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Functional Dissection of the Human Bcl2 Protein: Sequence Requirements for Inhibition of Apoptosis

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Overexpression of the cytoplasmic oncoprotein Bcl2 blocks programmed cell death (apoptosis) in many cellular systems. To map the sequences in Bcl2 that are necessary for its activity, we created a library of deletion-scanning mutants of this 239-amino-acid protein and tested their abilities to block staurosporine-induced fibroblast apoptosis, using a novel transient-transfection assay. Phenotypes of informative mutants were then confirmed by assaying for inhibition of steroid-induced apoptosis in stably transfected T-lymphoid cells. In accordance with earlier results, we found that Bcl2 activity was only partially reduced after deletion of the hydrophobic tail that normally anchors it in cytoplasmic membranes. Essential sequences were found in the remainder of the protein and appeared to be organized in at least two discrete functional domains. The larger, more C-terminal region (within residues 90 to 203) encompassed, but extended beyond, two oligopeptide motifs called BH1 and BH2, which are known to mediate dimerization of Bcl2 and related proteins. The second, more N-terminal region (within residues 6 to 31) was not required for protein dimerization in vivo, but its deletion imparted a dominant negative phenotype, yielding mutants that promoted rather than inhibited apoptotic death. Residues 30 to 91 were not absolutely required for function; by deleting most of this region along with the hydrophobic tail, we derived a 155-residue mini-Bcl2 that retains significant ability to inhibit apoptosis.

Timely suicide of individual cells plays an essential part in development and tissue homeostasis of most metazoan organisms. One specific form of cellular suicide, known as apoptosis, is characterized by the nuclear and cytoplasmic shrinkage, blebbing of the plasma membrane, karyorrhexis, and internucleosomal degradation of nuclear DNA. Apoptosis has been observed in many cell types and in organisms ranging from nematodes to humans (reviewed in references 13 and 23). Depending on the cells involved, it can occur either as the result of an inborn genetic program or in response to environmental stimuli such as growth factor deprivation, viral infection, hormones, cancer chemotherapeutic agents, or ionizing radiation. Though no universal signals or mediators of this phenomenon have as yet been identified, its stereotypic nature suggests that a common, evolutionarily conserved pathway underlies many or all instances of apoptotic death.

Support for this view comes in part from the discovery of a family of structurally related proteins that regulate the apoptotic response to diverse stimuli and in many different cell types (reviewed in references 37 and 45). Members of this family include the vertebrate Bcl2, Bax, Bcl-X_L, Bcl-X_S, A1, and Mcl-1 proteins, the *ced-9* gene product of *Caenorhabditis elegans*, and the Bhrf1 protein from Epstein-Barr virus (4, 17, 18, 24, 28, 35, 37, 45). All are cytoplasmic proteins with recognizably similar amino acid sequences, and most are known to function individually as either agonists or antagonists of apoptosis. For example, overexpression of Bax alone tends to promote apoptosis in susceptible cells (35), whereas Bcl-X_L alone tends to inhibit it (4). Many or all of these proteins can dimerize with themselves and with other family members, and there is evidence that the overall propensity of a cell to undergo apoptosis is dictated in part by the ratio in which proteins with different activities heterodimerize (35, 38, 46). This finding has led to the hypothesis that the relative expression levels of agonist and antagonist proteins of this class function as a rheostat controlling a distal, common step in the apoptotic pathway (34, 35).

The prototype of this family is Bcl2, a cytoplasmic membrane protein capable of inhibiting apoptosis in many (though not all) settings (1, 2, 16, 30, 37, 40–42). Bcl2 is widely expressed in mammalian tissues during embryogenesis (27) and is essential for maintenance and function of the mammalian immune system (32, 43). It is also expressed in certain neoplasms and is of particular interest because of its etiologic role in human follicular lymphoma (3, 10). The mechanism by which Bcl2 blocks apoptosis is unknown. The protein bears a C-terminal transmembrane segment and, when expressed at high levels, associates with membranous organelles throughout the cytoplasm, including the endoplasmic reticulum, mitochondria, and outer nuclear envelope (8, 25, 29, 31). Deletion of the transmembrane segment, however, reduces but does not eliminate Bcl2 activity (5, 35). The protein also contains two short (approximately 10-amino-acid) sequence motifs, called BH1 and BH2, which are also found in other members of its family and which are required for certain heterodimerization reactions among these proteins (35, 45, 46) and for Bcl2 activity (46). In addition, Bcl2 has been reported to bind in vitro to other mammalian proteins, including p23-R-Ras (14), Raf-1 (44), and a trio of proteins designated Nip1, Nip2, and Nip3 (6), but the physiologic significance of these interactions remains unproven.

