Bcl-2 is a cytoplasmic integral membrane protein with potent anti-apoptotic activity but whose mechanism of action is poorly understood. The purpose of this paper was to obtain large amounts of soluble Bcl-2 protein for structural and functional studies. Mouse Bcl-2(1–203) (missing the COOH-terminal hydrophobic tail) was produced in bacterial inclusion bodies, solubilized in guanidine, and refolded by dialysis. The resulting protein was monomeric in nondenaturing solution and was active in protecting mouse T hybridoma cells from glucocorticoid-induced apoptosis. Refolded Bcl-2(1–203) showed no tendency to homodimerize by gel filtration or analytical ultracentrifugation. Limited proteolysis experiments identified a region between the BH3 and BH4 homology domains of Bcl-2(1–203) which was extremely susceptible to digestion by several common proteases, but not by a cell extract known to contain CPP-32-like (interleukin-1β-converting enzyme family) protease activity. The protease-sensitive sites were located within a 50-residue stretch that contained most of the nonconserved and proline residues of Bcl-2(1–203). Trypsin-cleaved Bcl-2(1–203) eluted in the same position as the undigested protein on gel filtration in nondenaturing solution, indicating that the two portions of the molecule connected by the protease-sensitive region associate stably and noncovalently. The solution properties of Bcl-2(1–203) suggest that it consists of two noncovalently associated domains connected by a long protease-sensitive linker and that its structure is similar to that of Bcl-xL, which has been determined by x-ray and NMR analysis.

Bcl-2 was first detected as a t(14:18) chromosomal translocation in follicular B cell lymphomas/leukemias (1) which placed the Bcl-2 gene under the control of the Ig heavy chain promoter, resulting in its overproduction. Subsequent work indicated that overexpression of Bcl-2 prolonged cell survival (2) and inhibited programmed cell death induced by multiple, diverse stimuli including growth factor withdrawal, glucocorticoids, and γ-irradiation (3, 4). Bcl-2 is an intracellular transmembrane protein and was the first member described of a new family of structurally related proteins which now includes Bax, Bak, Bcl-x, ced-9, Mcl-1, A1, NR13, Bad, and Bik, all of which either promote or inhibit programmed cell death (5, 6). A number of studies have identified four domains that are highly conserved among Bcl-2 family members and which are required for Bcl-2 function. From the NH2 terminus, these Bcl-2 homology regions are designated BH4, BH3, BH1, and BH2 (see Fig. 6). Mutational analyses of Bcl-2 have shown that amino acid residues within BH4, BH1, and BH2 are required for its anti-apoptotic activity (7–9). In addition, immunoprecipitation or yeast two-hybrid experiments have demonstrated that Bcl-2 forms homodimers using residues within BH4, BH1, and BH2 (9–11). Bcl-2 also heterodimerizes with the pro-apoptotic protein Bax, resulting in enhanced cell survival (9, 12). Heterodimerization involves residues from BH1 and BH2 in Bcl-2 (9) and residues from BH3 in Bax (13) and the related protein, Bak (14).

Recently, the x-ray and NMR structures of the cytoplasmic portion of Bcl-xL, a molecule sharing 35% amino acid sequence identity with Bcl-2, have been published (15). The structure consists of seven associated α-helices and a long flexible region that connects the first and second helices. The first helix (α1) comprises most of BH4, whereas the remaining helices lie in the COOH-terminal part of the molecule which contains BH1, BH2, and BH3. The flexible domain corresponds to a region of the Bcl-xL and Bcl-2 molecules which exhibits the greatest variability in amino acid sequence between molecules from different species and Bcl-2 family members. Unlike the BH1–4 regions, the flexible domain can be deleted without loss of anti-apoptotic activity (7, 15).

Since most previous studies have focused on Bcl-2 rather than Bcl-x, it is important to establish that Bcl-2 and Bcl-x have similar structures. Previous work showed that mouse Bcl-2 truncated at residue 203 (approximately 12 residues NH2-terminal of the transmembrane portion) retained biologic activity (8, 16). In the current paper we have produced recombinant mouse Bcl-2(1–203) in bacteria. We show that refolded Bcl-2(1–203) is active, contains a long, protease-sensitive portion that corresponds to the Bcl-2 variable region, and that the NH2- and COOH-terminal portions remain noncovalently associated following digestion. Our data indicate that Bcl-2 and Bcl-x have similar structures.

