Evidence for α-Helical Conformation of an Essential N-terminal Region in the Human Bcl2 Protein*

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A region occupying approximately 24 amino acids near the N terminus of human Bcl2 is essential for this cytoplasmic membrane protein's ability to inhibit apoptosis. Systematic mutagenesis of this N-terminal region indicates that only five hydrophobic and aromatic residues within it are specifically required for function. Computerized secondary structure prediction, together with circular dichroism spectroscopy of synthetic peptides, indicates that the region encompassing these five residues has the propensity to take on an α-helical conformation in the presence of SDS micelles, which presumably mimic the hydrophobic surfaces of cellular membranes or polypeptides. The five critical residues are predicted to be clustered on one face of this putative helix, where they might serve to mediate protein-protein contacts involved in the multimerization of Bcl2 or in the interaction of Bcl2 with other, as yet unidentified components of the apoptotic pathway. Apparent structural homologues of this helical motif are also present in at least some other anti-apoptotic proteins from the Bcl2 family but not in those family members that tend to potentiate, rather than inhibit, apoptosis.

Programmed cell death often occurs through apoptosis, which is characterized in part by shrinkage and fragmentation of the cytoplasm and nucleus and by internucleosomal chromatin degradation (reviewed in Ref. 1). The ability to undergo apoptosis seems common to all differentiated cells in multicellular organisms, and though the conditions necessary to trigger its differ among cell types, the underlying biochemical pathway is thought to be evolutionarily conserved. Most details of this pathway are unknown, but recent studies suggest that critical early steps take place within the cytoplasm and that certain highly conserved cytoplasmic proteins participate in the response.

In particular, the propensity of cells to undergo apoptosis seems to be controlled by a family of structurally related cytoplasmic proteins whose prototype is the human bcl2 gene product (reviewed in Refs. 2 and 3). Bcl2 is a 239-amino acid cytoplasmic integral membrane protein with a wide tissue distribution that acts to inhibit apoptosis under a wide variety of circumstances. When expressed at high levels, it associates with mitochondrial membranes, the nuclear envelope, and the endoplasmic reticulum. The significance of these associations is uncertain, and at present, neither the physiologic site nor mechanism of Bcl2 action is known. Studies in other vertebrates, as well as in nematodes and animal viruses, have identified other proteins that share regions of significant sequence homology with Bcl2. When overexpressed individually in cells, these proteins may inhibit, promote, or modulate the apoptotic response. Several of these proteins form dimers or higher oligomers with themselves and with one another (4–10), and such interactions among Bcl2 family members may be important for their biological effects (10, 11).

Site-directed mutagenesis studies have delineated at least three discrete functional regions within human Bcl2 (10, 12–15). A hydrophobic domain at the C terminus (residues 203–239) confers membrane anchorage; deletion of this region reduces but does not eliminate Bcl2 activity (5, 12, 13). In contrast, activity can be completely eliminated by mutations in a second region (residues 90–203) that includes a pair of sequence motifs (termed BH1 and BH2) that are known to be required for Bcl2 homo- and heterodimerization (10, 12, 13). Bcl2 deletants that lack this third region (located near the N terminus within residues 6–31) not only fail to prevent apoptosis (12, 13) but can also function as trans-dominant inhibitors of wild-type Bcl2 (13). In this report, we present the results of a detailed analysis of this N-terminal region of human Bcl2, including functional testing of Bcl2 missense mutants and structural analysis of peptides corresponding to this region using CD spectroscopy. Our results provide new insights into the functional organization of Bcl2 and identify a structural motif that may prove to be shared by a subset of anti-apoptotic proteins.

MATERIALS AND METHODS

Cell Lines—Human GM701 fibroblasts and quail QT6 cells were grown as described previously (13).

Plasmids—Construction of the Bcl2 vector pSFFV-bcl2, which expresses human Bcl2 using a promoter from the spleen focus-forming virus, has been described previously (13). Mutations were introduced into the Bcl2 coding sequence by oligonucleotide-directed mutagenesis and confirmed by DNA sequencing. A truncated pSFFV-bcl2 mutant, designated ΔA1L, lacking all but the first six codons of the bcl2 sequence, served as negative control (13). The chloramphenicol acetyltransferase reporter plasmid pRSV-chloramphenicol acetyltransferase has been described (13).

Transient Transfection Assay for Bcl2 Activity—This assay has been described previously (13). Survival of cells transfected with a given plasmid was calculated as the ratio of chloramphenicol acetyltransferase activity in lysates of staurosporine-treated cells to that in untreated cells transfected with the same plasmid, expressed as a percentage.

