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Channel formation by antiapoptotic protein Bcl-2

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ABSTRACT **Bcl-2 is the prototypical member of a large family of apoptosis-regulating proteins, consisting of blockers and promoters of cell death. The three-dimensional structure of a Bcl-2 homologue, Bcl-X_L, suggests striking similarity to the pore-forming domains of diphtheria toxin and the bacterial colicins, prompting exploration of whether Bcl-2 is capable of forming pores in lipid membranes. Using chloride efflux from KCl-loaded unilamellar lipid vesicles as an assay, purified recombinant Bcl-2 protein exhibited pore-forming activity with properties similar to those of the bacterial toxins, diphtheria toxin, and colicins, i.e., dependence on low pH and acidic lipid membranes. In contrast, a mutant of Bcl-2 lacking the two core hydrophobic α-helices (helices 5 and 6), predicted to be required for membrane insertion and channel formation, produced only nonspecific effects. In planar lipid bilayers, where detection of single channels is possible, Bcl-2 formed discrete ion-conducting, cation-selective channels, whereas the Bcl-2 (Δh5, 6) mutant did not. The most frequent conductance observed (18 ± 2 pS in 0.5 M KCl at pH 7.4) is consistent with a four-helix bundle structure arising from Bcl-2 dimers. However, larger channel conductances (41 ± 2 pS and 90 ± 10 pS) also were detected with progressively lower occurrence, implying the step-wise formation of larger oligomers of Bcl-2 in membranes. These findings thus provide biophysical evidence that Bcl-2 forms channels in lipid membranes, suggesting a novel function for this antiapoptotic protein.**

Bcl-2 is the first identified member of a large family of cellular and viral apoptosis-regulating proteins (reviewed in refs. 1, 2). These proteins appear to block a distal step in a common pathway for apoptosis and programmed cell death, with some functioning as suppressors (Bcl-2, Bcl-X_L, Mcl-1, Ced-9, BHRF-1, E1b-19 kDa, A1, ASFV-5HL, Bcl-W, and NR13) and others as promoters (Bax, Bcl-X_S, Bak, Bad, Bik, and Bid) of cell death. In many cases, these proteins can interact with each other in a complex network of homodimers and heterodimers (1–5). Aberrant expression of Bcl-2 and some of its homologs has been described in association with several disease states characterized by either excessive accumulation of cells or inappropriate cell death, including cancer, autoimmunity, ischemic disease (stroke, myocardial infarction), HIV-associated immunodeficiency, and some neurodegenerative diseases (reviewed in refs. 6, 7). Thus, it is important to understand the biochemical mechanisms of action of this family of proteins, which share no amino acid sequence homology with other known proteins.

Bcl-2 and most of its homologs contain a stretch of hydrophobic residues near their carboxyl termini that anchor them in intracellular membranes, primarily the outer mitochondrial membrane, nuclear envelope, and parts of the endoplasmic reticulum (8, 9). These membrane locations, coupled with evidence that Bcl-2 can regulate Ca^{2+} fluxes and protein translocation across membranes (10–14), has prompted speculations that Bcl-2 family proteins may be involved in some aspect of either ion or protein transport (1). Recently, the three-dimensional structure of Bcl-X_L, an antiapoptotic homolog of Bcl-2, has been solved, revealing striking similarity to the pore-forming domains of diphtheria toxin (DT) and the bacterial colicins (15).

Bcl-X_L, DT, and the colicins A and E1 all contain a pair of central hydrophobic α-helices, arranged in a hairpin structure that is surrounded by 5–8 amphipathic α-helices. Studies of DT, colicin A, and colicin E1 suggest three steps to the process of pore formation: (*i*) association with the membrane in an orientation competent for subsequent integration, which requires low pH and negatively charged lipids; (*ii*) penetration of the two hydrophobic helices perpendicularly through the plane of the lipid bilayer, with folding upward of the surrounding amphipathic helices analogous to the opening of an umbrella; and (*iii*) channel formation by either dimerization/oligomerization of two or more molecules in the membrane, each contributing a pair of helices to the channel, or alternatively by integration into the membrane of additional amphipathic helices producing a monomeric channel (16–27). Though the core pair of helices is largely hydrophobic, they do contain some hydrophilic residues that presumably face the aqueous lumen of the channel. Also, studies with synthetic amphipathic peptides suggest that a minimum of four helices inserted perpendicularly through the membrane are required for formation of an aqueous channel (28–30).

Though Bcl-X_L is structurally similar to bacterial toxins, this does not necessarily imply functional similarity. The channel-forming domain of colicins, for example, exhibits a similar structural fold with myoglobins and phycocyanins, namely the globin fold, yet the three protein families fulfill entirely different functions (31). The predicted structural similarity of Bcl-2 to pore forming proteins therefore prompted us to explore whether Bcl-2 is capable of forming channels in lipid membranes.

METHODS

Plasmid Preparation. The His_6-Bcl-2 (ΔTM)-producing plasmid was constructed by liberating a *Eco*RI-*Xho*I cDNA encoding human Bcl-2 (1–218) followed by a termination codon from pEG202 and subcloning into pET-21 (32). For His_6-Bcl-2 (Δh5, 6)(ΔTM) [Bcl-2 (Δ143–184)(Δ219–239)], a Bcl-2-encoding cDNA in pRc/cytomegalovirus was PCR-amplified using PfuI DNA polymerase (Stratagene) by a two-step mutagenesis method (3, 32) using flanking (forward = 5′-GCGGAATTC*ATG*GCGCACGCTGGGAGAACA-3′ and reverse = 5′-CGCCTCGAG*TCA*CTTCAGAGACAGCCAGGAGAAATC-3′) (start/stop codons in italics) and internal mutagenic (forward = 5′-CTGCACACCTGGATCCAGGATAACGGA-3′ and re-

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Abbreviations: DT, diphtheria toxin; DOPC, dioleoyl phosphatidyl choline; DOPG, dioleoyl phosphatidyl glycerol.

