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BCL-2, A Novel Regulator of Apoptosis

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Abstract The *bcl-2* gene has a unique function among mammalian oncogenes as a negative regulator of apoptosis. Its expression pattern in embryonic and adult tissues is consistent with a role in maintaining in vivo survival of specific cell types.

The biochemical function of bcl-2 is unknown, but its localization to mitochondrial and microsomal membranes suggests several possibilities. bcl-2 is protective against oxidative stress in mammalian cells and can be replaced by antioxidants in a factor-deprivation model of apoptosis. These results are consistent with a model of apoptotic death involving oxidative stress in a central pathway.

The recent discovery of several bcl-2-related genes, some of which also inhibit apoptosis and others that unexpectedly promote apoptosis, has shed new light on several aspects of bcl-2 action. © 1996 Wiley-Liss, Inc.

Key words: *bcl-2* gene, localization, apoptosis, antioxidants, oxidative stress

The *bcl-2* gene was first characterized as the activated cellular oncogene in follicular lymphoma, a type of non-Hodgkin's lymphoma marked by slow growth and accumulation of mature resting B lymphocytes [1–3]. At least 85–90% of follicular lymphomas harbor an identical t(14;18) translocation, leading to the expectation that a previously unknown oncogene, designated *bcl-2* (B cell lymphoma/leukemia-2), was activated by the translocation.

The breakpoint on chromosome 14 interrupted the immunoglobulin heavy chain locus, providing a molecular tag to clone chromosome 18 sequences adjacent to the translocation breakpoints from genomic DNA libraries made from t(14;18)-bearing cell lines. Analysis of these sequences identified the *bcl-2* gene on the telomeric 18q chromosome fragment. In the majority of cases the chromosome 18 breakpoint occurred in the 3′ untranslated region of *bcl-2*, producing a *bcl-2–Ig* fusion gene [4]. Although the coding region of the *bcl-2* gene is not mutated, the significant effect of the translocation is overexpression of wild-type *bcl-2* RNA and protein [5].

The deduced amino acid sequence of the BCL-2 protein contains no recognized motifs or homologies that hint at its function. Initial transfection experiments also failed to demonstrate transforming activity with *bcl-2*. The first description of its cellular function by Vaux et al. [1988] employed factor-dependent cell lines for transfection studies of *bcl-2* [6]. Although overexpression of *bcl-2* did not yield factor-independent cell lines, prolongation of survival in the absence of an obligate growth factor, interleukin-3 (IL-3), was achieved. In this manner, *bcl-2* can substitute for the survival function of growth factors but does not promote cell cycle progression. Although these initial experiments suggested that *bcl-2* may perpetuate some downstream effects of specific growth factor–receptor interactions, subsequent tests of *bcl-2* function have demonstrated a broad survival effect in diverse models of apoptotic cell death [7].

The properties of *bcl-2* described above were unique for a putative oncogene and merited a careful examination of its oncogenic potential. Transgenic mice were created with a *bcl-2*-immunoglobulin enhancer transgene that targeted high levels of *bcl-2* expression to the B lymphocyte lineage [8]. These transgenic animals uniformly develop polyclonal B lymphocyte hyperplasia, likely due to the prolonged survival of B lymphocytes demonstrated in in vitro assays. After a latency period of about 1 year, 10–15% of *bcl-2*-transgenic mice develop diffuse immunoblastic B-cell lymphomas [9]. Approximately one-half of these tumors have clonal

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rearrangements of the c-*myc* proto-oncogene, supporting a synergistic relationship between *bcl-2* and c-*myc* that was first noted with bone marrow B–lymphocyte precursors [6].

bcl-2 is expressed in multiple lineages during development and in mature animals and high levels of expression are found in many types of malignancy [10–12]. In these tissues, *bcl-2* may serve as a regulator of programmed cell deaths in diverse cell types as well as a deregulated survival factor in cancer cells. Studies of *bcl-2* function and regulation should provide important clues to a basic cellular mechanism, cell death, that remains poorly understood.

INTRACELLULAR TARGETING OF BCL-2

Initial immunofluorescence microscopy studies with anti-BCL-2 antibodies showed an inhomogenous cytoplasmic and perinuclear pattern of staining [13]. The inferred amino acid sequence of BCL-2 was notable for a potential membrane spanning domain at the COOH-terminus and the lack of a recognizable signal peptide, suggesting an intracellular membrane localization [14].

