

The Protein bcl-2 α Does Not Require Membrane Attachment, but Two Conserved Domains to Suppress Apoptosis

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Abstract. Bcl-2 is a mitochondrial- and perinuclear-associated protein that prolongs the lifespan of a variety of cell types by interfering with programmed cell death (apoptosis). Bcl-2 seems to function in an antioxidant pathway, and it is believed that membrane attachment mediated by a COOH-terminal hydrophobic tail is required for its full activity. To identify critical regions in bcl-2 α for subcellular localization, activity, and/or interaction with other proteins, we created, by site-directed mutagenesis, various deletion, truncation, and point mutations. We show here that membrane attachment is not required for the survival activity of bcl-2 α . A truncation mutant of bcl-2 α lacking the last

33 amino acids (T3.1) including the hydrophobic COOH terminus shows full activity in blocking apoptosis of nerve growth factor-deprived sympathetic neurons or TNF- α -treated L929 fibroblasts. Confocal microscopy reveals that the T3 mutant departs into the extremities of neurites in neurons and filopodias in fibroblasts. Consistently, T3 is predominantly detected in the soluble fraction by Western blotting, and is not inserted into microsomes after in vitro transcription/translation. We further provide evidence for motifs (S-N and S-II) at the NH₂ and COOH terminus of bcl-2, which are crucial for its activity.

IN both vertebrates and invertebrates, cell numbers are controlled by a balance between cell renewal and cell death. Any disruption of this balance may result in neoplasia. Whereas cell proliferation and its aberrances have been extensively studied, relatively little is known about the regulation of cell death (20, 51). Cell death can occur by two distinct mechanisms, apoptosis or necrosis. In the former case, the cell actively participates in its own demise, characterized by plasma membrane blebbing, cell volume loss, nuclear condensation, and endonucleolytic degradation of DNA into nucleosomal intervals (DNA ladder) (67). It is now clear that apoptosis occurs under a wide range of primarily physiological processes, including the removal of interdigital cells during embryonic digit formation (32), the elimination of massive numbers of neurons generated in early developments to ensure refinement of connections at later stages (52), the deletion of autoreactive T and B cell clones during maturation (54), the formation of diverse immune cells from progenitor cells (54), the involution or self renewal of epithelial tissues/glands (28), and in tumor

regression (66). In all these cases, the purpose of apoptosis is to actively remove superfluous cells, cells with inappropriate specificity or developmental capacity, or cells that have already served their purpose (20, 52, 54). This removal of cells is achieved by changing the levels of hormones and growth factors in the cellular environment.

Although many of the extracellular signals that regulate apoptosis in different cell types have been identified, the biochemical mechanisms and identities of the gene products responsible for this process are only now being elucidated (16, 20, 24, 51). Genetic analysis of the nematode *Caenorhabditis elegans* has led to the identification of several genes that affect various steps in programmed cell death occurring during development. The ced-3 and ced-4 genes are thought to be necessary for apoptosis to occur because mutations that inactivate either of these result in the survival of cells that would otherwise die (19). In contrast, the ced-9 gene appears to function in preventing apoptosis because a gain-of-function mutation of this gene eliminates apoptosis (30).

This implies that both effector and repressor genes will also exist in mammalian cell death pathways. One such mammalian gene has been identified as bcl-2. Bcl-2 has been isolated from follicular B cell lymphomas where it is overexpressed as a result of a t(14;18) chromosomal translocation (4, 14, 62). It represents a novel oncoprotein that appears to predispose cells for carcinogenesis by enhancing their lifespan rather than their proliferation (36, 41). Gene transfer

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studies have indicated that bcl-2 blocks cell death induced by a variety of stimuli. It confers survival to certain hematopoietic cell lines after growth factor withdrawal (35, 49, 63), protects primary neuronal cell cultures (1, 26) as well as PC12 (5) from degradation induced by nerve growth factor depletion, spares cells from death induced by irradiation (53, 56), heat shock, ethanol, methotrexate, glucose and serum withdrawal, membrane peroxidation, free-radical induced damage (34, 39, 61, 68), TNF (31), and glucocorticoid (2, 53, 56), extends the survival of virally infected cells to allow viral persistence (3, 44) and renders tumor cells markedly resistant to killing by a wide variety of antineoplastic drugs (46, 64). Redirection of bcl-2 expression to cortical thymocytes in transgenic mice led to cells that were now resistant to glucocorticoid-, γ -irradiation-, and anti-T cell receptor-induced cell death (53, 56).

Recent results point to an important role of bcl-2 in an antioxidant pathway (34, 39). How bcl-2 exerts this function is, however, still poorly defined. A major hint could provide four recently identified proteins that interact with bcl-2 (bcl-xs, bcl-xL, bax, and R-ras) (7, 22, 50). Two of them, bcl-xs and bax, are bcl-2 homologues that can negatively regulate the survival activity of bcl-2 in a dominant fashion (7, 50). Since bax can form heterodimers with bcl-2 in vivo, it is believed that the death-inducing effect of the former is either caused by sequestering the latter or by directing the heterodimer to effectors of apoptosis instead of cell survival (50). It is, therefore, important to define the regions in bcl-2 that interact with bax/bcl-xs or downstream effectors.

Another unresolved issue is the intracellular site of action of bcl-2. In some cells, confocal microscopy and subcellular fractionation studies revealed an association of bcl-2 with the mitochondrial membrane (35). A recent study by Nguyen et al. (48) indicates that the COOH-terminal, hydrophobic 22 amino acids of bcl-2 serves as a signal/anchor segment to target bcl-2 to the outer mitochondrial membrane. In other cells, bcl-2 has mainly been localized to the endoplasmic reticulum and the nuclear membrane (3, 12, 38, 45a). Consistent with the latter observation, bcl-2 was still able to prevent the death of cells that lacked mitochondria DNA and, thus, an intact respiratory chain (38). These findings indicated that the action of bcl-2 may not be limited to one particular subcellular region but might occur at numerous sites, depending on the cell examined.

The bcl-2 gene can encode for two proteins, bcl-2 α and bcl-2 β , which differ only in their carboxyl tails because of an alternative splicing mechanism (60). Whereas overexpressed bcl-2 β predominantly resides in the cytoplasm and lacks survival activity, bcl-2 α is membrane bound and capable of conferring cell survival (58). This difference has been attributed to the presence of a hydrophobic domain in the carboxyl terminus of bcl-2 α (13), which is thought to act as a transmembrane anchor. Removal of this stretch has previously been shown to mitigate the survival activity of bcl-2 α (3). A recent report by Hockenbery et al. (34), however, suggested that a soluble bcl-2 lacking this stretch may still exert partial survival activity.

In an attempt to define bcl-2 functional motifs, regions required for subcellular localization and/or sites of interaction with other proteins, we generated bcl-2 mutants by site-directed mutagenesis and examined them for activity and

subcellular distribution by microinjection into the nuclei of nerve growth factor (NGF)¹-deprived neurons (26).

