Review

Bcl-2 family proteins as ion-channels

Sharon L. Schendel¹, Mauricio Montal² and John C. Reed^{1,3}

¹ The Burnham Institute, Program on Apoptosis and Cell Death Research, 10901 North Torrey Pines Road, La Jolla, California 92037, USA

² The University of California at San Diego, Department of Biology, 9500 Gilman Drive, La Jolla, California 92093-0366, USA

³ corresponding author: Dr Reed, tel: 619-646-3140; fax: 619-646-3194; email: jreed@burnham-institute.org

Received 30.9.97; revised 9.12.97; accepted 11.12.97

Edited by G. Melino

Abstract

The Bcl-2 protein family function(s) as important regulators of cellular decisions to heed or ignore death signals. The three-dimensional structure of the Bcl-2 homolog, Bcl-X_L, bears a strong resemblance to some pore-forming bacterial toxins. This similarity suggested that the Bcl-2 family proteins may also possess channel-forming capability. This review summarizes the recent initial studies on the *in vitro* channel activity of Bcl-2, Bcl-X_L and Bax and offers some speculation as to the physiological role that these channels may play in the cell death pathway.

Keywords: apoptosis; membrane; colicin

Abbreviations: BH, Bcl-2 homology; DT, diphtheria toxin; $\Delta\Psi_m$, mitochondrial membrane potential; PCD, programmed cell death; pS, picosiemens; PT, mitochondrial permeability transition

Introduction

Akin to a spy who carries a cyanide capsule with which to commit suicide upon capture, each cell in an animal species carries within it a program to direct its own death. An attenuated rate of programmed cell death (PCD) can result in disorders ranging from autoimmune disease to cancer, while an increased rate of PCD occurs in diseases such as AIDS or neurodegenerative disorders (e.g. Parkinson's disease) (Reed, 1994; Thompson, 1995). The gene encoding Bcl-2 (*B-cell lymphoma/leukemia-2*) was first identified on the basis of its involvement in B-cell malignancies where chromosomal translocations activate *bcl-2* in ~85% of follicular non-Hodgkin's B-cell lymphomas (Tsujimoto *et al*, 1985). Bcl-2 extends cell survival against apoptotic signals induced by a variety of treatments including: growth factor deprivation; ultraviolet and γ -radiation; heat shock; some cytotoxic lymphokines (i.e., tumor necrosis factor); calcium ionophores; viral infection; and agents that promote formation of free-radicals (Reed, 1994). The ability of Bcl-2 to protect

cells from such an assortment of insults, and the differing biochemical pathways which they utilize, suggests that Bcl-2 must control one or more of the distal steps in the cell death pathway.

Immunoprecipitation and yeast-two hybrid assays have aided in the expansion of the Bcl-2 family which now includes 16 cellular members and five viral homologs, among which are: Bcl-X_L, Bcl-X_S (Boise *et al*, 1993), Bax (Oltvai *et al*, 1993), Bak (Chittenden *et al*, 1995). The Bcl-2 family members are at cross-purposes; Bcl-2 and Bcl-X_L promote cell survival, while Bcl-X_S, Bax and Bak encourage cell demise. While Bcl-X_L shows the greatest degree of sequence identity with Bcl-2 (~45%), the pro-apoptotic proteins Bcl-X_S, Bax and Bak have pockets of sequence similarity, denoted BH1, 2, 3 and 4. The BH3 domain is present within all family members, while BH4 is found at the extreme N-terminus of primarily only anti-apoptotic proteins. Deletion mutagenesis has suggested that it is these regions of sequence similarity that govern protein-protein interactions between the family members to form either homo- or heterodimers (Yin *et al*, 1994; Sedlak *et al*, 1995; Zha *et al*, 1996a). The fate of the cell appears to rest with the relative amounts of these proteins, and the identity of the predominate protein complexes (Korsmeyer *et al*, 1993).

Cellular localization of Bcl-2, Bcl-X_L, Bax

With no readily identifiable sequence motifs, the biochemical mechanism by which the Bcl-2 family of proteins modulates apoptosis has remained elusive. Bcl-2, Bcl-X_L, and Bax are localized to the outer mitochondrial membrane via a C-terminal ~20-residue hydrophobic tail. (Krajewski *et al*, 1993; Gonzalez-Garcia *et al*, 1994; Zha *et al*, 1996b), although Bcl-2 and Bcl-X_L can be found in other sites as well, including the nuclear envelope and the ER. Immunoelectron microscopy revealed a non-uniform distribution of Bcl-2 in mitochondrial membranes, suggesting that it may be preferentially localized to zones of adhesion (ZOA) which join the outer and inner mitochondrial membrane (Krajewski *et al*, 1993; de Jong *et al*, 1994). Association of Bcl-2 with ZOAs, which are predicted to be sites of protein and ion import (Schatz, 1993), implies that Bcl-2 may be involved in regulating these activities.

Previously, Bcl-2 was predicted to have the ability to influence intracellular Ca²⁺ distribution (Baffy *et al*, 1993; Lam *et al*, 1994) and play a role in an antioxidant pathway (Hockenberry *et al*, 1993; Kane *et al*, 1993). However, the importance of these possibilities are questioned by studies which showed that the apoptotic pathway can proceed in an anaerobic environment and with decreased Ca²⁺ levels (Jacobson and Raff, 1995; Reynolds and Eastman, 1996; Shimizu *et al*, 1996). Alternatively, Bcl-2 is able to

counteract a decrease in mitochondrial membrane potential ($\Delta\Psi_m$), an early event in the apoptotic process, suggesting that Bcl-2 can perhaps balance perturbations in normal $\Delta\Psi_m$ (Zamzani *et al*, 1995). The mitochondrial membrane localization would thus place Bcl-2 at an advantageous site to participate in all of the above functions.

