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Invited Review

Mechanisms of Bcl-2 protein function

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Summary. The Bcl-2 protein blocks a distal step in an evolutionarily conserved pathway for programmed cell death and apoptosis. The gene encoding this protein was first discovered because of its involvement in the t(14;18) chromosomal translocations commonly found in B-cell lymphomas. Overexpression of Bcl-2 also occurs in many other types of human cancers, and prevents cell death induced by nearly all anticancer drugs and radiation. Since the discovery of Bcl-2 over ten years ago, several cellular and viral homologs have been identified, some of which suppress cell death and others which promote apoptosis. Many of these proteins can interact with each other through a complex network of homo- and heterodimers. Though functionally important, dimerization events still do not explain in a broader sense how these proteins actually control cell life and death. Recent findings that Bcl-2 can function both as an ion channel and as an adapter or docking protein however are beginning to provide insights into the molecular mechanisms through which these proteins regulate the programmed cell death pathway in normalcy and disease.

Key words: Bcl-2, Raf-1, Apoptosis

Introduction

Programmed cell death plays a critical role in normal development or tissue homeostasis, where it helps to maintain total cell numbers within physiologically appropriate ranges by ensuring that cell production by mitogenesis is offset by a commensurate amount of cell loss. In many cases, this form of cell death proceeds by apoptosis, a process that includes a characteristic set of morphological or biochemical features such as cell shrinkage, membrane blebbing, condensation and segmentation of nuclei, and fragmentation of chromosomal DNA. Dysregulation of the physiological cell death process can have profound consequences, and probably plays a major role in many types of diseases, including cancer, autoimmune diseases, and AIDS. One of the best studied survival genes is bcl-2, the first member identified of a growing family of genes that participate in the control of apoptosis.

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Discovery of *bcl-2* gene

The bcl-2 (B cell lymphoma/leukemia-2) gene was first discovered because of its involvement in B-cell malignancies, where t(14;18) chromosomal translocations activate the gene in about 80-90% of follicular non-Hodgkin's B-cell lymphomas (Tsujimoto et al., 1985). In these translocations, the *bcl-2* gene is moved from its normal location on chromosome 18q21 into juxtaposition with the immunoglobulin heavy chain locus on chromosome 14q32, thus placing the *bcl-2* gene under the influence of powerful transcriptional enhancer elements associated with the immunoglobulin heavy chain locus and leading to overproduction of bcl-2 mRNAs and their encoded proteins. In the majority of cases the chromosome 18 breakpoint occur in the 3' untranslated region of *bcl-2*, thus the breakpoints of these t(14;18) translocations do not result typically in an abnormal gene product.

The Bcl-2 protein is 239 amino acids in length in humans (25-26 kD) and contains no recognized motifs or homologies that hint at its function (Tsujimoto and Croce, 1986). The first description of its cellular function by Vaux et al. (1988) employed lymphokine Interleukin-3 (IL-3) dependent immature pre-B-cells for transfections of bcl-2. Overexpression of bcl-2 permitted prolonged cell survival in the absence of IL-3, but did not promote cell cycle progression (Vaux et al., 1988; Hockenbery et al., 1990). The similar results were obtained in IL-4- and GM-CSF-dependent cells (Nuñez et al., 1990) and in certain IL-2-dependent (Deng and Podack, 1993) and IL-6-dependent (Schwarze and Hawley, 1995) cells. This findings suggest that the *bcl-2* gene defines a new category of oncogenes, unlike oncogenes studied previously, which functions in preventing programmed cell death without necessarily affecting cellular proliferation. Bcl-2 has also been found to protect against neuronal cell death induced by nerve growth factor (NGF) withdrawal (Batistatou et al., 1993; Mah et al., 1993). Microinjection of bcl-2

expression plasmids into NGF-dependent sympathetic neurons and into NGF-dependent or brain-derived neurotrophic factor (BDNF)-dependent central nervous system (CNS)-derived sensory neurons resulted in the prevention of programmed cell death after removal of the neurotrophic factors from cultures (García et al., 1992; Allsopp et al., 1993). In addition to delaying cell death induced by growth factor or neurotrophic factor withdral, over-expression of bcl-2 can protect a wide variety of cell types from undergoing apoptosis in response to such diverse stimuli as ionizing radiation, viral infection, or chemotherapeutic agents (Reed, 1994).