Co-immunofluorescence experiments using organelle-specific probes and anti-BCL-2 antibodies have demonstrated overlapping distributions with mitochondria and endoplasmic reticulum [13,15]. Subcellular fractionations have confirmed that BCL-2 is associated with mitochondrial, nuclear, and endoplasmic reticulum compartments, in varying proportions in different studies [16]. It is unknown whether different cell types and expression levels of *bcl-2* may affect differential intracellular trafficking.

In vitro import of BCL-2 to mitochondria results in insertion into the outer mitochondrial membrane, compatible with results of immuno-electron microscopy and protease sensitivity assays of BCL-2 in situ [16,17]. Contrary data are obtained by hypotonic lysis of mitochondria, followed by separation of inner and outer mitochondrial membrane-enriched fractions [13]. BCL-2 co-purifies with a succinate dehydrogenase-rich inner mitochondrial membrane and matrix fraction, but not with an outer mitochondrial membrane marker, monoamine oxidase. A similar result has been observed with outer membrane proteins localized to contact sites between inner and outer membranes and would be consistent with the patchy distribution of BCL-2 at mitochondrial membranes by immuno-electron microscopy.

Nguyen et al. have compared BCL-2 targeting to mitochondrial and microsomal sites with reconstituted in vitro import assays [17]. BCL-2 inserts into both mitochondrial and microsomal membranes via the C-terminal hydrophobic domain of BCL-2, but with lower efficiency at microsomal sites [17,18]. Targeting to mitochondrial, but not microsomes, was dependent on ATP and sensitive to temperature. The signal anchor sequence for the yeast outer membrane protein Mas70p is homologous to the C-terminal domain of BCL-2. Like the Mas70p targeting sequence, the BCL-2-anchoring domain was sufficient to target a heterologous protein to the outer mitochondrial membrane in an ATP and temperature-sensitive reaction. Furthermore, a synthetic peptide containing the Mas70p signal-anchor sequence competed with BCL-2 for import to mitochondria but not microsomes. These results suggest that different mechanisms are used for BCL-2 localization at mitochondrial and microsomal sites, and that selective targeting to mitochondria occurs in vitro and possibly in vivo.

ROLE FOR BCL-2 IN OXIDATIVE METABOLISM

Membrane localization of BCL-2 is required for optimal function, although partial protection against apoptotic cell death is observed with truncated bcl-2 mutants that accumulate in the cytoplasm. BCL-2 may interact with necessary cofactors that reside at specific membrane sites, such as BAX or other BCL-2-related proteins that may form heterodimers [19,20]. Alternatively, the function of *bcl-2* may maximized be at certain intracellular locations. Mitochondria, endoplasmic reticulum, and, in some reports, the nuclear envelope, are sites of oxygen free radical generation and potentially, oxidative stress. Oxidative stress is causally associated with some models of apoptotic death, such as those induced by ionizing radiation. The ability of *bcl-2* to function as a general inhibitor of apoptosis strongly suggests that a common pathway is involved in apoptosis that is regulated by *bcl-2*. We have sought to determine whether oxidative stress is a common step in multiple models of apoptosis, and the possible involvement of *bcl-2* in an antioxidant pathway.

Direct application of oxidants to cells can result in an apoptotic process of cell death. Lymphoid cell lines were resistant to hydrogen peroxide-induced apoptosis, as well as most other triggers of apoptosis, if they expressed high lev-

els of *bcl-2* [21]. Cells incubated with 0.5 mM H_2O_2 were completely protected by *bcl-2,* but a twofold increase in hydrogen peroxide dose abolished the effect of *bcl-2.* These results suggest that there is a dosage effect in apoptotic pathways that can overcome a preset level of *bcl-2* survival–enhancing function.

If oxidative stress is participating in a general mechanism of apoptosis, antioxidants should protect cells from apoptotic death. In particular, antioxidant effects should be seen following triggers of apoptosis that do not involve oxidative stress as a proximal mechanism. We evaluated growth factor withdrawal in the IL-3-dependent pro-B lymphoid cell line, FL5.12, for the efficacy of antioxidants in a well-established model of apoptosis [21]. Moderate protection was observed with 50 mM N-acetylcysteine or 25 μM vitamin E. A stronger effect was seen following transfection and overexpression of glutathione peroxidase, an seleno-enzyme that metabolizes H_2O_2. Overexpression of manganese superoxide dismutase in this model had no effect on cell survival, however. Deleterious effects have been observed with expression of high levels of superoxide dismutase in cell lines that are correctable with supplemental catalase or glutathione peroxidase activity [22]. Thus, in the model of apoptosis triggered by IL-3 withdrawal, cellular capacity to metabolize peroxides or terminate lipid peroxidation chain reactions is unexpectedly capable of inhibiting cell death.

