Biochemical and Biophysical Research Communications **277,** 171–178 (2000)
doi:10.1006/bbrc.2000.3652, available online at http://www.idealibrary.com on IDEAL®

Analysis of the Role of Conserved Cysteine Residues in the Bcl-2 Oncoprotein

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Received September 12, 2000

The Bcl-2 oncoprotein is an integral membrane protein localized primarily to the outer membrane of the mitochondria. The precise molecular mechanism responsible for the antiapoptotic action of Bcl-2 remains unknown. Two cysteine residues are found in Bcl-2 and these residues are well-conserved across species. The first cysteine (cys[155]) is located in the α5 domain, a region important for the ion channel properties of Bcl-2, while the second cysteine (cys[226]) is located in the carboxyl-terminal membrane anchor domain. In this study, we found that replacement of both cysteines with serine residues generated a mutant protein that retained the ability to homodimerize and heterodimerize with proapoptotic Bax protein *in vitro.* In whole cells, the mutant protein efficiently heterodimerized with Bax, but exhibited impaired homodimerization relative to wild-type Bcl-2. The mutant protein was also less efficient than wild-type Bcl-2 at suppressing caspase activation, DNA fragmentation, and loss of viability during IL-3 withdrawal-induced apoptosis. Together, the data indicate that the cysteine residues in Bcl-2 contribute, but are not absolutely essential, to the ability of Bcl-2 to homodimerize, heterodimerize with Bax, and suppress apoptosis.

Key Words: **Bcl-2; Bax; apoptosis; cysteine; caspase; yeast two-hybrid.**

The Bcl-2 oncoprotein is a potent inhibitor of apoptosis and is overexpressed in a variety of human malignancies (1, 2). A number of proteins bearing considerable similarity with Bcl-2 have been identified and together these proteins comprise the Bcl-2 protein family (3). Members of this family exert either antiapoptotic (Bcl-2, Bcl-X_L, Mcl-1, A1, Bcl-W, CED-9) or proapoptotic (Bax, Bak, Bok, Bik, Blk, Hrk, Bad, Bid, Bim, Egl-1) activities in the cell. Sequence and functional comparisons have identified four homology domains, termed BH1, BH2, BH3, and BH4, in Bcl-2 family members (4–9). While all members of the Bcl-2 family contain at least one of these homology domains, Bcl-2 and the closely related protein Bcl-X_L contain all four (10). The BH1, BH2, and BH3 domains of Bcl-2 and Bcl-X_L are important for heterodimerization with other members of the Bcl-2 family (5, 6, 9, 11–13), while the BH4 domain is important for binding proteins such as Raf-1 and calcineurin (14–16). Several Bcl-2 family members, including Bcl-2 and Bcl-X_L, also contain carboxyl-terminal membrane anchoring domains. The membrane anchoring domain of Bcl-2 serves to localize this protein to the mitochondrial outer membrane, as well as membranes of the nucleus and endoplasmic reticulum (17–20).

The precise molecular mechanism responsible for the antiapoptotic action of Bcl-2 remains unknown. However, Bcl-2 has been shown to prevent release of cytochrome c from the mitochondria, an important event during apoptosis mediated by mitochondrial pathways (21–23). Additional studies have shown that Bcl-2, as well as Bcl-X_L and Bax, can form ion channels in artificial lipid membranes, indicating that Bcl-2 may regulate ion flow across the mitochondrial outer membrane (24–27). Evidence supporting a role for Bcl-2 as a membrane pore or channel has come from studies of the structure of Bcl-X_L. Crystallographic analyses have revealed that Bcl-X_L is composed of seven alpha helical regions which are separated by linker sequences (28). Corresponding alpha helices are easily identified in Bcl-2. Strikingly, the three dimensional structure of Bcl-X_L (and by analogy Bcl-2) closely resembles the

Abbreviations used: IL-3, interleukin-3; WCM, Wehi conditioned media; PCR, polymerase chain reaction; GST, glutathione-*S*-transferase; dblcys, double cysteine mutant; AD, activation domain; BD, DNA binding domain; Tag, T antigen; PARP, poly(ADP-ribose) polymerase.

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0006-291X/00 $35.00

structure of membrane pore domains from diptheria toxin and colicins (28). The pore domains of these bacterial toxins are defined by two, central alpha helical domains that span the membrane and are surrounded by other alpha helical sequences. In Bcl-X_L and Bcl-2, these central membrane spanning domains correspond to alpha helix 5 and 6, or $\alpha5\alpha6$. The importance of $\alpha5$ and $\alpha6$ to the function of Bcl-2 has been demonstrated through mutational analyses. Deletion of the $\alpha5\alpha6$ domains in Bcl-2 abrogates the ability of Bcl-2 to form ion channels, heterodimerize with Bax, and suppress Bax- and staurosporine-induced apoptosis (29). Moreover, deletion of amino acids 150–163 in human Bcl-2, which lie within the $\alpha5$ domain, also results in loss of anti-apoptotic function (8).

