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The Bcl-2 family of proteins: regulators of cell death and survival

Gabriel Nuñez and Michael F. Clarke

The Bcl-2 protein inhibits apoptosis induced by a variety of signals, in a range of cell types and in diverse organisms, and it is implicated in both normal development and oncogenesis. Despite this central role, the mechanism of action of Bcl-2 is not yet clear. Recent studies have uncovered a number of Bcl-2-related gene products that regulate apoptosis either negatively or positively, and Bcl-2 forms heterodimers with at least one of these proteins, Bax. This article discusses the role of the Bcl-2 family of proteins in the light of these findings.

The human *bcl-2* (B-cell lymphoma/leukaemia-2) gene was identified at the breakpoint site of the t(14;18) chromosomal translocation that is associated with follicular lymphoma. In this translocation, *bcl-2* moves from its normal location at 18q21 into juxtaposition with the immunoglobulin heavy-chain locus at 14q32 (Ref. 1), which results in its transcriptional activation and the overproduction of the 26 kDa Bcl-2 protein in lymphoma cells[2,3].

Bcl-2 is thought to contribute to oncogenesis by suppressing signals that induce apoptotic cell death. (For an introduction to apoptosis, see the box in the first article of this series in *TCB*, by McConkey and Orrenius.) For example, overexpression of *bcl-2* can prevent haematopoietic and neural cell apoptosis induced by growth factor withdrawal[4–6], and *bcl-2* also prevents or delays apoptosis induced by γ-irradiation, glucocorticoids, heat shock and multiple chemotherapeutic drugs[7–10].

The regulation of cell death by Bcl-2 has been conserved among divergent phyla. As discussed in the previous article on apoptosis by Osborne and Schwartz, the genes *ced-3* and *ced-4* are required for developmental cell death in the nematode *Caenorhabditis elegans*, whereas the *ced-9* gene is a negative regulator of such deaths. It is now known that *ced-3* is homologous to the gene encoding the mammalian interleukin-1β-converting enzyme (ICE), while *ced-9* is homologous to *bcl-2*. Indeed, overexpression of *bcl-2* in the worm prevents *ced-3*- and *ced-4*-dependent cell death; and overexpression of *bcl-2* in vertebrate cells prevents apoptosis induced by ICE overexpression.

This article summarizes recent progress in characterizing gene products related to Bcl-2, examines the role of Bcl-2 in normal development and in oncogenesis, and then discusses the possible mechanisms of action of Bcl-2 in regulating apoptosis.

The growing family of *bcl-2*-related genes

bcl-2 was the founding member of an expanding family of genes, which now includes *bcl-x*, *bax*, *mcl-1* and *A1*, that regulate apoptosis. The homology among the Bcl-2-related proteins is concentrated in two regions, termed BH1 and BH2 (Fig. 1). In addition, proteins of the Bcl-2 family contain a stretch of hydrophobic amino acids at their C-termini, which appear important for attachment to intracellular membranes[11,12].

bcl-x was recently isolated by hybridization to a *bcl-2* probe[13] and by functional expression cloning (M. González-García and G. Nuñez, unpublished). It gives rise to three transcripts by alternative splicing. The predicted Bcl-x_L protein displays high-level homology to Bcl-2. As with *bcl-2*, transfection of *bcl-x_L* into IL-3-dependent cells prevented their apoptotic cell death following growth factor deprivation[13,14]. The predicted Bcl-x_S protein lacks the internal region of 63 amino acids in Bcl-x_L that has greatest homology to Bcl-2. Surprisingly, transfection of *bcl-x_S* failed to inhibit cell death; rather, it

