, the pro-apoptotic effects of Bax may be elicited through an intrinsic pore-forming activity that can be antagonized by Bcl-2.

Members of the Bcl-2 family are intracellular, membrane-associated proteins that regulate apoptosis, or programmed cell death (*1*), although the mechanisms are unknown. Structural studies on Bcl-x, an anti-apoptotic protein, revealed structural analogies to the pore-forming bacterial toxins, colicins and diphteria toxin (*2*), and Bcl-x was subsequently shown to have pore-forming activity in synthetic lipid membranes (*3*). Bcl-2, another anti-apoptotic family member, was recently shown to form pores in lipid membranes (*4*). We now show that Bax, a pro-apoptotic member of the family, also is able to form pores, but with different properties, and that at physiological pH, Bcl-2 can block almost completely the pore-forming activity of Bax.

Full-length proteins of the Bcl-2 family are highly insoluble (*5*). Therefore, we used recombinant proteins that lack the COOH-terminal hydrophobic domain but that nevertheless retain biological activity (*6*) (Fig. 1, A and B). Both Bcl-2 and Bax were purified to apparent homogeneity (Fig. 1C).

We reasoned that if Bax and Bcl-2 could function in a manner similar to colicins or diphteria toxins, they may be able to form pores in plasma membranes and induce cell lysis. We therefore examined whether the presence of Bax or Bcl-2 in the culture medium of sympathetic neurons or in a suspension of sheep erythrocytes could affect cell integrity. When added to neuronal cultures at a concentration of 10 μM, Bax was lytic within 3 to 6 hours (Fig. 2A). At 5 μM or less the effect of Bax was less pronounced, the cytoplasm of the neurons became granular, and death was delayed, suggesting that below a critical Bax concentration, neurons could compensate for the toxic effects of Bax (Fig. 2B). Addition of 10 μM Bax together with an equimolar amount of Bcl-2 delayed neuronal lysis by ~12 hours (n = 2), whereas no effect was observed when ERK2 or stathmin proteins were added as a control. This result is consistent with an apparent decrease in Bax concentration, suggesting neutralization by Bcl-2. Ten micromolar Bax was also able to lyse red blood cells (Fig. 2D). In contrast, addition of Bcl-2 had no deleterious effect on neurons (Fig. 2C) or on red blood cells (Fig. 2D), suggesting that the membrane-interacting properties of these two proteins are intrinsically different.

To determine if Bax, indeed, was a pore-forming protein, we assessed whether Bax could trigger the release of liposome-encapsulated carboxyfluorescein (Fig. 3). Bax induced dye efflux from liposomes in a concentration-dependent manner at neutral pH, with a maximal effect at 120 nM (Fig. 3A). Under the same conditions, Bcl-2 triggered no carboxyfluorescein release at concentrations up to 360 nM (Fig. 3A). Channel formation by both the bacteria toxins and Bcl-x is favored by low pH (*3*). We therefore examined the ability of Bax and Bcl-2 to form pores in different pH environments. The release of carboxyfluorescein induced by 2.5 nM Bax increased in a pH-dependent manner and was eight times greater at pH 4.0 than at pH 7.5 (Fig. 3C). In contrast, although Bcl-2 was as efficient as Bax at pH 4, the channel-forming ability of Bcl-2 decreased at pH 5 and was abrogated at pH 6 (Fig. 3D). Thus, the pore-forming properties of Bax and Bcl-2 are different.

Bcl-2 has been suggested to antagonize the pro-apoptotic function of Bax by blocking Bax activity (rheostat model) (*7*). The delay seen in Bax-induced neuronal death after addition of Bcl-2 supported this model, and therefore we tested this hypothesis by studying whether Bcl-2 could inhibit the effect of Bax on liposomes at physiological pH. Accordingly, liposomes were first incubated with Bcl-2 or with a control protein (stathmin) before addition of Bax to the liposome solution. At a Bax:Bcl-2 ratio of 1:1 the Bax-triggered carboxyfluorescein efflux was decreased by 50%, and at a ratio of 1:10 the efflux was almost completely inhibited (Fig. 3B). The unrelated control protein stathmin had no adverse effect on Bax function (Fig. 3B). The mechanism by which Bcl-2 inhibits the ability of Bax to