In this report, we present the results of a systematic mutational analysis of human Bcl2. We have created a library of deletion mutants spanning the entire protein and have tested the abilities of these mutants to block apoptosis by using two

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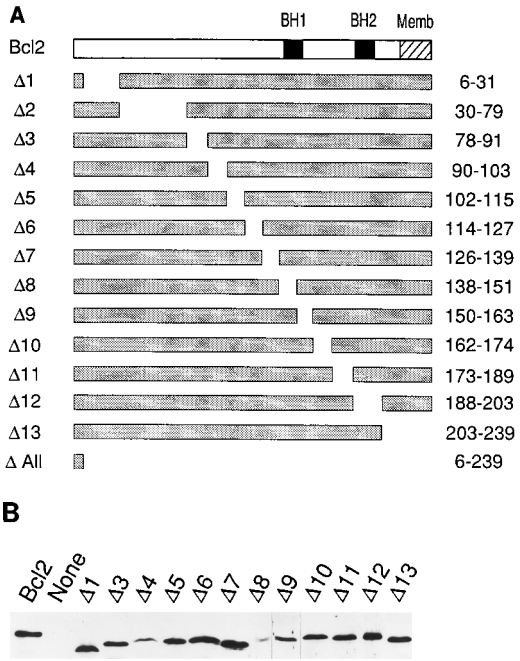

FIG. 1. Deletion-scanning mutants of human Bcl2. (A) Schematic organization of the 239-amino-acid Bcl2 protein (top). Black rectangles indicate the locations of the conserved BH1 and BH2 dimerization motifs (residues 138 to 150 and 188 to 196, respectively), and cross-hatching denotes the transmembrane segment (Memb; residues 205 to 239). Structures of the deletion mutants Δ1 to Δ13 and ΔAll are indicated below; in each case, the deleted residues indicated at right are replaced by the dipeptide Arg-Ser. (B) Immunoblot detection of wild-type and mutant Bcl2 proteins in transiently transfected QT6 quail cells, using an anti-Bcl2 monoclonal antibody. Mutant Δ2 (not shown) is not recognized by this antibody but was detected both by its biological activity (see Fig. 2C and 3B) and as an HA-tagged fusion protein in anti-HA immunoblots (data not shown).

different experimental systems. Our results provide new insights into the functional organization of Bcl2, uncover a novel dominant negative mutant, and define the minimal features of this protein that are necessary for inhibition of apoptosis.

MATERIALS AND METHODS

Cell lines. The human GM701 fibroblast (22) and murine WEHI-7.2 T-lymphoid cell lines were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and calf serum, respectively, with 100 U of penicillin per ml and 0.1 mg of streptomycin per ml. QT6 quail fibroblasts were grown in M199 medium with 5% (vol/vol) fetal calf serum and 1% (vol/vol) chicken serum. All cells were maintained at 37°C in a 5% CO_2 atmosphere at 90% relative humidity.

Plasmids. The parental expression vector pSFFV-*neo*-bcl2 (a generous gift from G. Nuñez) contained the wild-type human Bcl2 coding sequence under the control of a promoter from the spleen focus-forming virus, with downstream splicing and polyadenylation signals from simian virus 40, cloned into plasmid Bluescript KS along with a selectable *neo* marker (15, 20). We deleted the *neo* gene from this plasmid by partial *Bam*HI digestion to produce the nonselectable Bcl2 expression vector pSFFV-bcl2 for use in transient-transfection assays. Mutations were introduced into pSFFV-bcl2 by oligonucleotide-directed mutagenesis and were confirmed by DNA sequencing. We first created a series of *bcl2* missense mutants, each containing a single dicodon mutation, spaced at roughly 36-bp intervals along the coding sequence; in each case, a pair of adjacent native codons was replaced by a unique in-frame *Bgl*II site encoding Arg-Ser. By linking upstream and downstream restriction fragments from these missense mutants in various combinations, we then created the collection of deletion mutants depicted in Fig. 1A. The severely truncated pSFFV-bcl2 mutant designated ΔAll, which lacked all but the first six codons of the *bcl2* sequence, was used as a negative control. Some of the *bcl2* mutants were also substituted into pSFFV-*neo*-bcl2 to obtain selectable expression plasmids for stable transfection. A pSFFV-bcl2 derivative termed pBcl2-HA, in which sequences encoding an influenza virus hemagglutinin (HA) epitope were fused to the 3′ end of *bcl2*, was created by mutating the last two *bcl2* codons to a *Bgl*II site and ligating the 36-bp HA-coding sequence into this site. The chloramphenicol acetyltransferase (CAT) reporter plasmid pRSV-CAT has been described elsewhere (21).