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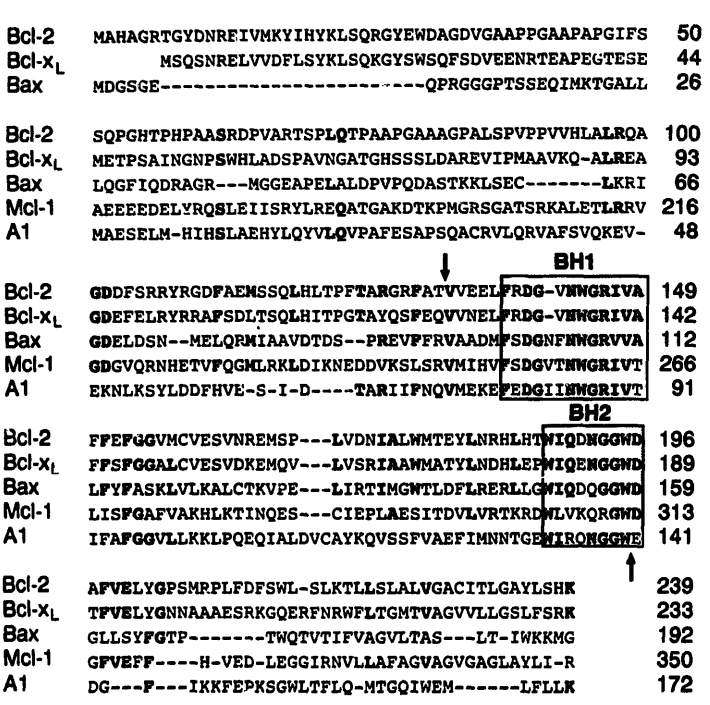

FIGURE 1

Regions of homology between Bcl-2 and related proteins. The amino acid sequences for human Bax$_\alpha$, Bcl-x$_L$ and Mcl-1 and murine A1 are aligned with human Bcl-2$_\alpha$. Identical amino acids shared by three or more sequences are shown in bold, and stippled. The regions of greatest homology among Bcl-2-related proteins, BH1 and BH2, are boxed. The arrows indicate a region of 63 amino acids of Bcl-x$_L$ that is not present in Bcl-x$_S$ (Ref. 13).

facilitated apoptosis by inhibiting the death suppressor activity of Bcl-2 (Ref. 13), perhaps by competing for its targets or positive regulators. The predicted Bcl-x$_\beta$ protein lacks the hydrophobic C-terminal domain present in Bcl-x$_L$ and Bcl-x$_S$ (Ref. 14). *bcl-x$_\beta$* also inhibits apoptosis induced by growth factor deprivation (M. González-García *et al.*, unpublished).

The Bax protein was identified by immunoprecipitation of Bcl-2 (Ref. 15). Bax is a 21 kDa protein with 21% homology to Bcl-2 and forms heterodimers with Bcl-2 (Ref. 15). Importantly, expression of *bax* does not block apoptosis; instead it appears to inhibit the function of Bcl-2, perhaps by forming Bcl-2–Bax complexes or by competing with other Bcl-2 targets (see below).

The *mcl-1* and *A1* genes were isolated by differential hybridization. *mcl-1* encodes a 37 kDa protein[16] and *A1* encodes a 20 kDa protein[17], both of which display significant homology to Bcl-2 and Bcl-x$_L$, particularly in the BH1 and BH2 domains (Fig. 1). *mcl-1* and *A1* are early-response genes that are induced transiently by differentiation signals or growth factors[16,17]. *mcl-1* appears to inhibit apoptosis (R. Craig and A. Eastman, pers. commun.). Overexpression of *A1* in IL-3-dependent cell lines delays their apoptotic death induced by growth factor withdrawal (M. Benedict and G. Nuñez, unpublished).

Thus, the regulation of apoptosis in vertebrates by *bcl-2* and related genes is complex and involves positive and negative functions. It is possible that further *bcl-2*-related genes will be identified in the near future.