**MATERIALS AND METHODS**

**Cloning of Bcl-2(1–203)–DNA encoding Bcl-2(1–203) was prepared by polymerase chain reaction amplification of a plasmid containing a full-length murine Bcl-2 cDNA (a kind gift from Dr. Jeff Huth, NIDDK, Bethesda, MD) using 5’ primer CTGGCAGAAGGATATACATATGGCGCAGGCGGGAGAACA and 3’ primer GTTAGCAGCCCTCGAGT TAATCACATGGCGGGCCATATA. The polymerase chain reaction product was cloned directly into pCR® II with an Invitrogen (San Diego, CA) TA cloning kit. The insert encoding Bcl-2(1–203) was removed by digestion with NdeI and Xhol and subcloned into NdeI/Xhol sites of pET21a (Novagen, Madison, WI). Sequence analysis using the dyeodeoxy chain termination procedure confirmed that the insert contained the correct Bcl-2(1–203) sequence. The BL21(DE3) strain of Escherichia coli was transformed with the pET21aBcl-2(1–203) plasmid for protein expression.**

**Induction and Purification of Bcl-2(1–203) Protein—**Transformed bacteria were grown to an OD600 of 0.4–0.8 and induced with isopropyl-β-D-}

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Expression and Folding of Bcl-2(1–203) Protein—Mouse Bcl-2(1–203), a 22.5-kDa protein that lacks the membrane-spanning region, was produced in E. coli as insoluble inclusion bodies. Fig. 1A shows that induced bacteria produced large amounts of a 25-kDa protein that was easily purified by washing the inclusion bodies. After solubilization in guanidine and dialysis, the resulting protein eluted on gel filtration in nondenaturing solution as a single peak with an $M_s$ of 22,000 (Fig. 1B). Column-purified Bcl-2(1–203) gave a single band by SDS-PAGE (Fig. 1A) and two very closely spaced bands by IEF near the predicted isoelectric point of 6.05 (Fig. 1C). The IEF results revealed minor heterogeneity, perhaps due to partial deamidation of Asn or Gln residues. NH$_2$-terminal sequence analysis confirmed that the refolded protein was indeed Bcl-2 (see Fig. 1). Bcl-2(1–203) was solubilized at 1–2 mg/ml in physiological buffers but precipitated at higher concentrations; typical yields were 5–10 mg of refolded Bcl-2(1–203)/liter of bacteria. Since misfolded proteins tend to aggregate in nondenaturing solvents (21) and precipitate on IEF gels, these data suggest that the refolded protein was indeed Bcl-2 (see Fig. 1). Bcl-2(1–203) was solubilized at 1–2 mg/ml in physiological buffers but precipitated at higher concentrations; typical yields were 5–10 mg of refolded Bcl-2(1–203)/liter of bacteria. Since misfolded proteins tend to aggregate in nondenaturing solvents (21) and precipitate on IEF gels, these data suggest
that Bcl-2,1–203, was folded correctly.

Recombinant Bcl-2 Protein Is Active—Overexpression of Bcl-2 or Bcl-2,1–203 inhibits dexamethasone-induced, but not Fas-mediated, apoptosis in the mouse T cell hybridoma 2B4.11 (Ref. 18 and data not shown). To determine if the bacterially expressed and refolded Bcl-2,1–203 protein was functionally active, 2B4 cells were electroporated with either bovine serum albumin or Bcl-2,1–203 protein (Table I). A plasmid expressing active, 2B4 cells were electroporated with either bovine serum albumin or Bcl-2,1–203 protein was functionally expressed and refolded Bcl-2,1–203 protein was functionally (Ref. 18 and data not shown). To determine if the bacterially expressed Bcl-2,1–203 protein showed qualitatively the same anti-apoptotic activity as the transfected full-length Bcl-2 gene.