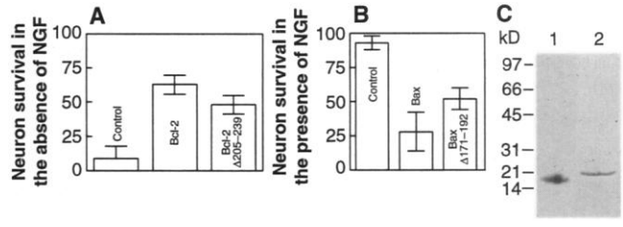

Fig. 1. Characterization and purification of biologically active Bax and Bcl-2 lacking the hydrophobic COOH-terminal domain. (**A**) Sympathetic neurons were microinjected with cDNAs (*13*, *14*) encoding either Bcl-2 or Bcl-2 lacking the 34 COOH-terminal amino acids. Twenty-four hours later nerve growth factor (NGF) was withdrawn from the culture medium. Neuron survival was assayed after 48 hours of NGF deprivation and is expressed as the percentage of neurons at 6 hours after injection (neurons that survived injection). Results are the mean ±SEM (n = 3) with 100 to 150 neurons injected in each experiment. (**B**) Neurons were microinjected with cDNAs encoding either Bax or a Bax mutant lacking the 20 COOH-terminal amino acids. Neuron survival was assayed 48 hours later. Results are the mean SEM (n = 3) with 100 to 150 neurons injected in each experiment. (**C**) The purified proteins (*15*) were analyzed on 10 to 15% SDS–polyacrylamide gel electrophoresis (PAGE) (PhastSystem, Pharmacia). Proteins were detected by Coomassie blue staining. The identity of the proteins was confirmed by NH_2-terminal amino acid sequencing and by mass spectrometry. Lane 1, Bax; lane 2, Bcl-2.

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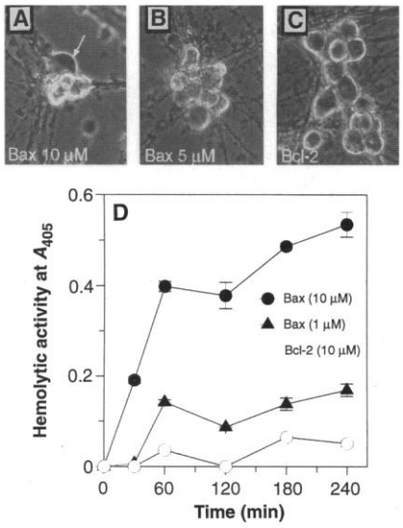

Fig. 2. Lytic effects of Bax on neurons and red blood cells. (**A**) Bax protein (10 μM) was added to serum-free medium of sympathetic neurons. Three to 6 hours later, neurons began to swell and finally lysed (arrow). (**B**) When 5 μM Bax was added under the same conditions, neurons became granular and died after 24 hours. (**C**) Bcl-2 (10 μM) did not affect neuronal integrity. (**D**) Bax and Bcl-2 were added to sheep red blood cells and incubated at 37°C with continous shaking, and hemoglobin release was measured at different time points (*16*).

induce membrane permeability is currently unknown.

The following data support the notion that pore-forming activity is an intrinsic property of Bax. Five different, highly purified preparations of Bax, from either glutathione-S-transferase (GST)–fused or His-tagged Bax, all produced similar results. In contrast, ERK-2 (extracellular-regulated kinase), thyroglobulin, stathmin, or SCG10 did not show pore-forming activity, nor did Bcl-2 at physiological pH. Bax activity was antagonized by addition of Bcl-2 (*8*), whereas addition of control proteins had no effect. These data support the hypothesis that pore-forming activity is intrinsic to Bax and is not due to contaminants in the Bax preparations.

Pore formation by the Bax protein was further investigated and characterized electrophysiologically with planar lipid bilayers (Fig. 4). Currents were measured in bilayers (2 mm^2) separating symmetrical or asymmetrical salt solutions into which Bax was added at concentrations between 5 and 500 nM. Within 10 min to 1 hour after addition of Bax, or alternatively after membrane formation in solution containing Bax, an increase in membrane conductance (g_m) was consistently observed in 17 experiments performed at pH 7 and in three experiments performed at pH 4. At pH 7, we observed elementary channels of 5.6 ± 0.2 pS occurring at early times after exposure of the membrane to Bax (observed in three experiments, Fig. 4A). The openings had a fast flickering in the millisecond range, and their duration could not be described by a single time constant. These small pore openings were usually swamped by long bursts of larger conductance fluctuations between 26 ± 7 pS (at 100-Hz bandwidth) and a predominant opening of 250 ± 25 pS with occasional residencies at two main sublevels (80 ± 25 pS and 180 ± 25 pS) (Fig. 4B). The mean dwell time (τ_o) at levels above 125 pS was 240 ± 20 ms. Further superimposed on this activity were abrupt changes of g_m in multiples of about 450 pS resulting in conductances up to 2 nS (Fig. 4C). This was particularly evident at later times and with high voltages. At pH 4, the Bax channel activity was modified in two main respects (Fig. 4D). First, conductance levels were about threefold lower (g_m = 77 ± 10 pS and 27 ± 4 pS) and openings were much shorter-lived (τ_o above 40 pS was 85 ± 6 ms). Large conductance changes as observed at pH 7 (Fig. 4C) were also detected at pH 4, although their levels were lower (*9*).