Transient-transfection assay for Bcl2 activity. Confluent GM701 cells were split 1:6 in 100-mm-diameter plates 24 h before transfection and then transfected overnight by the calcium phosphate method, each plate receiving 15 μg of wild-type or mutant pSFFV-bcl2, 0.1 μg of pRSV-CAT, and 25 μg of salmon sperm DNA. Cells were split 1:2 at 40 h after transfection, and one plate from each pair received 1 μM staurosporine. Cells from each plate were harvested 24 to 28 h later and were lysed by three freeze-thaw cycles in 250 μl of 250 mM Tris (pH 7.5). Debris was pelleted by a 5-min microcentrifugation at 4°C, and a 5- to 10-μl aliquot of supernatant was assayed for CAT activity by thin-layer chromatography and scintillation counting as previously described (21). Background activity measured in parallel from lysates of untransfected GM701 cells was subtracted from each value obtained. Survival of cells expressing each plasmid was then calculated as the ratio of activities in the staurosporine-treated and untreated plates, expressed as a percentage. As expected, the survival of untransfected or ΔAll-transfected cells depends in part on the duration of staurosporine treatment; in the experiments presented here, it ranged from 2 to 5%.

Immunoblot assay. Confluent QT6 cells were split 1:8 at 24 h before transfection and then were transfected with 20 μg of the indicated pSFFV-bcl2 variant by the calcium phosphate method. Cells were shocked 16 h later with 10% (vol/vol) dimethyl sulfoxide in phosphate-buffered saline for 2 min. Cells harvested 44 h after transfection were then lysed by nutation for 30 min at 4°C in lysis buffer (1% [vol/vol] Nonidet P-40, 150 mM NaCl, 5 mM $MgCl_2$, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.5], 2 μM phenylmethylsulfonyl fluoride, 5 μg of leupeptin per ml, 5 μg of pepstatin per ml). Debris was pelleted by microcentrifugation for 10 min at 4°C, and aliquots of supernatant were analyzed on denaturing 14% acrylamide–0.1% (wt/vol) sodium dodecyl sulfate-containing electrophoretic gels and then transferred electrophoretically onto Hybond C-Extra membranes (Amersham) overnight at 35 mA. Membranes were preblocked for 15 min in BLOTTO (2.5% [wt/vol] nonfat dry milk–0.1% [vol/vol] Tween 20 in phosphate-buffered saline) and then probed for 2 h with a commercial mouse monoclonal immunoglobulin G antibody against Bcl2 (DAKO) diluted 1:200 in BLOTTO, all at room temperature. Following three 10-min washes in phosphate-buffered saline containing 0.1% Tween 20, membranes were incubated for 1 h at room temperature with a secondary sheep anti-mouse immunoglobulin G antibody conjugated to horseradish peroxidase (Amersham), diluted 1:10,000 in BLOTTO. Membranes were again washed as described above, treated with ECL (enhanced chemiluminescence) substrate (Amersham), and exposed to film.

Immunoprecipitations. QT6 cells were transfected as above with 10 μg of pBcl2-HA and 20 μg of wild-type or mutant pSFFV-bcl2. Lysates were prepared as for immunoblots except that 1% (vol/vol) Brij 69 was substituted for Nonidet P-40 in the lysis buffer. A 20-μl aliquot of each sample was used for immunoblotting; the remainder was combined with 0.02 volume of commercial anti-HA antiserum (Babco) for 1 h on ice, followed by 10% (vol/vol) protein A-Sepharose beads with nutation for 1 h at 4°C. The beads were pelleted by a 10-min microcentrifugation, washed once with Brij 69 lysis buffer, resuspended in gel-loading buffer, incubated in boiling water for 5 min, and then analyzed by immunoblotting as described above.

Stable transfection. To prepare singly transfected lines, 10^7 logarithmically growing WEHI-7.2 cells were washed twice with HEPES-buffered saline (pH 7.1), resuspended in 0.7 ml of HEPES-buffered saline, and electroporated at 220 mV and 960 μF with 20 μg of a pSFFV-*neo*-bcl2 derivative. Cells were placed in growth medium for 24 h, then transferred to 96-well plates, and placed under selection with 500 μg of G418 per ml for 14 days, with addition of fresh G418 and medium every 4 days. Single colonies were picked manually, expanded, and tested for Bcl2 expression by immunoblotting. Doubly transfected lines were prepared in the same manner but were electroporated with 15 μg of wild-type pSFFV-bcl2 along with 5 μg of the indicated mutant form of pSFFV-*neo*-bcl2. Survival of stable lines was assayed by manually scoring trypan blue exclusion in 200 cells from each plate.

RESULTS

We began by creating a series of deletion-scanning mutants of the Bcl2 coding sequence in the mammalian expression plasmid pSFFV-bcl2. Each contained an in-frame deletion of 14 to 60 native codons, which were replaced by a *Bgl*II restriction site encoding the dipeptide Arg-Ser. Together, these deletions spanned virtually the entire Bcl2 coding sequence (Fig. 1A). To confirm their integrity, the mutant plasmids were first transfected transiently into QT6 quail fibroblasts, and extracts from these cells were then examined by immunoblotting with a monoclonal antipeptide antibody against Bcl2 residues 41 to 54. As shown in Fig. 1B, each plasmid gave rise to a single immunoreactive protein of somewhat smaller apparent mass than wild-type Bcl2. We designate these mutant proteins Δ1 to

Δ13. Expression of Δ4 and Δ8 was reproducibly lower than that of the other mutants, and Δ2 protein could not be detected by this assay since it lacks the necessary epitope sequences. All other mutants were expressed at comparable levels in these cells.