Bcl-X_L structure and implications for function

A new possibility for the biochemical function of the Bcl-2 family proteins is suggested by the recently determined 3-dimensional structure of Bcl-X_L (Muchmore *et al*, 1996). Bcl-X_L is comprised of 7 α -helices arranged in three layers. The outer two layers of amphipathic helices sandwich between them two long central α -helices (each approximately 20 residues) with a bias toward hydrophobic residues. A long loop devoid of secondary structure intervenes between the first and second helices. Interestingly, the sequence corresponding to this proline-rich 'loop' region of Bcl-X_L is absent in the pro-apoptotic members of the Bcl-2 family and poorly conserved among anti-apoptotic proteins such as Bcl-2 and Bcl-X_L. Although this loop is dispensable for the anti-apoptotic function of Bcl-X_L and Bcl-2, this region may be vulnerable to protease digestion (Strack *et al*, 1996) and contains phosphorylation sites (Chang *et al*, 1997). Post-translational modifications or conformational changes mediated via this domain may serve as a means for modulating the anti-apoptotic activity of Bcl-2 and Bcl-X_L (Minn *et al*, 1997).

The BH1, 2 and 3 domains fold together to give one face of the Bcl-X_L molecule a pronounced hydrophobicity. This structural feature, coupled with site-directed mutagenesis studies, suggests that hydrophobic protein-protein interactions may be the 'glue' that holds the Bcl-2 family homo- and heterodimers together. Indeed, a peptide corresponding to the BH3 region of Bak was found to be able to nestle into a crevice formed by the apposition of the BH1, BH2 and BH3 regions of Bcl-X_L (Sattler *et al*, 1997). This crevice is just wide enough to accommodate an α -helix and the Bak BH3 peptide obliges this structural constraint by undergoing a transition from random coil to α -helix upon binding to Bcl-X_L *in vitro*. This interaction is governed by both hydrophobic and electrostatic forces, and disruption of either by introduction of alanine at several sites along the Bak peptide's length was sufficient to abolish binding. BH3 peptides also disrupt dimerization among other members of the family, including Bcl-2 and Bax (Diaz *et al*, 1997).

A bacterial connection?

The Bcl-X_L structure bears a striking resemblance to the previously solved structures of the diphtheria toxin membrane-translocation domain (Choe *et al*, 1992) and the pore-forming domains of colicins A and E1 (Parker *et al*, 1992; Elkins *et al*, 1997). The diphtheria toxin, produced by *Corynebacterium diphtheriae*, is composed of two parts: the A and B fragments, which possess ADP-ribosylation and pore-forming activity, respectively (London, 1992). The diphtheria toxin gains access to the cell by receptor-mediated endocytosis and upon encountering the acidic pH inside the

endosome, the B fragment forms a channel in the endosomal membrane through which the A-fragment passes.

Colicins are plasmid-encoded protein antibiotics that are produced by and active against *Escherichia coli* and related strains. The ability to produce colicin is not a rare trait among *E. coli* inhabiting the human gut. Out of a survey of 234 *E. coli* strains isolated from healthy or diseased humans, more than 60% of the strains had the ability to produce at least one form of colicin (Achtman *et al*, 1983). Within populations of colicinogenic bacteria, a small fraction of the cells are producing colicin. Colicin expression is strongly repressed under normal conditions, but this repression can be relieved when the cell's DNA is irretrievably damaged (Pugsley and Oudega, 1987). Colicin producing cells are non-viable and thus colicin production is assumed to be a lethal event wherein some cells are sacrificed so that the community may survive, not unlike the clearing of aged or defective cells by apoptosis in animal species.

The pore-forming colicins kill sensitive cells via the formation of a highly conductive voltage-gated ion channel in the target cell's cytoplasmic membrane. This channel is non-specific and conducts monovalent ions with diffusion rates ($>10^6$ ions-channel⁻¹-sec⁻¹) that exceed the rate of cellular proton extrusion and results in depolarization of the cytoplasmic membrane. There are several consequences of this depolarization: inhibition of active transport; and depletion of intracellular K⁺, phosphate, and ATP stores either through hydrolysis or leakage through the colicin pore (Cramer *et al*, 1995).

Although colicins and diphtheria target very different organisms, the structures of both these bacterial toxins share a similar 'cloak and dagger' strategy in which the hydrophobic 'dagger' is shielded within a shell of amphipathic helices, allowing these proteins to lead 'dual lives' in that they may exist in a soluble state, but under certain conditions, will insert into membranes to form a channel (Oh *et al*, 1996; Shin *et al*, 1993; Donovan *et al*, 1982). This characteristic has made them the subject of numerous studies having the goal of determining how proteins insert into a membrane bilayer.

The structural similarity between Bcl-X_L and the pore-forming domains of bacterial toxins suggests that the Bcl-2 family of proteins should have pore-forming potential. The features of the Bcl-X_L structure should be applicable to the other Bcl-2 family members, an inference supported by the example of the pore-forming domains of colicins A, E1, and Ia, which share at most 30% sequence identity (Cramer *et al*, 1995), yet have highly similar 3-dimensional structures. Figure 1 shows the predicted structures of Bcl-2 and Bax which were modeled using the coordinates of Bcl-X_L.

Bcl-X_L, Bcl-2, and Bax pore formation *in vitro*

For the Bcl-2 protein family members to form pores they must contain helices that are long enough to span the membrane bilayer and these helices must be largely devoid of charged residues. Each residue of an α -helix contributes ~1.5 Å to its overall length. If an average lipid bilayer has a hydrophobic cross-section of ~30 Å (Montal and Mueller, 1972), then for an α -helix to span a membrane bilayer and participate in channel

formation, it must be ~20 residues long. Bcl-X_L has two helices that meet these criteria, and they are the two central helices (i.e., $\alpha 5$ and $\alpha 6$; Figure 1, as indicated). Although two helices are insufficient to enclose an aqueous lumen, the propensity for the Bcl-2 protein family members to form dimers suggests that two or more molecules could come together, each contributing their hydrophobic helices to create a pore.

To explore the pore-forming capability of the Bcl-2 protein family, several groups have undertaken studies of Bcl-2, Bcl-X_L, and Bax effects on planar lipid bilayers and lipid vesicles. Large, unilamellar vesicles that encapsulate either ions or fluorescent dyes are useful for observing channel formation. This technique monitors channel formation on a macroscopic level, in that for solute efflux from these vesicles to be detected, (particularly measurement of ion efflux) a majority of protein molecules must participate in channel formation. Demonstration of channel activity in vesicles discounts the possibility that only a very small fraction of molecules are inserting into the membrane bilayer. However, this method lacks the sensitivity to yield specific information concerning channel conductance, voltage-gating, or ion selectivity. These specific channel characteristics are best studied using the planar lipid bilayer configuration. In the planar bilayer approach, two chambers are divided by a septum having a small (50–200 μ M diameter) aperture in which the membrane bilayer is formed (for reviews see Montal and Mueller, 1972; Kagan and Sokolov, 1994). Consistent with the structural prediction, Bcl-2, Bcl-X_L, and most recently, Bax, have demonstrated avid pore-forming capability *in vitro* when assayed under conditions similar to those employed previously for assessing DT and colicin channel activity *in vitro*.