Transgenic mice with bcl-2 overexpression were used for investigation of the in vivo effects of bcl-2. When the *bcl-2* transgene is expressed in T cells, approximately one third of the mice develop peripheral T-cell lymphomas (Linette et al., 1995). The transgenic mice bearing a bcl-2-immunoglobulin enhancer transgene that targeted high levels of bcl-2 expression to the B lymphocyte lineage developed polyclonal B lymphocyte hyperplasia, likely due to the prolonged survival of B lymphocytes (McDonnell et al., 1989). In about one-half of lymphomas arising in bcl-2-immunoglobulin transgenic mice, a common second hit is rearrangement of the *c-myc* oncogene, supporting a synergistic relationship between bcl-2 and c-myc (McDonnell and Korsmeyer, 1991). Interstingly, Chen et al. (1997) recently reported that overexpression of bcl-2 in trangenic mice promotes retinal axon regeneration, which is unlikely to be due entirely to its anti-apoptotic activity. Thus, Bcl-2 may have other functions in some types of cells.

Though the *bcl-2* gene was first discovered because of its involvement in the t(14;18) chromosomal translocations commonly found in B-cell lymphomas, bcl-2 is expressed in multiple lineages during development and in mature animals. High levels or aberrant patterns of expression have also been reported in a wide variety of human cancers (Reed, 1997). In these tissues, bcl-2 may serve as a physiological regulator of programmed cell deaths in diverse cell types, as well as a deregulated survival factor in cancer cells. Studies of the biochemical mechanism of action by which the Bcl-2 protein protects cells from apoptotic cell death seem likely to not only tell us how the Bcl-2 protein works, but also will provide critical insights into the still poorly understood phenomenon of apoptosis.

Intracellular localization of Bcl-2 protein

Bcl-2 protein contains a stretch of hydrophobic amino acids at its C-terminus (transmembrane domain [TM]) that allows it to post-translationally insert into intracellular membranes. Subcellular fractionation, confocal, and electron microscopy studies using anti-Bcl-2 antibodies indicate that Bcl-2 resides in the outer mitochondrial membarne, nuclear evelope, and endoplasmic reticulum (Monaghan et al., 1992; Jacobson et al., 1993; Krajewski et al., 1993). Delection of the TM domain from Bcl-2 partially abolishes or diminishes its ability to protect against apoptosis in most types of cells examined thus far (Hockenbery et al, 1993; Tanaka et al., 1993; Nguyen et al., 1994). On balance, these studies have suggested that Bcl-2's function, at least partially, depends on its intracellular membrane localization. Most other members of the Bcl-2 family also contain a transmembrane (TM) domain near the C-termini and, where examined to date, appear to reside within approximately in the same intracellular membrane compartments (Yang et al., 1995; Zha et al., 1996a).

Function of Bcl-2 on anti-oxidant pathways

Data have been presented which argue both in favor and against the possibility of an effect of Bcl-2 on an anti-oxidant pathway in cells (Hockenbery et al., 1993; Kane et al., 1993; Jacobson and Raff, 1995; Shimizu et al., 1995). In favor of a role for Bcl-2 on oxidative stress pathways are the findings that: (1) Bcl-2 prevents induction of apoptotic and (in some cases) necrotic cell death induced by agents that either result in oxygen free radical production or that deplete intracellular glutathione; (2) Overexpression of certain antioxidant enzymes such as forms of superoxide dismutase (SOD) or glutathione peroxidase can also render cells more resistant to induction of cell death analogous to Bcl-2; and (3) Bcl-2 prevents the accumulation of lipid peroxides, suggesting that Bcl-2 somehow nullifies damage to membranes by reactive oxygen species (ROS) (Hockenbery et al., 1993; Kane et al., 1993). The relevance of these findings to the intracellular locations of the Bcl-2 protein could be that mitochondria, and to some extent ER and nuclear envelope, are the major sites of free-radical generation in cells. Additional evidence supporting a possible role for a redox mechanism for Bcl-2 comes from studies of SOD-deficient yeast, where expression of the human Bcl-2 protein was shown to restore growth under aerobic conditions (Kane et al., 1993). However, in a cell-free system for "apoptosis" in which Bcl-2 can function to prevent nuclear breakdown and DNA degradation, chemicals that modulate redox conditions had no significant effects on either induction of apoptotic-like changes in nuclei or the ability of Bcl-2 to function (Newmeyer et al., 1994). Also, Bcl-2 is able to rescue cells from programmed cell death occurring under hypoxic conditions in which the generation of ROS is greatly reduced, suggesting ROS are not required for programmed cell death (Jacobson and Raff, 1995; Shimizu et al., 1995).