In this report we have examined the role of cysteine residues in the function of Bcl-2. Cysteine residues often play key roles in protein function. Cysteine residues can comprise the active site of enzymes, as is the case in cysteine proteinases. Alternatively, cysteine residues can participate in intramolecular disulphide bond formation, helping to determine tertiary structure, and hence protein function. Cysteine residues also can form intermolecular disulphide bonds with other proteins, leading to the assembly of multiprotein complexes, which may stabilize or facilitate the activities of component proteins. In addition, cysteine residues can contribute to intra- or intermolecular structure through hydrogen bonding with other polar residues. The Bcl-2 protein contains only two cysteine residues; one lies in the critically important $\alpha5$ domain and the other in the carboxyl-terminal membrane anchoring domain. Both cysteine residues are conserved between human (2, 30), mouse (31), and chicken Bcl-2 (32). Using site-directed mutagenesis, we find that these conserved cysteine residues contribute, but are not absolutely essential for Bcl-2 function.

MATERIALS AND METHODS

Chemicals and reagents. All tissue culture media, additives, and antibiotics were purchased from Gibco/BRL (Gaithersburg, MD). Taq polymerase was purchased from Perkin Elmer Cetus (Norwalk, CT) and restriction and cloning enzymes were purchased from Boehringer Mannheim (Indianapolis, IN). DNA sequencing kits were obtained from PanVera (Madison, WI). Yeast strains, and vectors for yeast two-hybrid studies were purchased from Clontech (Palo Alto, CA). Amino acids and yeast media supplements were purchased from Sigma (St. Louis, MO). All other reagents obtained were molecular biology grade.

Cell culture. 32D (clone 23) is an interleukin-3 (IL-3)-dependent murine myeloid progenitor cell line (33, 34). Wehi 3B is an IL-3-producing murine myelomonocytic cell line (35). Both cell lines were generously provided by Dr. Robert Redner at the University of Pittsburgh (Pittsburgh, PA), and were maintained in an atmosphere of 5% CO_2 and 37°C in RPMI 1640 media containing 10% heat-inactivated fetal bovine serum (FBS), L-glutamine, penicillin, streptomycin-sulfate, and fungizone. In addition, media for 32D cells was supplemented to 10% with Wehi conditioned media (WCM) as a source of IL-3. Transfected cell lines were maintained in media supplemented with 0.5 mg/ml G418.

Generation of mutant Bcl-2. Mutant dblcysBcl-2 was generated by sequentially changing cysteine^{155} and cysteine^{226} to serine residues using a polymerase chain reaction (PCR) strategy (36). For mutation of cysteine^{155}, an N-terminal-encoding mutant fragment was amplified using the 5′ primer (primer A) 5′-CTGACCCGGGGAATTCGTACCTGCAGCTTCTTTTCGGGGAACCATGGCGCAAGCCGGGAGAACA-3′ and a 3′ primer 5′-CTCTCCACAGACATGACCCCACCGA-3′, and a C-terminal-encoding mutant fragment was amplified using a 5′ primer 5′-GGTCATGTCTGTGGAGAGCGTCAA-3′ and the 3′ primer (primer Z) 5′-CTGTAAGCTTCCCGGGTCTAGATCAAGTTTCCGGTTCCGGCGGCGGAGTCGGCGGTTTCTTGTGGCCCAGGTATGCA-3′. One microliter each of the N-terminal- and C-terminal-encoding fragments were then mixed, and full-length cys^{155}ser was generated by amplifying with primer A and primer Z. For mutation of cysteine^{226} and generation of the dblcysBcl-2 mutant, the cys^{155}ser PCR fragment was used as template DNA. Again, an N-terminal-encoding mutant fragment was amplified using primer A and a 3′ primer 5′-CCAGAGTGATGGAGGCCCCGACCA-3′, and a C-terminal-encoding mutant fragment was amplified using a 5′ primer 5′-GGGGCCTCCATCACTCTGGGTGCA-3′ and primer Z. One microliter each of the N-terminal- and C-terminal-encoding fragments were then mixed, and full-length dblcysBcl-2 was generated by amplifying with primer A and primer Z. The resulting full-length dblcysBcl-2 fragment was then subcloned into Bluescript vector (Stratagene, La Jolla, CA) and the entire mutant fragment was verified by DNA sequencing (37).