Materials and Methods

Cell Culture

Sympathetic neurons from superior cervical ganglia of newborn rats were cultured as previously described (29). L929 murine fibroblasts and their derivatives were maintained in RPMI medium supplemented with 10% fetal calf serum (Gibco Laboratories, Grand Island, NY).

Antibodies

A polyclonal antiserum against a 14-amino acid peptide encompassing domain 2 of murine bcl-2 (GAAPTPGIFSFQPE) was generated in rabbits. The peptide was synthesized by the TASP method (23). For Western immunoblotting, the bcl-2 antiserum dilution was 1:2,000, for immunocytochemistry 1:200. Part of the antibody was affinity purified on nitrocellulose strips containing a GST-bcl-2 fusion protein as described before (21). The monospecific 27-4 antibody obtained was used at dilutions 1:500 for Western blots and 1:100 for immunocytochemistry. Secondary antibodies were an alkaline phosphatase-conjugated goat anti-rabbit whole antibody for Western blots (Sigma Immunochemicals, St. Louis, MO) and a FITC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Labs., Inc., West Grove, PA).

cDNA Cloning and Construction of Expression Vectors

The murine bcl-2 cDNA was obtained by PCR amplification of a murine B6.1 T cell library using the primers GGTACCTGCAGCTTCTTTTCGGGG and CGCTGCAGGTTTGTGACCTCACTTGTG. Whereas a PstI site was introduced into the 3' primer, the 5' primer already contained a PstI site upstream of the ATG start codon. The amplified cDNA was cloned into the PstI site of pSK⁺ Bluescript (Stratagene, La Jolla, CA), and the correct sequence confirmed by sequencing. Subsequently, the bcl-2 cDNA was cloned into the HindIII site of the retroviral vector, pMV12 for eukaryotic expression (kindly provided by I. B. Weinstein and R. S. Krauss, Columbia-Presbyterian Cancer Center, Columbia University, College of Physicians and Surgeons, New York). Bcl-2/psK⁺ was cut with HindIII and Sma and ligated at the 5' end into the HindIII site of pMV12. For site-directed mutagenesis, the bcl-2 cDNA was cloned into the EcoRV/XbaI site of pUC19M (Clontech, Palo Alto, CA), a derivative of pUC19 having the EcoRI site replaced by EcoRV. After mutagenesis and for microinjection into neurons and in vitro transcription/translation, wild-type and mutant constructs were all subcloned into the EcoRI/XbaI sites of pcDNA1/amp (Invitrogen, San Diego, CA). In this vector, the expression of the bcl-2 cDNA is driven by the cytomegalovirus promoter.

Site-directed Mutagenesis

Site-directed mutagenesis was carried out using the Transformer™ site-directed mutagenesis kit from Clontech, according to the protocol provided by the manufacturer. All the mutant bcl-2 cDNAs were subcloned into the EcoRI/XbaI site (for PT3, EcoRV/XbaI) of the pcDNA 1 amp expression vector for in vitro transcription/translation and microinjection into neurons. The mutagenic primers for bcl-2 mutagenesis were the following:

DEL1: (deletion of domain 1), resulting in the deletion of 78 bp (26 amino acids) between nucleotides (nt) 332 and 408 (Gly4-Glu29): CGGGGA-AGGATGCGCAATGGGATGCTGGAGATGCG;

DEL2: (deletion of domain 4), resulting in the deletion of 195 bp (65 amino acids) between nt698 and 894 (Phe127-Gly191): TTCACGCGAGG-GGACGCTGGATGCCTTTGTGGAA;

DEL3: (deletion F4.1 and F5), resulting in the deletion of 63 bp (21 amino acids) between nt 878 and 942 (Gln187-Phe207): introduces a unique ClaI site: CATCTGCACACCTGGATCGATTTCCTCGGTGCT;

DEL4: (deletion of the putative nuclear pore sequence), resulting in the deletion of 18 bp (6 amino acids) between nt 758 and 777 (Phe147-Gly152): TGGGGGAGGATTGTGGCCGCGGTCAATGTGTGGAGAGC

1. *Abbreviations used in this paper:* NGF, nerve growth factor; nt, nucleotides; Rb, retinoblastoma gene product.

P11: (point mutation of Cys155 to His155): TTCGGTGGGGCTATGCAT-GTGGAGAGCGTCAAC
P12: (point mutation of Cys226 to His226): GCCCTGGTCGGGGCCCCAC-ATCACTCTGGGTGCA
P13: (point mutation of Tyr177 to Phe177): GCCCTGTGGATGACTGAA-TTCCTGAACCGGCATCTG
T3.2 (truncation at nt 929 or Met203): CTATATGGCCCCAGCATGTGA-GGTCGACAAACCTGC
T4: (truncation at nt 899 or Asp193, removes F5): GATAACGGAGGCTGG-GATTGAGGTCGACAAACCTGC
T5: (truncation at nt 908 or Val196): GGCTGGGATGCCTTTGTGTGA-GGTCGACAAACCTGC
T6: (truncation at nt 926 or Ser202): GAACTATATGGCCCCAGCTGA-GGTCGACAAACCTGC
T7: (truncation at nt 923 or Pro201): GTGGA ACTATATGGCCCCCTGA-GGTCGACAAACCTGC

Three bcl-2 mutations were generated as follows: For T1 (truncation at nt 878 or Ile186), the bcl-2/pSK⁺ was digested with BamHI, and the resulting 576-bp fragment was directly cloned into the BamHI site of the pcDNA I/amp expression vector. Because the cloning site was not at a stop codon, T1 obtained one additional amino acid (H) encoded by 3' residing vector sequences. T2 and T3.2 were similarly generated, but via HindII and SphI digestions, respectively. T2 (truncation at nt 797 or Val159) obtained the additional amino acids IHHTGGRSSMHLEGPIL, whereas T3.1 (truncation at nt 929 or Met203) was extended by the amino acids HLEGPIL.

Construction of L929 Stably Overexpressing bcl-2

CsCl-purified sense orientation pMV12 plasmids alone or carrying either full-length wild-type or T3.1 truncated mutant bcl-2 cDNA (10 µg) were transfected into subconfluent ψ 2 cells with 25 µg of lipofectin (GIBCO BRL, Gaithersburg, MD) to produce recombinant murine Moloney retroviruses (21). After 48 h, the culture medium containing viruses was collected, filtered, and stored at -70°C. 5×10^5 recipient L929 cells (subconfluent) were infected with the virus-containing medium in the presence of 8 µg/ml of polybrene. 48 h later, the cells were trypsinized and replated into medium containing 400 µg/ml of hygromycin B (Calbiochem-Novabiochem Corp., La Jolla, CA). Resistant clones of L929 cells were picked by ring isolation after 2 wk of selection. Clones and mixed cell populations were expanded and analyzed for bcl-2 expression by Western blots. Two cell lines overexpressing either wild-type bcl-2 (L929bcl-2/5) or the T3.1 mutant (L929T3/3) were used for further studies. In addition, vector control lines were established (L929pMV12/2).