In vitro channel formation by the pore forming domains of both colicins and diphtheria toxin requires that several requirements be met: (i) the pH must be less than 4.0; (ii) the membrane must contain at least 10% negatively charged lipids; and (iii) a *trans*-negative membrane potential (i.e. the side of the membrane opposite to which the protein is added has an excess negative charge) must be present. With these requirements in mind, an assay used to measure *in vitro* colicin channel activity was carried out, except with the substitution of Bcl-X_L or Bcl-2 for colicin E1. Bcl-X_L and Bcl-2 induce solute efflux from KCl-loaded vesicles composed of 70% and 30% neutral and anionic lipid, respectively. Interestingly, Bcl-2 induced Cl[−]-efflux from KCl-loaded vesicles required sixfold less protein compared to Bcl-X_L (300 vs 1800 ng/mL). This variation could be attributable to the fact that the Bcl-2 protein preparation used for these studies was soluble only at acidic (pH 3.3) pH, while Bcl-X_L was soluble at pH 7. In the case of colicin E1, incubation of the protein at acidic pH prior to addition to liposomes results in a dramatically increased activity, as if the protein were 'primed' to interact with the membrane (S.L. Schendel and W.A. Cramer, unpublished observations). A similar situation could occur for Bcl-2 as well. Side-by-side comparison of colicin E1 channel peptide activity and that of Bcl-2 showed that only ~5-fold more Bcl-2 was necessary to induce an efflux rate similar to that of colicin E1, highlighting the pore-forming capability of Bcl-2. As with colicin E1, Bcl-X_L-induced significant ion efflux only when the pH was lower than 4.0. Channel activity was improved in the presence of the K⁺-specific ionophore valinomycin, which transports K⁺ ions out of the liposome, creating a *trans*-negative membrane

Figure 1 Ribbon diagrams of Bcl-2 and Bax modeled on the coordinates of Bcl-X_L using the QUANTA software package (Molecular Simulations, San Diego, CA). The putative membrane-spanning helices, $\alpha 5$ and $\alpha 6$, are the darker ones. The predicted loop of Bcl-2 is not shown

potential. When the same assay was performed with liposomes composed solely of neutral lipids, both Bcl-X_L and Bcl-2 lost all channel-forming activity.

Most studies of colicin and DT channel formation utilized the fragments of the toxins containing the pore-forming region which lacks domains required for binding to the integral membrane protein receptors on the surface of the target cells. The removal of these other regions from the toxin adds additional constraints for channel activity which are probably irrelevant *in vivo*. In the case of the colicin pore-forming domain, the low pH and acidic lipid requirements for *in vitro* channel activity can be explained in terms of lipid-protein interactions. The colicin E1 pore-forming domain has a number of lysine residues which are vital in docking the protein at the negatively charged membrane surface (Zakharov *et al*, 1996). The low pH also may induce a conformational change in the colicin molecule, presumably a loosening of the outer amphipathic shell that would expose the central hydrophobic helical hairpin, freeing it to insert into the membrane bilayer (van der Goot *et al*, 1991; Schendel and Cramer, 1994). Analogously, the Bcl-X_L and Bcl-2 proteins used in all studies to date lack the hydrophobic C-terminal anchor, which facilitates purification, but also relieves the protein of its normal means of membrane tethering. Therefore *in vitro*, it may be necessary to have acidic pH to increase the likelihood that the protein will 'find' the membrane surface. Bcl-X_L and Bcl-2 have a slightly net negative charge at neutral pH and therefore the acidic pH could be necessary to protonate these negatively charged residues and the resultant charge neutralization could increase the hydrophobicity of the protein, making it more likely to insert into the membrane bilayer.

To gain information about the details of Bcl-X_L and Bcl-2 channel formation, their activities on planar bilayers were examined (Minn *et al*, 1997; Schendel *et al*, 1997). Upon addition of the proteins to one side of the planar bilayer apparatus, a voltage can be applied at a constant value. Any resultant current can be plotted as a function of the applied voltage which yields the channel conductance. The channel conductance is expressed in terms of siemens (S), and the channel conductances observed for the Bcl-2 protein family are in the picosiemen (pS) range. The

conductance (g) is readily obtained using Ohm's law from a plot of current vs voltage (I-V plot) by dividing the current (expressed in Ampere, A) by the voltage difference across the membrane (V) ($I=gV$) (Hille, 1984) (Box 1).

At pH 4.0, after imposition of 20 mV potential, Bcl-X_L rapidly (<5 min) incorporated into the lipid bilayer to give a conductance that increased in a step-wise fashion, eventually to levels where resolution of single-channel conductance was difficult. In contrast to the liposome measurements, channel formation by Bcl-X_L could be detected at neutral pH. Channel formation at this pH was a much rarer event, with a lag time between addition of protein to observation of channel formation increasing to ~30 min. At both pH 7.2 and pH 4.0, the relationship between voltage and current was Ohmic in that a plot of current vs applied voltage is linear. The Bcl-X_L channel displayed multiple conductance states with the most common conductance value being 276 pS for Bcl-X_L and three other conductance states of 80, 134, and 179 pS in 0.15 M KCl (Table 1).

Box 1: Definitions of channel parameters obtained from single channel recordings using planar lipid bilayers.

- * Channel conductance is expressed in units of siemens: the reciprocal of an ohm.
- * Channel conductance (g) is given by $g=I/V$ where I is the current flowing through an open channel in response to an applied voltage V (the membrane potential).
- * In the case of a channel having a 20 pS conductance: a current of 2 pA (picoamperes) would flow across the lipid bilayer at a membrane potential of 100 mV. Since an ampere is the flow of 6.24×10^{18} charges/sec. it follows that about 10 million ions flow through the open 20 pS channel.