Bcl-2 function *in vivo*

The expression of the *bcl-2* gene is widespread in many fetal tissues[18], but in adult tissues *bcl-2* appears to be expressed in cells that are rapidly dividing and differentiating into mature components (Table 1). Such cells include stem cells of the crypts of the gut epithelia or the skin, and early haematopoietic progenitors[19]. *bcl-2* expression declines in cells as they mature, or at stages when cells may be eliminated. For example, *bcl-2* is downregulated during keratinocyte or myeloid differentiation[19]; and transiently during B and T cell differentiation, at a stage when such cells are prone to undergo clonal elimination by apoptosis[20–22]. Taken together, these observations have led to the hypothesis that Bcl-2 is a survival factor for early progenitor cells and for fully differentiated cells that are long-lived. Conversely, downregulation of Bcl-2 during tissue differentiation appears to facilitate cell death that occurs during clonal selection or that is coupled to terminal differentiation of epithelial or haematopoietic tissues.

bcl-x mRNA is produced in a variety of tissues in the chicken, human and mouse[13,14]. In the mouse, *bcl-x$_L$* is the major *bcl-x* mRNA form expressed during embryonic and postnatal development, and in the adult it is highly expressed in the brain, kidney, bone marrow and thymus[14]. The expression of *bcl-x$_L$* in the adult central nervous system (CNS)[14] contrasts with the low or undetectable expression of *bcl-2* in most neurons of the CNS. Both murine *bcl-x$_L$* and *bcl-x$_\beta$* are highly expressed in bone marrow and thymus but downregulated in spleen and lymph nodes[14].

Mice lacking functional Bcl-2 (*bcl-2* 'knockouts') have given further insight into the biological functions of *bcl-2*. Surprisingly, such mice developed normally to birth, but died at two to ten weeks of age due to fulminate apoptosis of lymphoid tissues and polycystic kidney disease[24,25]. Why other tissues that normally express large amounts of *bcl-2*, such as the nervous system or the bone marrow, are unaffected in such mice is not known. One plausible explanation is that there is a redundancy built into the *bcl-2* pathway, an idea supported by the overlapping expression of *bcl-x* and *bcl-2* in many tissues. It is possible that *bcl-x*, or other *bcl-2* family members, are involved in the normal homeostasis of tissues that are not affected in the *bcl-2* knockouts. Ongoing experiments with mice containing disrupted mutations of the various *bcl-2* family members should clarify this issue.

bcl-2 and oncogenesis

Malignant transformation can be thought of as arising from activation of the cell's mitogenic machinery and inactivation of the growth-inhibitory and apoptosis machinery, through multiple mutations. For example, in breast cancer, two of the most common abnormalities are deregulated expression of c-*myc* and inactivation or mutation of the p53 tumour suppressor gene[26]. Expression of the gene encoding p53 or certain oncogenes such as c-*myc* can induce some cancer cells to undergo apoptosis[27–30], which suggests that the cell death programme is a critical defence mechanism against malignant

TABLE 1 – CHARACTERISTICS OF THE Bcl-2 AND RELATED GENE PRODUCTS

Gene	Gene product	Function	Expression[d]	Subcellular location	Refs
bcl-2	Bcl-2_α	Blocks apoptosis	Widespread in embryonic tissues Highly restricted in postnatal tissues	Outer mitochondria, nuclear envelope, ER	18 19–23
bcl-x	Bcl-x_L	Blocks apoptosis	Widespread in embryonic tissues Highly restricted in postnatal tissues	Outer mitochondria, nuclear envelope	14 13,14
	Bcl-x_s	Promotes apoptosis	Human thymus	Unknown	13
	Bcl-x_β	Blocks apoptosis[a]	Same as Bcl-x_L	Unknown	14
bax	Bax$_\alpha$	Promotes apoptosis	Widespread	Unknown	15
A1	A1	Blocks apoptosis[b]	Hematopoietic tissues	Unknown	16
mcl-1	Mcl-1	Blocks apoptosis[c]	Myeloid cells[e]	Unknown	17

[a]González-García, M. *et al.*, unpublished.
[b]M. Benedict and G. Nuñez, unpublished.
[c]R. Craig and A. Eastman, pers. commun.
[d]Expression of *bax*, *bcl-x*, *A1* and *mcl-1* at the RNA level.
[e]Detailed analysis in tissues not yet carried out.

transformation. Many types of tumours, including most breast carcinomas, produce Bcl-2 (Ref. 31). Although *bcl-2* alone does not stimulate cell proliferation or cause transformation, it cooperates with c-*myc*[4,32,33] and members of the *ras* family[34] to transform cells. Recently it has been shown that *bcl-2* protects cells from c-*myc*- and p53-induced apoptosis[33,35–38], which suggests that Bcl-2 suppresses apoptosis signals that occur with transformation.