Yeast two-hybrid analyses. Yeast two-hybrid analyses were performed using reagents from the Matchmaker kit from Clontech (Palo Alto, CA). Briefly, PCR was used to amplify cDNA fragments corresponding to amino acid 2 through the stop codon for wild-type Bcl-2, dblcysBcl-2, or wild-type Bax. The amplified fragments were verified by DNA sequence analysis, then subcloned at appropriate restriction sites downstream from and in-frame with sequences encoding the activation domain (vector pGAD424) or the DNA binding domain (vector pAS2) of GAL4. For wild-type and mutant Bcl-2, the 5′ PCR primer was 5′-GACCCGGGTGCGCAAGCCGGGAGAACA-3′ and the 3′ primer was 5′-GTAGAATTCTCACTTGTGGCCCAGGTAT-3′. Wild-type Bax was amplified using the 5′ primer 5′-GACCCGGGTGACGGGTCCGGGGAGCAGCTT-3′ and the 3′ primer 5′-CGGATCCTCAGCCCATCTTCTTCCAGAT-3′. Final constructs encoding activation domain and DNA binding domain fusion proteins were introduced into the yeast strain CG-1945. Protein-protein interactions were assessed by the ability of the transformed yeast to grow on plates lacking histidine, as described by the manufacturer of the Matchmaker kit (Clontech).

Production of glutathione-S-transferase (GST) fusion proteins. Fusion proteins were generated by fusing the second amino acid of wtBcl-2, dblcysBcl-2, or Bax with the C-terminal sequence of GST in the bacterial expression vector pGex-2T (Pharmacia, Peapack, NJ). First, the wtBcl-2 or dblcysBcl-2 cDNAs were PCR amplified using the 5′ primer 5′-GTGAATTCATGCGCAAGCCGGGAGA-3′ and the 3′ primer 5′-GTAGAATTCTCACTTGTGGCCCAGGTAT-3′, and Bax cDNA was amplified using the 5′ primer 5′-GACCCGGGTGACGGGTCCGGGGAGCAGCTT-3′ and the 3′ primer 5′-CGGATCCTCAGCCCATCTTCTTCCAGAT-3′. The resulting PCR fragments were subcloned into Bluescript vector, verified by DNA sequencing, excised, and then subcloned into the *Eco*RI (Bcl-2 and dblcysBcl-2) or *Sma*I (Bax) sites of pGex-2T vector. Fusion constructs were then introduced individually into competent DH5α bacteria (Gibco/BRL). To produce the fusion proteins, bacterial colonies expressing the desired protein were grown in LB media until the $O.D._{595}$ reached 0.6. IPTG was then added to 0.5 mM and the bacteria were allowed to grow for an additional 3 h. The bacteria were then harvested by centrifugation and resuspended in buffer consisting of 25 mM Tris

(pH 8.0), 120 mM NaCl, 0.5% NP40, 1 mM DTT, 1.5 mM PMSF, 3 μg/ml leupeptin, 20 μg/ml aprotinin. The resuspended bacteria were then lysed using 0.1 mm glass beads and a bead-beater instrument (Biospec Products, Bartlesville, OK). To ascertain the concentrations of fusion proteins in lysate samples, fusion proteins were purified from an aliquot of the lysate using glutathione-agarose beads (Sigma), according to manufacturers instructions. The quantity of purified protein was then determined using the Bio-Rad protein assay kit II (Hercules, CA).

In vitro translation reactions. Full-length *in vitro* translated Bcl-2, dblcysBcl-2, or Bax were generated in the presence of ^{35}S-methionine using the TNT Coupled Reticulocyte Lysate System from Promega (Madison, WI). Full-length cDNAs, subcloned in Bluescript vector, were used as templates for the *in vitro* transcription/translation reactions.

In vitro binding assays. For *in vitro* binding assays, 125,000 cpm of *in vitro* translated protein was incubated for 3 h at 4°C with bacterial lysate containing 5 μg of fusion protein. Incubations were set up in a total volume of 800 μl of the bacterial lysis buffer. Following incubation, 100 μl of glutathione-agarose beads, prewashed in lysis buffer, were added, and incubation was continued at 4°C for an additional 45 min. The beads were then pelleted and washed three times in ice cold lysis buffer. The final pellets, containing bound proteins, were resuspended in sample buffer containing 100 mM DTT, boiled for 5 min, centrifuged to remove beads, electrophoresed on 12.5% SDS/PAGE gels, and transferred to nitrocellulose filters. The filters were dried and exposed to film.

Generation of expression constructs and transfection of 32D cells. Wild-type Bcl-2 or dblcysBcl-2 were expressed in 32D cells as epitope-tagged proteins, with the 11-amino acid KT3 epitope tag (38) fused to the carboxy-terminal ends of the proteins. The KT3 epitope tag is derived from SV40 virus T antigen protein (38). Briefly, wild-type or mutant Bcl-2 cDNAs were amplified and sequenced as previously described (39), then subcloned downstream from a B19 parvoviral p6 promoter in the mammalian expression vector B19/neo (40). The B19/neo vector also contains the neomycin phosphotransferase gene, encoding resistance to G418. The B19/neo, B19/neo/Bcl-2-KT3, and B19/neo/dblcysBcl-2-KT3 expression constructs were introduced individually into 32D cells by electroporation (250 V, 960 μF). Following electroporation, cells were selected in media containing 0.5 mg/ml G418. After 2 weeks of selection, independent clones were isolated by limiting dilution.