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verse = 5′-CCAGGTGTGCAGCACCCCGTGCCTGAAGAGCTC-3′) primers. The *Eco*RI- and *Xho*I-digested, gel-purified PCR product was subcloned into pSKII, its proper construction confirmed by DNA sequencing, and then transferred to pET-21.

Purification of Bcl-2 Proteins. The His_6-Bcl-2 (ΔTM) and mutant His_6-Bcl-2 (Δh5, 6) (ΔTM) proteins were produced from pET vectors in *Escherichia coli* BL21(DE3) cells. A single colony was cultured at 37°C in Luria–Bertani medium with 100 μg/ml ampicillin. Induction was carried out at an OD_{600} of 0.8 with 1 mM isopropyl β-D-thiogalactoside at 37°C for 6–8 hr before recovering cells by centrifugation. The pelleted cells were stored at −20°C until used. Cells from 2 liters of culture were resuspended in 50 ml of lysis buffer (50 mM Tris, pH 9.2/150 mM NaCl/5 mM EDTA/10 mM 2-mercaptoethanol/1% Triton X-100/1 mM phenylmethylsulfonyl fluoride/0.5 mg/ml lysozyme), and incubated at room temperature for 30 min before brief sonication to reduce the viscosity. Pellets were collected by centrifugation at 13,000 rpm for 15 minutes in a JA14 rotor (Beckman), washed once with 200 ml of 20 mM Tris, pH 8.0/1% Triton X-100/150 mM NaCl/1 mM EDTA/5 mM 2-mercaptoethanol, and once with 200 ml of 20 mM Tris, pH 8.0/150 mM NaCl. The washed pellets, which contain most of expressed protein, were solubilized in 20 ml of 50 mM phosphate-buffered (pH 6.5) 6 M guanidinium·HCl (GdmHCl). Clear supernatants were collected by centrifugation at 17,000 rpm for 15 min in JA20 rotor (Beckman), and incubated with rotation at 4°C for 12–16 hr with 15 ml of nickel-agarose (Qiagen), which had been washed with 50 mM phosphate-buffered (pH 6.5) 4 M GdmHCl. The resin was packed into a column of 3.2 cm diameter and washed with 50 mM phosphate-buffered (pH 6.5) 4 M GdmHCl, 20 mM imidazole at 6.4 ml/min until the A_{280} reached 0.01. Immobilized protein then was eluted with 0.2 M acetic acid, 4 M GdmHCl, dialyzed against 20 mM acetic acid at 4°C for 12–16 hr, and stored at −80°C at pH 3.3 at 10–15 mg/ml. Purified proteins were characterized by SDS/PAGE (15% gels), followed by Coomassie staining. Concentrations were determined by A_{280} using $\varepsilon = 1.5$ and 0.71 mg/ml for Bcl-2 and Bcl-2 (Δh5, 6), respectively.

Liposome Preparation and Cl^- Efflux Measurements. Large unilamellar vesicles composed of either 70% or 100% dioleoyl phosphatidyl choline (DOPC) and 30% or 0% dioleoyl phosphatidyl glycerol (DOPG) were prepared in 10 mM dimethyl glutaric acid/100 mM KCl/2 mM $Ca(NO_3)_2$, pH 5.0, according to the method of Peterson and Cramer (24). The liposomes were diluted 200-fold to a final concentration of 0.05 mg/ml in 10 mM dimethyl glutaric acid/100 mM choline nitrate/2 mM $Ca(NO_3)_2$, titrated to the given pH with NaOH. Valinomycin was added (15 nM) to the vesicle suspension before Bcl-2 addition to generate an inside-negative potential. Bcl-2 or Bcl-2(Δ5, 6) was added to a final concentration (200–600 ng/ml) sufficient to release at least 50% of encapsulated KCl, the remainder of which was released by the addition of Triton X-100 (0.1%). The total amount of K^+ or Cl^- released was compared against a calibration curve produced by successive additions of 10 mM KCl. Bcl-2-induced K^+ or Cl^--efflux was measured with either a K^+ or Cl^--selective electrode (Orion, Boston) coupled to a double junction reference electrode (Orion) (24).

Planar Bilayer Preparation and Single-Channel Recordings. Lipid bilayers were formed at the tip of patch pipets by apposition of two monolayers, using 4:1 diphytanoyl phosphatidylethanolamine:diphytanoyl phosphatidylcholine (Avanti Biochemicals, Alabaster, AL) as described (33, 34). Purified proteins were added to the aqueous subphase (0.5 M KCl, 1 mM $CaCl_2$ adjusted to pH 5.4 or buffered with 5 mM Hepes to pH 7.4) after bilayer formation. Acquisition and analysis of single-channel currents at 24 ± 2°C were as described (20, 21, 30, 33). The channel recordings illustrated are representative of the most frequently observed conductances under the specified experimental conditions. Single-channel conductance, γ, was calculated from Gaussian fits to current histograms and the channel open (τ_o) and closed (τ_c) lifetimes were calculated from exponential fits to probability density functions (33) using data from segments of continuous recordings lasting $t > 45$ s and with $n \geq 500$ events (mean ± SEM). Openings with $\tau_o \leq 0.3$ ms were ignored. The data reported include statistical analysis of > 8,000 single-channel openings.

RESULTS

To explore the possibility that Bcl-2 forms channels in membranes, we produced Bcl-2 protein having an N-terminal His_6-tag in bacteria and devoid of its C-terminal membrane-anchoring tail (ΔTM). Removal of the TM domain was necessary to produce soluble recombinant protein. Bcl-2 (ΔTM) protein has been shown to retain antiapoptotic activity in mammalian cells (35). After purification by Ni-chelation affinity chromatography (Fig. 1*A*), His_6-Bcl-2 (ΔTM) protein was tested for pore-formation using KCl-loaded large unilamellar liposomes under conditions typically required for the assay of pore-formation by DT and colicins (24, 27, 36). Cl^- efflux was measured for most experiments, but similar results were also obtained using a K^+ selective electrode (not shown). The macroscopic channel activity detected by this method requires that the bulk of the protein molecules participate in channel formation, thus excluding the possibility that a rare subpopulation of denatured molecules with aberrant conformations is responsible for ion efflux.