Bcl-2,1–203, Does Not Self-associate—Because Bcl-2 has been reported to homodimerize (9, 10), we asked whether recombinant Bcl-2,1–203 would form aggregates in solution. Initial studies using size exclusion chromatography at different Bcl-2,1–203 concentrations failed to reveal stable self-association. Analytical equilibrium ultracentrifugation was therefore used as a more sensitive probe of aggregation. At equilibrium, the mass distribution data were best fit by a single species with a molecular mass of 23 kDa, with less than 3% aggregate, over the full concentration range tested (Fig. 2). The highest concentration of Bcl-2,1–203 in the centrifuge cell exceeded 0.5 mg/ml (22 μM). At this concentration we would have detected self-association if the $K_d$ had been 200 μM or less.

Limited Proteolysis of Bcl-2,1–203—We next probed the structure of Bcl-2,1–203, by limited proteolysis using three relatively nonspecific proteases. When Bcl-2,1–203, was incubated with trypsin, chymotrypsin, or papain, discrete bands were produced by each of the proteases at low protease:Bcl-2 ratios (Fig. 3). Tryptic digestion at a weight ratio as low as 1:6,400 gave two major fragments migrating at about 15 and 10 kDa. Chymotrypsin also produced two bands, one larger than the 15-kDa tryptic fragment, and one smaller than the 10-kDa peptide. Finally, papain produced a 12-kDa band and a larger fragment that disappeared rapidly. The fact that very low concentrations of three different proteases each produced one or two large fragments suggests that Bcl-2,1–203 contains one or more regions that are highly exposed, with the remaining regions being protected from proteolytic attack. In view of its high susceptibility to cleavage by common proteases, Bcl-2,1–203, was also tested with extracts from Jurkat cells that contained CPP-32-like (interleukin-1β-converting enzyme family) protease activity. After a 90-min incubation at 37 °C, undiluted cell extract did not cleave Bcl-2,1–203, (data not shown).

Time Course of Digestion and Noncovalent Association of Tryptic Fragments—Bcl-2,1–203 was digested with trypsin for various times, and the reaction products were examined by SDS-PAGE and size exclusion chromatography. SDS-PAGE (Fig. 4B) showed that by 10 min, most of the Bcl-2,1–203, was digested into the two major fragments described above. Both bands appeared simultaneously, suggesting that they were produced by a single cleavage of the intact Bcl-2 molecule. In contrast, these same samples, when analyzed by gel filtration in nonnaturating solution (Fig. 4A) showed little evidence of fragmentation into peptides of the size observed by SDS-PAGE, indicating that the two tryptic peptides remained associated by noncovalent interactions. To confirm this, Bcl-2,1–203, was digested with trypsin, fractionated by gel filtration, and analyzed by SDS-PAGE (Fig. 5). Two gel filtration peaks were analyzed. The fraction (Fig. 5A, peak B) eluting at 21 kDa (the same position as intact, monomeric Bcl-2,1–203) consisted of cleaved protein (Fig. 5B, lane 4), clearly demonstrating that the tryptic fragments associated noncovalently. The high molecular weight fraction (Fig. 5A, peak A), probably consisted of aggregated low molecular weight peptides since no large fragments were seen by SDS-PAGE with either Coomassie (Fig. 5B, lane 3) or silver (not shown) staining.

Location of Protease-sensitive Sites—To identify the protease-sensitive sites of Bcl-2,1–203, three tryptic fragments were NH$_2$-terminally sequenced (Fig. 6). Fragment 2, the 15-kDa band, was produced by cleavage at Arg$^{68}$ and fragment 3, the 10-kDa band, had the same NH$_2$-terminal sequence as intact Bcl-2,1–203, Thus it is likely that cleavage at Arg$^{68}$ produced fragments 2 and 3. Fragment 1 was a very faint band that migrated at the same rate as the high molecular weight chymotryptic fragment. It arose by cleavage at Phe$^{69}$ and was probably due to contaminating chymotrypsin in our trypsin preparation. In view of the fact that Bcl-2,1–203 contains potential trypsin and chymotrypsin sites throughout the molecule, it is clear that residues 49 and 68 were particularly susceptible to proteolytic attack.