Influence of Bcl-2 on intracellular Ca^{2+} homeostasis

Another possible functional implication of the intracellular locations of the Bcl-2 protein is suggested by data showing that Bcl-2 may regulate the homeostasis of Ca^{2+} in cells, based on experiments which have shown an ability of Bcl-2 overexpression to inflence the sequestration of Ca^{2+} within the ER, prevent the release

of Ca^{2+} from mitochondria of cells treated with uncouplers of oxidative phosphorylation, and block entry of Ca^{2+} into nuclei (Vanhaesebroeck et al., 1993; Lam et al., 1994; Marin et al., 1996). The presence of Bcl-2 in nuclear and ER membranes, therefore, may have some relevance to the fact that most of the Ca^{2+} in cells is sequestered in the lumen of the ER and, by extension, the space between the inner and outer nuclear membranes. Furthermore, in most types of cells, the mitochondria represent the next largest intracellular storage site for Ca^{2+}, again suggesting that Bcl-2 is at least located in the right places to function either directly or indirectly as a regulator of intracellular Ca^{2+} homeostasis.

Complex network of Bcl-2 family proteins

Since the discovery of bcl-2, several homologs of this gene and its encoded protein have been identified (Table 1). Interestingly, many of these proteins can interact with each other through a complex network of homo- and heterodimers that regulate programmed cell death (Sato et al., 1994; Sedlak et al., 1995). To date, 15 cellular homologs of Bcl-2 have been reported, including the anti-apoptotic proteins Bcl-XL, Mcl-1, A1/Bfl-1, Bcl-W, Nr-13 (avian), and Ced-9 (nematode) and the pro-apoptotic proteins Bax, Bcl-X_S, Bad, Bak, Bik, Bid and Hrk (Boise et al., 1993; Kozopas et al., 1993; Lin et al., 1993; Oltvai et al., 1993; Hengartner and Horvitz, 1994; Boyd et al., 1995; Chittenden et al., 1995a; Choi et al., 1995; Farrow et al., 1995; Gillet et al., 1995; Kiefer et al., 1995; Yang et al., 1995; Gibson et al., 1996; Wang et al., 1996c; Inohara et al., 1997). The Bcl-X_L and Bcl-

Table 1. Bcl-2 Family Proteins

PROTEIN	SOURCE	PRIMARY FUNCTION
Bcl-2	mammalian	anti-apoptotic
Bcl-X_L	mammalian	anti-apoptotic
Bcl-X_S	mammalian	pro-apoptotic
Bax	mammalian	pro-apoptotic
Mcl-1	mammalian	anti-apoptotic
A1/Bfl-1	mammalian	anti-apoptotic
Brag-1	mammalian	anti-apoptotic*
Bcl-w	mammalian	anti-apoptotic
Bad	mammalian	pro-apoptotic
Bak	mammalian	pro-apoptotic
Bik	mammalian	pro-apoptotic
Bid	mammalian	pro-apoptotic
Hrk	mammalian	pro-apoptotic
Nr13	avian	anti-apoptotic*
Ced9	nematode	anti-apoptotic
E1b-19kDa	adenovirus	anti-apoptotic
BHRF1	EBV	anti-apoptotic
LMW5-HL	African swine fever virus	not tested
ORF-16	herpesvirus	not tested
KSbcl-2	human herpesvirus 8	anti-apoptotic

*: function not formally tested but over-expression in tumors and presence of BH4 domains suggest anti-apoptotic.