A general property of Bax channels observed under all conditions was their membrane potential (V_m)–dependent formation or activation (Fig. 4E). Upon asymmetrical addition of Bax to one chamber, channel activity was facilitated when a negative V_m was applied to the same side. However, a marked sidedness of the channel activity was also often observed when Bax was added symmetrically. This result implies that the channel-forming structures have an intrinsic asymmetry.

Ion selectivity of the Bax channels at pH 7 was studied in asymmetric NaCl solutions by applying voltage ramps between −30 and +30 mV during periods of high channel activity. In two experiments with 145 mM Na^+ and 125 mM Cl^- on the cis side and 1 M Na^+ Cl^- on the trans side, the reversal potential of the channels was about −15 mV, whereas in two experiments with 145 mM Na^+ and 125 mM Cl^- on the cis side and 40 mM Na^+, 20 mM Cl^- on the trans side, we estimated the reversal potential to be about +10 mV. Both estimates are consistent with channels that are slightly cation selective with a permeability ratio of Na^+ to Cl^- of about 2.1 (2.25 and 1.95 in either case).

In conclusion, Bax can form pH-dependent channels in lipid membranes, as reported for the anti-apoptotic proteins Bcl-x (*3*) and Bcl-2 (*4*). The intrinsic channel properties of Bax and those of Bcl-x and Bcl-2 reveal differences, especially with respect to pH. These differences may be re-

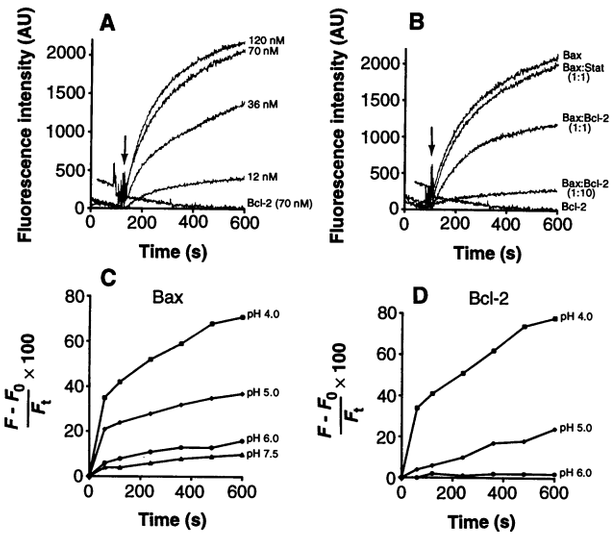

Fig. 3. Release of liposome-encapsulated carboxyfluorescein (*17*). (**A**) Fluorescence was measured every second with a spectrofluorimeter (FP-777, Jasco) with excitation at 488 nm and emission at 520 nm. In each test, 20 μl of liposomes containing 20 mM carboxyfluorescein was diluted in 1 ml of PBS (pH 7.5), and 5 μl of purified protein was added at the time indicated by the arrow. Incubation was done at room temperature. Final Bax concentrations are indicated for each curve. Bcl-2 was tested at 70 nM. Each curve was normalized by subtraction of the initial fluorescence value obtained at time zero. (**B**) Bcl-2 inhibits Bax-induced carboxyfluorescein efflux at pH 7.5. Bcl-2 was first incubated for 15 min at room temperature with 20 μl of liposomes in 1 ml of PBS at the indicated concentrations. Bax (70 nM) was then added to the solution at the time shown by the arrow. (**C** and **D**) Effect of pH on Bax- and Bcl-2–induced carboxyfluorescein efflux. Measurements were performed in 5 mM sodium citrate, 150 mM NaCl buffers at pH 4.0, 5.0, and 6.0 and in PBS at pH 7.5. Twenty microliters of the liposomes were diluted into 1 ml of the buffers and incubated for different time periods at room temperature with 2.5 nM Bax (C) or Bcl-2 (D). Background fluorescence was measured in the absence of added protein. Fluorescence measurements were performed immediately after pH neutralization of the samples by addition of 100 μl of 1 M tris-HCl, pH 7.5 (pH correction was required to eliminate fluorescence quenching at pH < 7.5). The fluorescence signal is expressed as percentage of total fluorescence after correction for the background. F, fluorescence in the sample; F_0, background fluorescence; and F_t, total fluorescence as measured after addition of Triton X-100.