When added to KCl-loaded liposomes composed of 70% neutral (DOPC) and 30% acidic (DOPG) lipids, Bcl-2 formed pores in a pH-dependent fashion (Fig. 1*B*). The release of Cl^- after Bcl-2 (ΔTM) protein addition was not due to lysis of the liposomes, based on light scattering studies of liposomes before and after addition of Bcl-2 versus Triton X-100. Comparisons of the pore-forming activities of colicin E1 and Bcl-2 demonstrated that only ≈5- to 10-fold more Bcl-2 protein was required to produce equivalent Cl^- efflux at pH 4.0 (Fig. 1*B*), suggesting that Bcl-2 is quite active as a pore-forming molecule.

Progressively less Cl^- efflux was induced by equimolar amounts of Bcl-2 at higher pH, a behavior typical of bacterial toxins such as colicin E1 in these bulk ion efflux assays (24, 27, 36). Fig. 1*C* shows an analysis of Bcl-2-mediated pore formation over the pH range of 4.0 to 6.0, demonstrating markedly reduced Cl^- efflux at pH 4.5 and 5.0 compared with pH 4.0 and undetectable pore activity at pH 5.5 and 6.0. Immunoblot analysis revealed that >95% of Bcl-2 (ΔTM) was liposome associated at pH 4.0 compared with <5% at pH 6.0, consistent with the idea that low pH protonates certain residues in Bcl-2 (ΔTM) thereby overcoming electrostatic repulsions with the anionic lipids and augmenting Bcl-2 insertion into membranes (not shown).

Bcl-2-mediated pore formation in liposomes requires acidic lipids. Fig. 1*D* shows a comparison of Cl^- efflux induced by Bcl-2 in liposomes composed of 70% DOPC/30% DOPG versus 100% DOPC at pH 4.0, demonstrating no pore-forming activity of Bcl-2 protein on neutral lipid vesicles. Bcl-2 also formed pores efficiently in liposomes prepared with neutral DOPC and 15% (wt/wt) of the anionic mitochondrial lipid cardiolipin (which carries two negative charges compared with only one for DOPG) (not shown). The failure of Bcl-2 to create pores in neutral lipids provides further evidence that this protein does not nonspecifically lyse vesicles. In this regard, the internal biological membranes where Bcl-2 is localized contain acidic lipids, implying that these observations obtained with synthetic lipids are physiologically relevant. Taken together, these results indicate that Bcl-2 can form ion-conducting pores in a pH- and acidic lipid-dependent manner, similar to bacterial toxins with which it shares structural similarity (19, 24, 27).

To further explore the mechanism of pore formation by Bcl-2, a mutant was constructed that lacks the two core hydrophobic helices (h5, 6) that are predicted to be required for penetration of the lipid bilayer and subsequent channel

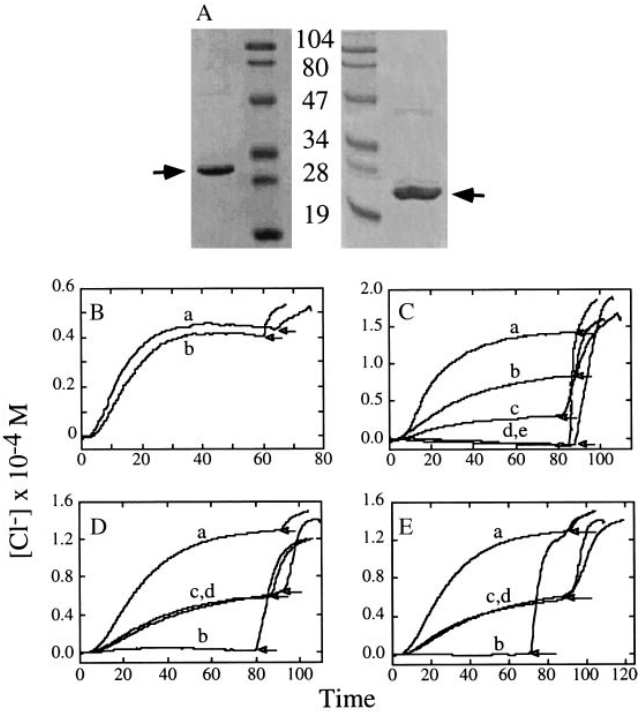

FIG. 1. Bcl-2 forms pH-dependent pores in acidic-lipid containing liposomes. (*A*) The His_6-Bcl-2 (ΔTM) protein (*left*) and mutant His_6-Bcl-2 (Δh5, 6) (ΔTM) protein (*right*) were purified using Ni-chelation affinity chromatography. Coomassie-stained SDS/PAGE gels of the His_6-Bcl-2 (ΔTM) and His_6-Bcl-2 (Δh5, 6) (ΔTM) proteins are shown, demonstrating >95% purity of the intact recombinant proteins (molecular mass markers are shown in kilodaltons). (*B*) Bcl-2 and colicin E1 proteins are contrasted with regards to Cl^- efflux from KCl-loaded 70% DOPC/30% DOPG liposomes at pH 4.0. His_6-Bcl-2 (ΔTM) (*b*) or colicin E1 (*a*) were added at t_0 in dimethyl glutaric acid buffer at ≈350 ng/ml and 50 ng/ml, respectively. Arrows indicate addition of 0.1% Triton X-100 to release residual Cl^- from the vesicles. (*C*) Bcl-2-mediated (300 ng/ml) Cl^- efflux was compared at various pHs, including pH 4.0 (*a*), 4.5 (*b*), 5.0 (*c*), 5.5 (*d*), and 6.0 (*e*). (*D*) Wild-type (*a*, *b*) and mutant (*c*, *d*) Bcl-2 proteins were evaluated for ability to induce Cl^- efflux from acidic 70% DOPC/30% DOPG (*a*, *c*) and neutral 100% DOPC (*b*, *d*) vesicles. Arrows indicate addition of 0.1% Triton X-100 to induce release of residual Cl^-. (*E*) Wild-type His_6-Bcl-2 (ΔTM) (*a*, *b*) and mutant His_6-Bcl-2 (Δh5, 6) (ΔTM) (*c*, *d*) proteins (300 ng/ml) were compared in 70% DOPC/30% DOPG liposomes. Cl^- efflux was measured at pH 4.0 (*a*, *c*) and 5.5 (*b*, *d*).