Each mutant was then tested for its ability to block staurosporine-induced killing of human GM701 fibroblasts, a well-characterized model of apoptosis (22). Testing was performed in a transient-transfection assay (Fig. 2A) which is rapid and reproducible and avoids the potential selection biases that can occur during isolation of stably transfected clones. For this assay, individual plates of GM701 cells are cotransfected with a wild-type or mutant Bcl2 expression vector along with the CAT reporter plasmid pRSV-CAT; 40 h later, each plate is subdivided into two, one of which is then treated with the protein kinase inhibitor staurosporine. Cell lysates are subsequently prepared at 64 to 68 h after transfection, and total CAT activities in the two plates are compared for each plasmid. Exposure to staurosporine induces apoptosis within 24 h in untransfected GM701 cells (22), including the many cells on each transfected plate that fail to take up plasmid DNA. Cells transfected with a nonfunctional Bcl2 mutant (such as ΔAll) also undergo apoptosis, and this is manifested in a time-dependent decrease in CAT activity in the treated plate compared with the untreated control (Fig. 2B; compare lanes 1 and 2). By contrast, cells expressing wild-type Bcl2 or a functional mutant are protected from apoptosis, so that CAT activity remains more nearly equivalent in the two plates (lanes 3 and 4).

Applying this assay to our Bcl2 mutants, we found that two (Δ2 and Δ3) were fully protective, two others (Δ4 and Δ8) could not be interpreted reliably because of low protein expression, and the remaining nine were functionally impaired (Fig. 2C). In particular, deletion of the transmembrane segment alone yielded a protein (Δ13) that was approximately 25% as effective as wild-type Bcl2 in blocking apoptosis; this result was in agreement with results from other laboratories using comparable mutants in stable transfectants and so tended to confirm the validity of our assay (5, 35). Similarly, deletion of the BH2 region (mutant Δ12) essentially abolished activity, as has been observed previously (46) with certain missense mutations in this region and also in BH1.

Our analysis of the entire protein, however, revealed that activity was also completely eliminated by all deletions either between BH1 and BH2 or in the region extending as far as 37 amino acids upstream from BH1. Thus, to a first approximation, the inactive mutants Δ5 to Δ12 (and perhaps also Δ4) appeared to define a single, continuous functional domain that encompassed, but extended beyond, the BH1 and BH2 motifs. This interpretation is consistent with the pattern of evolutionary sequence conservation observed when the human, mouse, rat, and chicken Bcl2, Bax, and Bcl-X_L proteins are compared: although BH1 and BH2 are especially well conserved, they are embedded within a larger region of significant conservation spanning approximately residues 96 to 200 (references 4, 7, 12, 33, 35, and 39 and data not shown).

The only other severely defective mutant in our series was Δ1, which lacked Bcl2 residues 6 to 31. The inactivity of this mutant in our assay echoed an earlier study (5) in which deletion of residues 4 to 29 was found to abolish murine Bcl2's ability to prevent neuronal cell apoptosis induced by growth factor deprivation or tumor necrosis factor alpha. We found, moreover, that the 63 residues separating the Δ1 region from the larger downstream domain appeared functionally dispensable, since the intervening mutants Δ2 and Δ3 each gave complete protection. These results indicate that at least some of the residues missing from Δ1 are critical for Bcl2 activity and may function as an independent domain. Consistent with this view, Bcl2 sequence comparisons among species reveal a small island of conserved residues corresponding to residues 11 to 28