TNF- α -induced Apoptosis

L929 or their derivatives expressing either wild-type or T3.1 bcl-2 were seeded into 24-well plates at a concentration of 5×10^5 cells/ml. After 12 h, the cells were treated with 3 ng/ml mouse TNF- α (Boehringer-Mannheim Biochemicals, Indianapolis, IN). At the times indicated, half of the cells per well were trypsinized, stained with trypan blue, and counted under the microscope to determine cell viability. In parallel, the other half of the cells was collected by centrifugation and washed in 20 mM Tris-HCl (pH 8.0). DNA was phenol/chloroform extracted, treated with RNase (Boehringer-Mannheim Biochemicals) for 15 min at 37°C and electrophoresed on a 2% agarose gel to determine the degree of DNA ladder formation.

Microinjection of bcl-2 cDNAs into Neurons and Cell Survival Assay

Neurons were cultured in 3.5-cm diameter dishes for 7 d. Rectangles (10 \times 3 mm) containing 150–200 neurons were drawn on the bottom of each dish to allow localization of injected neurons. Doubly CsCl-purified circular pcDNA I amp plasmids containing various bcl-2 mutant cDNAs were dissolved in H₂O at a concentration of 0.05 µg/ml. Approximately 500 DNA molecules were introduced into one cell nucleus with a low pressure microinjection system (automatic injector; Inject+Matic, Geneva) to ensure high neuronal survival (25). 3 h after injection, the NGF-containing medium was replaced with NGF-free medium. The medium was changed daily. Survival of neurons was analyzed by morphological criteria. Neuronal death was clearly recognized by shrinkage and fragmentation of cell body and neurites (26).

In Vitro Transcription and Translation

In vitro transcription and translation was carried out exactly as described previously (13). cDNA fragments were cloned into the pcDNA I/amp expression vector. After linearization by NheI, the plasmids served as templates for in vitro transcription of RNA using SP6 RNA polymerase (37) (Promega Corp., Madison, WI). RNA products (0.5 µg) were translated in vitro using a rabbit reticulocyte lysate cell-free translation system (Promega Corp.) at 30°C for 45–60 min in the presence of 1 mCi/ml [³⁵S]methionine, as previously described (37). Microsomal vesicles (Promega Corp.), at a concentration of 3.0 eq/0.5 mg of initial RNA in a 25-µl translation reaction, were included in the reticulocyte translation reaction, and samples were incubated for an additional 30 min at 30°C. Translation was terminated by the addition of cycloheximide at a final concentration of 20 µg/ml. At this point, a membrane-stabilizing agent, tetracaine (27), was added to a final concentration of 2 mM, followed by 5 min of incubation at 24°C. All subsequent steps were carried out at 4°C. Sedimentation of the membrane vesicles was performed at pH 8.0 and 11.5 as described (27) by layering the reaction mixture over a 0.5-M sucrose cushion and centrifuging in an air-fuge (A100/18 rotor; Beckman Instruments, Inc., Fullerton, CA) for 5 min at 20 p.s.i. The entire supernatant solution (including the sucrose cushion) was removed, the membrane pellet was resuspended into the same volume, and both samples were analyzed by SDS-PAGE. As a positive control for translocation into microsomes, we concomitantly in vitro transcribed/translated a β -lactamase cDNA.

Subcellular Fractionation

Proteins were extracted as follows: Cells were washed three times in phosphate-buffered saline, and either immediately lysed in preheated (95°C) extraction buffer A (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 2 mM EGTA, 6 mM β -mercaptoethanol, 2 µg/ml aprotinin, and 10 µg/ml leupeptin) containing 1% SDS followed by sonication (total extracts) or scraped into ice-cold buffer A, lysed by sonication, and centrifuged at 100,000 g for 1 h, yielding the soluble cytosol and the particulate membrane pellet (8, 9). The particulate fraction was solubilized in preheated buffer A containing 1% SDS. The protein content in these extracts was determined according to a modified method of Bradford (10).

Immunoblot and Immunocytochemical Analysis

Immunoblot analysis of the cytosolic, membrane, and total extracts were performed exactly as described previously (8, 9). Briefly, after SDS-polyacrylamide gel electrophoresis on a 12% acrylamide gel (43), proteins were electrotransferred (Bio Rad Laboratories, Richmond, CA) as described by Towbin et al. (59). The membrane was subsequently stained with 0.2% Ponceau red to assure equal protein loading and transfer. Membranes were then incubated for 90 min with primary bcl-2 antibodies in a 1:2,000 dilution (for monospecific antibodies, 1:500) in blocking buffer. The immunocomplex was visualized by colorization with alkaline phosphatase-conjugated goat anti-rabbit secondary antibodies and nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate as substrates. For immunocytochemical analysis, microinjected neurons were kept in NGF-rich medium for 3 d, and L929 fibroblasts were grown in serum-containing medium overnight on coverslips before bcl-2 immunoreactivity was measured. Both types of cells were fixed in 3% paraformaldehyde, permeabilized in 0.05% saponin in PBS, and incubated with anti-bcl-2 and then FITC-conjugated goat anti-rabbit IgG antibodies. Immunolocalization of bcl-2 was performed by a Bio Rad confocal microscope.

Results

Generation of bcl-2 Mutants

To decipher functional domains in bcl-2, we considered the following regions as targets for mutagenesis: (a) the COOH terminus previously implicated in survival activity and membrane targeting; (b) stretches of amino acids including domain II (50) and two cysteines that are highly conserved between human, mouse, and chicken homologues of bcl-2 (18); and (c) three motifs that have been shown in other pro-