formation, based on structural similarities to bacterial toxins (16, 17, 26). This mutant is analogous to the Bcl-X_S protein, a proapoptotic isoform of Bcl-X_L that is generated by alternative mRNA splicing (37). Like Bcl-X_S, the Bcl-2 (Δh5, 6) protein promotes rather than suppresses cell death when expressed in mammalian cells with the C-terminal TM domain, binds to Bcl-2 in yeast two-hybrid assays, but fails to homodimerize or bind to Bax (refs. 3, 38 and data not shown). The His_6-Bcl-2 (Δh5, 6) (ΔTM) protein was produced in bacteria, purified (Fig. 1*A*), and added to KCl-loaded liposomes in side-by-side comparisons with Bcl-2 (ΔTM). Unlike the His_6-Bcl-2 (ΔTM) protein, the mutant His_6-Bcl-2 (Δh5, 6) (ΔTM) protein produced only nonspecific Cl^- efflux. For example, though some Cl^- efflux was induced by the Bcl-2 (Δh5, 6) protein, this occurred regardless of pH and lipid composition of membranes (Fig. 1 *D* and *E*). This promiscuous stimulation of Cl^- efflux at elevated pH and in neutral membranes is indicative of nonspecific membrane disruption as might be caused by detergents. In this regard, the reported structure of Bcl-X_L predicts that the Bcl-2 (Δh5, 6) protein is essentially entirely amphipathic, with the remaining five helices all having a hydrophobic and a hydrophilic side. Thus, Bcl-2 (Δh5, 6), presumably interacts with membranes in a fashion analogous to detergents, resulting in nonspecific membrane disruption.

To further characterize the pore-forming activity of Bcl-2, experiments were performed using planar bilayer membranes (33). This sensitive technique monitors the activity of single channels. The sensitivity of this method permits channel recordings to be performed at neutral pH, where protein association with membranes and subsequent integration are rarer events. When the His_6-Bcl-2 (ΔTM) protein was applied to planar bilayers at neutral pH in symmetric 0.5 M KCl, discrete channel activity was observed with random openings and closings. The most common channels detected exhibited conductances of 18 ± 2 pS and 41 ± 2 pS (Fig. 2 *A–F*), with the smaller conductance channel occurring more frequently.

The channels formed by Bcl-2 exhibited ohmic behavior (i.e., voltage-independent), in that the current conducted over the range from +100 mV to −100 mV obeyed a linear current-voltage relation, without evidence of rectification. Fig. 2 *A–F* and *G–I* contrast the currents conducted by Bcl-2 at +100 mV with −100 mV, demonstrating essentially identical amplitudes and durations of the open state of channels but with the current flowing in opposite directions. This symmetric activity may reflect the random orientation of Bcl-2 channels in bilayers.

In addition to the primary ≈20 pS and secondary ≈40 pS conductances, a third discrete channel conductance of 90 ± 10 pS occurred far less frequently than the smaller conductance channels (Fig. 2 *J–L*). All three of these channel activities (≈20 pS, ≈40 pS, and ≈90 pS) were independent. In particular, careful inspection of hours of channel recordings excluded the possibility that the larger ≈40 pS and ≈90 pS conductance channels merely represented the sum of two or three, respectively, of the smaller ≈20 pS channels opening in membranes simultaneously.

Recordings at lower pH demonstrated that acid pH promotes Bcl-2 channel insertion into planar bilayers. At pH 5.4, the membrane conductance tended to shift from high to low conductance, reflecting the progressive insertion of more channels into the membrane. The single-channel conductances of 20, 40, and ≈90 pS were clearly discerned at pH 5.4 (Fig. 3). These channel events appeared in bursts, and the channel activity was significantly more heterogeneous than that recorded at pH 7.4 (Fig. 2). At V = 100 mV, a conductance of 26 pS was very frequent and homogeneous (Fig. 3*A*). This channel was far more frequent than the 40 pS events. At V = −100 mV, 10 and 20 pS channels were the most frequent (Fig. 3*B*). The 80–90 pS conductance also occurred, but infrequently and predominantly during transitions between low and high conductance levels.

The Bcl-2 channel, at pH 5.4, is cation-selective. Current-voltage relationships for Bcl-2 channels in symmetric 0.5 M KCl and under a 2-fold concentration gradient of KCl (0.5 M in pipette and 1 M in bath) were obtained from membranes containing multiple channels (≈4). The transference number for cations, determined from reversal potential (V_r) measurements under single KCl concentration gradients, is 0.94 ± 0.05 (n = 3). This indicates that Bcl-2 channels are ≈90% cation-selective under these conditions. To ascertain that the current was conducted by cations, the signals were calibrated by using valinomycin (a potassium-selective ionophore) under identical conditions, yielding equivalent results.

In contrast to the wild-type Bcl-2 protein, the Bcl-2 (Δh5, 6) mutant failed to form discrete channels in lipid bilayers when applied at equimolar concentrations (Fig. 4). Only irregular stray fluctuations were observed in the membrane current, and these conductances did not have the typical square-wave appearance of bona fide channels (Fig. 4*B*). This behavior is consistent with the idea that the Bcl-2 (Δh5, 6) protein interacts with the membrane in a nonspecific fashion, typical of surfactants and other amphipathic molecules, but fails to form discrete channels. Taking the results obtained by the single-channel analysis of Bcl-2 and the Bcl-2 (Δh5, 6) mutant together with the findings derived from the liposome perme-