We subsequently examined directly for evidence of oxidative stress or damage in cells during apoptosis. The 2B4 T-cell hybridoma undergoes dexamethasone-induced apoptosis in a relatively synchronized fashion, representive of the sensitivity of normal thymocytes to glucocorticoids. The fluorescent polyunsaturated fatty acid, cis-parinaric acid, was used in a flow cytometric assay for lipid peroxidation following dexamethasone treatment [21]. 2B4 cells have detectable increases in lipid peroxidation by 4 h after dexamethasone treatment that progressively increases over the next 7 h. By contrast, 2B4 cells that were transfected with *bcl-2* were protected from dexamethasone-induced apoptosis and showed no evidence of lipid peroxidation over the same time interval. The occurrence of measurable lipid peroxidation after 4 h of dexamethasone suggests that an oxidative stress begins early during the apoptosis process. This biochemical measurement precedes DNA degradation to oligonucleosome-sized fragments by 6–7 h and supports the concept of an oxidative component to the apoptotic mechanism.

2B4 cells do not appear to undergo an oxidative burst in response to glucocorticoids. Incubation of 2B4 cells with 5,6 carboxy-2′,7′-dichlorofluorescein-diacetate, an oxidation-sensitive fluorescent probe responsive to several reactive oxygen and free radical species failed to detect an increase in oxidant generation with dexamethasone treatment over basal levels [21]. It can be postulated that the lipid peroxidation observed in this model represents a pro-oxidant effect from a primary decrease in cellular antioxidant protection. It is tempting to speculate that *bcl-2* function may be a target of specific apoptotic triggers in some models of apoptosis. In both dexamethasone-induced apoptosis and in response to the pro-oxidant compound menadione, *bcl-2* overexpression did not diminish rates of oxidant generation, suggesting that it may function further downstream in an antioxidant pathway to prevent lipid peroxidation and potentially other secondary effects of oxidative stress.

COMPLEX FAMILY OF CELL DEATH REGULATORS

An important development in understanding *bcl-2* function and its regulation has been the discovery of a family of *bcl-2*-related genes [23]. The predicted amino acid sequences of these proteins are most similar in two short domains, designated BH1 and BH2 [24]. There are currently seven genes identified in mammalian species [25–32] (Fig. 1). Certain members of this family of genes are capable of promoting cell death. BAX, a protein identified by its coimmunoprecipitation with BCL-2, sensitizes cells to cell death when overexpressed [25]. The *bcl-x* gene has two splice forms with opposing functions: *bcl-*x_L promotes survival while *bcl-*x_S promotes cell death [26].

The advantages of having multiple cell survival genes expressed in an organism are unclear. Apart from the current assays for apoptosis, there are no functional discriminators available. Perhaps they function in different cell types. The expression patterns of *bcl-2* and related genes are different in some instances. As an example *bcl-*x_L is highly expressed in adult central nervous system (CNS), while *bcl-2* expression is highest in fetal CNS and downregulated in adults [26]. We have recently examined the expression of *bcl-2* and *bcl-x* in primitive hematopoietic progenitors [33]. We identified a