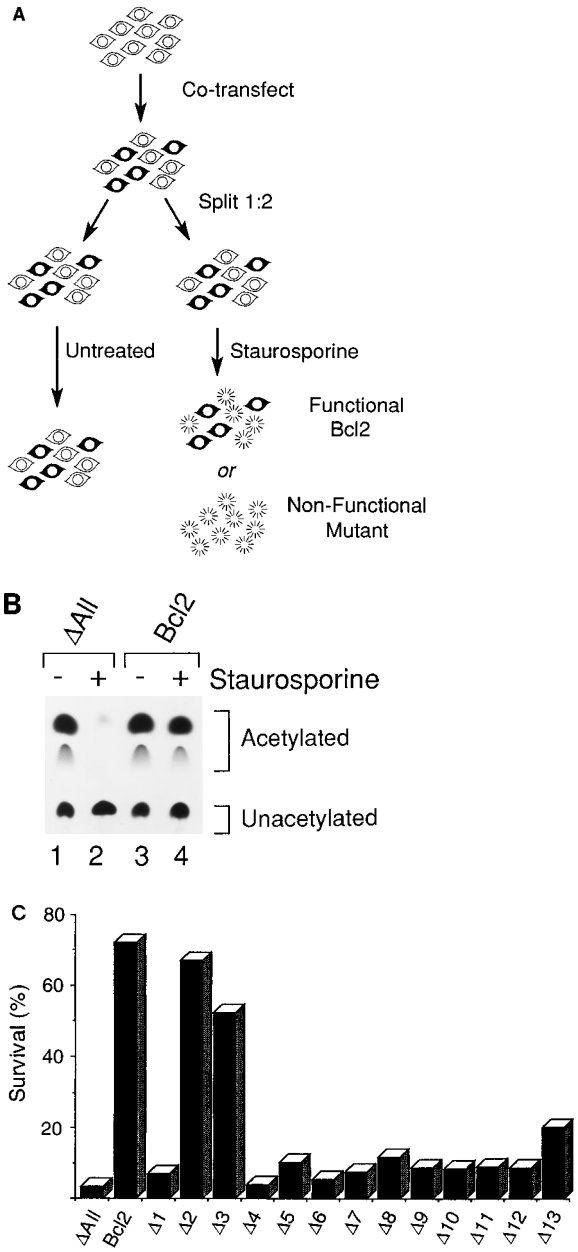

FIG. 2. Antiapoptotic activities of Bcl2 mutants in a transient-transfection assay. (A) Assay protocol. Two identical plates of GM701 fibroblasts which transiently express both a Bcl2 variant and CAT are prepared; one plate in each pair is treated with staurosporine, and any resulting apoptosis among the transfected cells is detected by a relative decrease in CAT activity recovered from that plate. CAT-expressing transfected cells are denoted by black cytoplasm. (B) Representative data from GM701 cells expressing either wild-type Bcl2 or the nonfunctional mutant ΔAll. A chromatographic CAT assay was performed on extracts from staurosporine-treated (+) and untreated (−) plates expressing each construct; positions of unacetylated and acetylated chloramphenicol are indicated at the right. These data are from the experiment depicted in panel C; in this experiment, CAT activity declined by approximately 95% after 24 h of staurosporine treatment in ΔAll transfectants but by less than 30% in Bcl2 transfectants. (C) Biological activities of the Bcl2 mutants used for panel A, expressed as percent survival in the transient-transfection assay. Results shown are from one representative experiment; each mutant was tested at least three times with similar results.

in the human sequence, whereas the sequences at positions 30 to 90 have diverged widely (39). Interestingly, this conserved N-terminal region is absent from Bax, a Bcl2 family member that promotes apoptosis (35).

To test the results of the transient-transfection assay, we transfected some of the informative mutants from our collection along with a selectable *neo* marker into the mouse thymoma cell line WEHI-7.2 and isolated stably transfected clones. WEHI-7.2 cells rapidly undergo apoptosis when exposed to the steroid hormone dexamethasone (Dex) but can be protected completely from this agent by stable transfection with wild-type Bcl2 (26). For the present studies, we isolated WEHI-7.2 transfectants that stably expressed mutants Δ1, Δ2, Δ6, or Δ13, isolating two or more independent clones of each. As shown by the representative immunoblot in Fig. 3A, levels of stable expression varied among constructs: whereas Δ13 was relatively abundant, Δ1 and Δ6 were always expressed at a somewhat lower level. We therefore designated these as high and low expressors, respectively, and selected as controls two additional stable clones (Bcl2-high and Bcl2-low) that expressed wild-type Bcl2 protein at a level corresponding to each. Thus, each of these three mutants was compared with an expression-matched control.

The effect of Dex on the stable transfectants is depicted in Fig. 3B. Whereas untransfected WEHI-7.2 cells died rapidly (75% failed to exclude trypan blue after 65 h of Dex treatment), the Bcl2-high control was almost completely protected and the Bcl2-low control also showed significant resistance. Among the mutants, only Δ2 gave complete protection; this result confirms that Δ2 is biologically active, though its specific activity cannot be quantified because its level of protein expression could not be determined by immunoblot. The C-terminal mutant Δ13, though expressed at least as abundantly as Bcl2-high, gave somewhat less protection, confirming that it is only partially active. In marked contrast, mutants Δ1 and Δ6 completely lacked the protection conferred by the Bcl2-low control, and indeed clones expressing Δ1 reproducibly died more rapidly than untransfected parental cells. Electrophoretic analysis (Fig. 3C) showed a nucleosomal pattern of nuclear DNA degradation occurring after Dex treatment only in the untransfected cells and in clones expressing Δ1 or Δ6 and thus confirmed that these cells died through apoptosis.