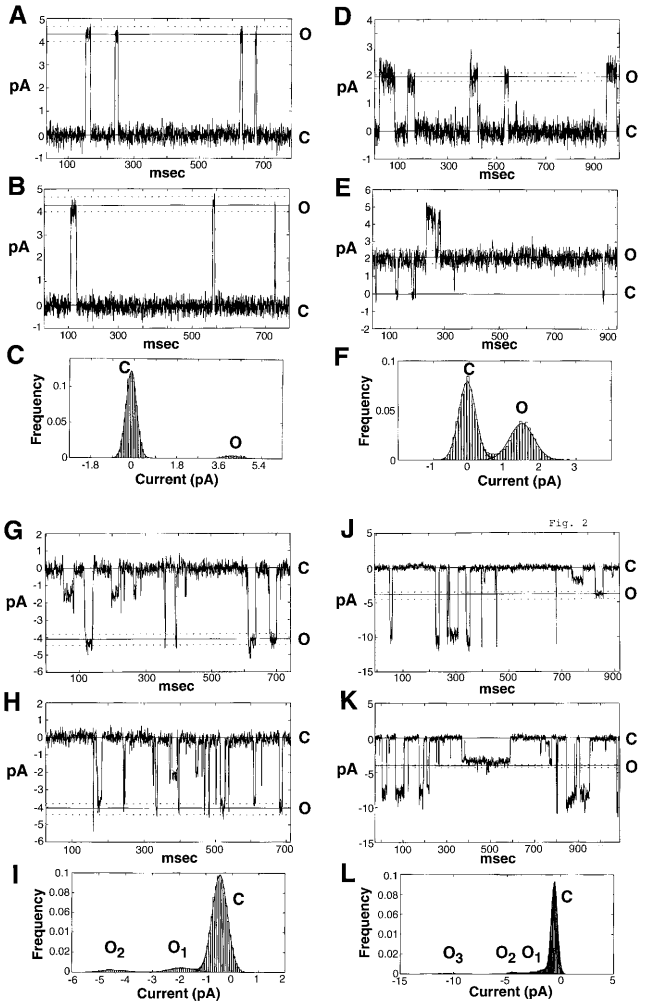

FIG. 2. Bcl-2 forms discrete channels in planar lipid bilayers. Single-channel recordings were obtained from preformed lipid bilayers in symmetric 0.5 M KCl supplemented with 1 mM $CaCl_2$ and 10 mM Hepes, pH 7.4 after addition of 50 μg/ml His_6-Bcl-2 (ΔTM) protein. The currents of the closed (C) and open (O) states are indicated by the solid lines. The dotted lines define the range set to discriminate the transitions between states and were based on signal to noise ratio measurements (33). For the records obtained at + 100 mV (*A–F*), an upward deflection indicates channel opening, whereas a downward deflection represents channel opening at −100 mV (*G–L*). Current histograms and Gaussian fits (*C*, *F*, *I*, *L*) reflect results obtained in the range $-100\ \text{mV} \leq V \leq 100\ \text{mV}$ from continuous segments of a record lasting several minutes. (*A–C*) Records chosen to demonstrate the 41 ± 2 pS channel, where *A* and *B* are representative current records and *C* is the current histogram with Gaussian fit of the data, showing relative occurrence of the open (O) and closed (C) channel states. The record was derived from a burst of 40 pS channel activity occurring in the absence of 20 pS channels. The probability of the channel being closed (P_c) ≥ 0.9, under these experimental conditions. The open channel lifetimes (τ_o) for the γ= 41 pS conductance channel were well fitted by a sum of two exponentials, $\tau_{o1} = 6.4 \pm 1$ msec, $\tau_{o2} = 11.2 \pm 3$ msec and the closed times by, $\tau_{c1} = 0.6 \pm 0.2$, $\tau_{c2} = 17.7 \pm 3.0$ msec ($n = 3$). *D–F* demonstrate the 18 ± 2 pS channel, where *D* shows a record where the channel is mostly closed (five openings over the time course presented), *E* an example where channel is mostly open over the time shown (note transient openings of a second ≈20 pS channel after ≈250 msec), and *F* represents the current histogram. The data are derived from a burst of purely ≈20 pS channel activity. The region of the record selected for histogram preparation was biased toward a period of high-frequency channel opening and is intended to show the high signal-to-noise ratio even for this small ≈20 pS channel. (*G–I*) Examples of the ≈40 pS (marked by solid line and flanking dashed lines) and ≈20 pS (not marked) channels recorded at −100 mV (downward deflections), demonstrating similar character to the recordings at +100 mV. The mixture of ≈20 pS and ≈40 pS channel activities shown in this record is more typical of Bcl-2 channel formation than the monoconductance channel activities that were presented in *A–F* for purposes of illustration. The current histogram (*I*) notes the frequency of occurrence of the open states for the ≈20 pS (O_1) and ≈40 pS (O_2) channels, and is representative of the relative times the ≈20 pS and the ≈40 pS channels occupy the closed and opened states. (*J–L*) The less common 90 ± 10 pS channel. Note interspersed ≈20 pS and ≈40 pS conductances. (The ≈40 pS channel is denoted by the solid line for comparison.) The current histogram (*L*) demonstrates the relative frequency of occurrence of the open states for the ≈20 pS (O_1), ≈40 pS (O_2), and ≈90 pS (O_3) channels. The ≈90 pS channel occurrence is too infrequent to be visually discerned in the plot. The particular records presented (*J*, *K*) are biased to show examples of this larger conductance channel activity.

ability assay, we conclude that the His_6-Bcl-2 (ΔTM) protein can form discrete channels in membranes, whereas the Bcl-2 mutant lacking two core hydrophobic helices does not.