Table 1 Single channel characteristics of Bcl-2 family proteins reconstituted in planar lipid bilayers

Protein	pH	Voltage (mV)	Salt (mM)	Conductance States (pS)	Ion Selectivity	Reference
Bcl-2	7.4	100	500 KCl	20 40 90	Cation	Schendel <i>et al</i> , 1997
Bcl-X _L	7.2	20	150/50 KCl (cis:trans)	276 179 134 80	Cation	Minn <i>et al</i> , 1997
Bax	7.0	100	125 NaCl	26 80 180 250 2000	Cation	Antonsson <i>et al</i> , 1997
Bax	7.0	40	450/150 KCl (cis:trans)	329	Anion	Schlesinger <i>et al</i> , 1997

When assayed in planar bilayers, Bcl-2 also displayed a linear relationship between current and voltage, in that the current conducted at +100 and -100 mV was identical except for opposite direction of current flow. However, the Bcl-2 channel's conductance states were much different than those detected for Bcl-X_L. The primary conductance levels for Bcl-2 were ~1/10 that of Bcl-X_L, with the most common channel events having conductance of 20 and 40 pS in 0.5 M KCl (Table 1). A larger conductance of 90 pS was also observed, albeit only rarely. Each of these conductance levels was a unique channel activity and not representative of several simultaneous openings of 20 pS channels (Schendel *et al*, 1997). The conductances of Bcl-2 channels were nearly the same at neutral and low pH, but at lower pH the Bcl-2 channels opened more frequently, suggesting that channel insertion increases markedly.

Structural implications of conductance measurements

The 20 pS conductance level of Bcl-2 is highly similar to that observed for colicin E1 in planar lipid bilayers (Bullock *et al*, 1983). The colicin E1 channel is predicted to consist of a bundle of four α -helices, two hydrophobic, and two amphipathic, which penetrate the membrane bilayer spontaneously and upon imposition of a *trans*-negative membrane potential, respectively (Elkins *et al*, 1997). Although Bcl-2 would be predicted to possess only two helices of sufficient length to span a membrane bilayer, two Bcl-2 monomers could come together to form a four helix bundle, with each contributing two α -helices to the channel. Alternatively, studies employing synthetic peptides which insert into membranes to form pentameric helical bundles gave rise to ~40 pS channel conductances (Montal *et al*, 1993). For this situation to occur, additional helices of Bcl-2 could insert into the membrane, although the other predicted helices, while possibly long enough, contain residues which would entail significant energy costs to insert into the hydrophobic membrane bilayer (e.g. Lys, Arg) or residues traditionally considered to disrupt helices (e.g. Pro, Gly). Although these studies were performed with Bcl-2 lacking its C-terminal anchoring region, it is conceivable that this region of the protein, while dispensable for function *in vivo* (Borner *et al*, 1994), could contribute to channel formation. More likely, however, the larger conductances measured for Bcl-2 and particularly for Bcl-X_L could result from oligomerization of three or more molecules integrated into the lipid bilayer.

Ion selectivity of Bcl-2 and Bcl-X_L channels

Ion selectivity of a channel can also be determined using planar bilayer measurements. The selectivity is a preferential ability of a channel to conduct a given ionic species more efficiently than others and can be expressed in terms of the ratio of permeability or conductance values for the ions under comparison. The pore selectivity can be affected by the residues lining the mouth and lumen of the pore, e.g., the presence of negatively charged residues in the lumen of the pore would be expected to impede the passage of anions through the pore. When channel activity is measured in a

planar bilayer apparatus in which the ion concentration of the *cis*-compartment differs from that in the *trans*-, there occurs a potential at which the current is zero, which appears as the y-intercept on a current vs voltage plot.

This zero-current potential can be related to the permeability ratios of the ions under consideration by the following equation:

$$E_{rev} = 59.1 \frac{\log P_A [A]}{P_B [B]}$$

where E_{rev} is the zero-current potential and P_A and P_B represent the permeabilities of the ion A and B, where both ions have identical valences. Using a variety of solutes and concentration gradients, and measuring the point at which no net current flows, it is possible to determine the ion selectivity.

The ion selectivity of the Bcl-X_L channel varied as a function of pH, having a permeability ratio between K⁺ and Cl⁻ (P_K/P_{Cl}) of 1.05 and 4.31 at pH 4.0 and 7.2, respectively. This indicates that at pH 4.0 there exists no barrier within the lumen that would impede the passage of either cations or anions, but at pH 7.2, cations pass

Figure 2 Helical wheel analysis of predicted α -helices 5 and 6 of Bcl-2, Bcl-X_L, and Bax. Residues for Bcl-2 and Bax were selected on the basis of sequence alignment with Bcl-X_L. The plots were constructed based on the α -helical periodicity of $i \dots i+4$. Positive and negative residues are highlighted with a circle and square, respectively. The charged face of the helix would be predicted to line the channel lumen

through the channel with much greater ease. This result supports the prediction that Bcl-X_L should prefer to pass cations, as a α -helical wheel analysis of the putative membrane insertion domain shows a bias toward acidic residues on one face of the helices (Figure 2). Presumably at pH 4.0, these residues would be protonated and would not interfere with the passage of either cations or anions, but at pH 7.2 the negative charge on these residues should discourage anion passage. At pH 5.4, Bcl-2 was also 90% cation selective (Schendel *et al*, 1997). Again, this agrees well with a α -helical wheel analysis of the putative fifth and sixth helices of Bcl-2, which suggested that the faces of these helices predicted to line the channel lumen have residues with acidic side chains (Figure 2).

To explore whether the predicted 5th and 6th helices of Bcl-2 were indeed responsible for its observed ion channel activity, this region was deleted resulting in a protein that is highly similar to the alternatively spliced variant of Bcl-X_L, Bcl-X_S. The loss of this region converted Bcl-2(Δ h5,6) to a pro-apoptotic protein, similar to Bcl-X_S. Bcl-2(Δ h5,6) promiscuously promoted low-level ion efflux from KCl-loaded vesicles in that identical amounts of ion flux were detected under all conditions tested, even those where Bcl-2 failed to display activity, such as on 100% neutral lipid vesicles and at pH values above pH 4.5 (Schendel *et al*, 1997). This non-specific behavior may be due to the amphipathic nature of this shortened Bcl-2 molecule, in that it may be interacting with the membrane surface in much the same way an amphipathic detergent would.