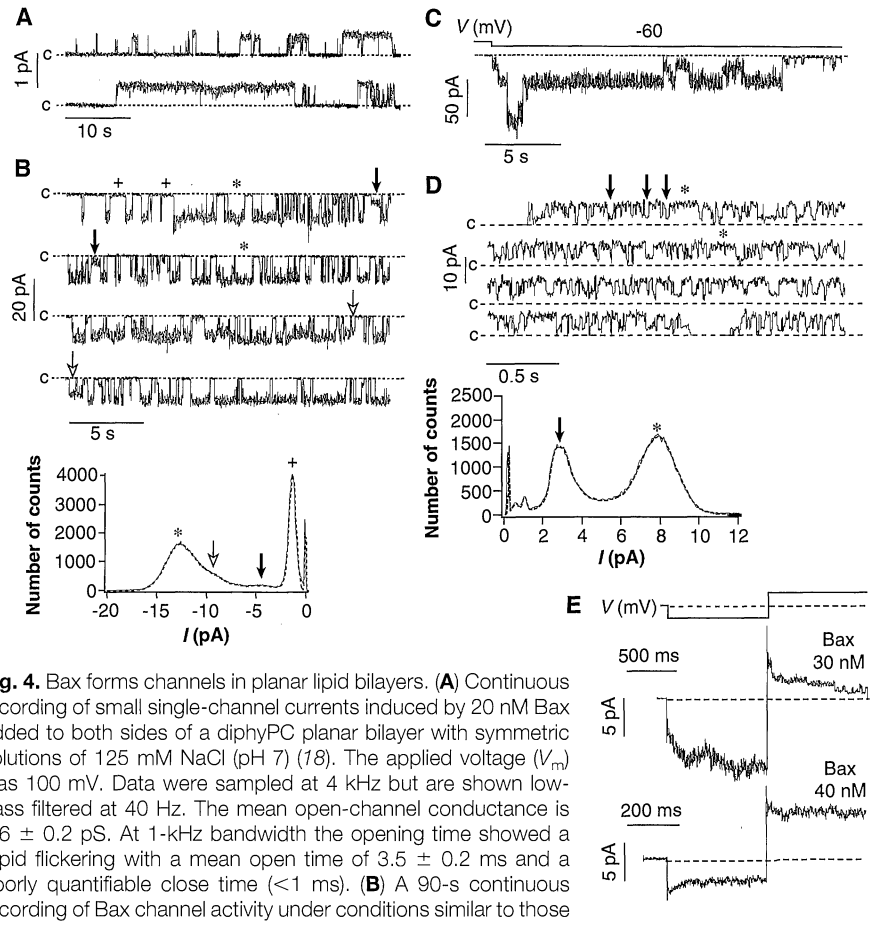

Fig. 4. Bax forms channels in planar lipid bilayers. (**A**) Continuous recording of small single-channel currents induced by 20 nM Bax added to both sides of a diphyPC planar bilayer with symmetric solutions of 125 mM NaCl (pH 7) (*18*). The applied voltage (V_m) was 100 mV. Data were sampled at 4 kHz but are shown low-pass filtered at 40 Hz. The mean open-channel conductance is 5.6 ± 0.2 pS. At 1-kHz bandwidth the opening time showed a rapid flickering with a mean open time of 3.5 ± 0.2 ms and a poorly quantifiable close time (<1 ms). (**B**) A 90-s continuous recording of Bax channel activity under conditions similar to those in (A) at V_m = −50 mV. Asterisks indicate conductance of 250 ± 25 pS. Other peaks represent levels of lower amplitude of 180 ± 25 (open arrows), 80 ± 25 (filled arrows), and 26 ± 7 pS (plus signs). The dwell time at any level above 125 pS was fitted by a single exponential distribution with a mean time of 240 ± 20 ms. (**C**) Large steps of membrane conductance recorded during 30 s at −60 mV. The largest conductance level is ~1.6 nS; the predominant level is 700 pS. Several other conductance levels in steps of ~450 pS and 900 pS are readily observable. Smaller openings at the beginning and end of the trace have characteristics similar to those shown in (B). (**D**) An 80-s continuous recording of single-channel activity at pH 4 of the type shown in (B) for pH 7. Bax (150 nM) was added on both sides at V_m = 100 mV. The data were analyzed after low-pass filtering at 200 Hz. The histogram shows two main conductance levels at 77 ± 10 pS (asterisks) and 27 ± 4 pS (arrows) and two minor small-conductance channels (8.5 ± 1 and 4 ± 2 pS). The dwell time at any level above 40 pS was fitted by a single exponential distribution with a mean time of 85 ± 6 ms [(B) and (D) have different time scales]. (**E**) Incorporation or formation of Bax channels is facilitated by negative voltages when applied to the side to which the protein was added. Traces show the average response to a series of 30-V pulses according to the double-step protocol (top). In the experiment with Bax in trans, the voltage steps were ±40 mV; otherwise, the voltage steps were ±60 mV.