DISCUSSION

Here we present biophysical evidence that the antiapoptotic protein Bcl-2 forms channels in membranes. The primary channel conductance at neutral pH of 18 ± 2 pS is consistent with pore formation by Bcl-2 homodimers, with each monomer contributing a pair of helices (presumably helices 5 and 6) to the channel, based on studies of synthetic amphipathic helices that assemble into tetrameric four-helix bundles in membranes and create channels of ≈20 pS conductance (29, 30). Interestingly, colicin E1 also has been reported to produce ≈20 pS conductance channels, through a mechanism believed to involve creation of a four-helix bundle upon colicin E1 insertion into membranes (reviewed in ref. 17). For colicin E1, however, channels appear to arise from monomers, with the two core hydrophobic helices from a single molecule of colicin E1 inserting into the membrane followed by two additional flanking amphipathic helices from the same molecule. Though additional biophysical studies are required to distinguish between channel formation by monomers versus dimers of Bcl-2, we favor the dimerization hypothesis based on the reputation for Bcl-2 and its homologs to homodimerize and heterodimerize. The second most common Bcl-2 channel exhibited a conductance of 41 ± 2 pS. Channels with similar conductance have been measured using synthetic peptides that form pentameric helical bundles in membranes (39), raising the possibility that these larger channels may be produced by Bcl-2 trimers or by insertion of additional amphipathic helices from Bcl-2 into the membrane (24). Moreover, the presence of three discrete channel activities with progressively greater conductances (≈20 pS, ≈40 pS, and ≈90 pS) but occurring with progressively lesser frequency raises the possibility of step-wise oligomerization of Bcl-2 protein molecules in planar bilayers.

The pore-forming motif of ion-conducting channels such as the acetylcholine receptor appears to be comprised of α-helical bundles. These α-helical channels tend to assume a closed conformation until appropriately triggered to open, whereas large pore-forming proteins such as porin and hemolysin have β-barrel structures and assume a mostly open state under conditions similar to those used here (reviewed in ref. 40). The finding that the Bcl-2 channel is mostly in a closed state *in vitro* at neutral pH raises the question of what controls opening and closing of Bcl-2-mediated channels *in vivo*. The pH-dependence of pore formation in lipid vesicles and the augmentation of channel insertion into planar bilayers at acid pH, suggest that pH may act as a channel modulator. In this regard, it could be relevant that one of the prominent locations of Bcl-2 is the outer mitochondrial membrane. Immunoelectron microscopic and subcellular fractionation studies suggest that Bcl-2 is particularly abundant at the contact sites that adjoin the inner and outer mitochondrial

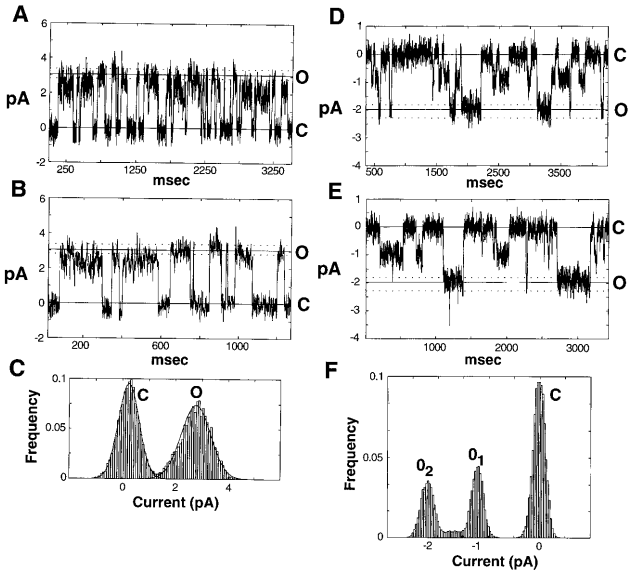

FIG. 3. Bcl-2 channels in lipid bilayers at pH 5.4. Single-channel recordings are shown from lipid bilayers in symmetric 0.5 M KCl, 1 mM $CaCl_2$ adjusted to pH 5.4 after addition of 50 μg/ml His_6-Bcl-2 (ΔTM) protein. (*A–C*) Sample records obtained at $V = 100$ mV, demonstrating the occurrence of the 26 pS channels. *D-F* correspond to records at $V = -100$ mV, showing channels of 10 pS and 20 pS. Other conditions are as for Fig. 2.

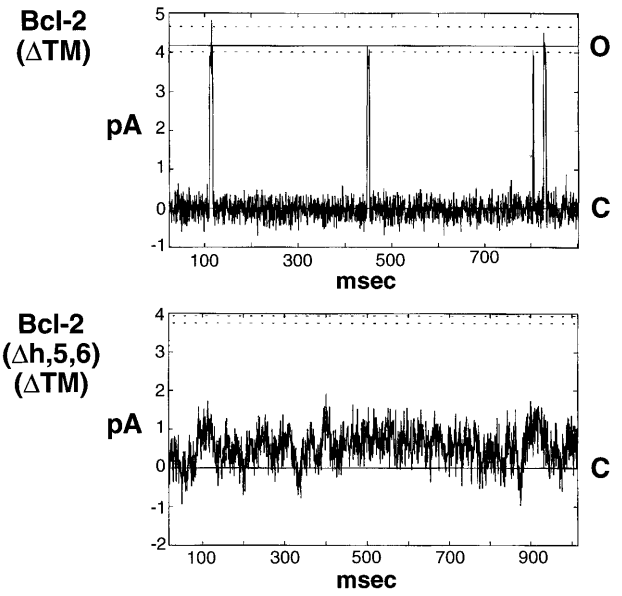

FIG. 4. The Bcl-2 (Δh5, 6) mutant fails to form channels. Membrane currents from lipid bilayers containing His_6-Bcl-2 (ΔTM) (*A*) and the deletion mutant lacking helices 5 and 6 (*B*). The final protein concentrations were 50 μg/ml. Currents recorded at 100 mV in symmetric 0.5 M KCl, 1 mM $CaCl_2$, 5 mM Hepes, pH 7.4. Note the occurrence of stray, erratic fluctuations in membrane current produced by the deletion mutant contrasted with the square events produced by the wild-type Bcl-2. Data representative of seven experiments.

membranes where a substantial proton gradient exists (8, 41, 42). Analogous to Bcl-2, colicin E1 appears to enter sensitive *E. coli* at the inner and outer membrane contact sites in bacteria, where it functions as a bactericidal pore (43). However, because low pH is believed to merely promote association of the pore-forming fragment of colicin E1 with the membrane surface *in vitro* (by protonating residues and increasing electrostatic attraction with acidic lipids in membranes) rather than actually inducing pore formation (27, 36), channel formation *in vivo* by the full-length Bcl-2 protein with its C-terminal membrane anchoring domain may be substantially less pH-dependent.