IL-3 withdrawal. Twenty-four hours prior to IL-3 withdrawal, cells were plated at a density of 3×10^5 cells/ml in media containing 0.5 mg/ml G418, 10% FBS, and 10% WCM (as a source of IL-3). Following this preincubation, cells were pelleted by centrifugation, then washed three times with cold phosphate-buffered saline (13 mls/wash). The washed cells were resuspended at a density of 1×10^6 cells/ml in media containing 0.5 mg/ml G418 and 10% FBS alone, then placed at 37°C. At various timepoints after resuspension in IL-3-deficient media, aliquots of cells were removed for preparation of whole cell lysates or genomic DNA.

Whole cell lysate preparation and Western blotting. To prepare whole cell lysates, cells were pelleted, washed once in PBS, then lysed in buffer consisting of 50 mM Tris (pH 8.0), 150 mM NaCl, 0.1% SDS, 1.0% NP40, 1.5 mM PMSF, 3 μg/ml leupeptin, 20 μg/ml aprotinin. Protein samples (25 μg/lane) were then electrophoresed on 12.5% SDS/PAGE gels and transferred to nitrocellulose filters. The filters were probed with anti-KT3 monoclonal antibody, anti-mouse Bcl-2 or anti-PARP monoclonal antibodies (Pharmingen, San Diego, CA).

RESULTS AND DISCUSSION

To study the role of the two cysteine residues found in Bcl-2, we used site-directed mutagenesis to convert these amino acids to serine residues in the mouse Bcl-2 protein. The first cysteine residue is found at position 155 in the mouse protein (158 in human), just downstream from the BH1 domain (a.a. 133–152 in mouse) which is important for homo- and heterodimerization interactions (2, 30, 31, 9). Importantly, cysteine^{155} resides within the α5 helix (a.a. 141–160 in mouse) of Bcl-2, a region that likely spans the mitochondrial outer membrane and contributes to the channel- or pore-forming properties of Bcl-2 (28, 29). Deletion of sequences within α5 that encompass this cysteine residue, namely amino acids 150–163 in human Bcl-2 (corresponding to 147–160 in mouse), has been shown to completely abrogate the antiapoptotic function of Bcl-2 (8). The potential importance of cysteine^{155} is further suggested by the fact that it is conserved across species (human, mouse, chicken), and that a corresponding cysteine is found in the same location in Bcl-X_L (2, 10, 30–32). The second cysteine residue in the mouse Bcl-2 protein is found at position 226 (229 in human), and this cysteine is also conserved across species. However, a corresponding cysteine residue is not found in the same location in Bcl-X_L (10). Cysteine^{226} resides in the carboxyl-terminal membrane anchoring domain of Bcl-2. Previous studies have shown that deletion of this domain results in a protein with significantly diminished, albeit some, antiapoptotic activity (41–44, 8).

In preliminary studies we attempted to focus exclusively on cysteine^{155}, by generating mutants only at this site. However, we found that proteins with only the cys^{155}ser mutation were expressed at very low levels in transfected cells (not shown), suggesting that this mutant, containing only one cysteine residue, was unstable in the cell. For this reason we generated and studied a double cysteine mutant (dblcys), where both cysteine^{155} and cysteine^{226} were converted to serines. This double mutant, containing no cysteine residues, appeared to be more stable, as it was expressed at much higher levels in transfected cells.

We first asked whether mutation of the cysteine residues in Bcl-2 would impact the ability of this protein to homodimerize and to heterodimerize Bax. Bax is a proapoptotic member of the Bcl-2 protein family (4). When Bcl-2 heterodimerizes with Bax it abrogates the ability of Bax to promote apoptosis (4). In Fig. 1 wild-type Bcl-2 (Bcl-2) and dblcysBcl-2 were produced in bacteria as GST fusion proteins, then assayed for their ability to bind *in vitro* translated ^{35}S-Bcl-2, ^{35}S-dblcysBcl-2, or ^{35}S-Bax proteins. In control experiments, we determined that GST protein alone did not exhibit detectable binding to any of these *in vitro* translated proteins. By contrast, both GST-Bcl-2 and GST-dblcysBcl-2 bound all three *in vitro* translated proteins. Furthermore, GST-Bcl-2 and GST-dblcysBcl-2 were essentially indistinguishable in their ability to homo- or heterodimerize. This indicated that