These findings together suggested that Bcl2 is organized in at least two functional domains: an N-terminal region (domain A) defined by mutation Δ1, and a larger C-terminal region (domain B) defined by mutations Δ4 or Δ5 through Δ12 (Fig. 4A). The remaining 40% of the protein did not appear to be absolutely required. To test this interpretation, we created one additional mutant (Δ14) lacking residues 30 to 90 and another (Δ15) lacking both residues 30 to 79 and the C-terminal tail. As with Δ2, expression of these mutants could not be quantitated by immunoblotting. Nevertheless, both mutants protected against apoptosis at least as well as Δ13 in the transient assay, i.e., at least 25% as effectively as wild-type Bcl2 (Fig. 4B). Confirmatory results were obtained in stable transfectants, in which the minimal Δ15 mutant conferred partial but significant protection (Fig. 4C).

The unusually rapid apoptotic response of cells expressing Δ1 (Fig. 3B) was reminiscent of findings in cells that overexpress Bax (4, 35). This suggested that, like Bax, the Δ1 mutant might have the ability to block the activity of wild-type Bcl2 in *trans*. To test that possibility, we cotransfected WEHI-7.2 cells with vectors encoding Bcl2 and the Δ1 mutant, respectively, along with a *neo* marker gene and then selected clones that stably expressed both the wild-type and mutant proteins. An immunoblot of one such clone is depicted in Fig. 5A, and this

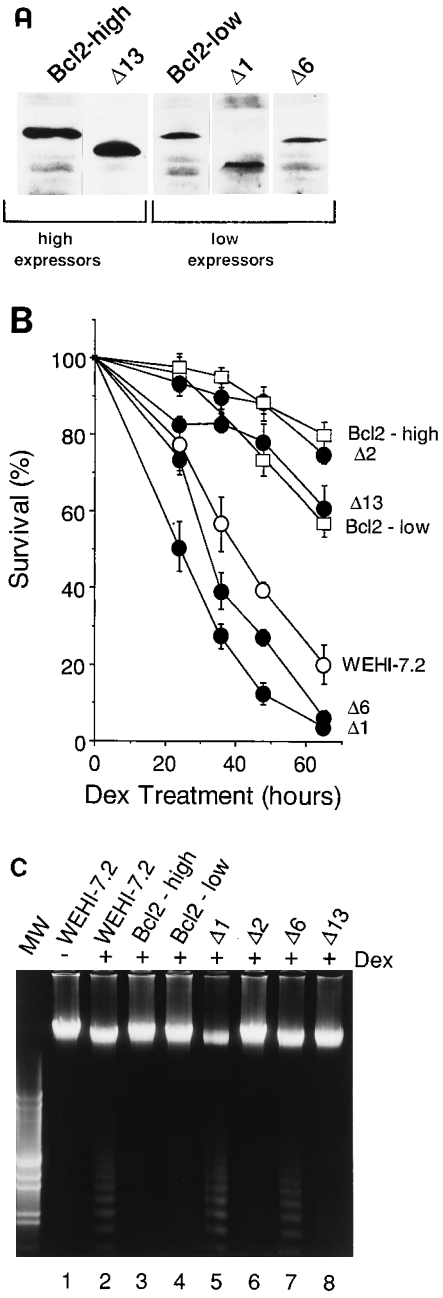

FIG. 3. Antiapoptotic activities of selected Bcl2 mutants in stably transfected T-lymphoid cells. (A) Immunoblot detection of Bcl2 variants in untransfected cells (WEHI-7.2) and in clones stably transfected with the indicated mutants. All samples contain equal amounts of total protein and were analyzed on a single gel. (B) Time course of Dex-induced apoptosis in the indicated clones. Survival (%) refers to the percentage of cells from each clone that excluded trypan blue after exposure to 1 μM Dex for the indicated times. Each point corresponds to the mean ± standard deviation for triplicate plates in a single representative experiment. (C) Chromosomal DNA degradation in stable transfectants undergoing Dex-induced apoptosis. Nuclear DNA was harvested from the indicated clones either without pretreatment (−) or following 20 h of exposure to 1 μM Dex (+) and was fractionated electrophoretically on a 1% agarose gel containing ethidium bromide. The molecular weight marker (MW) was a combined *Eco*RI and *Hin*dIII digest of lambda phage DNA.

clone's response to Dex is shown in Fig. 5B. The doubly transfected cells exhibited a brisk, Dex-dependent apoptotic response comparable to that seen in untransfected WEHI-7.2 cells, which indicated that coexpression of Δ1 overcomes the