In planar bilayers, Bcl-2(Δ h5,6) failed to form discrete channels, but produced only random stray conductances. These results suggest that pore formation of Bcl-2, and presumably Bcl-X_L, absolutely requires the presence of the hydrophobic central 5th and 6th helices.

Bax *in vitro* channel formation

The most recent member of the Bcl-2 protein family documented to display pore-forming activity is the pro-

apoptotic protein Bax (Antonsson *et al*, 1997). As with Bcl-X_L and Bcl-2, Bax lacking its C-terminal hydrophobic anchor was used. As with Bcl-2, only nanomolar concentrations of Bax were necessary to induce a healthy efflux of liposome-encapsulated fluorescent dye carboxyfluorescein at pH 4.0. But in contrast to Bcl-2 and Bcl-X_L, detectable levels of dye efflux from liposomes were noted even up to pH 7.4, although at ~8-fold lower amounts. Whether or not this result indicates that the channel properties of Bax differ from that of Bcl-2 and Bcl-X_L is unclear, as the inactivity of Bcl-2 at pH 7.4 could be attributable to its low solubility in the absence of detergents at this pH. Moreover, it should be noted that the colicin E1 pore-forming domain has detectable activity using a similar dye-encapsulation method, although it shows no appreciable activity with measurements of ion-efflux at neutral pH using electrodes. Thus, the capability of this fluorescence-based assay to detect much lower amounts of solute loss prevents a direct comparison with the findings obtained by other groups who employed methods relying on electrodes to detect ion-efflux.

In planar bilayer measurements, Bax was able to increase membrane conductance at both pH 4 and 7 within 10–60 min after Bax addition (Antonsson *et al*, 1997). At pH 4.0, unitary conductance steps similar to those detected for Bcl-2 were seen: 27 and 77 pS in 125 mM NaCl (Table 1). These conductances were transient, consistent with the behavior of Bcl-2 and Bcl-X_L. The conductances increased as the pH was raised to 7.0 and sub-populations of channels having conductances at 26, 80, 180 and 250 pS were detected. As the measurement progressed with time, larger conductances occurred in steps of 450 pS resulting in a final conductance near 2 nS. Recent studies of Bax channel activity in planar bilayers were in agreement with these results in that a predominant conductance state of 300 pS was detected at pH 7.0 (Schlesinger *et al*, 1997).

Unlike the channels observed for Bcl-2 and Bcl-X_L, the Bax channel did not display a linear relationship between current and voltage (Antonsson *et al*, 1997; Schlesinger *et*

Figure 3 Schematic representation of Bcl-2 tethered to a membrane via its hydrophobic C-terminal region. The BH4 and BH3 domains involve α -helices 1 and 2, respectively. The loop connecting α -helices 1 and 2 is shown devoid of secondary structure, as suggested by the Bcl-X_L structure (Muchmore *et al*, 1996). Upon membrane integration, α -helices 5 and 6 are postulated to insert into the membrane bilayer. This insertion could be accompanied by a conformational change which could free the BH4 and BH3 domains for interactions with other proteins. Presumably, at least two Bcl-2 molecules would participate to form a conductive channel consistent with a four-helix bundle

al, 1997). Application of a *cis*-negative voltage enhanced channel formation, suggesting that channel formation by Bax is voltage-dependent. It must be borne in mind that this voltage dependence could reflect either the steps of Bax protein insertion into membranes or opening/formation of channels after insertion. The membrane potential dependence is opposite that observed for the colicins, which insert into planar bilayers upon imposition of a *cis*-positive voltage (Cramer *et al*, 1995). Bax channels were more weakly ion selective at pH 7.0 than were either Bcl-2 or Bcl-X_L having a permeability ratio of Na⁺ to Cl⁻ of 2.1, nearly half that of Bcl-X_L. This result differs from a recent study of Bax channel characteristics wherein Bax channels were anion-selective at pH 7.0 ($P_K/P_{Cl}=0.5$) (Schlesinger *et al*, 1997). The actual ion preference of the Bax channel thus remains unsettled, however the bias towards positively charged side chains in the predicted 5th and 6th helices of Bax (Figure 1) would appear to support the latter study's findings.

Though highly controversial, it is tempting to speculate that the presence of a proton gradient and membrane potential across the inner mitochondrial membrane may somehow impact on the processes of protein insertion or channel formation/opening, particularly at the contact sites where the inner and outer membranes come together and where Bcl-2 family proteins tend to be concentrated.

Antonsson *et al* (1997), reported that in lipid vesicles at neutral pH Bcl-2 was able to prevent Bax-channel formation. When Bcl-2 was incubated with carboxyfluorescein-loaded vesicles at pH 7.0 (where Bcl-2 would have no activity) prior to the addition of Bax at a 1:10 Bax-Bcl-2 ratio, Bax channel activity was abolished (Antonsson *et al*, 1997). This antagonism of Bax channel activity was specific to Bcl-2 as control proteins had no effect on channel formation. It is unclear as to the nature of the interaction between Bcl-2 and Bax that impedes channel formation, and at what point the interaction occurs. For example, Bcl-2 might interfere with Bax by binding to it in solution before it contacts membranes or inserts into membranes. Assays of Bcl-2 vesicle association done in this laboratory indicate that at pH 7.0, Bcl-2 has an affinity for the membrane surface. This result would suggest that Bcl-2 would be bound to the membrane surface, but unable to insert into the membrane bilayer at pH 7.0. Bax-Bcl-2 interactions at the membrane surface could prohibit Bax from undergoing the transition from the soluble to membrane-inserted state (Figure 3). At pH 4.0, however, where Bcl-2 displays its own channel-forming capability, the addition of Bcl-2 and Bax together to a vesicle suspension causes an enhanced rate of efflux, presumably due to an additive effect (our unpublished results). Thus, further investigations of the interactions between Bax and Bcl-2 channels are required to gain better understanding of how Bcl-2 might antagonize Bax channel formation in some cases and not others. In particular, studies of interactions of Bcl-2 and Bax channels in planar bilayers should be very informative.