### Table I

**Bcl-2,1–203 inhibits apoptosis**

| Programmed cell death inducer | % Cytotoxicity |
|------------------------------|---------------|
|                               | BSA           | Bcl-2          |
| Experiment 1                  |               |               |
| Dex (10 μM)                   | 34            | 34            |
| Dex (100 μM)                  | 56            | 19            |
| Anti-Fas mAb (5 ng/ml)        | 46            | 41            |
| Experiment 2                  |               |               |
| Dex (10 μM)                   | 33            | 16            |
| Dex (100 μM)                  | 57            | 26            |
| Anti-Fas mAb (5 ng/ml)        | 28            | 22            |

Bovine serum albumin (control) and Bcl-2,1–203 proteins (approximately 1 mg/ml) were electroporated into 2B4.11 T hybridoma cells along with a plasmid encoding β-galactosidase. Cells were then treated with dexamethasone (Dex) or anti-Fas monoclonal antibody (mAb) as indicated, and the numbers of viable, transfected cells were determined by β-galactosidase expression. Cytotoxicity is expressed as the percentage decrease of β-galactosidase signal in samples treated with programmed cell death inducer relative to the untreated controls. Two representative experiments are shown.
DISCUSSION

Refolded mouse Bcl-2(1–203) is a biologically active protein that is soluble and monomeric in nondenaturing solution. By contrast, misfolded proteins almost invariably aggregate in solution (21) and lack biological activity. It is highly likely, therefore, that the recombinant Bcl-2(1–203) used in this study was folded correctly. Bacterially produced mouse Bcl-2(1–203) has thus provided a source of soluble protein suitable for probing the structure and function of the Bcl-2 cytoplasmic domain.

Previous reports showed that Bcl-2 homodimerizes in mammalian cells or in yeast two-hybrid systems (9–11); in contrast, Bcl-xL fails to homodimerize (22). It is therefore not surprising that recombinant Bcl-xL does not dimerize either in solution or in the crystal (15). However, the failure of recombinant mouse Bcl-2(1–203) to self-associate even at relatively high protein concentrations appears to be inconsistent with previous results. One reason for this inconsistency could be that residues COOH-terminal to 203 are required for homodimerization. Handa et al. (10) showed by yeast two-hybrid analysis that residues between 196 and 218 in human Bcl-2 (193–215 in the mouse) are necessary for homodimerization, but whether residues between 203 and 215 are required for self-association is not known. Another reason might be that bacterially produced Bcl-2 lacks post-translational modifications, present in yeast or mammalian cells, which are required for homodimerization. Bcl-2 can be phosphorylated (23–25), but other modifications are also possible.

The most striking feature of the Bcl-2(1–203) molecule is its extraordinary susceptibility to a variety of proteases. Trypsin, chymotrypsin, and papain all rapidly produce one or two well-defined fragments at low protease:Bcl-2 ratios, even though multiple potential cleavage sites were present throughout the molecule. The sites cleaved by trypsin and chymotrypsin were located within a region of Bcl-2 which is highly variable in amino acid sequence between species and between Bcl-2 and Bcl-x (shaded residues in Fig. 6). The Bcl-2 variable region is also rich in the helix-breaking residue, proline, which varies from 20 to 24% in the variable regions of mouse, chicken, and human Bcl-2 compared with 3–4% in the conserved regions. Interestingly, this region also contains very few aspartate residues, the cleavage site for interleukin-1β-converting enzyme family proteases, which may explain why Bcl-2(1–203) was not susceptible to proteolysis by the Jurkat cell extracts.

The exquisite sensitivity of the Bcl-2(1–203) molecule is its extraordinary susceptibility to a variety of proteases. Trypsin, chymotrypsin, and papain all rapidly produced one or two well-defined fragments at low protease:Bcl-2 ratios, even though multiple potential cleavage sites were present throughout the molecule. The sites cleaved by trypsin and chymotrypsin were located within a region of Bcl-2 which is highly variable in amino acid sequence between species and between Bcl-2 and Bcl-x (shaded residues in Fig. 6). The Bcl-2 variable region is also rich in the helix-breaking residue, proline, which varies from 20 to 24% in the variable regions of mouse, chicken, and human Bcl-2 compared with 3–4% in the conserved regions. Interestingly, this region also contains very few aspartate residues, the cleavage site for interleukin-1β-converting enzyme family proteases, which may explain why Bcl-2(1–203) was not susceptible to proteolysis by the Jurkat cell extracts.