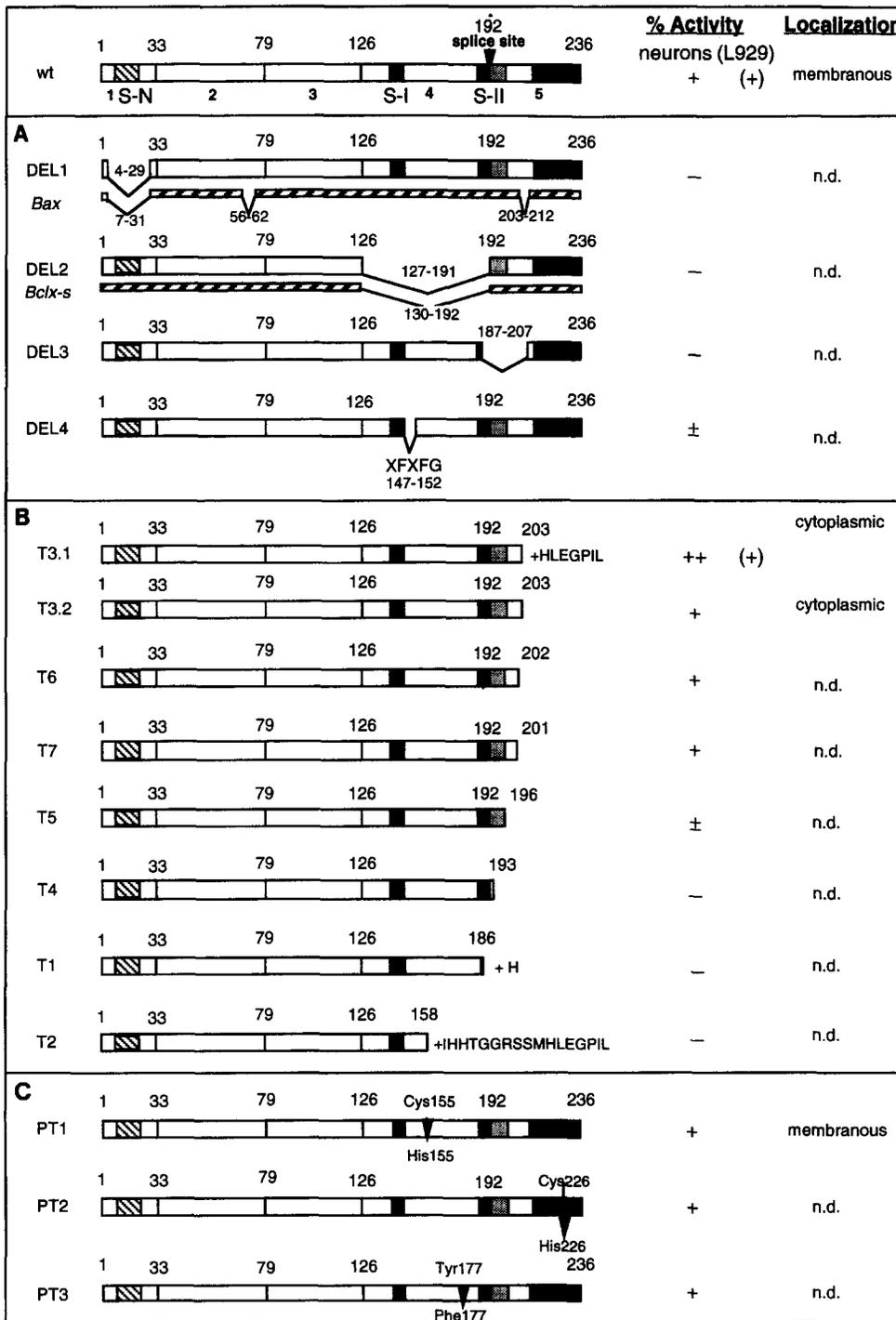


Figure 1. Strategy of the in vitro mutagenesis of *bcl-2* and survival activity of the mutants. (A) Murine wild-type *bcl-2* has 236 amino acid residues and can be divided into five domains, based on the varying homologies with *bcl-2* of other species. Particularly high sequence homologies are found at the NH₂ terminus (*hatched region*, domain *S-N*) and in domain 4 (*filled boxes*, *S-I* and *S-II*). C-terminal to *S-II* is found a region (*S-II-C*) required for functional activity of *bcl-2* (*stippled box*). The filled box at the COOH terminus represents the membrane interacting segment. (B and C) Structure of *bcl-2* mutants including various deletions (*DEL*), truncations (*T*), and point mutants. *DEL1* and *DEL2* mutants exhibit structural similarities with *bax* and *bclx-s*, respectively. Survival activity was measured in neurons deprived of NGF or in L929 fibroblast exposed to TNF- α (between parenthesis). Localization of *bcl-2* was determined by confocal microscopy in neurons and in 3T3 fibroblasts (see Fig. 3). *n.d.*, not determined.

teins to serve as protein-protein interaction modules. The latter include a variation of the so-called retinoblastoma gene product (Rb) binding "pocket" (LxCxE -DxxxLxDL/MxxL; in *bcl-2*: VxCxE -ExxxLxDNxxL) present in the adenovirus E1A, SV40 largeTAg, papillomavirus HPV E7, cyclin D, and Elf proteins (17, 65), the sequence EYLN found in the human EGF receptor which, when phosphorylated on tyrosine, interacts with the signaling molecule Sem-5/GRB2 (55), and a putative nuclear pore degenerate sequence xFxFG present in pore proteins, such as p62, NUP1, nup153, and NSP1 (57).

Survival Activity of *bcl-2* Mutants

The survival activities of the above described *bcl-2* mutants were examined by microinjecting their cDNAs into the nuclei of sympathetic neurons. We have previously shown that microinjection of the wild-type human *bcl-2* cDNA markedly enhanced the lifespan of these neurons in the absence of NGF (26). Here, we show that this is also the case for the murine *bcl-2* counterpart (Figs. 1 and 2). Whereas NGF-deprived neurons injected with the expression vector alone died by apoptosis within 3 d, neurons injected with