In addition to a role in the genesis of cancers, the modulation of apoptosis may also influence the outcome of cancer treatment. Elegant studies with p53 'knockout' mice showed that X-irradiation and certain chemotherapeutic agents kill cells through the p53-dependent apoptosis pathway[39,40]. In contrast, the expression of *bcl-2* has been shown to protect cells from both chemotherapy and radiation-induced apoptosis[7,9,10,13]. Because Bcl-2 production is a common feature of many carcinomas, lymphomas and leukaemias, it is thought that Bcl-2 might play a role in the resistance to therapy. Indeed, expression of *bcl-2* in leukaemia, carcinoma of the prostate andneuroblastoma has been shown to be a marker for poor prognosis[41–44].

How does Bcl-2 prevent apoptosis?

The global changes in many cellular processes that occur during apoptosis make it difficult to distinguish cause from effect. Nevertheless, several models have been proposed to explain the inhibition of apoptosis by Bcl-2. Here we discuss clues to the function of Bcl-2 from knowledge of its cellular distribution, and then turn to possible mechanisms of Bcl-2 action.

Cellular distribution of the Bcl-2 protein

Bcl-2 is a stable protein[21] associated with organelles, especially mitochondrial membranes. It is now known to reside primarily in the outer mitochondrial membrane, nuclear envelope and endoplasmic reticulum (ER)[45,46]. Recent electron microscopy studies indicate that Bcl-x_L has a subcellular distribution similar to that of Bcl-2 (Ref. 14), which suggests that both proteins function in a similar manner to prevent cell death (Fig. 2). *In vitro* and *in vivo* studies showed that amino acids 187 to 216 of Bcl-2 are crucial for correct integration of its hydrophobic C-terminus into the membrane[11,12]. The N-terminus of the integrated protein is exposed to the cytosol[47]. Interestingly, during mitosis Bcl-2 may contact chromosomes[48], although the importance of this finding remains unknown.

The functional significance of Bcl-2 targeting to the outer membrane of cell organelles is also unclear. Deletion of the hydrophobic C-terminus of Bcl-2 abrogates much, but probably not all, of the

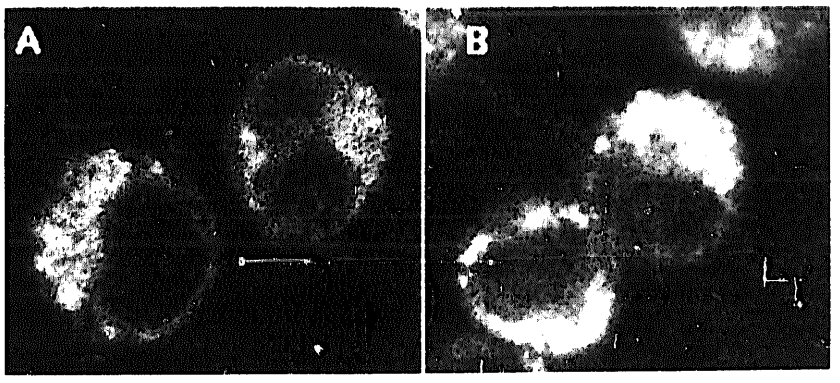

FIGURE 2

Localization of Bcl-2 and Bcl-x_L to intracellular membranes. Confocal images of FL5.12 cells transfected with human *bcl-2* or FLAG-tag-*bcl-*x_L are shown. Cells were permeabilized, and stained with (A) 6C8 (anti-Bcl-2) or (B) M2 (anti-FLAG) antibodies. The staining pattern of Bcl-2 and Bcl-x_L is granular and extranuclear, consistent with localization of Bcl-2 and Bcl-x_L to cytoplasmic organelles. Dual labelling experiments and electron microscopy have shown that Bcl-2 and Bcl-x_L localize primarily to the outer membrane of mitochondria, the ER and the nuclear envelope (see Table 1 for details and references).