Immunoblot Assay—Expression of wild-type and mutant Bcl2 protein was assayed in transiently transfected quail QT6 fibroblasts using a mouse monoclonal antibody against Bcl2 residues 41–54 (DAKO Corp.) as described (13). Unlike GM701 fibroblasts, QT6 cells contain no...
Peptide Synthesis and Purification—The 30-, 21-, and 17-residue Bcl2 peptides were synthesized using standard 1-fluorenylmethoxycarbonyl chemistry and were purified by preparative reverse-phase high performance liquid chromatography. Their purities were confirmed by analytical reverse-phase chromatography and mass spectral analysis.

Computerized Structural Prediction—Peptide secondary structure was predicted using the computer facilities of the European Molecular Biology Laboratory, Heidelberg and the neural network algorithm developed by Rost and Sander (16–18). The results were displayed using the facilities of the Computer Graphics Laboratory at the University of California at San Francisco and the MidasPlus graphics package (K. Gallo, C. Huang, T. E. Ferrin, and R. Langridge, University of California at San Francisco, 1989).

CD Spectroscopy—Samples containing 50 μM of the 30-mer peptide were assayed at 15°C at pH 4.0 (NaOAc buffer) and pH 7.0 (MOPS1 buffer), with 20 mM NaCl and 20 mM SDS; samples containing 50 μM of the 17- and 21-mer peptides were examined at pH 7.0 only, under the same salt and SDS conditions. A Jasco J500A CD spectropolarimeter with 1-mm pathlength cells was used to record each spectrum. Each was the average of 20 scans over the wavelength range of 200–260 nm with a step resolution of 0.4 nm.

RESULTS

This analysis began with the construction of 12 mutant forms of the Bcl2 expression plasmid pSFFV-Bcl2 using oligonucleotide-directed mutagenesis. In each mutant, sequences encoding a pair of adjacent residues in the N-terminal domain were replaced by glycine (or, where glycine was normally present, alanine) codons; together, the mutations spanned the entire 24-amino acid domain (Fig. 1A). The size and stability of the corresponding mutant proteins were confirmed by transiently transfecting each plasmid into QT6 avian cells and examining the extracts from these cells by immunoblot using a monoclonal anti-Bcl2 antibody. Each plasmid gave rise to a single immunoreactive peptide of the same apparent mass as the wild-type protein (Fig. 1B).

A previously described transient transfection assay (13) was used to test the ability of these Bcl2 mutants to block staurosporine-induced apoptosis in the human fibroblast line GM701. In this assay, individual plates of GM701 cells are co-transfected with a wild-type or mutant Bcl2 plasmid along with a chloramphenicol acetyltransferase reporter plasmid. After a period of growth, cell populations are split into treatment and control groups. The latter are exposed to the protein kinase

1 The abbreviation used is: MOPS, 4-morpholinepropanesulfonic acid.
inhibitor staurosporine, which induces apoptosis within 24 h in untransfected GM701 cells and in those cells transfected with a nonfunctional Bcl2 mutant. This results in a time-dependent decrease in chloramphenicol acetyltransferase activity in the treated plate compared with its untreated counterpart. In contrast, cells transfected with wild-type Bcl2 or with a functional mutant do not undergo apoptosis, and consequently, chloramphenicol acetyltransferase activities within each pair of plates remain more nearly equal (13).

Three mutants involving residues 14–15, 18–19, and 22–23, respectively, were found whose ability to inhibit apoptosis was totally abrogated (Fig. 1C). Chloramphenicol acetyltransferase activity was reduced by more than 95% after staurosporine treatment in cells transfected with these mutants, indicating that they conferred almost no protection against apoptosis. The remaining mutants provided levels of protection comparable with that of the wild-type protein; in each case, chloramphenicol acetyltransferase activity in the drug-treated plates averaged at least 73% of that observed in untreated plates. The three inactive mutants were each expressed at levels approximately 2-fold lower than those of the other mutants, suggesting either that these mutations partially destabilized the protein or that cells expressing functional Bcl2 have a growth or survival advantage in the transient assay (to compensate for this disparity, 2-fold more total protein was loaded for each of these mutants on the gel shown in Fig. 1B). However, one other mutant (residues 24–25) that was expressed at comparably low levels conferred essentially wild-type activity in the drug-treated plates (Fig. 1A and B), implying that the functional defect in the three inactive mutants was not entirely due to reduced protein expression. This interpretation was borne out in the following experiment.