The exquisite sensitivity of the Bcl-2 variable region to proteolysis is readily understood from the Bcl-xL NMR and x-ray results (15), assuming that Bcl-2 and Bcl-xL have similar structures. Both NMR and x-ray analyses indicate that the Bcl-xL variable region is highly mobile, flexible, and exposed to solvent. The conserved regions, on the other hand, are rigid, compact, and mostly inaccessible to solvent. It is therefore not surprising that the variable region exhibits a high preferential susceptibility to proteolytic attack. In Bcl-xL, the two portions of the molecule which are joined by the variable loop form a globular structure consisting of seven interacting α-helices with the helix in the NH2-terminal portion (BH4), making extensive hydrophobic contacts with helices in the COOH-terminal region (15). Our observation that the cleaved Bcl-2 molecule behaves as it were the undigested, monomeric protein in nondenaturing solution demonstrates that the two halves of
terminal region within a molecule would have to be disrupted. Regulation of the internal association between these regions, perhaps by post-translational modification, might therefore be an important aspect of Bcl-2 structure and function.

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Note Added in Proof—A recent paper by Strack et al. (Strack, P. R., Frey, M. W., Rizzo, C. J., Cordova, B., George, H. J., Meade, R., Ho, S. P., Corman, J., Tritch, R., and Korant, B. D. (1996) _Proc. Natl. Acad. Sci. U. S. A._ **93**, 9571–9576) demonstrated that Bcl-2 is cleaved by HIV protease subsequent to apoptosis.

REFERENCES

1. Tsujimoto, Y., Cossman, J., Jaffe, E., and Croce, C. M. (1985) _Science_ **228**, 1440–1442.
2. Reed, J. C. (1994) _J. Cell Biol._ **124**, 1–6.
3. Hockenberg, D. M. (1995) _Bioessays_ **17**, 631–638.
4. Yin, X. M., Olvai, Z. N., Vess-Navock, D. J., Linette, G. P., and Korsmeyer, S. J. (1994) _Cold Spring Harbor Symp. Quant. Biol._ **59**, 387–393.
5. Boise, L. H., Gotteskalck, A. R., Quintans, J., and Thompson, C. B. (1995) _Curr. Top. Microbiol. Immunol._ **200**, 107–121.
6. Hunter, J. J., Bond, B. L., and Parslow, T. G. (1996) _Mol. Cell. Biol._ **16**, 877–883.
7. Borner, C., Martinou, I., Mattmann, C., Imler, M., Schaerer, E., Martinou, J. C., and Tschopp, J. (1994) _J. Cell Biol._ **126**, 1059–1068.
8. Yin, X-M., Olvai, Z. N., and Korsmeyer, S. J. (1994) _Nature_ **369**, 321–323.
9. Hanada, M., Aimé-Symphé, C., Sato, T., and Reed, J. C. (1995) _J. Biol. Chem._ **270**, 11982–11989.
10. Sato, T., Hanada, M., Bodrug, S., Sato, T., and Reed, J. C. (1995) _Proc. Natl. Acad. Sci. U. S. A._ **92**, 9238–9242.
11. Olvai, Z. N., Milliman, C. L., and Korsmeyer, S. J. (1995) _Cell_ **74**, 609–619.
12. Zhu, H., Aimé-Symphé, C., Sato, T., and Reed, J. C. (1996) _J. Biol. Chem._ **271**, 7440–7444.
13. Momm, A., Moreno, M. B., Petrak, D., and Zacharkou, C. M. (1995) _J. Immunol._ **155**, 4644–4652.
14. Schlegel, J., Peters, L., and Orrenius, S. (1995) _FEBS Lett._ **364**, 139–142.
15. Jaeckel, R. (1995) _Biophys. Mol. Biol._ **49**, 117–237.
16. Sedlak, T. W., Olvai, Z. N., Yang, E., Wang, K., Boise, L. H., Thompson, C. B., and Korsmeyer, S. J. (1995) _Proc. Natl. Acad. Sci. U. S. A._ **92**, 7834–7838.
17. Haldar, S., Jena, N., and Croce, C. M. (1996) _Proc. Natl. Acad. Sci. U. S. A._ **92**, 4507–4511.