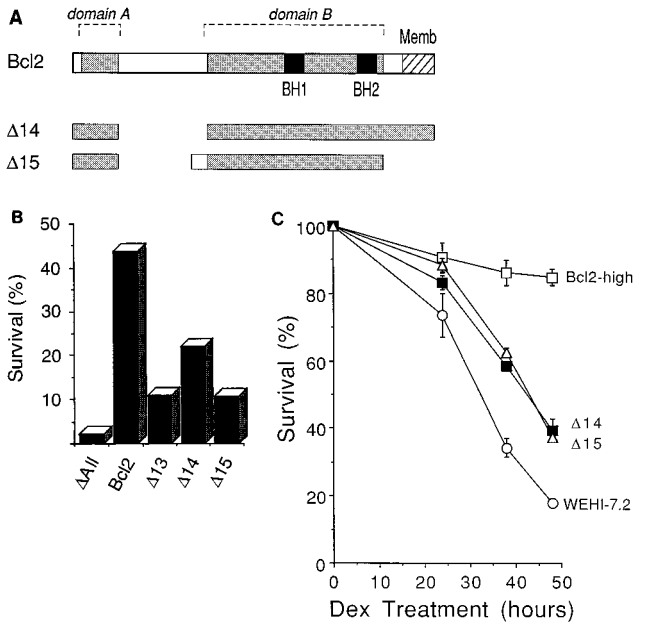

FIG. 4. Functional organization of the human Bcl2 protein and derivation of a biologically active mini-Bcl2. (A) Schematic view of functional domains in Bcl2 (top) based on data from Fig. 2 and 3. Approximate boundaries of domains A and B (shaded) are residues 6 to 31 and 90 to 203, respectively. The transmembrane segment (Memb; cross-hatched) and BH1 and BH2 motifs (black rectangles) are also shown. Shown below are the structures of mutants Δ14 (which lacks residues 30 to 91) and Δ15 (which lacks residues 30 to 79 and 203 to 239). In each case, deleted residues are replaced by the dipeptide Arg-Ser. (B) Biological activities of Δ13, Δ14, and Δ15 were determined in the transient GM701 transfection assay, as described for Fig. 2C. (C) Biological activity of Δ14 and Δ15 in stably transfected clones of WEHI-7.2 cells, assayed as described for Fig. 3B. Data shown are for one of two independent Δ15 clones that yielded similar results.

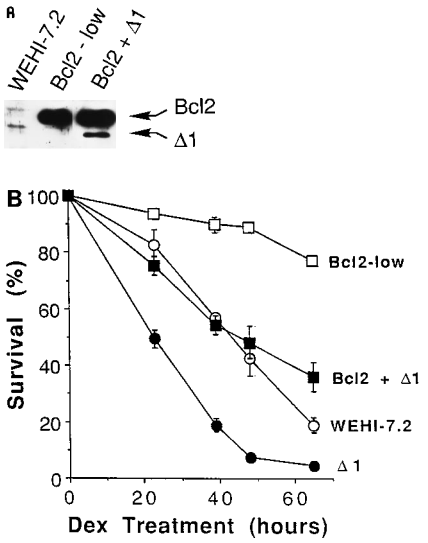

FIG. 5. The Δ1 protein is a dominant negative mutant of Bcl2. (A) Immunoblot detection of Bcl2 and/or Δ1 proteins in WEHI-7.2 cells and stably transfected clones, using an anti-Bcl2 antibody. (B) Time course of Dex-induced apoptosis in the indicated clones, determined as described for Fig. 3B. Three independent doubly transfected clones were tested, results for only one of which are shown; the other two clones expressed lower ratios of Δ1 to Bcl2, as judged by immunoblotting, and showed intermediate susceptibility to apoptosis in the transient-transfection assay (data not shown).

protective effect of Bcl2. Thus, Δ1 is a dominant negative mutant of Bcl2.

One mechanism by which dominant negative mutants can exert their effects is by forming nonfunctional multimers with the wild-type protein (19). We therefore carried out coimmunoprecipitation studies to determine whether our Δ1 mutant could multimerize with intact Bcl2. We prepared a vector (called pBcl2-HA) which encoded a modified Bcl2 bearing a 12-amino-acid C-terminal extension comprising an epitope from influenza virus HA. QT6 cells were transiently transfected with pBcl2-HA and a vector encoding Δ1, individually or in combination; cell lysates were then prepared, and the HA-tagged protein was immunoprecipitated from these lysates by using an anti-HA antibody. The immunoprecipitates and crude cell lysates were then probed for Bcl2 protein sequences by immunoblotting. As shown in Fig. 6, the anti-HA antibody readily precipitated Bcl2-HA but failed to recognize Δ1 protein in cells transfected with Δ1 alone. Δ1 was, however, efficiently precipitated from lysates of cells that coexpressed Bcl2-HA, implying that the dominant negative Δ1 mutant forms stable heterodimers with Bcl2 within cells.

DISCUSSION

We have carried out a systematic deletion analysis of human Bcl2 in order to map peptide sequences required for its antiapoptotic effect. Functional properties of the deletion mutants were assayed in two different cell culture models of apoptosis, and these two assays yielded results that were generally in agreement with each other and with the more limited mutational analyses reported previously by other labs (5, 35, 38, 46). The results are also consistent with the pattern of sequence conservation among Bcl2 and related proteins from various species, as would be expected if functionally important residues are evolutionarily conserved.