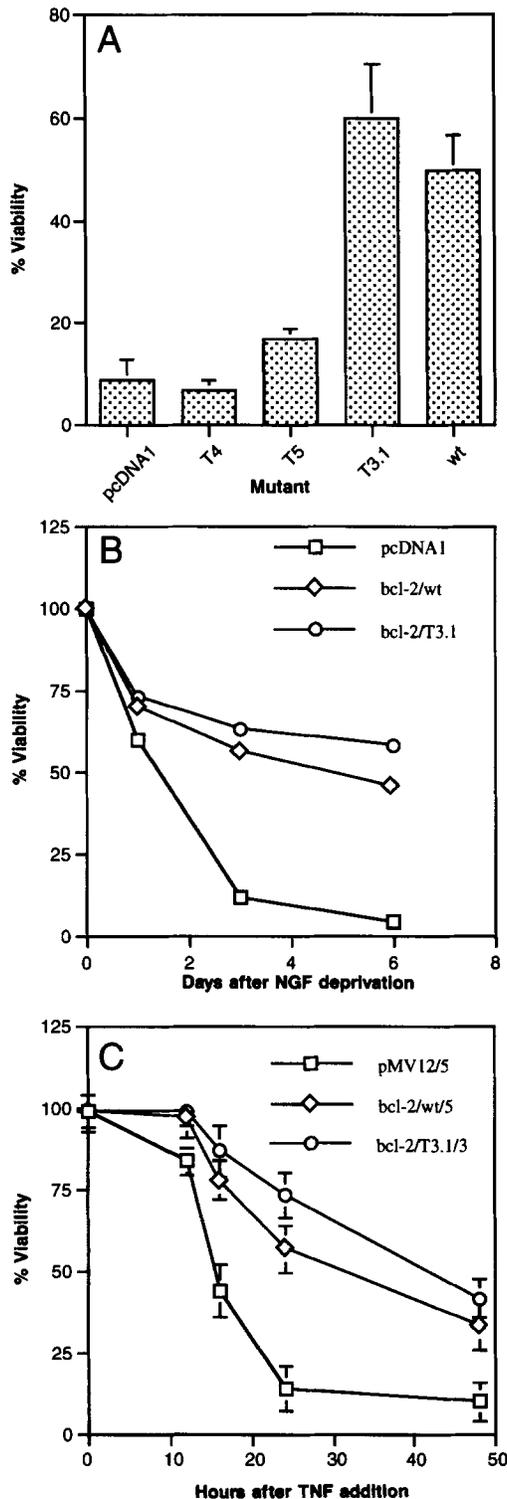


Figure 2. Inhibition of neuronal and fibroblast cell death by bcl-2 mutants. (A) Viability assays: Neurons were microinjected with pcDNA1/amp(vector control), murine wild-type bcl-2 α , and T4, T5, and T3.1 mutants were deprived of NGF. The percent viability was assessed by morphological criteria (shrinkage, fragmentation of cell bodies) after 6 d and represented as the mean \pm SEM ($n = 3$, 100–200 neurons each experiment). (B) Kinetics of cell viability: At several timepoints post NGF deprivation, the viability of neurons was assayed as described in A. Data are shown as the mean of three experiments. (C) Effects of bcl-2 on L929 cell survival in the presence of TNF- α . Cells were stably transfected with

bcl-2 fully retained viability. The survival activity of bcl-2 was maintained when the two conserved cysteines, Cys155 (PT1) or Cys226 (PT2), were mutated to histidines (Fig. 1). Cys155 is contained within a putative Rb-binding pocket (17, 65). According to previous findings with classical Rb-binding proteins, this mutation should have ablated survival activity, if an interaction between bcl-2 and Rb would be necessary for bcl-2 function (17, 65). Cys226 is part of the COOH-terminal hydrophobic stretch. This residue has been implicated in the interaction of bcl-2 with membrane proteins (11). Our data indicate that such an interaction, if it indeed occurs, has no significance for bcl-2 activity. No role for survival activity also appears to play a putative SH2 binding domain in the central part of bcl-2. This is because point mutation of tyr177 (PT3), the critical residue for a possible interaction with an SH2 domain (55), does not alter the capacity of bcl-2 to protect neurons from cell death (Fig. 1). In contrast, deletion of a putative nuclear pore degenerate sequence (xFxFG) (DEL4) (57) did partly mitigate the survival activity of bcl-2 (Fig. 1).

An interesting picture was obtained when we created deletions and truncations within highly conserved regions of bcl-2. We focused our analysis on the NH₂ terminus, the most conserved (up to 100%) internal part (domain 4), and the COOH terminus of bcl-2 (Fig. 1). Deletion of the first NH₂-terminal 29 amino acids (S-N domain, DEL1), as well as the removal of the internal domain 4 of bcl-2 (DEL2), fully destroyed the ability of bcl-2 to confer cell survival to NGF-deprived neurons (Fig. 1). Because in vitro translation analysis revealed no stable protein product of the DEL2 mutant (in contrast to DEL1, data not shown), we cannot exclude that the lack of activity is simply caused by translation inefficiency.

As previously described (3), removal of the last COOH-terminal 50 amino acids (truncation at Ile 186, T1) fully ablates the survival activity of bcl-2 (Fig. 1). A further truncation at amino acid 159 (T2) had the same effect (Fig. 1). Truncation at Asp 193 (T4) had a mitigating effect on cell survival (Fig. 1). This mutated bcl-2 essentially resembles bcl-2 β without its unique carboxyterminal tail (Fig. 1). When truncations were applied further carboxyterminal to T4, i.e., in the region unique to bcl-2 α , survival activity of bcl-2 was at least partly retained. T5, a truncation mutant at Val196 rescued 18% (Fig. 2 A), whereas T7 (truncation at Pro201), T6 (truncation at Ser202) rescued NGF-deprived neurons from cell death as efficiently as wild-type bcl-2 (Fig. 1). Consistent with these truncation mutants, deletion of Gln 187-Phe207 (DEL3) fully ablated survival activity of bcl-2 (Fig. 1). The deletion mutant was in vitro translated (data not shown) into a protein of correct size.

The most intriguing mutant was T3, a truncation at Met203. This mutant was constructed by two means (see Materials and Methods): (a) by restriction digest cloning, adding seven amino acids coded by the vector sequence before the stop signal (T3.1), and (b) by site-directed mutagenesis, introducing a stop codon after Met203 (T3.2). As expected from the analysis of T7 and T6, T3.1 and T3.2 were

pMV 12 (vector control), bcl-2 wild type, and its soluble mutant T3.1. The percent of cell viability was assessed by trypan blue exclusion. Data are shown as mean \pm SEM of three experiments.