In order to determine which of the six implicated residues were essential, we constructed another series of mutant Bcl2 plasmids that substituted glycine for individual codons. Immunoblots confirmed the size and stability of each mutant protein (Fig. 2A); within this series, the levels of protein expression varied no more than 2-fold among mutants. Using the transient transfection assay (Fig. 2B), we found that individual mutations of residues Ile-14, Val-15, Tyr-18, Ile-19, and Leu-23 each reduced Bcl2 activity by at least 80%. The mutant K22G was less seriously impaired, retaining roughly half the wild-type activity. Together, these results indicated that residues Ile-14, Val-15, Tyr-18, Ile-19, and Leu-23 were indispensable for anti-apoptotic activity of Bcl2, whereas the remaining 19 residues in the N-terminal domain were not specifically required.

We next analyzed the primary sequence of Bcl2 using the neural network-based protein secondary structure prediction algorithm of Rost and Sander (16–18). This algorithm was used to generate structural predictions for the full-length sequence of Bcl2 and also for the sequences of residues 2–31, 6–26, and 10–26 in isolation. In every case, the algorithm predicted with at least 72% confidence that residues 11–26 would adopt an α-helical conformation (data not shown), with the region of predicted helicity encompassing the five functionally critical residues. Fig. 3(A and B) shows two views of a space-filled model of the N-terminal region (residues 2–31) based on this structural prediction, with residues 11–26 modeled as α-helix and residues 2–10 and 27–31 modeled as random coil. In this model, the five critical residues (light gray) are positioned together on one face of the helix.

To search for physical evidence of such a helix, we synthesized a 30-mer peptide corresponding to Bcl2 residues 2–31 (Fig. 4A) and evaluated its conformation in solution under different temperatures, pHs, and salt concentrations using CD spectroscopy. In aqueous solution, the peptide exhibited only a single spectral minimum at 198 nm, suggesting a random coil conformation (data not shown). However, when we added 20 mM of SDS micelles, we obtained markedly different spectra that included minima at 208 and 222 nm (Fig. 4B); these features indicate the presence of significant α-helical structure.

The extent of helicity, as reflected in the values of these spectral minima, was approximately 27% and seemed similar at pH 4.0 and 7.0 (Fig. 4B). Further CD spectra in the presence of SDS suggested that the extent of helicity remained constant as NaCl concentration varied from 0 to 50 mM but decreased with increasing temperature (data not shown).

FIG. 3. Predicted conformation of the Bcl2 N terminus. Two perpendicular views are shown of a computer-generated water-accessible surface model of residues 2–31 of human Bcl2, with residues 11–26 in helical conformation. The N terminus is oriented upward in each case; side chain conformations are free energy minimized. Light gray shading indicates the five functionally essential residues identified in this study.

N-terminal Helix in Bcl2
In an effort to identify the regions of highest helical propensity, we then examined two shorter synthetic peptides corresponding to Bcl2 residues 6–26 and 10–26, respectively (Fig. 4A). Like the larger peptide, each seemed to be a random coil in the absence of detergent (data not shown) but acquired significant helicity in the presence of 20 mM SDS (Fig. 4C). Of note, the CD spectral minima of the 17-residue peptide were some-what more negative than those of the larger peptides, suggesting that the region of greatest helical propensity lies, as predicted, within residues 11–26.

**DISCUSSION**

Sequence comparisons among Bcl2 family members reveal a cluster of conserved residues corresponding to Bcl2 residues 6–26 and 10–26, respectively (Fig. 4A). Like the larger peptide, each seemed to be a random coil in the absence of detergent (data not shown) but acquired significant helicity in the presence of 20 mM SDS (Fig. 4C). Of note, the CD spectral minima of the 17-residue peptide were somewhat more negative than those of the larger peptides, suggesting that the region of greatest helical propensity lies, as predicted, within residues 11–26.

**A** Predicted Helix

| Bcl2   | 10 - D N R E - I V M K Y I H Y K L S Q R G Y | 28 |
|--------|------------------------------------------|----|
| BclXL  | 4  - S N R E - L V V D F L S Y K L S Q K G Y | 22 |
| A1     | 8  - H I H S - L A E H Y L Q Y V L Q V P A F | 26 |
| Ced-9  | 79 - D I E G - F V V D Y F T H R I I R O N G M | 97 |
| Bhrf-1 | 4  - S T R E I I L A I C I R D S R I V H G N G | 23 |
| Bax    | 14 - T S S E Q I V K T * G A F L L Q * G * F | 30 |

**FIG. 5. N-terminal sequence alignments for selected members of the Bcl2 protein family.** The alignments shown are from Ref. 2 and represent the maximal global similarities to residues 10–28 of human Bcl2; dots indicate gaps introduced to optimize the alignments. Solid rectangles enclose the five critical residues identified in this study for Bcl2 and corresponding residues in BclXL (4) and A1 (27). Dashed rectangles indicate possible counterparts of these residues in the nematode Ced-9 and Epstein-Barr virus Bhrf-1 proteins. No convincing match to this five-residue motif was detected in Bax nor in Bad or Bak (data not shown).