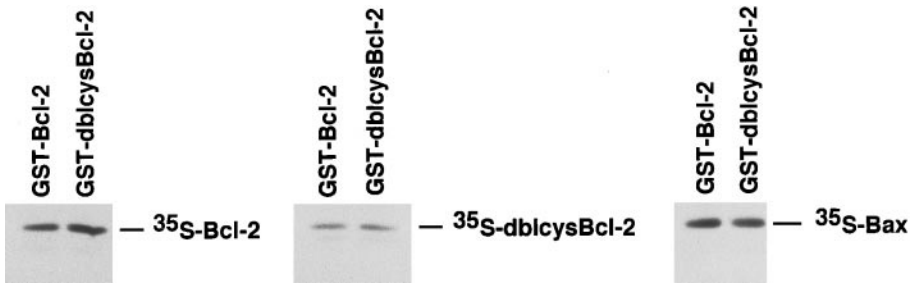

FIG. 1. The dblcysBcl-2 mutant retains the ability to bind to wild-type Bcl-2 or Bax *in vitro.* Bacterial lysates containing 5 μg of GST-Bcl-2 (lane 1) or GST-dblcysBcl-2 (lanes) were incubated with 125,000 cpm of *in vitro* translated ^{35}S-Bcl-2 (A), ^{35}S-dblcysBcl-2 (B), or ^{35}S-Bax (C) for 3 h at 4°C. GST fusion proteins, and associated binding partners, were then purified on glutathione-agarose as described under Materials and Methods. The purified proteins were electrophoresed on a 12.5% SDS/PAGE gel, transferred to nitrocellulose, and exposed to film. A and B were exposed to film for 6 days and C was exposed for 2 days. In control experiments, *in vitro* translated proteins did not bind to GST alone. The figure shows that dblcysBcl-2 protein retains the ability to bind to Bcl-2 and Bax *in vitro.*

cysteine residues 155 and 226 were not essential for homodimerization or heterodimerization with Bax *in vitro.*

The ability of the dblcysBcl-2 mutant to homo- and heterodimerize in whole cells was tested using a yeast two-hybrid system (Fig. 2). For these experiments, Bcl-2, dblcysBcl-2, and Bax were engineered as fusion proteins with the activation domain (AD) or DNA binding domain (BD) of the GAL4 transcription factor. The fusion proteins were then coexpressed with various other proteins in yeast, and positive interactions were scored by the ability of the yeast to grow on plates lacking histidine (see Materials and Methods). As shown in Fig. 2A, the Bcl-2, dblcysBcl-2, and Bax proteins did not show any nonspecific interactions with the control proteins p53 or T antigen (Tag). By contrast, strong interaction was detected between p53 and Tag, two proteins that are known to associate inside the cell. In Fig. 2B, we detected strong interaction between dblcysBcl-2 and Bax, similar to that found between Bcl-2 and Bax. dblcysBcl-2 also interacted with Bcl-2, albeit perhaps to a reduced extent relative to homodimerization between two Bcl-2 molecules. Homodimerization interactions between two dblcysBcl-2 molecules (dblcysBcl-2-AD/dblcysBcl-2-BD) appeared to be quite weak. These findings suggested that the cysteine residues in Bcl-2, while not essential for intracellular homo- or heterodimerization, may contribute to the stability of the homodimer complex.

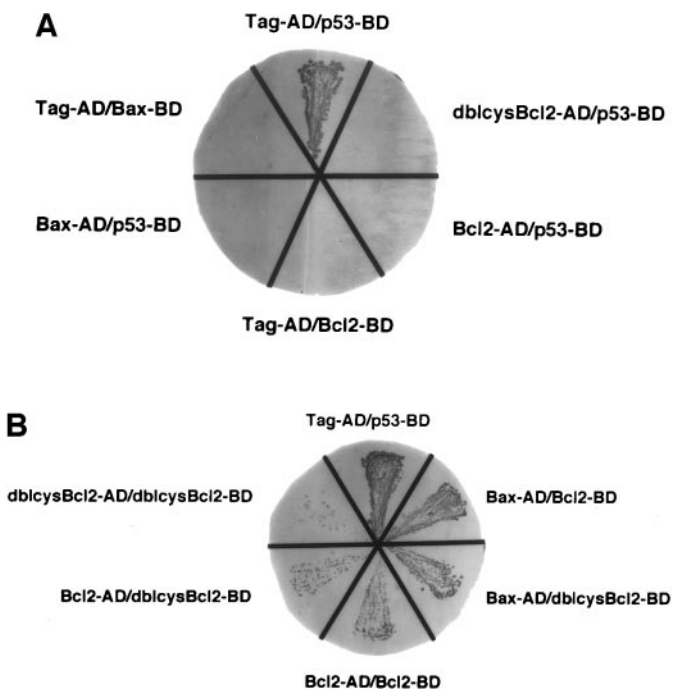

FIG. 2. Intracellular interactions of dblcysBcl-2 using yeast two-hybrid system. (A) Wild-type Bcl-2, dblcysBcl-2, Bax, SV40 T antigen (Tag), or p53 proteins fused with the GAL4 activation domain (AD) or DNA binding domain (BD) were expressed in various combinations in the CG-1945 yeast strain. Intracellular interactions were determined by the ability of the transformed yeast to grow on plates lacking histidine. As shown, wild-type Bcl-2, dblcysBcl-2, and Bax do not interact nonspecifically with Tag or p53. (B) Successful growth of transformed yeast on plates lacking histidine demonstrates that dblcysBcl-2 associates with wild-type Bcl-2 and Bax inside the cell.