lated to the pro-apoptotic and anti-apoptotic functions, respectively, of these proteins. Membrane insertion and pore formation of Bax might promote cell death by allowing the passive flux of ions and small molecules across intracellular membranes in which the protein is localized. Localization of Bax to mitochondrial membranes may trigger a permeability transition and consequent disruption of the transmembrane potential, two critical events during the early stages of apoptosis (*10*, *11*). Our results with neurons and liposomes suggest that at normal physiological pH this activity may be antagonized by the presence of the anti-apoptotic Bcl-2 protein. Neurons from Bax-knockout mice are resistant to naturally occurring cell death and to neurotrophic factor deprivation (*12*). Development of specific Bax channel blockers may therefore be of therapeutic utility in the treatment of diseases of the nervous system associated with neuronal apoptosis.

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15. Human Bax-α lacking 20 amino acids at the COOH-terminus was expressed as a GST fusion protein or a His-tagged protein in *Escherichia coli*, and the protein was purified from the soluble cell fraction (B. Antonsson, in preparation). In brief, the GST-Bax fusion protein was applied to a glutathione-Sepharose column, and Bax was released by cleavage with thrombin (0.6 U/ml). Bax was subsequently purified on heparin-Sepharose, followed by fast protein liquid chromatography (FPLC) Mono Q. His-tagged Bax was purified on a Ni–nitrilotriacetic acid–agarose column followed by FPLC Mono Q. Human Bcl-2 lacking 34 amino acids at the COOH-terminus was expressed in *E. coli*. Bcl-2 was purified from the soluble cell fraction by sequential chromatography on Q-Sepharose, phenyl-Sepharose, heparin-Sepharose, and FPLC Mono Q. The COOH-terminal truncations were necessary because yields and solubility of full-length recombinant protein were too low.
16. Sheep red blood cells were incubated with Bax or Bcl-2 at 37°C in tris-buffered saline composed of 10 mM tris-HCl (pH 7.4), 155 mM NaCl, 5 mM KCl, 0.5 mM $CaCl_2$ [B. Kenny, C. Cherveaux, I. A. Holland, *Mol. Microbiol.* **11**, 99 (1994)].
17. Liposomes containing 20 mM 6,7-carboxyfluorescein (Sigma) were prepared as described [R. Sadoul, M. Hirn, H. Deagostini-Bazin, G. Rougon, C. Goridis, *Nature* **304**, 347 (1983)] with 400 μg of phosphatidylserine from bovine brain (Sigma), 400 μg of phosphatidylcholine (Sigma), and 230 μg of cholesterol (Fluka). The liposomes were dialyzed for 24 hours against phosphate-buffered saline (PBS) and diluted to 8 ml. For analysis at acidic pH, fluorescence of released dye was measured after adjustment of pH to 7.5 by addition of 1 M tris-HCl (pH 7.5).
18. Planar lipid bilayers were formed by apposition of two phospholipid monolayers initially formed at the air-water interface as described [M. Montal, *Methods Enzymol.* **32**, 545 (1974)]. The monolayers at the interface were spread from a solution of 2 mg/ml lipid [100% diphytaneoyl-phosphatidycholine (diphyPC) or 60% diphyPC, 40% phosphatidylserine in hexane]. The composition of the buffered salt solutions was 10 mM NaH_2PO_4-NaOH, 10 mM sodium citrate, 125 mM NaCl, 0.5 mM EDTA (pH 7.0) and 10 mM sodium citrate, 125 mM NaCl (pH 4.0). Membrane currents were recorded under voltage-control by a patch-clamp amplifier (EPC-7, List, Darmstadt, Germany). Voltage stimulation and data acquisition were controlled by a Macintosh microcomputer (Cupertino, CA) interfaced to the recording amplifier with a 16-bit AD/DA converter (Instrutech, Elmond, NY). Off-line analysis was done with special purpose IGOR software (Wavemetrics).

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