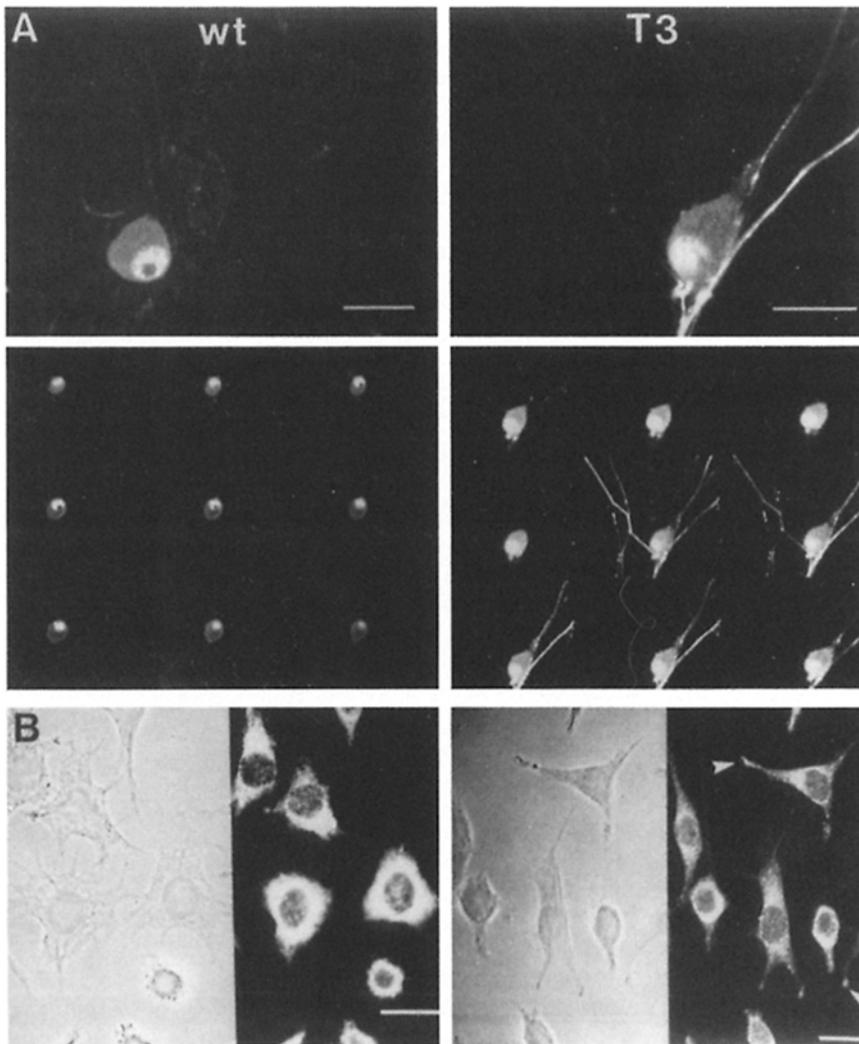


Figure 3. Subcellular distribution of wild-type bcl-2 and its COOH terminal truncated form. (A) Immunofluorescent confocal microscopy of neurons microinjected with wild-type bcl-2 (left panel) and the truncated mutant T3.1 (right panel). Serial section of 0.2 μm are shown in the lower panels. Bcl-2 was detected using affinity purified mouse bcl-2-specific antibody 27.4. (B) Immunofluorescent and phase contrast confocal microscopy of L929 fibroblast stably transfected with wild-type bcl-2 (left panels) and the truncated mutant T3.1 (right panels). The arrows point to the tip of the processes that accumulate the soluble form of bcl-2. Bars, 25 μM .

at least equally active as wild-type bcl-2 when injected into neurons (Figs. 1 and 2). Taken together, our mutagenesis studies revealed that the carboxy-terminal membrane attachment domain is not required for bcl-2 activity.

To obtain further evidence that truncation mutants lacking the hydrophobic COOH-terminal tail of bcl-2 exhibit survival activity, we stably overexpressed T3.1 in L929 fibroblasts. Consistent with a previous report (31), treatment of L929 cells with 3 ng/ml of mouse TNF- α triggers apoptosis (Fig. 2). Based on trypan blue exclusion staining, we found that only 18% of the cells survived such treatment for 24 h. Overexpression of the wild-type murine bcl-2, however, extended survival to 60% (Fig. 2). As in the neuronal assay, the T3.1 mutant was as active as wild-type bcl-2. Its overexpression protected up to 75% of the fibroblasts from TNF-induced cell death after 24 h of treatment (Fig. 2 C). This was seen with three independent cell clones expressing high levels of the construct as well as in a mixed cell population of overexpressors (data not shown). Overexpression of wild-type bcl-2 and the T3.1 mutant also led to a drastic delay of TNF-mediated DNA fragmentation in these cells (data not shown).

Bax was shown to regulate the activity of bcl-2 in a domi-

nant negative fashion, most likely by forming heterodimers (50). Although bax and bcl-2 display a high overall sequence similarity, bax is lacking 25 residues close to the NH₂ terminus. Because our DEL1 mutant resembled the structure of bax with respect to this deletion (Fig. 1, *DEL1*, residues 4–29; *BAX*, residues 7–31), we tested whether the absence of the corresponding amino acids could explain the inhibitory activity of Bax on bcl-2. Bcl-2 remained, however, equally active when coinjected into neurons with an excess of DEL1 mutant, indicating that additional structural features are required for heterodimerization (data not shown). Negative results were also obtained when the bcl-x_s-like mutant DEL2 was assayed for its inhibitory activity on bcl-2 (data not shown).

Association of bcl-2 Mutants with Subcellular Structures In Vivo

Since the hydrophobic 26 amino acids at the carboxy terminus were found to constitute a transmembrane domain that anchors bcl-2 in the intracellular membranes (11), it was important to investigate whether its absence in the active truncation mutants was associated with an altered subcellular

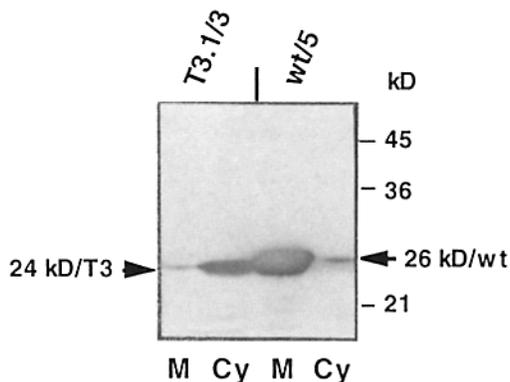


Figure 4. Western blot analysis of wild-type bcl-2 and the truncated T3.1 mutant. L929 fibroblasts stably overexpressing wild-type or T3.1 mutant bcl-2 were separated into heavy membranes (*m*) and cytoplasm (*cy*), and the bcl-2 was detected by Western blot analysis.

distribution. We embarked on two strategies to monitor the subcellular distribution of our mutants: (a) immunolocalization in intact cells; and (b) detection by Western blot analyses of fractionated cellular extracts.

For immunocytochemical studies, we injected the various mutant cDNAs into sympathetic neurons or analyzed L929 fibroblasts stably overexpressing the mutants. We recognized the previously reported (35), punctuated staining characteristic of mitochondria in cells injected with wild-type bcl-2 (Fig. 3 A). This was best observed in L929 cells where the nuclear background staining was weak (Fig. 3 B). By contrast, the T3.1 mutant bcl-2 associated with cellular structures not previously seen with wild-type bcl-2 immunofluorescence: neurite extensions in neurons (Fig. 3 A) and filopodias in fibroblasts (Fig. 3 B). This is consistent with a release of T3.1 from intracellular membrane structures and a departure to cellular extremities.

Consistent with this notion, Western blot analyses of cytosolic and membrane extracts from L929 cells stably overexpressing either wild-type or T3.1 bcl-2 demonstrated that while the 26-kD wild-type bcl-2 mainly associated with the particulate fraction, the 24-kD T3.1 is predominantly soluble (Fig. 4). It should be noted, however, that association with the respective compartment is not absolute. Approximately 10–20% of wild-type bcl-2 appears to be soluble un-

der normal growth conditions (Fig. 4). A small portion of T3 also remains membrane attached (Fig. 4).