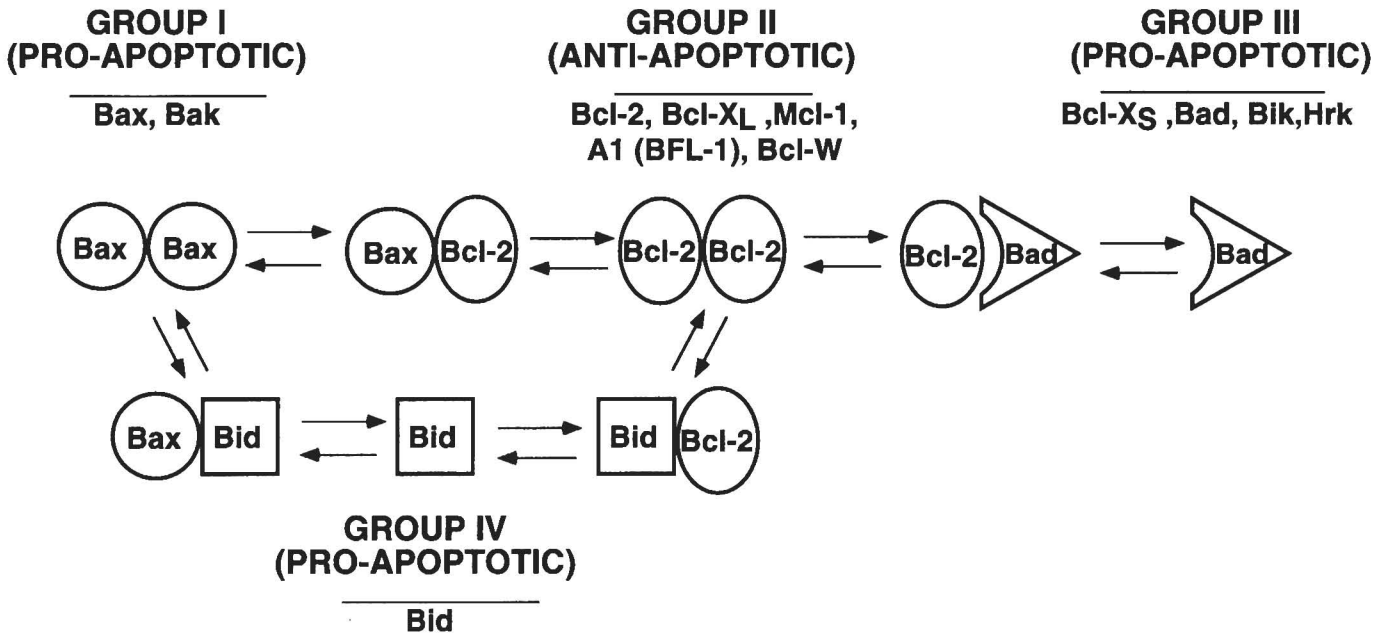

Fig. 1. Model for the physical interactions among Bcl-2 family proteins. Many of the Bcl-2 family proteins can be grouped into one of four categories based on their impact on cell death and dimerization properties. The group I proteins promote cell death and can both homodimerize with themselves and heterodimerize with anti-apoptotic proteins. The group II proteins suppress cell death and can form both homodimers with themselves and heterodimers with pro-apoptotic proteins. The group III proteins can heterodimerize with anti-apoptotic proteins presumbly sequestering them from cell death suppression activity, but appear to be incapable of forming homodimers with themselves or heterodimers with pro-apoptotic proteins. The group IV is presented by Bid which anologous to the group III proteins, except that Bid can both dimerize with anti-apoptotic (group II) proteins and with pro-apoptotic (group III) proteins.

X_S protein arise through alternative mRNA splicing from the same gene (Boise et al., 1993). An additional human homolog BRAG-1 and two xenopus homologs have also been described whose effects on cell life and death have not been assessed, but they most likely are suppressors of apoptosis (Cruz-Reyes and Tata, 1995; Das et al., 1996). Finally, several homologs of Bcl-2 have been discovered in viruses, including the E1b-19 kD protein of adenovirus, bhrf-1 protein of Epstein-Barr virus, LMH-5W protein of African Swine Fever virus, ORF-16 protein of Herpes Saimiri virus, and KSbcl-2 of Herpes virus (Rao et al., 1992; Henderson et al., 1993; Neilan et al., 1993; Smith, 1995; Cheng et al., 1997).

Many of the Bcl-2 family proteins can be grouped into one of four categories based on the impact they have on cell death and their dimerization properties (Fig. 1). One group consists of pro-apoptotic proteins such as Bax and Bak, which can homodimerize with themselves and heterodimerize with anti-apoptotic proteins such as Bcl-2 and Bcl-X_L. A second group is comprised of anti-apoptotic proteins such as Bcl-2, Bcl-X_L, A1, and Mcl-1. These proteins suppress cell death, and can typically homodimerize with themselves and heterodimerize with pro-apoptotic proteins such as Bax or Bak. The third group is presented by Bcl-X_S, Bad, Bik and Hrk which appear to function essentially as trans-dominat inhibitors of the anti-apoptotic proteins such as Bcl-2 and Bcl-X_L. They can heterodimerize with anti-apoptotic members of the family, presumably sequestering them so that they cannot dimerize with pro-apoptotic proteins such as Bax and Bak, but appear to be incapable of homodimerizing with themselves. The pro-apoptotic protein Bid may constitute a fourth group of Bcl-2 family proteins. Unlike Bcl-X_S and Bad, the Bid protein can dimerize with both anti-apoptotic proteins (Bcl-2 and Bcl-X_L) and pro-apoptotic proteins (Bax). However, in contrast to the group I proteins such as Bax and Bak that are suspected to directly promote cell death, Bid cannot homodimerize with itself (Sato et al., 1994; Yang et al., 1994; Hanada et al., 1995; Boyd et al., 1995; Zha et al., 1996a).