We next examined the ability of the dblcysBcl-2 mutant to suppress apoptotic cell death in mammalian cells. For these experiments, murine myeloid 32D cells were engineered to overexpress Bcl-2 or dblcysBcl-2 proteins. The 32D cell line is dependent on the cytokine interleukin-3 (IL-3) for survival (33, 34). When IL-3 is withdrawn, 32D cells activate caspase proteases and undergo apoptotic cell death, as characterized by fragmentation of genomic DNA and loss of cell viability (45–47). Overexpression of wild-type Bcl-2 has previously been shown to inhibit IL-3 withdrawal-induced apoptosis (48, 49). The Bcl-2 and dblcysBcl-2 proteins were expressed in 32D cells as epitope-tagged proteins, with the 11-amino acid KT3 epitope fused to their carboxyl-terminal ends ((38), see Materials and Methods). Clonal transfected lines were used for all analyses, and expression of the exogenous, engineered pro-

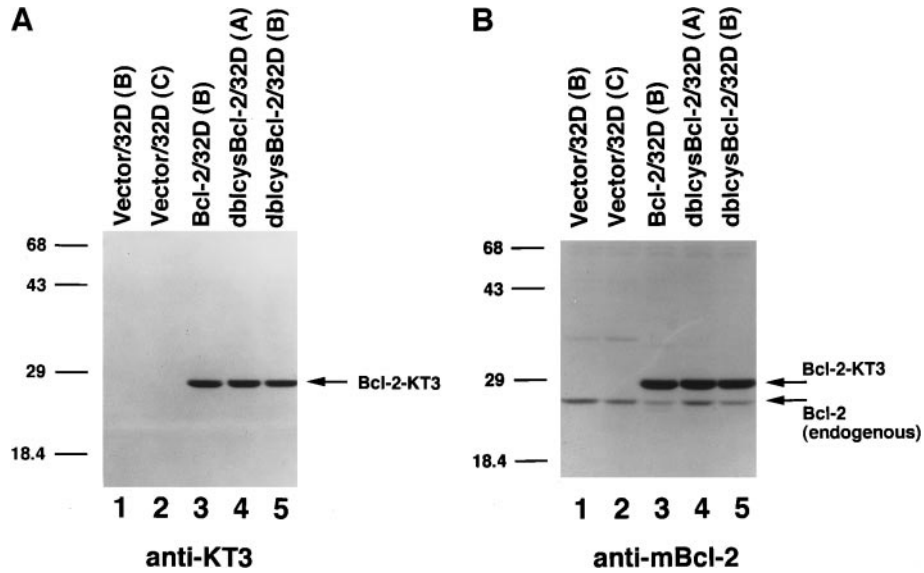

FIG. 3. Expression of epitope-tagged Bcl-2-KT3 or dblcysBcl-2-KT3 in 32D cells. Western blotting was used to analyze whole cell lysates (25 μg protein per lane) of two clonal 32D cell lines transfected with B19/neo vector alone (Vector/32D; lanes 1–2), one clonal cell line transfected with vector containing a cDNA encoding Bcl-2-KT3 (Bcl-2/32D; lane 3), and two clonal cell lines transfected with vector containing a cDNA encoding dblcysBcl-2-KT3 (dblcysBcl-2/32D; lanes 4–5). The clone names are indicated in parentheses. Blots were probed with anti-KT3 monoclonal antibody (A) or anti-murine Bcl-2 monoclonal antibody (B).

teins was demonstrated by Western blotting with anti-KT3 monoclonal antibody (Fig. 3A). As shown, the epitope-tagged Bcl-2 and dblcysBcl-2 proteins were expressed at nearly equal levels in the clonal transfected cell lines. No anti-KT3 immunoreactivity was seen in vector-transfected control cells. In Fig. 3B, Western blots were probed with a monoclonal antibody against murine Bcl-2, in order to detect both the exogenous proteins (29 kDa) as well as endogenous Bcl-2 (26 kDa). Although 32D cells express low levels of endogenous Bcl-2, the endogenous Bcl-2 does not block IL-3 withdrawal-induced apoptosis in these cells (perhaps due to downregulation of the endogenous protein following IL-3 withdrawal). In the transfected cell lines the expression levels of epitope-tagged exogenous proteins was at least 5-fold higher than endogenous protein levels.