In vivo significance of pore formation

The idea of the Bcl-2 family proteins functioning as pore-forming proteins is still in its infancy and explanations of a

physiological role for pore formation by these proteins are at best highly speculative. Although pore forming propensity appears to be a trait that is shared by several of the Bcl-2 family members, it remains to be demonstrated whether these proteins actually form pores *in vivo*, and if they indeed do, what is the functional significance of the resultant pore. It is also unclear why both pro- and anti-apoptotic proteins should share pore forming capability, although the recent demonstration that under certain conditions Bcl-2 can block Bax pore formation partially relieves this dilemma. Other than what can be indirectly gleaned from ion conductance measurements, there is as yet no information regarding the *in vivo* channel lumen diameters of the Bcl-2 family channels and this characteristic will be important in defining just what sorts of molecules these channels can accommodate in their lumens. Also, it is unclear what the molecularity of the channels are, although it seems clear that the channels must be formed by more than one molecule, as the two central hydrophobic helices are insufficient to completely line a channel lumen. Several possibilities for how Bcl-2, Bcl-X_L, and Bax can regulate apoptosis through pore formation can be envisioned.

Bcl-2 regulation of mitochondrial permeability transition

One early event associated with apoptosis is a decrease in the mitochondrial membrane potential (Zamzani *et al*, 1995), and this increase involves the so-called mitochondrial 'permeability transition'. This phenomenon is characterized by an increased permeability of the inner mitochondrial membrane to solutes ≤ 1500 Da (Zoratti and Szabo, 1995) which is caused by the opening of pores formed by multiprotein complexes situated at mitochondrial membrane contact sites. The PT may play an important role in regulation of Ca²⁺ homeostasis, as recently demonstrated (Schinder *et al*, 1996; Ichas *et al*, 1997). One important feature of the PT is its voltage dependence and its openings and closing are highly dependent on the mitochondrial membrane potential ($\Delta\Psi_m$). Therefore, a drop in $\Delta\Psi_m$ such as that which occurs early during apoptosis could result in PT opening. The overexpression of Bcl-2 prevents this early perturbation of $\Delta\Psi_m$ and the subsequent PT opening. Bcl-2 pore formation could serve to offset ion imbalances that may result in a lowered $\Delta\Psi_m$, and thus restore $\Delta\Psi_m$ to normal levels, preventing PT opening. Interestingly, patch clamp measurement of channel conductances present in a mitochondrial fraction enriched in contact sites yielded evidence for a variety of channels, some in the 10–80 pS range, others conducting at 475 and 550 pS (Moran and Sorgato, 1992), which corresponds to channel conductances observed for Bcl-2, Bax, and Bcl-X_L, respectively. A new method to study intracellular ion channels *in situ* using double-barreled patch clamp electrodes may provide access to record directly the PT channels and their regulation by Bcl-2 family proteins (Jonas *et al*, 1997).

Another possibility for the function of the Bcl-2 family pores is as conduits for proteins. The role of cytochrome (Cyt c) release from mitochondria during apoptosis has taken on an increased importance. Cyt c, which normally skitters across the mitochondrial inner membrane as part of the electron transport chain is somehow freed from the confines of the

mitochondrial inter-membrane space and after gaining access to the cytosol, plays a role in the activation of caspases, a family of proteases involved in the ultimate degradation of the cellular framework (Kluck *et al*, 1997). Bcl-2 was able to block the release of Cyt *c* from the mitochondria in intact cells and in a cell-free extract, and it did so at the level of the mitochondria, as addition of exogenous Cyt *c* circumvented this blockage. Perhaps Cyt *c* is escaping the mitochondria using the PT pore as its portal, and Bcl-2 with its ability to block PT is shutting this escape hatch. Alternatively, Bax, which also situated in the outer mitochondrial membrane, perhaps also at contact sites, could provide the gateway to cytosol for Cyt *c*. Again, Bcl-2 with its ability to block Bax pore formation under certain conditions could conceivably block release of Cyt *c* into the cytosol.

Lastly, the insertion of Bcl-2, Bax, and Bcl-X_L proteins into membranes may be of greater importance to their function as adaptor or docking proteins than for pore formation. Bcl-2 and Bcl-X_L have shown affinity for a host of proteins, among them the protein kinase Raf-1, the protein phosphatase calcineurin, as well as other proteins with ill-defined functions such as CED-4 and the Hsp70/Hsc90 modulating protein BAG-1 (reviewed in Häcker and Vaux, 1995). In contrast, Bax shows no affection for these proteins, perhaps due to its lack of a BH4 domain, which is important for several of these interactions (Wang *et al*, 1996). It is possible that the BH4 domain is tucked out of reach in the uninserted state of Bcl-2 and Bcl-X_L, but becomes accessible upon insertion of the central hydrophobic helices (Figure 2). Similarly, recent structural studies (Sattler *et al*, 1997) of the Bak BH3 peptide bound to the aforementioned groove suggest that the peptide insertion could prevent access or freeing of the BH3 domain for interaction with other BH3-containing proteins.

Much remains to be explored concerning pore formation by the trio of Bcl-2, Bcl-X_L, and Bax, and even whether the other members of the continually growing Bcl-2 family share the channel-forming trait. In this regard, it seems highly unlikely based on sequence comparisons and secondary structure predictions that the 'BH3-only' branch of the Bcl-2 family (Bik, Bad, Bak and Hrk) will form channels. Thus, these members of the Bcl-2 family seem likely to be relegated to the role of trans-dominant inhibitors of anti-apoptotic proteins such as Bcl-2 and Bcl-X_L. Of utmost importance will be the elucidation of the *in vivo* importance of the pores created by Bcl-2, Bcl-X_L, Bax and other structurally similar members of the Bcl-2 family. How these proteins make the transition from a soluble to membrane-inserted state, the dynamics and reversibility of the process, as well as which protein-protein interactions regulate this event are also critical questions. With continued investigations, however, the answers to these and other related questions are likely to shed bright light on one of the most important points for regulating the pathways of programmed cell death and apoptosis.

Acknowledgements

Research at the University of California San Diego is supported by NIH (GM-49711). We thank R Kodandapani for the modeling of Bax and Bcl-2

and S McDonald and M Hasham for their help in the preparation of Figure 2. SLS is supported by NIH Postdoctoral Training grant (1-T32 AG00252-01).