Sequencing alignment and mutagenesis studies have identified up to four evolutionarily conserved domains (BH1, BH2, BH3, and BH4) within Bcl-2 family proteins, which can be important for their function (Zha et al., 1996b) (Fig. 2). A suggestion of functional importance of some of the homo- and heterodimerization events that Bcl-2 family proteins participate in has been made by experiments where small deletions or single aminoacid substitution mutants have been created that alter the ability of Bcl-2 or some of its homologs to interact with themself or with other proteins. For example, deletion of the BH3 domain from Bax or mutations in conserved residues in this domain have been shown to prevent Bax/Bax homodimerization and abrogate Bax's function as a promoter of cell death in both mammalian cells and in yeast (Han et al., 1996; Zha et al., 1996a). Thus, for Bax to promote cell death, homodimerization may be required. It should be noted, however, that these same mutations which abolish Bax/Bax homodimerization also prevent Bax/Bcl-2 heterodimerization. It is, therefore, entirely possible that Bax promotes cell death both directly as a Bax/Bax homodimer and indirectly by binding to and neutralizing Bcl-2. Similar results have been obtained for Bak, which seems to prefer Bcl-X_L as its heterodimerization partner rather than Bcl-2 (Chittenden et al., 1995b), though less is known about the effects of BH3 domain mutations on Bak/Bak homodimerization. In contrast, Bax (ΔBH3) mutants have been reported to retain their ability to counter the death repressor activity of Bcl-X_L at least during chemotherapy-induced apoptosis (Simonian et al., 1996). Thus, in some circumstances, Bax may be able to function independent of its homodimerization or heterodimerization with Bcl-2 or Bcl-X_L. Moreover, some single amino-acid substitution mutants within the BH1 and BH2 domains of Bcl-2 and Bcl-X_L can prevent binding to Bax and abrogate Bcl-2's and Bcl-X_L's protective action against apoptosis, while other specific mutations in the BH1 and BH2 domains of Bcl-X_L have been shown to abolish or diminish interactions with Bax,

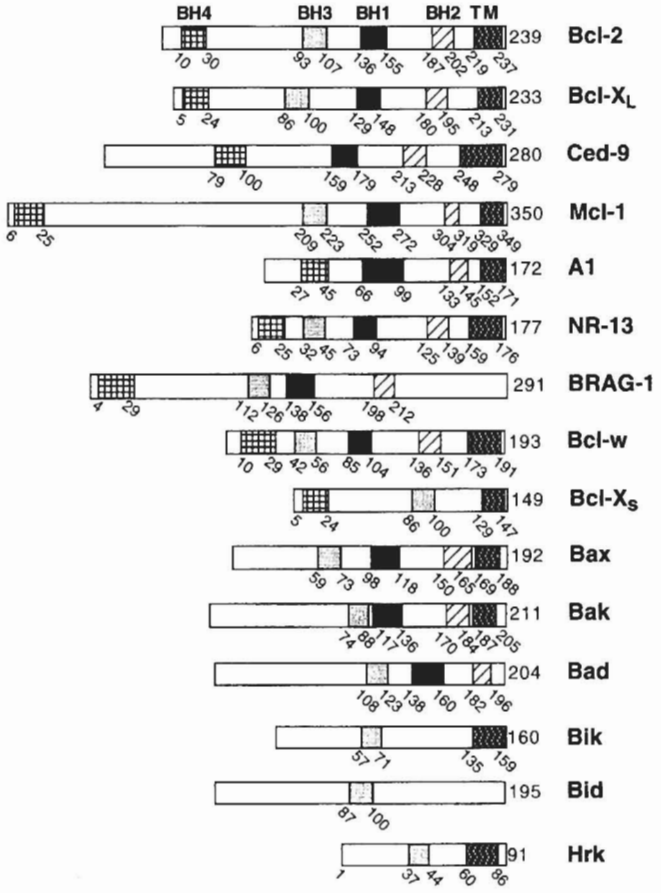

Fig. 2. Domain-structures of Bcl-2 family proteins. The structures of human Bcl-2 protein and the known cellular homologs of Bcl-2 are depicted in linear form. The conserved domains with a high degree of sequence homology are indicated as BH1, BH2, BH3, and BH4, as well as the locations of the transmembrane (TM) domains.

without impairing the ability of Bcl-X_L to suppress cell death (Yin et al., 1994; Sedlak et al., 1995; Cheng et al., 1996). Thus, the significance of homo- and hetero-dimerization remains unclear, despite considerable effort by several groups.

Deletion mutants of Bcl-2 lacking the BH4 domain exhibit either loss of function or dominant-inhibitory activity, paradoxically promoting apoptosis (Borner et al., 1994; Hanada et al., 1995; Hunter et al., 1996), thus indicating the functional significance of the BH4 domain. BH4 is not, however, required for binding to either Bax or wild-type Bcl-2 protein (Hanada et al., 1995). Consequently, it appears that while binding to Bax may be important for Bcl-2 function, under some circumstances, it is not the only requirement.