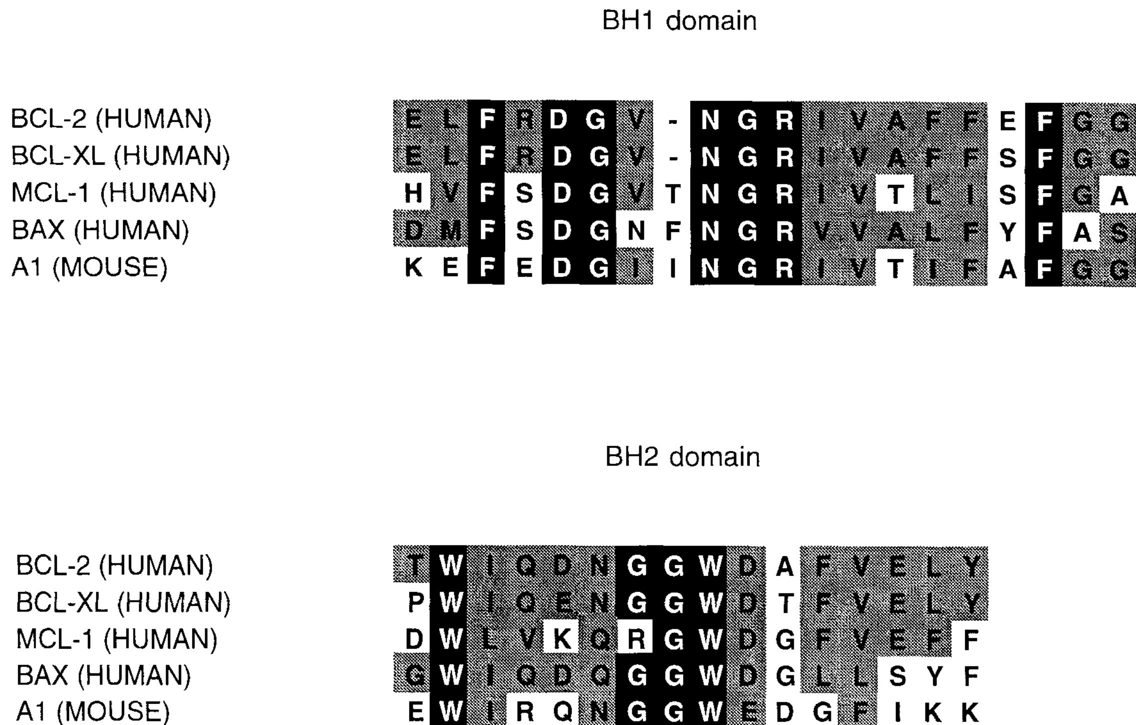

Fig. 1. Conserved nucleotide sequences in the BH1 and BH2 regions are shown. Amino acids identical to human bcl-2 are in black and conservative substitutions are shaded.

primitive human myeloid precursor population in which *bcl-2* is not expressed, in contrast to the majority of bone marrow precursors. These cells have in vitro characteristics of quiescent pluripotent stem cells. Induction of *bcl-2* expression occurs during activation and differentiation in response to hematopoietic growth factors. In contrast to *bcl-2, bcl-x* is expressed in all subsets at comparable levels. The discordant expression of *bcl-2* and *bcl-x* suggests important differences in the regulation of these genes by physiologic signals. The importance of *bcl-x* for early hematopoietic function and survival is borne out by the phenotype of *bcl-x* gene-ablated mice recently published [34]. These mice die at embryonic day 13 with widespread apoptotic cell death of hematopoietic cells in fetal liver.

There are other important differences in the regulation of the expression of these genes within the same cell type. *bcl-2* mRNA is induced within 6–12 h after mitogenic stimuli in lymphocytes. *bcl-x*$_S$ mRNA increases within 6 h after lymphocyte activation. *Mcl-1* was identified by differentially screening for early response genes expressed during myeloid differentiation and has been shown to be transiently upregulated in response to differentiating stimuli [27].

BCL-2 and BAX interact as heterodimers and each also can form homodimers. The opposing cellular functions of *bcl-2* and *bax* have suggested the possibility that one dimer partner acts as a dominant negative inhibitor of the other partner's active function. Mutations in either BH1 or BH2 domains of *bcl-2* can disrupt dimer formation and characteristically eliminate *bcl-2* function. The presence of these domains in other members of the *bcl-2* gene family suggest that dimerization with multiple competing partners may be occurring [35]. Yin et al. [24] created and analyzed a single amino acid substitution in the BH1 domain of *bcl-2* that selectively interfered with BAX-BCL-2 heterodimer formation yet retained the capacity for BCL-2 homodimer formation. This mutant *bcl-2* had lost its survival function, suggesting that BAX–BCL-2 heterodimerization is essential for *bcl-2* function. If the dominant negative model of BAX–BCL-2 function is correct, this result suggests that *bcl-2* operates by inhibiting a prodeath function of bax.

The availability of *bcl-2* homologs, some of which act to decrease the effective *bcl-2* survival function through competing protein–protein interactions, will provide a stringent test for pro-

posed mechanisms of *bcl-2* action. Alterations of cell survival by experimentally manipulating expression levels of *bcl-2*-related genes should have similar effects on oxidative stress, for example, if *bcl-2* function directly affects this pathway. Similarly, analysis of the effects of overexpressed *bax* on oxidative stress compared with *bcl-2* may yield further information about the opposing functions of these dimerization partners.

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