Another potential regulator of Bcl-2's channel activity besides pH is protein–protein interactions involving the various homologous and nonhomologous proteins that have been reported to bind to Bcl-2 (reviewed in refs. 1, 2). Thus, interactions with proteins such as BAG-1, which enhance Bcl-2's function as a antiapoptotic protein, may promote pore formation, while interactions with antagonistic proteins such as Bcl-X_S and BAD may interfere with pore formation.

Many questions remain unanswered about the relation of Bcl-2's *in vitro* channel activity to its function as a suppressor of apoptosis. For example, what is Bcl-2 intended to transport across membranes in cells? While the colicins form nonspecific ion channels and are used as a mechanism by some strains of *E. coli* to kill competing bacteria by inducing depolarization of the target cell's membrane potential, DT is thought to form a channel involved in protein transport across lysosomal membranes—namely, providing a conduit through which the ADP ribosylating A-subunit of the toxin gains access to the cytosol (20–22, 44). Another question is how proapoptotic proteins such as Bax oppose the cytoprotective effects of Bcl-2. Do they merely shut off the pore-forming activity by Bcl-2 by heterodimerizing with it, or do they alternatively form counteracting pores? Our preliminary data suggest that Bax also forms pores in a pH-dependent fashion in anionic lipid-containing liposomes and does not merely abrogate pore formation by Bcl-2 (unpublished data). The implication, therefore, is that Bcl-2 allows transport of an ion or a protein across membranes in a direction that is cytoprotective, whereas Bax does the opposite. It is of interest, therefore, that the predicted membrane penetrating fifth α-helix of Bcl-2 contains two acidic residues (glutamic acids) whereas Bax contains two basic residues (lysines). Thus, these proteins very likely have different selectivities (45). The presence of glutamic acid residues that presumably face the lumen of Bcl-2 channels is also consistent with the observed selectivity of Bcl-2 for cations.

Though highly speculative, a testable hypothesis that integrates a variety of observations in the literature is that Bcl-2 forms ion channels that result directly or indirectly in hyperpolarization of the mitochondrial membrane potential ($\Delta\psi$). Predicted secondary consequences of this hyperpolarization of mitochondria, which have been reported for Bcl-2 over-expressing cells, include: (*i*) increased mitochondrial uptake of cationic fluorescent dyes such as rhodamine 123 (46); (*ii*) enhanced Ca^{2+} uptake by mitochondria (13); and (*iii*) increased resistance to stimuli that induce mitochondrial permeability transition and dissipation of $\Delta\psi$, with attendant release of matrix-stored Ca^{2+}, generation of oxygen free-radicals, and swelling of the matrix resulting in rupture of the outer mitochondrial membrane and liberation into the cytosol of apoptogenic proteins, such as cytochrome *c* and AIF, which can in turn activate cell death effector proteases (caspases) (47–49).

The striking similarities in the three-dimensional structures of Bcl-X_L and colicins A and E1, together with the evidence presented here and elsewhere (50) showing that Bcl-2 and Bcl-X_L have highly similar pore-forming properties when compared with these bacterial toxins, raise the possibility that Bcl-2 family proteins and bacterial colicins may share a common ancestral origin in evolution or may represent an example of convergent evolution. This is particularly evident when one considers that mitochondria are believed to have descended from intracellular bacteria that developed a symbiotic relation with eukaryotic cells. Thus, the pore-forming colicins, with their associated immunity proteins that bind to colicins and prevent pore formation as a means of conferring cytoprotection to resistant strains of bacteria (17, 51), could have provided the framework for the subsequent emergence of the Bcl-2 family of proteins, many of which are localized to

mitochondrial membranes and which (like the colicins) are inextricably involved in the regulation of cell life and death.