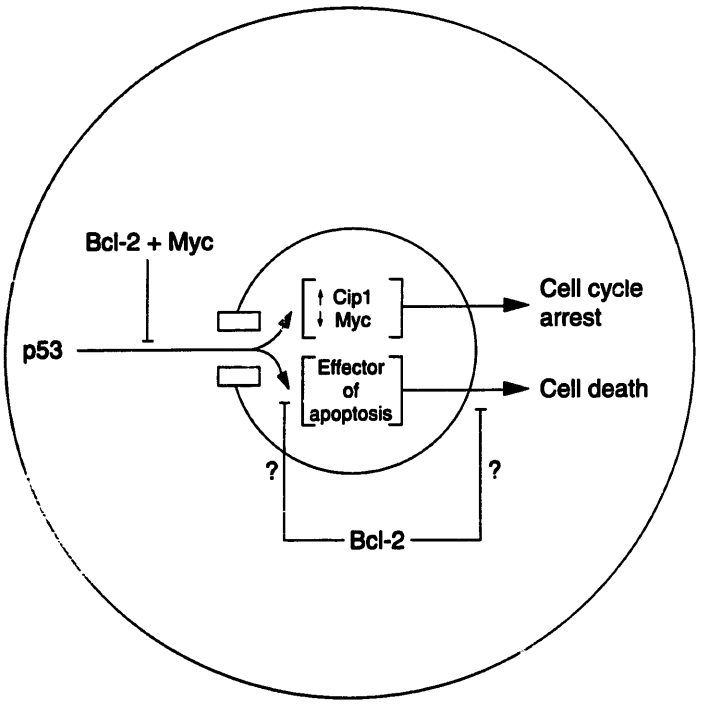

FIGURE 3

Model of apoptosis based on the interaction of Bcl-2 and Myc with p53. In mouse erythroleukaemia cells, p53 arrests cells in the G1 phase of the cell cycle, and induces apoptosis. [Arrest results from downregulation of c-*myc* expression, which is obligate for cell cycle progression, and upregulation of *Cip1* expression (Cip1 binds to cyclins and inhibits cell cycle progression).] The ability of p53 to induce apoptosis is independent of its cell cycle arrest function. Cells that coexpress p53 and *bcl-2* are growth arrested, but apoptosis is delayed[38]. p53 is nuclear in these cells. We suggest that p53 activates an effector of apoptosis that is inhibited by Bcl-2. By contrast, cells that coexpress genes encoding p53, Bcl-2 and Myc continue to proliferate and do not undergo apoptosis[38]. In such cells, p53 is cytoplasmic during G1 when cells are susceptible to p53-induced apoptosis and growth arrest[38]. We postulate that Myc induces or activates a factor that cooperates with Bcl-2 to block p53 transport across the nuclear membrane.

protective effects of the Bcl-2 protein[11,12]. It is not clear whether the residual function of the mutant proteins results from interaction with cytoplasmic targets or integration of a small amount of the mutant protein into organelle membranes. Interestingly, Bcl-2 prevents the appearance of morphological changes characteristic of apoptosis in cells that do not contain mitochondrial DNA[49] or nuclei[50].