Bcl-2 functions as a regulator of mitochondrial membrane permeability transition

Recently, evidence has accumulated that Bcl-2 family proteins can regulate the phenomenon of membrane permeability transition (PT) in mitochondria. PT results from the opening of a large megachannel located at the contact sites in mitochondria where the inner and outer membranes abutt and where various transport processes involving ions and proteins occur (Bernardi et al., 1994). In intact cells, a wide variety of apoptotic insults can induce mitochondrial PT, as defined by reduced uptake of cationic fluorescent dyes into mitochondria and subsequent generation of reactive oxygen species (Marchetti et al., 1996; Muchmore et al., 1996; Zamzami et al., 1996a,b; Zhu et al., 1996). Over-expression of Bcl-2 prevents the loss of the electro-chemical gradient ($\Delta\psi$) across mitochondria and suppresses the subsequent production of ROS and release of apoptogenic proteins under conditions where Bcl-2 also prevents apoptosis, but not when the apoptotic stimulus is such that Bcl-2 fails to prevent cell death (Kroemer et al., 1996; Susin et al., 1996). In experiments employing isolated mitochondria where the mitochondria are derived from Bcl-2 over-expressing cells or their control transfected counterparts that contain only low levels of Bcl-2, it has been shown that Bcl-2 can prevent the induction of PT by oxidants, Ca^{2+}, and atractyloside (Kroemer et al., 1996). It is tempting therefore to speculate that the ability of Bcl-2 to prevent PT induction by a wide variety of insults, including elevated cytosolic Ca^{2+} and oxidative injury, may provide an explanation for reports that Bcl-2 can also prevent necrotic cell death under some circumstances (Kane et al., 1995; Shimizu et al., 1995, 1996).

The induction of PT can also result in release of mitochondrial proteins into the cytosol. Among the proteins released are cytochrome c (Cyt c) and an unidentified molecule called AIF for Apoptosis-Inducing Factor (Liu et al., 1996; Susin et al., 1996). Both Cyt c and AIF reside normally in the inter-membrane space of mitochondria. When added to cytosolic extracts derived from healthy non-apoptotic cells, both Cyt c and AIF can induce rapid activation of ICE-family proteases (Caspases) and trigger apoptotic like destruction of naive nuclei added to these extracts (Liu et al., 1996; Susin et al., 1996). Recent evidence indicates that Bcl-2 can prevent the efflux of Cyt c from mitochondria, based on experiments using both intact cells and isolated mitochondria (Kluck et al., 1997; Yang et al., 1997). In human acute myeloid leukemia (HL60) cells, the release of Cyt c from mitochondria into cytosol can be detected as quickly as 1 hour after treatment with staurosporine or etoposide, followed by CPP32/caspase activation and cell death. Overexpression of Bcl-2 prevented the translocation of Cyt c from mitochondria to cytosol and the initiation of apoptosis. In a cell-free system in which *Xenopus* egg extracts were used, baculovirus-produced Bcl-2 added to the mitochondrial fraction blocked Cyt c release, caspase activation, and the apoptotic effects on added nuclei (Kluck et al., 1997). Bcl-2 had no effect when added after mitochondrial Cyt c release, suggesting that Cyt c release is required, together with other cytosolic factors, for subsequent caspase activation and apoptotic cell death.

The three-dimensional structure of Bcl-X_L was recently shown to be similar to the structures of the pore-forming domains of the bacterial toxins, diphtheria toxin (DT) and the colicins (Muchmore et al., 1996). Studies of the bacterial toxins suggest that a key feature of these pore-forming domains is the ability to form ion channels in biological membranes (Cramer et al., 1995). True to its structural similarity to these bacterial toxins, recent data indicate that recombinant Bcl-X_L protein, as well as Bcl-2, can insert into either synthetic lipid vesicles or planar lipid bilayers and form ion-conducting channels (Minn et al., 1997; Schendel et al., 1997). Moreover, a mutant of Bcl-2 lacking the two core hydrophobic α-helices (helix 5 and 6) failed to form specific ion-conducting channels, suggesting the central hydrophobic helicies are important for membrane insertion and pore formation (Schendel et al., 1997). Although both Bcl-2 and Bcl-X_L have been demonstrated to form an ion-selective channel, it remains unclear at present how this pore-forming activity relates to the bioactivities of these proteins. The interaction of Bcl-2 or Bcl-X_L with other Bcl-2 family proteins may be further required to regulate membrane insertion and membrane permeability.