Our data support the view that Bcl2 is organized in at least two separate functional domains, which we designate domains A and B (Fig. 4A). Domain B, the larger of the two, appears to extend over most or all of the region defined by mutants Δ4 to Δ12, i.e., residues 90 to 203. It therefore includes the BH1 and BH2 motifs that have been noted in earlier studies (35, 46) but also encompasses the region between these motifs and as many as 49 residues immediately upstream of BH1. Although we cannot exclude the possibility that this region actually comprises two or more closely packed elements that each have distinct functions, the relatively uniform sequence conserva-

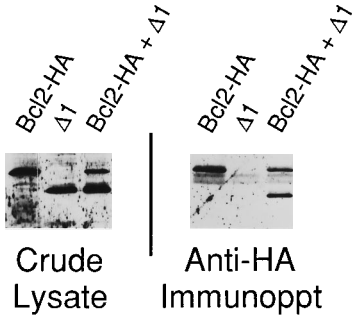

FIG. 6. Coimmunoprecipitation of the Δ1 mutant with a wild-type Bcl2-HA fusion protein. Bcl2 and Δ1 were detected by immunoblotting with an anti-Bcl2 antibody in crude extracts of QT6 cells transfected with the indicated constructs (crude lysate) and material immunoprecipitated from the same extracts by an anti-HA antiserum (Anti-HA Immunoppt).

tion across this region in various Bcl2-like proteins (45) suggests that it may represent a single functional entity. Most strikingly, sequence alignments between human Bcl2 and Bcl-X_L proteins (4) show conservation spanning the entire region corresponding to Bcl2 residues 96 to 203, with 68% sequence identity across this region. The functions of domain B certainly include a role in protein-protein interactions among Bcl2 family members, as point mutations in the BH1 or BH2 motifs alone have been shown to abolish heterodimerization of Bcl2 with Bax in vitro (46). It will be of interest to determine whether mutations elsewhere in domain B have a similar effect.

The second essential region of Bcl2 (domain A) is located within residues 6 to 31 and likely coincides with an evolutionarily conserved region detected previously (39) at residues 11 to 28. As might be predicted from the pattern of sequence conservation, we find that domain A is separated from domain B by more than 60 nonessential amino acids (residues 30 to 91). Our data confirm that deletion of domain A inactivates Bcl2 (5) but that it does not prevent dimerization with wild-type Bcl2 (38). The latter finding is consistent with data from Sato et al. (38), who have proposed that Bcl2 can multimerize through head-to-tail linkages involving domains A and B. We find that in our system, a Bcl2 mutant lacking domain A (mutant Δ1) exerts a potent dominant negative effect, in that it is capable of inhibiting activity of wild-type Bcl2 in *trans*, even when expressed in relatively low stoichiometric amounts (Fig. 5). Although an earlier study (5) of a similar mutant failed to detect such inhibitory activity, this may reflect differences in the assay systems used; in particular, we find that inhibition can be demonstrated much more readily in stable transfectants than in transient transfectants (data not shown) as were used in the earlier study. The inhibitory activity that we observed may result from the mutant's ability to form mixed dimers with full-length Bcl2 (Fig. 6) and thereby sequester the wild-type protein in an inactive form. This mutant would, moreover, be incapable of participating in head-to-tail multimerization (38), which might be strictly required for Bcl2 function. Interestingly, no obvious counterpart of domain A is present in the Bcl2-related protein Bax, a naturally occurring agonist of apoptosis which also can function as a dominant Bcl2 inhibitor (35).

Bcl2 has recently been reported to associate in vitro with three previously unrecognized mammalian proteins designated Nip1, Nip2, and Nip3 (6). All three of those proteins were also found to bind the 19-kDa adenovirus E1B gene product, whose functions include inhibiting apoptosis in infected cells (11, 36). These findings raised the possibility that the Nip proteins are components of the cellular apoptotic pathway that serve as proximate targets of Bcl2 action. However, whereas Bcl2 residues 42 to 48 were shown to be required for interaction with the Nip proteins (6), our data now reveal that those residues can be deleted from Bcl2 without abolishing its capacity to block apoptosis (Fig. 2C, 3B, and 4B). This implies that interaction with the Nip proteins is not an absolute requirement for Bcl2 activity, though these proteins may contribute to optimal Bcl2 function.

An additional outcome of our study was the finding that combined deletion of both the C-terminal transmembrane segment (residues 203 to 239) and most of the region separating domains A and B yielded a 155-amino-acid mutant (Δ15) that retained significant antiapoptotic activity. Though we could not specify the level of expression of this mutant protein, its effect in the transient-transfection assay was comparable to that of mutant Δ13, which lacks only the transmembrane segment. This finding demonstrates that at least 35% of the residues in Bcl2 are not strictly required and that residues 30 to 79, in particular, may play little or no part in blocking apoptosis. Because of its small size, Δ15 could prove to be a useful model for studying physiological binding interactions of Bcl2, as well as an accessible source of structural information on a biologically active Bcl2 derivative.

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