Bcl-2-interacting proteins

Recently, Bcl-2 has been shown to bind to two proteins: Bax and R-Ras. These observations have given insight into how Bcl-2 functions. As discussed above, increased levels of *bax* expression appear to counteract the effects of Bcl-2 and to promote, rather than inhibit, apoptosis[15]. Substitution of Gly145 of the BH1 domain or Trp188 of the BH2 domain of Bcl-2 functionally inactivates the protein[51], and these mutants cannot bind Bax but can still form Bcl-2 homodimers[52]. These results can be interpreted in three ways. First, it is possible that the Bcl-2 homodimer is normally the functional complex, but that the mutated homodimers are not fully active. Second, the Bcl-2–Bax heterodimer may be the functional complex and the Bax–Bax homodimer may be a negative regulator[51]. Third, the domains of Bcl-2 that interact with Bax might allow Bcl-2 to dimerize with another factor to form an active complex. In this latter case, neutralization of Bcl-2 by *bax* overexpression may be explained by competition between Bax and the other Bcl-2 partner for Bcl-2. The modulation of Bcl-2 activity by its partners may explain why Bcl-2 fails or is very poor in inhibiting some forms of apoptosis[5,8].

The Bcl-2 protein also associates with the C-terminal 60 amino acids of the R-Ras protein, a 23 kDa membrane protein that is 55% homologous to Ha-Ras[52]. The Ras protein family consists of more than 50 proteins that regulate many diverse processes, such as cellular proliferation and differentiation, cytoskeletal control, and intracellular vesicular trafficking. Understanding of the consequences of the Bcl-2–R-Ras interaction awaits discovery of the function of the R-Ras protein.

Bcl-2 and oxygen free radical metabolism

As discussed in the article by McConkey and Orrenius, reactive oxygen species are thought to be involved in apoptosis. Recently, it has been postulated that Bcl-2 inhibits cell death by acting as an antioxidant[53] or inhibiting the generation of oxygen free radicals[54]. In haematopoietic cells undergoing apoptosis after exposure to various apoptotic signals, overexpression of *bcl-2* was associated with decreased oxidative damage to cellular constituents, but was not associated with decreased formation of reactive oxygen intermediates[53]. However, in neuronal cells undergoing necrosis as a result of glutathione depletion, expression of *bcl-2* was associated with both decreased generation of oxygen free radicals and decreased oxidative damage to cellular constituents[54]. Thus, Bcl-2 appears to function as an antioxidant. However, it is unclear whether Bcl-2 protects against death specifically induced by reactive oxygen species and, indeed, whether reactive oxygen intermediates are required for apoptosis.

Bcl-2 and intracellular Ca^{2+}

Bcl-2 may inhibit apoptosis by altering Ca^{2+} fluxes through intracellular organelles. In some experimental systems, the release of Ca^{2+} from a mobilizable pool located in the ER has been associated with apoptosis (see Orrenius and McConkey's article). Bcl-2 is located in ER membranes and its overproduction interferes with the efflux of Ca^{2+} across ER membranes in cells undergoing apoptosis[55,56].

Bcl-2 and modulation of subcellular trafficking

The idea that Bcl-2 may play a role in nuclear transport came from immunohistochemistry studies suggesting that Bcl-2 might be localized to nuclear pores[45]. Bcl-2 has recently been shown to alter the nucleocytoplasmic trafficking of p53 (Ref. 38) and the cdc2 and CDK2 cell cycle regulatory proteins[57]. Coexpression of *bcl-2* with the gene encoding p53 delayed p53-induced apoptosis, but did not affect

p53 trafficking to the nucleus nor p53-induced growth arrest; however, Bcl-2 and Myc cooperated to block p53 entry into the nucleus and thus prevented p53-induced apoptosis and growth arrest[38]. We speculate that Bcl-2 inhibits an effector of apoptosis that is activated by p53, and that Myc induces or activates afactor that cooperates with the membrane protein Bcl-2 to modulate the transport pathway responsible for the entry of p53 into the nucleus (see Fig. 3).

Future directions

Remarkable progress has been made in understanding the role that Bcl-2 and its family members play in regulating apoptosis and how this contributes to embryonic development, adult tissue homeostasis, and carcinogenesis. Apoptosis is characterized by changes in multiple cellular processes. It follows that Bcl-2 must block a single critical step that initiates these many events, or that it blocks all of the multiple events that together trigger apoptosis. A complete understanding of the factors with which Bcl-2 family members interact, and how these interactions regulate the mediators of apoptosis, will distinguish between these possibilities and lead to new insight into this unique cell viability regulatory system.

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