Bcl-2 functions as an adapter protein

In addition to the interactions between Bcl-2 family proteins, Bcl-2 has also been reported to bind several other non-homologous proteins, including the kinase Raf-1, the GTPases R-Ras and H-Ras, the p53-binding protein p53-BP2, the Prion protein Pr-1, and several novel proteins including CED-4, BAG-1, Nip-1, Nip-2, and Nip-3 (Boyd et al., 1993; Fernández-Sarbia and Bischoff, 1993; Wang et al., 1994; Kurschner and Morgan, 1995; Takayama et al., 1995; Chen and Faller, 1996; Naumovski and Cleary, 1996; Chinnaiyan et al., 1997; Spector et al., 1997; Wu et al., 1997). In most

cases, neither the functional significance of these interactions with Bcl-2 nor the regions on the Bcl-2 protein required for binding to these proteins has been explored. However, it has been recently reported that both Raf-1 and probably the BAG-1 protein bind to Bcl-2 in a BH4-dependent manner and that these proteins can cooperate with Bcl-2 in the suppression of cell death (Wang et al., 1994, 1996a,b; Takayama et al., 1995).

The BH4 domain is uniquely found in the anti-apoptotic members of the Bcl-2 family with the exception of Bcl-X_S and represents the first α helix in Bcl-2 based on comparisons with the reported structure of Bcl-X_L (Muchmore et al., 1996). Depending upon the cellular context, deletion mutants of Bcl-2 lacking the BH4 domain either exhibit a loss-of-function phenotype or alternatively act as dominant inhibitors of the wild-type Bcl-2 protein, thereby promoting cell death (Borner et al., 1994; Hunter et al., 1996). It has been shown that Bcl-2 can target the cytosolic protein kinase Raf-1 to mitochondrial membranes in a BH4-dependent manner and that artificially targeting Raf-1 to mitochondrial membranes by fusing the catalytic domain to the trans-membrane domain of a mitochondrial outer membrane protein (Mas70p) protected cells from apoptosis (Wang et al., 1996b).

How does the BH4-dependent interaction of Bcl-2 with Raf-1 and BAG-1 enhance protection from apoptosis? Recently it has been shown that BAG-1 not only binds to Bcl-2 but also can specifically interacts with Raf-1. Moreover, BAG-1 can activate this kinase, apparently through a protein-protein interaction that involves binding of BAG-1 to the catalytic domain of Raf-1 (Wang et al., 1996a). Thus, the concept emerges of Bcl-2 as a docking or adapter protein around which "signal-transduction" events occur. In the case of Raf-1 and BAG-1, dimers of Bcl-2 could be viewed as a docking site that allows Raf-1 and BAG-1 to meet each other in cells, interact, and result in transient reversible activation of the Raf-1 kinase locally in the vicinity of Bcl-2 on the surface of mitochondria, ER, or nuclear envelope membranes.

Once activated locally in the vicinity of Bcl-2, the kinase Raf-1 appears to mediate directly or indirectly phosphorylation of the pro-apoptotic protein BAD (Wang et al., 1996b) (Fig. 3). The unphosphorylated BAD protein can bind to anti-apoptotic proteins such as Bcl-2 and Bcl-X_L, preventing them from dimerizing with Bax and abrogating their function as blockers of cell death (Yang et al., 1995). Studies in which phosphorylation of BAD was induced by stimulating a hemopoitic cell line with the growth factor Interleukin-3 (IL-3) suggest that once phosphorylated, BAD no longer binds to Bcl-2 and Bcl-X_L (Zha et al., 1996c). Moreover, the two serines that become phosphorylated in response to IL-3 stimulation reside within consensus binding sites for 14-3-3, an abundant cytosolic protein that sequesters the phosphorylated BAD protein in the cytosol where it cannot interact with Bcl-2 or Bcl-X_L. In this regard, it is important to note that BAD represents one of the few members of Bcl-2 family that does not posses a C-terminal membrane anchoring domain. Though it remains to be determined whether the same two serines in BAD become phosphorylated within the context of Raf-1 targeting to Bcl-2 at the surface of mitochondria or other organelles (as opposed to Raf-1 activation at the plasma membrane via Ras-dependent mechanisms after growth factor stimulation), the implication is that the interaction of Bcl-2 with Raf-1 provides a mechanism for eradicating the suppression of BAD.

Interestingly, Bcl-2 has also been found to interact with the serine/threonine protein phosphatase calcineurin. Co-expression of Bcl-2 and calcineurin results in targeting of calcineurin to the same membraneous locations where Bcl-2 normally resides, in a BH4-dependent manner. The functional significance of the Bcl-2/calcineurin interaction remains unclear, but overexpression of an active version of calcineurin induces apoptosis which can be blocked by Bcl-2 (Shibasaki and McKeon, 1995; Shibasaki et al., 1997). It has been shown that calcineurin dephosphorylation of NF-AT, a transcription factor first identified because of its ability to stimulate expression of lymphokine and lymphokine receptor genes in lymphocytes, is required for NF-AT translocation from the cytosol into the nucleus (Shibasaki et al., 1996). Thus, the ability of Bcl-2 to bind calcineurin, sequestering it at membranes where it cannot dephosphorylate NF-AT in the cytosol, may contribute to cell cycle arrest. The potential for Bcl-2 to modulate the cell cycle, causing a slowing in either entry into (G0→G1) or progression through (G1→S) the cell cycle, may represent a protective function that allows cells more time, for example, to repair damaged DNA prior to S-phase. It also is entirely possible that targeting calcineurin to intracellular membranes by Bcl-2 contribute to cell servival via blocking de-