Membrane Association of bcl-2 Mutants In Vitro

The removal of the carboxyl terminal hydrophobic stretch has been shown to create a form of bcl-2 that is incapable of inserting into microsomes after in vitro translation (13). Wild-type and mutant bcl-2 cDNAs used for microinjection into neurons (cloned into pcDNA I amp) were, therefore, subjected to T7 polymerase-directed in vitro transcription/translation in the presence and absence of microsomes (cotranslational insertion) (Fig. 5). In this method, β -lactamase, a signal peptide-containing protein normally translocates into the microsomes (Fig. 5). Surprisingly, in contrast to a previous report (13), wild-type bcl-2 did not partition completely into the microsomal fraction. At least 30% of the in vitro-translated wild-type protein and even 50% of the T1, T2 mutants were recovered in the supernatant (Fig. 5). In contrast, the T3.1 mutant was fully recovered in the supernatant (Fig. 5).

Discussion

Domain 4 of bcl-2 contains two highly conserved segments (S-I and S-II) and three regions with similarity to known functional motifs, i.e. an Rb-binding pocket, an SH2 binding domain and a nuclear pore degenerate motif. Our mutagenesis studies speak against an involvement of the Rb- and SH2-binding motifs in the survival activity of bcl-2. In contrast, the excision of the putative nuclear pore sequence led to a partial loss of activity ($\sim 20\%$). This raises the possibility of a role for bcl-2 in some aspect of nuclear transport, nuclear pore complex formation, or nuclear envelope assembly or maintenance. Consistent with this notion is the recent immunolocalization of bcl-2 to patches of the nuclear envelope that are highly reminiscent of nuclear pore complexes (42). We can, however, not exclude that the removal of the putative nuclear pore sequence simply distorts the structure of the adjacent extremely conserved S-I segment, which is thought to be of functional importance (50).

The NH₂-terminal region (S-N) is highly conserved between the bcl-2 of different species and bcl-x, suggesting a role in protein-protein interaction. Indeed, the deletion of 26 amino acids (residues 4–29) at the NH₂ terminus com-

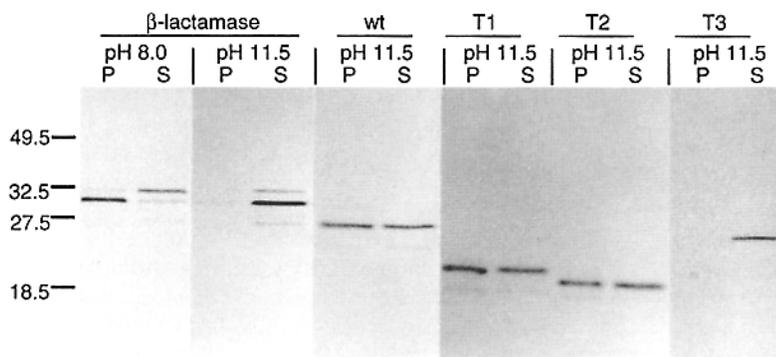


Figure 5. In vitro synthesis of wild-type and mutant bcl-2. In vitro synthesis of bcl-2 and its truncated mutants, T1, T2, and T3. cDNAs were transcribed, and the resulting RNAs were translated in vitro using rabbit reticulocyte lysate containing [³⁵S]methionine in the presence of microsomal vesicles. Membrane associated protein (*p*) was separated from soluble proteins(*s*) by high speed centrifugation at pH 11.5. Samples were analyzed by SDS-PAGE and by fluorography. The two left lanes are controls with β -lactamase, which translocates into the lumen of microsomes thereby liberating its signal peptide (reduction of molecular mass). At pH 11.5, microsomes lose their luminal content, and β -lactamase is recovered in the soluble, nonmembranous fraction.

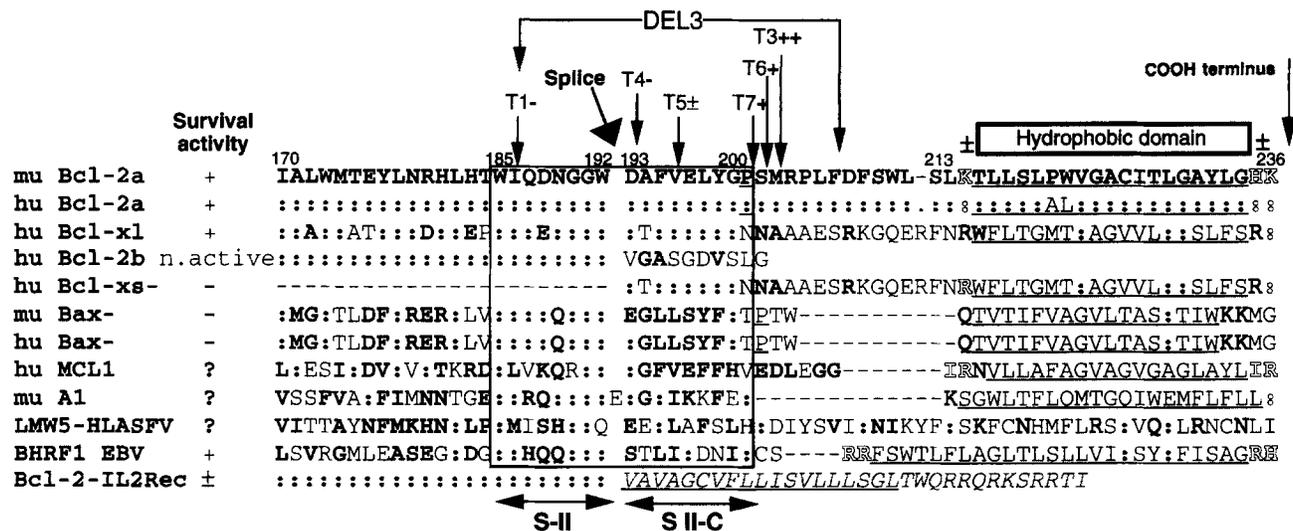


Figure 6. Structure-function analysis and regions of homology between mouse bcl-2 and related mammalian and viral genes. The COOH-terminal amino acid sequences for mouse and human bcl-2 α , mouse bcl-2 β , mouse and human Bax, human bcl-x α and bcl-x β , human mcl-1, mouse A1, African swine fever virus LMW5-HL, Epstein-Barr virus BHRF1, and a hybrid human bcl-2-IL-2 receptor transmembrane region were aligned (references 7, 15, 40, 45, 47, 50, 60, see text). The region with highest homology (S-II) and its adjacent functionally important COOH-terminal extension (S-II-C) are boxed. A predicted hydrophobic domain and adjacent charged residues are present in most members of the superfamily and are indicated by underlining and outlined characters, respectively. Residues identical to the mouse sequence are shown with (:), while conserved (hydrophobic and charged) amino acids are bold typed.

pletely impaired the survival activity of bcl-2. Because the translation of this mutant was efficient in vitro, a crucial role of the NH₂-terminal residues for the survival activity is likely. Interestingly, bax, which induces rather than prevents cell death, lacks this particular S-N domain.