These observations imply that the N-terminal region plays an essential role in Bcl2 function. In the present study, we conducted a detailed mutational analysis to map the essential residues of this region and found five amino acid residues, Ile-14, Val-15, Tyr-18, Ile-19, and Leu-23, that are essential for anti-apoptotic activity. Individual mutations in any one of these five hydrophobic or aromatic residues profoundly inhibited Bcl2 activity in a transient transfection assay, whereas every other residue could be replaced by glycine or alanine without more than a 50% loss of activity.

The periodic spacing of the critical residues, coupled with a computerized structural prediction for the entire N-terminal region, suggested that this region might be α-helical. CD spectral analysis provided support for this conjecture by revealing that peptides encompassing the critical sequences from the Bcl2 N terminus readily adopt a helical conformation in the presence of SDS micelles, conditions that may be presumed to mimic the amphiphilic environment found at hydrophobic surfaces of cellular membranes or in the cores of globular proteins (19–23). Provided that such micelles were present, the peptides maintained their helical properties across a fairly broad range of pH and salt concentration. The fact that helical folding occurred in the absence of flanking peptide sequences, coupled with the evidence that deleting residues 30–79 from Bcl2 has only minimal effect on its activity (13), suggests that residues 6–29 might constitute an autonomous structural domain. Modeling of this domain further suggests that its function may depend upon clustering of the five critical hydrophobic and aromatic residues on one surface of the helix, as depicted in Fig. 3.

Though the exact biological role of this apparently helical domain is not yet known, one possibility is that its hydrophobic surface mediates contacts that might be necessary for proper folding of the Bcl2 monomer or for interacting with other components of the apoptotic pathway. An alternative, though not mutually exclusive, hypothesis is that this domain mediates the formation of Bcl2 multimers within cells. Support for the latter hypothesis comes from studies by Reed and co-workers (14, 15) that indicate that Bcl2 may homomultimerize through sequential “head-to-tail” contacts between N-terminal and C-terminal sequences of consecutive monomers. According to this model, Bcl2 variants that carry mutations or deletions affecting the critical N-terminal residues would retain the ability to dimerize with wild-type Bcl2 (as they have been observed to do, see Refs. 13–15) but would be unable to form higher multimers. Presuming that multimerization is essential for Bcl2 function, this might account for the inability of such mutants to protect against apoptosis. Moreover, because incorporation of even one such mutant would block the formation of a wild-type multimer through chain termination, this model would also account for
the finding that N-terminal deletion mutants exert their dominant negative effects even at relatively low molar ratios (near 1:1) of mutant to wild-type protein (13). Activity at such low stoichiometric ratios is in fact typical of dominant negative mutants that act by blocking formation of higher order multimers (24–26).

Amino acid sequence comparisons suggest that analogues of the critical five-residue motif from Bcl2 may also be present in related proteins. Earlier published alignments (e.g. Ref. 2) have identified regions resembling the Bcl2 N terminus in the anti-apoptotic proteins BclX<sub>L</sub>, A1, and Ced-9, as well as in the proapoptotic protein Bax. Those analyses, however, were based on the entire N-terminal sequence and thus include a preponderance of nonessential residues. Reevaluation of the alignments focusing only on the critical residues identified in this study (Fig. 5) reveals that the five-residue motif is well conserved in BclX<sub>L</sub> and A1. Moreover, an analysis of potential secondary structures in these two proteins using the Rost and Sander algorithm suggests that, in both cases, the conserved residues lie within a region of predicted α-helicity and thus may not be true counterparts of the Bcl2 motif. Less satisfactory sequence matches may also be present in the nematode Ced-9 and Epstein-Barr virus BHRF1 proteins, but these were not predicted to lie within regions of helicity and thus may not be true counterparts of the Bcl2 N-terminal motif. Most importantly, sequences matching this motif could not be identified anywhere within Bax, Bak, or Bad, three Bcl2-related proteins that promote, rather than antagonize, apoptosis. In contrast, close homologues of the BH1 and BH2 motifs are present in all three of these proteins (5–9).

Taken together, these results suggest that the functional domain at the N terminus of Bcl2, which consists of a putative α-helix bearing five critical spaced hydrophobic and aromatic residues, may have counterparts in at least a subset of related anti-apoptotic proteins, and that its presence distinguishes these from related proteins that act to favor programmed cell death.

Addendum—While this manuscript was under review, Muchmore et al. (20) reported a crystallographic and NMR structure for the Bcl2 homologue BclX<sub>L</sub>. That structure includes an α-helix at the location predicted in the present study.

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