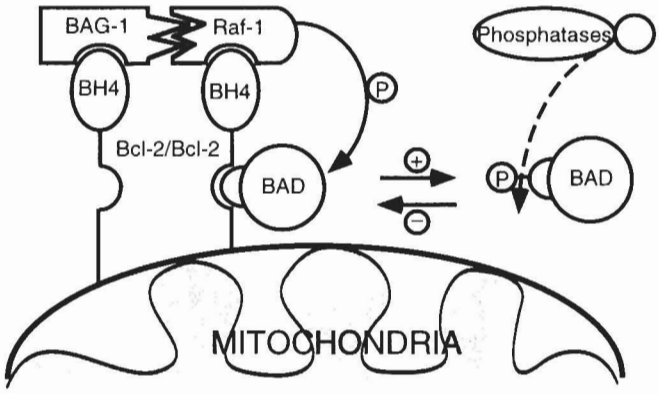

Fig. 3. Model for Bcl-2/Raf-1 mediated phosphorylation of BAD. Bcl-2 can target Raf-1 and probably BAG-1 to mitochondria via its BH4 domain. BAG-1 binds to and activates the kinase Raf-1, resulting in induction of BAD phosphorylation. Once the BAD protein is phosphorylated, it no longer binds to Bcl-2 or Bcl-X_L. BAD lacks a C-terminal membrane anchoring domain and thus it is free to float between cytosol and membrane locations, depending on its phosphorylation state.

phosphorylation of Bcl-2 family proteins or other apoptotic regulators in the cytosol.

It has been recently shown that the worm homolog of Bcl-2 (CED-9) as well as Bcl-X_L can bind to worm CED-4, which in turn binds to CED-3 or other Caspases such as FLICE (Caspase-8) and ICE (Caspase-1). Moreover CED-9, CED-4 and CED-3 appear to form a trimolecular complex (Chinnaiyan et al., 1997; Spector et al., 1997; Wu et al., 1997). Genetic studies in the free-living nematode *Caenorhaditis elegans* have demonstrated that CED-3 and CED-4 are necessary for programmed cell death, whereas CED-9 protects cells from death during development of this multicellular organism (Shaham and Horvitz, 1996). One of CED-9's functions appears to be as an inhibitor of CED-4, presumbly preventing CED-4 from activating the worm homolog of the caspases CED-3 (Chinnaiyan et al., 1997; Spector et al., 1997; Wu et al., 1997). In mammalian cells, Chinnaiyan et al. (1997) have shown that wild-type of Bcl-X_L and a functionally active mutant of Bcl-X_L that fails to bind Bax coimmunoprecipitated with worm CED-4 and blocked CED-4-induced killing. Thus, binding to CED-4 may be an important feature of Bcl-2 and Bcl-X_L function, presumably preventing CED-4 from promoting activation of CED-3 family proteases in the cytosol. Moreover, the pro-apoptotic members of Bcl-2 family, such as Bax, Bak and Bik, not only bind to Bcl-X_L but also attenuate Bcl-X_L binding to CED-4 (Chinnaiyan et al., 1997). Thus, disrupting the interaction between Bcl-X_L and CED-4 may be an important function for Bax and the other death-promoting members of Bcl-2 family. Though many details remain unexplored, it seems likely that the interaction of anti-apoptotic Bcl-2 family proteins with the caspases via bridging CED-4 homologs will prove to be one of the major mechanisms by with Bcl-2, Bcl-X_L, and their related proteins suppress apoptosis. However, this hypothesis has yet to be proven for human and mammalian Bcl-2 family proteins.

Conclusions

Over ten years have passed since the predicted amino-acid sequence of the Bcl-2 protein was first reported (Tsujimoto and Croce, 1986). Though many details are missing, we are beginning to understand the biochemical functions of Bcl-2 and its homologs with regards to regulation of cell life and death. Bcl-2 and several of its related proteins seem likely to have multiple functions. These proteins not only interact with each other through a complex network of homo- and heterodimers, but also have important interactions with other types of proteins such as Raf-1, calcineurin, and CED-4. The hope is that with advances in our knowledge of the molecular details of how Bcl-2 and its various interacting proteins biochemically function, it may one day improve our ability to treat cancer and many other diseases that involve the programmed cell death pathway.

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