References

- Achtman M, Mercer A, Kusecek B, Pohl A, Heuzenroeder M, Aaronson W, Surron A and Silver RP (1983) Six widespread bacterial clones among *Escherichia coli* K1 isolates. *Infect. Immun.* 39: 315–335
- Antonsson B., Contin F, Ciavatta A, Montessuit S, Elwis S, Martinou I, Bernasconi L, Bernard A, Mermoud J-J, Mazzei G, *et al.* (1997) Inhibition of Bax channel-forming activity by Bcl-2. *Science* 277: 370–372
- Baffy G, Miyashita T, Williamson JR and Reed JC (1993) Apoptosis induced by withdrawal of interleukin-3 [IL-3] from an IL-3 dependent hematopoietic cell line is associated with repartitioning of intracellular calcium and is blocked by enforced Bcl-2 production. *J. Biol. Chem.* 268: 6511–6519
- Boise LH, Gonzales-Garcia M, Postema CE, Ding L, Lindsten T, Turka LA, Mao X, Nunez G and Thompson CB (1993) Bcl-x a Bcl-2 related gene that functions as a dominant regulator of apoptotic cell death. *Cell* 74: 597–608
- Borner C, Martinou I, Mattmann C, Irmier M, Schaefer E, Martinou J-C and Tschoop J (1994) The protein bcl2 does not require membrane attachment, but two conserved domains to suppress apoptosis. *J. Cell Biol.* 126: 1059–1068
- Bullock JO, Cohen FS, Dankert JR and Cramer WA (1983) Comparison of the macroscopic and single channel conductance properties of colicin E1 and its COOH-terminal tryptic peptide. *J. Biol. Chem.* 258: 9908–9912
- Chang BS, Minn AJ, Muchmore SW, Fesik SW and Thompson CB (1997) Identification of a novel regulatory domain in Bcl-X_L and Bcl-2. *EMBO J.* 16: 968–977
- Chittenden T, Harrington EA, O'Connor R, Flemington C, Lutz RJE and Guild BC (1995) Induction of apoptosis by the Bcl-2 homologue Bak. *Nature* 374: 733–736
- Choe S, Bennett MJ, Fujii G, Curmi PMG, Kantardjieff KA, Collier RJ and Eisenberg D (1992) The crystal structure of diphtheria toxin. *Nature* 357: 216–222
- Cramer WA, Heymann JB, Schendel SL, Deriy BN, Cohen FS, Elkins PA and Stauffacher CV (1995) Structure-function of the channel-forming colicins. *Annu. Rev. Biophys. Biomol. Struct.* 24: 611–641
- de Jong D, Prins FA, Mason DY, Reed JC, van Ommen GB and Kluin PM (1994) Subcellular localization of the bcl-2 protein in malignant and normal lymphoid cells. *Cancer Res.* 54: 256–260
- Diaz J-L, Oltersdorf T, Horne W, McConnell M, Wilson G, Weeks S, Garcia T and Fritz LC (1997) A common binding site mediates heterodimerization and homodimerization of Bcl-2 family members. *J. Biol. Chem.* 272: 11350–11355
- Donovan JJ, Simon MI, and Montal M (1982) Insertion of diphtheria toxin into and across membranes: role of phosphoinositide asymmetry. *Nature* 298: 669–672
- Elkins P, Bunker A, Cramer WA and Stauffacher CV (1997) A mechanism for toxin insertion into membranes is suggested by the crystal structure of the channel-forming domain of colicin E1. *Structure* 5: 443–458
- Gonzalez-Garcia M, Perez-Ballesteros RP, Ling L, Duan L, Boise LH, Thompson CB and Nunez G (1994) bcl-xl is the major bcl-x mRNA form expressed during murine development and its product localizes to mitochondria. *Development* 120: 3033–3042
- Häcker G and Vaux DL (1995) A sticky business. *Curr. Biol.* 622–624
- Hille B. (1984) *Ionic Channels of Excitable Membranes*. Sunderland, Massachusetts, Sinauer Associates Inc.
- Hockenberry DM, Oltvai ZN, Yin X-M, Millman CL and Korsmeyer SJ (1993) Bcl-2 functions in antioxidant pathway to prevent apoptosis. *Cell* 75: 241–251.
- Ichas F, Jouaville S and Mazat J-P (1997) Mitochondria are excitable organelles capable of generating and conveying electrical and calcium signals. *Cell* 89: 1145–1153
- Jacobson MD and Raff MC (1995) Programmed cell death and Bcl-2 protection in very low oxygen. *Nature* 374: 814–816
- Jonas EK, Knox RJ, and Kaczmarek LK. (1997) Giga-Ohm seals on intracellular membranes: a technique for studying intracellular ion channels in intact cells. *Neuron* 19: 7–13
- Kagan BL and Sokolov Y. (1994) Use of lipid bilayer membranes to detect pore formation by toxins. *Meth. Enzymol.* 235: 691–705
- Kane DJ, Sarafin TA, Auton S, Hahn H, Gralla FB, Valentine JC, Ord T and Bredesen DE (1993) Bcl-2 inhibition of neural cell death: decreased generation of reactive oxygen species. *Science* 262: 1274–1276