Our results provide clear evidence that a stretch of amino acids within domain 4 (S-II) and its COOH-terminal extension delineated by Asp186-Val196 at the beginning of domain 5 (S-II-C) is crucial for its function (Fig. 5). The S-II region is highly conserved not only among different bcl-2 homologues (bax, bcl-x, and bcl-2 β) (7, 50, 60) but also among other putative cell survival factors. These include MCL1 (40), a gene product involved in programmed myeloid cell differentiation, A1 (45), a hemopoietic-specific early response gene, and BHRF1 (15) and LMW5-HL (47), two viral genes involved in the survival of host cells during productive and/or persistent infections of Epstein-Barr virus and African swine fever viruses, respectively. The S-II region (Trp185-Trp192) is especially intriguing because it is located just before the site where bcl-2 α and bcl-2 β are differentially spliced (Fig. 6). Given the finding that our mutant T4 (ends at Asp193) is inactive in conferring cell survival, it is likely that this amino acid stretch alone is not sufficient for survival activity. It becomes fully active only when joined to at least five to nine additional residues. This extension probably assures the structural and functional integrity of the S-II segment. Indeed, the mutant T7 (additional nine residues, Asp193-Pro201) was as functional as the wildtype, whereas the addition of only 4 amino acids (mutant T5, 193-196) led to only partial recovery. Considering that the S-II-C segments of the highly active BHRF1 protein (Fig. 5) and bcl-2 display only little sequence similarity, no or little sequence requirements seem to exist for this segment.

In view of the recent identification of bcl-2 homologues that regulate the extent of bcl-2 α activity through hetero- and

homodimer formation (50), it is conceivable to propose that the herein defined functional S-II/S-II-C domain could mediate dimerization. This region is well situated to generate splice variants that would still dimerize but exhibit different activities. Varied dimerization between different partners is a common mechanism for regulating cellular functions (e.g., transcriptional activity; reference 6). Such a mechanism would also provide a satisfactory explanation for the dominant negative action of bax (50) on the survival activity of bcl-2. In this respect, bax would form an inert heteromeric complex with bcl-2 similar to transcription factors that lack a DNA-binding or transactivation domain ("squenching"). Interestingly, bax has retained the S-II domain but exhibits a distinct putative S-II-C domain (50) (Fig. 6). In contrast to bax, bcl-x α has retained the putative effector domain S-II-C, but lost the entire domain S-II (7) (Fig. 6).

Previously, it was believed that the removal of membrane attachment site of bcl-2 leads to at least a partial impairment of its function (3), but here, we show that the membrane interaction is not obligatory. T3, a truncated form of bcl-2 lacking the COOH-terminal hydrophobic tail (amino acids 204-236) is soluble in intact cells and after in vitro synthesis, and yet is fully active in sparing neurons and fibroblasts from apoptosis. A major concern with this result could be that the T3 mutant is only marginally active but expressed at a much higher level than wild-type bcl-2. According to our immunocytochemical (Fig. 3 B) and immunoblot (Fig. 4) studies, this appears, however, not to be the case because the T3 mutant was actually less expressed than its wild-type counterpart.

The survival activity of the soluble T3 mutant is not necessarily in conflict with previously published structure-function analyses, but rather, it extends the results published to date: First, a bcl-2 mutant lacking the last COOH-terminal 50 amino acids (our T1) failed to block apoptosis in

baculovirus-infected Sf9 insect cells (3). In view of our data, this is presumably caused by the lack of the two putative functional domain S-II (Fig. 6) rather than to the absence of the COOH-terminal transmembrane stretch. Second, deletion of the last 26 amino acids including the transmembrane domain led to a failure of bcl-2 to be inserted into microsomes after in vitro transcription/translation (13). However, the impact of this mutation on survival activity was not reported. This bcl-2 mutant was less truncated (by seven amino acids) than the herein-described T3 construct. Third, a recent study demonstrated that bcl-2 β , which has a minimal, nonconserved S-II-C domain but lacks the COOH-terminal hydrophobic stretch, is inactive in sparing 32D cells from apoptosis induced by IL-3 removal (58). Surprisingly, the addition of a heterologous transmembrane stretch of the IL-2 receptors renders bcl-2 β at least partially active. It is possible that the activity of the S-II segment may be unveiled through cooperation with additional residues derived from the IL-2 receptor transmembrane domain (Fig. 6). The observed partial regain of activity of the bcl-2-TM-IL-2 receptor chimeric protein would, therefore, be caused by a gain of function of the S-II domain rather than result from the membrane association. In agreement with this hypothesis are the negative results obtained with our DEL3 mutant, which still has the transmembrane domain but lacks the S-II/S-II-C domain. Fourth, Oltvai et al. (50) recently discussed in their study that a bcl-2 mutant lacking the COOH-terminal 22 amino acids is cytoplasmic and partially protects cells from death. This mutant is even larger than T3 (by 11 amino acids), indicating that removal of only the transmembrane stretch already renders bcl-2 soluble. Interestingly, the authors mention that their mutant still associates with bax (50), further strengthening the notion that dimerization motifs must lie upstream of the transmembrane region. This finding further suggests that membrane attachment may not be required for the interaction of bcl-2 with other bcl-2 homologues.

Recent results (34, 39) indicate a role for bcl-2 in an antioxidant pathway to prevent cellular damage including lipid peroxidation. The protein acts either before or after the generation of O₂^{·-} and its conversion to peroxides. It was proposed that bcl-2 could act as a trap for oxygen radicals similar to glutathione. Glutathione contains a cysteine residue that acts as a hydrogen/electron donor in the reduction of oxygen radicals. Our mutagenesis studies speak against a possible analogous involvement of Cys155 or Cys226 in the trapping of oxygen radicals by bcl-2. A third cysteine (Cys89) that was not mutated here is not conserved between human, chicken, and mouse bcl-2 (11, 18).

O₂^{·-} radical production in vivo is localized to distinct organelles including mitochondria, ER, and nuclear membranes. Consistent with this presumptive function is the close association of bcl-2 with mitochondrial (35) or perinuclear membranes (3, 12, 38). Our study provides evidence that bcl-2 may also fulfill its function in the cytoplasm. The soluble active T3.1 mutant migrates to neurites in neurons and filopodias in fibroblasts (Fig. 3). These structures are known to be especially vulnerable to apoptosis and to H₂O₂ exposure: neurites rapidly retract (33) and fibroblasts detach from their substrate. Because of its presence in cellular extensions, the T3.1 mutant may therefore efficiently protect these structures from degradation.

In summary, our data presented here demonstrate that bcl-2 can also exert its survival activity in the cytoplasm. In addition, they point towards putative functional motifs S-N and S-II/S-II-C, which lie in highly conserved domains. This finding sets the stage to more precisely define the functional domains of bcl-2, and to investigate their role in binding other bcl-2 homologues, other regulators, and effectors crucial for cell survival.

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