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1. Reed, J. C. (1994) *J. Cell Biol.* **124,** 1–6.
2. Yang, E. & Korsmeyer, S. J. (1996) *Blood* **88,** 386–401.
3. Hanada, M., Aimé-Sempé, C., Sato, T. & Reed, J. C. (1995) *J. Biol. Chem.* **270,** 11962–11968.
4. Sedlak, T. W., Oltvai, Z. N., Yang, E., Wang, K., Boise, L. H., Thompson, C. B. & Korsmeyer, S. J. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 7834–7838.
5. Yin, X. M., Oltvai, Z. N. & Korsmeyer, S. J. (1994) *Nature (London)* **369,** 321–333.
6. Reed, J. C. (1996) in *Behring Institute Mitteilungen*, eds. Krammer, P. H. & Nagata, S. (Behring Institute Mitteilungen, Marburg), Vol. 97, pp. 72–100.
7. Thompson, C. B. (1995) *Science* **267,** 1456–1462.
8. Krajewski, S., Tanaka, S., Takayama, S., Schibler, M. J., Fenton, W. & Reed, J. C. (1993) *Cancer Res.* **53,** 4701–4714.
9. Lithgow, T., van Driel, R., Bertram, J. F. & Strasser, A. (1994) *Cell Growth Differ.* **3,** 411–417.
10. Baffy, G., Miyashita, T., Williamson, J. R. & Reed, J. C. (1993) *J. Biol. Chem.* **268,** 6511–6519.
11. Lam, M., Dubyak, G., Chen, L., Nuñez, G., Miesfeld, R. L. & Distelhorst, C. W. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 6569–6573.
12. Marin, C. M., Fernandez, A., Bick, R. J., Brisbay, S., Buja, L. M., Snuggs, M., McConkey, D. J., von Eschenbach, A. C., Keating, M. J. & McDonnell, T. J. (1996) *Oncogene* **12,** 2259–2266.
13. Murphy, A. N., Bredesen, D. E., Cotopassi, G., Wang, E. & Fiskum, G. (1996) *Neurobiology* **93,** 9893–9898.
14. Ryan, J. J., Prochownik, E., Gottlieb, C. A., Apel, I. J., Merino, R., Nuñez, G. & Clarke, M. F. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 5878–5882.
15. Muchmore, S. W., Sattler, M., Liang, H., Meadows, R. P., Harlan, J. E., Yoon, H. S., Nettesheim, D., Changs, B. S., Thompson, C. B., Wong, S., Ng, S. & Fesik, S. W. (1996) *Nature (London)* **381,** 335–341.
16. Cleveland, M. V., Slatin, S., Finkelstein, A. & Levinthal, C. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 3706–3710.
17. Cramer, W. A., Heymann, J. B., Schendel, S. L., Deriy, B. N., Cohen, F. S., Elkins, P. A. & Stauffacher, C. V. (1995) *Annu. Rev. Biophys. Biomol. Struct.* **24,** 611–641.
18. Cramer, W. A., Zhang, Y.-L., Schendel, S., Merrill, A. R., Song, H. Y., Stauffacher, C. V. & Cohen, F. S. (1992) *FEMS Microbiol. Immunol.* **105,** 71–82.
19. Davidson, V. L., Brunden, K. R. & Cramer, W. A. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 1386–1390.
20. Donovan, J. J., Simon, M. I., Draper, R. K. & Montal, M. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 172–176.
21. Donovan, J. J., Simon, M. I. & Montal, M. (1982) *Nature (London)* **298,** 669–672.
22. Kagan, B. L., Finkelstein, A. & Colombini, M. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 4950–4954.
23. Parker, M. W., Postma, J. P. M., Pattus, F., Tucker, A. D. & Tsernoglou, D. (1992) *J. Mol. Biol.* **224,** 639–657.
24. Peterson, A. A. & Cramer, W. A. (1987) *J. Membr. Biol.* **99,** 197–204.
25. Shin, Y.-K., Levinthal, C., Levinthal, F. & Hubbell, W. L. (1993) *Science* **259**, 960–963.
26. Silverman, J. A., Mindell, J. A., Finkelstein, A., Shen, W. H. & Collier, R. J. (1994) *J. Biol. Chem.* **269,** 22524–22532.
27. Zakharov, S. D., Heymann, J. B., Zhang, Y.-L. & Cramer, W. A. (1996) *Biophys. J.* **70,** 2774–2783.
28. Montal, M. (1995) *Annu. Rev. Biophys. Biomol. Struct.* **24,** 31–57.
29. Montal, M., Montal, M. S. & Tomich, J. M. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 6929–6933.
30. Oblatt-Montal, M., Buhler, L. K., Iwamoto, T., Tomich, J. M. & Montal, M. (1993) *J. Biol. Chem.* **268,** 14601–14607.
31. Holm, L. & Sanders, C. (1993) *Nature (London)* **361,** 309.
32. Zha, H., Fisk, H. A., Yaffe, M. P. & Reed, J. C. (1996) *Mol. Cell. Biol.* **16,** 6494–6508.
33. Montal, M., Anholt, R. & Labarca, P. (1986) in *Ion Channel Reconstitution*, ed. Miller, C. (Plenum, New York), pp. 157–204.
34. Oblatt-Montal, M., Reddy, G. L., Iwamoto, T., Tomich, J. M. & Montal, M. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 1495–1499.
35. Borner, C., Martinou, I., Mattmann, C., Irmler, M., Scharer, E., Martinou, J.-C. & Tschopp, J. (1994) *J. Cell Biol.* **126,** 1059–1068.
36. Shiver, J. W., Cramer, W. A., Cohen, F. S., Bishop, L. J. & de Jong, P. J. (1987) *J. Biol. Chem.* **262,** 14273–14281.
37. Boise, L. H., Gonzalez-Garcia, M., Postema, C. E., Ding, L., Lindsten, T., Turka, L. A., Mao, X., Nuñez, G. & Thompson, C. B. (1993) *Cell* **74,** 597–608.
38. Sato, T., Hanada, M., Bodrug, S., Irie, S., Iwama, N., Boise, L. H., Thompson, C. B., Golemis, E., Fong, L., Wang, H.-G. & Reed, J. C. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 9238–9242.
39. Montal, M. O., Iwamoto, T., Tomich, J. M. & Montal, M. (1993) *FEBS Lett.* **320,** 261–266.
40. Montal, M. (1996) *Curr. Opin. Struct. Biol.* **6,** 499–510.
41. de Jong, D., Prins, F. A., Mason, D. Y., Reed, J. C., van Ommen, G. B. & Kluin, P. M. (1994) *Cancer Res.* **54,** 256–260.
42. Hockenbery, D. M., Nuñez, G., Milliman, C., Schreiber, R. D. & Korsmeyer, S. J. (1990) *Nature (London)* **348,** 334–336.
43. Guihard, G., Boulanger, P., Benedetti, H., Lloubes, R., Besnard, M. & Letellier, L. (1994) *J. Biol. Chem.* **269,** 5874–5880.
44. Donovan, J. J., Simon, M. I. & Montal, M. (1985) *J. Biol. Chem.* **260,** 8817–8823.
45. Reddy, L. G., Iwamoto, T., Tomich, J. M. & Montal, M. (1993) *J. Biol. Chem.* **268,** 14608–14615.
46. Hennet, T., Bertoni, G., Richter, C. & Peterhans, E. (1993) *Cancer Res.* **53,** 1456–1460.
47. Liu, X., Kim, C. N., Yang, J., Jemmerson, R. & Wang, X. (1996) *Cell* **86,** 147–157.
48. Susin, S. A., Zamzami, N., Castedo, M., Hirsch, T., Marchetti, P., Macho, A., Daugas, E., Geuskens, M. & Kroemer, G. (1996) *J. Exp. Med.* **184,** 1331–1342.
49. Zamzami, N., Susin, A., Marchetti, P., Hirsch, T., Gomez-Monterrey, I., Castedo, M. & Kroemer, G. (1996) *J. Exp. Med.* **183,** 1533–1544.
50. Minn, A. J., Valez, P. R., Schendel, S. L., Liang, H., Muchmore, S. W., Fesik, S. W., Fill, M. & Thompson, C. B. (1996) *Nature (London)* **385,** 353–357.
51. Guihard, G., Combettes, L. & Capiod, T. (1996) *Biochem. J.* **318,** 849–855.