- Gluck RM, Bossy-Wetzel E, Green DR and Newmeyer DD (1997) The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* 275: 1132–1136
- Korsmeyer SJ, Shutter JR, Veis DJ, Merry DE and Oltvai ZN (1993) Bcl-2/Bax: a rheostat that regulates an anti-oxidant pathway and cell death. *Canc. Biol.* 4: 327–332
- Krajewski S, Tanaka S, Takayama S, Schibler JJ, Fenton W and Reed JC (1993) Investigation of the subcellular distribution of the bcl-2 oncoprotein: residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes. *Canc. Res.* 53: 4701–4714
- Lam M, Dubyak G, Chen L, Nunez G, Miesfeld RL and Distelhorst CW (1994) Evidence that Bcl-2 represses apoptosis by regulating endoplasmic reticulum-associated Ca^{2+} -fluxes. *Proc. Natl. Acad. Sci. U.S.A.* 91: 6569–6573
- London E. (1992) Diphtheria toxin: membrane interaction and membrane translocation. *Biochim. Biophys. Acta.* 1113: 25–51
- Minn AJ, Velez P, Schendel SL, Liang H, Muchmore SW, Fesik SW, Fill M and Thompson CB (1997) Bcl-X_L forms an ion channel in synthetic lipid membranes. *Nature* 285: 353–357
- Montal MO, Iwamoto T, Tomich JM and Montal M (1993) Design, synthesis and functional characterization of a pentameric channel protein that mimics the presumed pore structure of the nicotinic cholinergic receptor. *FEBS Lett* 320: 261–266
- Montal M and Mueller P. (1972). Formation of bimolecular membranes from lipid monolayers and a study of their electrical properties. *Proc Natl Acad Sci U.S.A.* 69, 3561–3566
- Moran O and Sorgato MC (1992) High-conductance pathways in mitochondrial membranes. *J. Bioenerg. Biomembr.* 24: 91–98
- Muchmore SW, Sattler M, Hwang H, Meadows RP, Harlan JE, Yoon HS, Nettesheim D, Chang BS, Thompson CB, Wong S-L, *et al.* (1996) X-ray and NMR structure of human BclX_L, an inhibitor of programmed cell death. *Nature* 381: 335–341
- Oh KJ, Zhan H, Cui C, Hideg K, Collier RJ, and Hubbel WL (1996) Organization of diphtheria toxin T domain in bilayers: a site-directed spin labeling study. *Science* 273: 810–812
- Oltvai ZN, Millman CL and Korsmeyer SJ (1993) Bcl-2 heterodimerizes with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* 74: 609–619
- Parker MW, Postma JPM, Pattus F, Tucker AD and Tsernoglou D (1992) Refined structure of the pore-forming domain of colicin A at 2.4 Å resolution. *J. Mol. Biol.* 224: 639–657
- Pugsley AP and Oudega B (1987) Methods for studying colicins and their plasmids. In *Plasmids: a practical approach*. (ed.) Oxford: IRL Press.
- Reed JC (1994) Bcl-2 and the Regulation of Programmed Cell Death. *J. Cell Biol.* 124: 1–6
- Reynolds JE and Eastman A (1996) Intracellular calcium stores are not required for Bcl-2-mediated protection from apoptosis. *J. Biol. Chem.* 271:
- Sattler M, Liang H, Nettesheim D, Meadows RP, Harlan JE, Matthias E, Yoon HS, Shuker SB, Chang BS, Minn AJ, *et al.* (1997) Structure of Bcl-Bak peptide complex: recognition between regulators of apoptosis. *Science* 275: 983–986
- Schatz G (1993) The protein import machinery of mitochondria. *Prot. Sci.* 2: 141–146
- Schendel SL and Cramer WA (1994) On the nature of the unfolded intermediate in the *in vitro* transition of the colicin E1 channel domain from the aqueous to the membrane phase. *Prot. Sci.* 3: 2272–2279
- Schendel SL, Xie Z, Montal MO, Matsuyama S, Montal M and Reed JC (1997) Channel formation by anti-apoptotic protein Bcl-2. *Proc. Natl. Acad. Sci. U.S.A.* 93: 511–5118
- Schlesinger PH, Gross A, Yin X-M, Yamamoto K, Saito M, Waksman G, Korsmeyer SJ. (1997) Comparison of the ion channel characteristics of proapoptotic BAX and antiapoptotic BCL-2. *Proc. Natl. Acad. Sci. U.S.A.* 94: 11357–11362
- Schinder AF, Olson EC, Spitzer NC, and Montal M. (1996) Mitochondrial dysfunction is a primary event in glutamate neurotoxicity. *J. Neurosci.* 16: 6125–6133
- Sedlak TW, Oltvai ZN, Yang E, Wang K, Boise LH, Thompson CB and Korsmeyer SJ (1995) Multiple Bcl-2 family members demonstrate selective dimerizations with Bax. *Proc. Natl. Acad. Sci. U.S.A.* 92: 7834–7838
- Shimizu S, Eguchi Y, Kamiike W, Waguri S, Uchiyama Y, Matsuda H, and Tsujimoto Y. (1996) Bcl-2 blocks loss of mitochondrial membrane potential while ICE inhibitors at a different step during inhibition of death induced by respiratory chain inhibitors. *Oncogene* 13: 21–29
- Shin YK, Levinthal C, Levintahl F, and Hubbel WL. (1993) Colicin E1 binding to membranes: time-resolved studies of spin-labeled mutants. *Science* 259: 960–963
- Strack PR, Frey MW, Rizzo CJ, Cordova B, George HJ, Meade R, Ho SP, Corman J, Tritch R, Korant BD. (1996) Apoptosis mediated by HIV protease is preceded by cleavage of Bcl-2. *Proc. Natl. Acad. Sci. U.S.A.* 93: 9571–9576
- Thompson CB (1995) Apoptosis in the pathogenesis and treatment of disease. *Science* 267: 1456–1462
- Tsujimoto Y, Cossman J, Jaffe E and Croce C (1985) Involvement of the bcl-2 gene in human follicular lymphoma. *Science* 228: 1440–1443
- van der Goot FG, González-Mañas JM, Lakey JH and Pattus F. (1991) A 'molten-globule' membrane-insertion intermediate of the pore-forming domain of colicin A. *Nature* 354: 408–410
- Wang HG, Rapp UR and Reed JC (1996) Bcl-2 targets the protein kinase Raf-1 to mitochondria. *Cell* 87: 629–638
- Yin X-M, Oltvai ZN and Korsmeyer SJ (1994) BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax. *Nature* 356: 312–323
- Zakharov SD, Heymann JB, Zhang Y-L and Cramer WA (1996) Membrane binding of the colicin E1 channel: activity requires an electrostatic interaction of intermediate magnitude. *Biophys. J.* 70: 2774–2783
- Zamzani N, Marchetti P, Castedo M, Decaudin D, Macho A, Hirsch T, Susin SA, Petit PX, Mignotte B and Kroemer G. (1995) Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. *J. Exp. Med.* 182: 367–377
- Zha H, Aime-Sempe C, Sato T and Reed JC (1996a) Proapoptotic protein bax heterodimerizes with Bcl-2 and homodimerizes with Bax via novel domain (BH3) distinct from BH1 and BH2. *J. Biol. Chem.* 271: 7440–7444
- Zha H, Fisk HA, Yaffe MP, Mahajan N, Herman B and Reed JC (1996b) Structure-function comparisons of the proapoptotic protein Bax in yeast and mammalian cells. *Mol. Cell. Biol.* 16: 6494–6508
- Zoratti M and Szabo I (1995) The mitochondrial permeability transition. *Biochim. Biophys. Acta. Rev. Biomembr.* 1241: 139–176