To examine apoptosis induction in the transfected 32D cell lines, we first studied the activation of caspases proteases. The activation of cellular caspases is a hallmark biochemical feature of apoptosis, and can be assessed by observing the cleavage of caspase substrate proteins (50, 51). One such substrate is poly(ADP-ribose) polymerase (PARP), whose cleavage by caspases results in the appearance of an 85-kDa proteolytic fragment (52). In the experiment shown in Fig. 4, cells were deprived of IL-3 for varying lengths of time, followed by preparation of whole cell lysates and Western blotting with anti-PARP monoclonal antibody. In vector-transfected control cells the majority of full-length PARP was degraded to the 85-kDa fragment by 12 h after IL-3 withdrawal (lane 2). In sharp contrast, in cells overexpressing wild-type Bcl-2, the majority of PARP was detected as full-length protein, even 48 h after IL-3 withdrawal (lane 8). Cells overexpressing the dblcysBcl-2 mutant also exhibited markedly delayed PARP cleavage, with relatively little

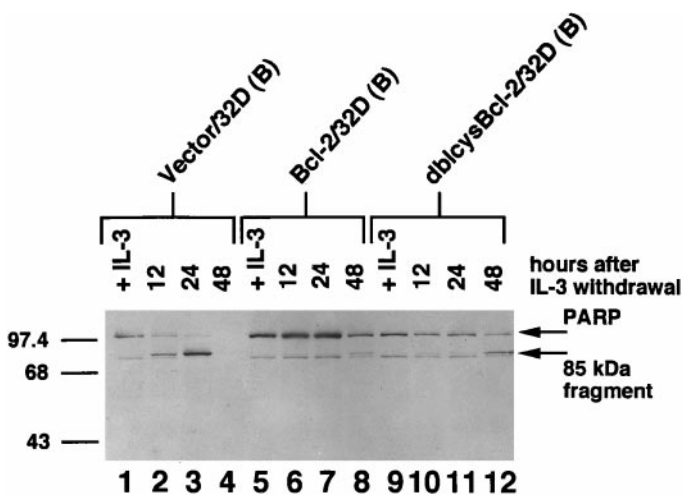

FIG. 4. Inhibition of IL-3 withdrawal-induced PARP cleavage by Bcl-2 and dblcysBcl-2. Clones of Vector/32D, Bcl-2/32D, and dblcysBcl-2/32D cells were deprived of IL-3 for varying lengths of time and whole cell lysates were prepared as described under Materials and Methods. The letters in parentheses indicate the clone name. Protein aliquots (25 μg/lane) were electrophoresed on a 12.5% SDS/PAGE gel, transferred to nitrocellulose, and probed with anti-PARP monoclonal antibody. The locations of intact PARP and the 85-kDa proteolytic fragment are indicated. A nonspecific band located just below the 85-kDa fragment is also visible in all lanes.

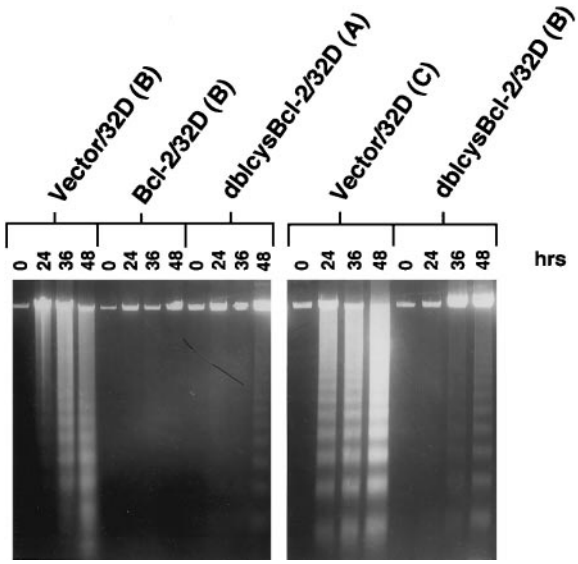

FIG. 5. Inhibition of genomic DNA fragmentation by Bcl-2 and dblcysBcl-2. Genomic DNA was purified from two independent clones of Vector/32D cells, one clone of Bcl-2/32D cells, and two clones of dblcysBcl-2/32D cells following deprivation of IL-3 for varying lengths of time. DNA was prepared as previously described (45). Aliquots of the DNA (2 μg/lane) were electrophoresed on 2% agarose gels, then stained with ethidium bromide.

cleavage seen at 24 h (lane 11). However, by 48 h the majority of PARP was cleaved in these cells. This indicated that the mutant protein, while able to delay caspase activation, was not as efficient at doing so as wild-type Bcl-2.

Downstream from the activation of caspase proteases during apoptosis is the activation of nuclear DNAses and resulting degradation of genomic DNA to oligonucleosomal-length fragments (53–55). To investigate the impact of the dblcysBcl-2 mutant on DNA fragmentation, we analyzed genomic DNA harvested from cells deprived of IL-3 for varying lengths of time (Fig. 5). DNA from two clones of vector-transfected cells showed considerable fragmentation by 24 h after IL-3 withdrawal. On the other hand, cells overexpressing wild-type Bcl-2 showed little evidence of DNA fragmentation as late as 48 h after removal of IL-3. In two different clones of cells overexpressing the dblcysBcl-2 mutant only modest fragmentation of DNA was seen. For one clone, fragmentation was first detected after 48 h, while in the other clone fragmentation was seen by 36 h. Thus, similar to what was seen in PARP cleavage experiments, the dblcysBcl-2 mutant protein, while functionally able to inhibit DNA fragmentation, was less effective than wild-type Bcl-2.

One of the final endpoints of apoptotic cell death is the loss of membrane integrity and cell viability. Loss of viability and membrane integrity can be assessed by examining uptake of the vital dye trypan blue. The ability of the dblcysBcl-2 mutant to prevent IL-3 withdrawal-induced loss of viability was studied in Fig. 6. In several experiments, two independent clones of vector-transfected cells demonstrated viabilities of less than 10 percent 48 h after IL-3 withdrawal. Cells overexpressing the wild-type Bcl-2 protein, on the other hand, retained greater than 80 percent viability even72 h after removal of IL-3. Interestingly, the dblcysBcl-2 mutant also delayed loss of viability, although to a lesser extent. By 48 h after IL-3 withdrawal, the two clones of dblcysBcl-2-expressing cells showed viabilities exceeding 60 percent. However, by 72 h the viabilities of these cell lines were in the range of 15 to 30 percent. Comparison of the dblcysBcl-2 lines with the vector-transfected control cells revealed that overexpression of the dblcysBcl-2 mutant delayed IL-3 withdrawal-induced loss of viability by at least 24 h.

Taken together, our data show that the dblcysBcl-2 mutant binds efficiently to Bax, but is only partially effective, relative to wild-type Bcl-2, at preventing IL-3 withdrawal-induced caspase activation, DNA fragmentation, and loss of viability. The dblcysBcl-2 protein also may have impaired ability to homodimerize in the cell, as demonstrated using the yeast two-hybrid assay. Nonetheless, the partial activity of this mutant clearly shows that cysteine residues in the Bcl-2 molecule are not absolutely essential for heterodimerization with Bax or suppression of apoptosis. This likely rules out a role for the sulfhydryl moieties of these residues as active site constituents, or as required sites of interaction with critical binding partners. Moreover, since cysteine^{155} is found in the α5 helical domain it is probable that the sulfhydryl group of this amino acid does not play a critical role in facilitating passage of ions or molecules through the channels formed by the α5 and α6 helices. However, the fact that the dblcysBcl-2 mutant exhibits less antiapoptotic activity than wild-type Bcl-2 suggests that the cysteine residues may contrib-

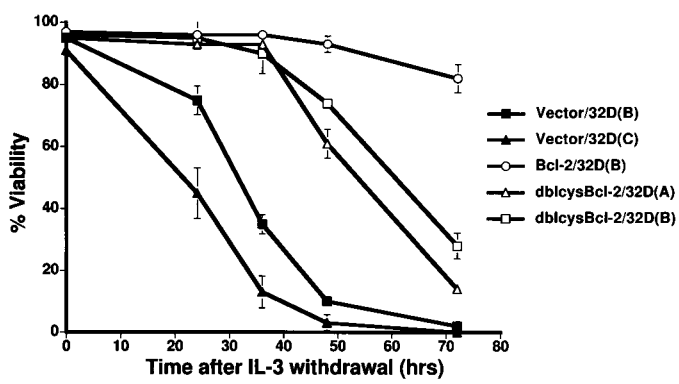

FIG. 6. Loss of cell viability in IL-3-deprived cells expressing Bcl-2 or dblcysBcl-2. Cells were deprived of IL-3 as described under Materials and Methods. At various timepoints after IL-3 withdrawal, cell viabilities were determined by trypan blue exclusion. Each data point represents the mean of triplicate samples, with a minimum of 100 cells counted per sample. Error bars depict standard deviations.

ute to formation of proper secondary or tertiary structure in the Bcl-2 molecule. In future experiments it will be important to clarify how these cysteine residues may contribute to homodimerization interactions, and whether efficient homodimerization or oligomerization of Bcl-2 is essential for maximal antiapoptotic function.

ACKNOWLEDGEMENTS

This work was supported by National Institutes of Health Grant CA66044 to D.E.J. We also thank L. T. Williams for helpful discussions and the gift of oligonucleotide primers.

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