Transient Expression of Wild-type or Mitochondrially Targeted Bcl-2 Induces Apoptosis, whereas Transient Expression of Endoplasmic Reticulum-targeted Bcl-2 Is Protective against Bax-induced Cell Death*

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Bcl-2 protein family members function either to promote or inhibit programmed cell death. Bcl-2, typically an inhibitor of apoptosis, has also been demonstrated to have pro-apoptotic activity (Cheng, E. H., Kirsch, D. G., Clem, R. J., et al. (1997) Science 278, 1966–1968). The pro-apoptotic activity has been attributed to the cleavage of Bcl-2 by caspase-3, which converts Bcl-2 to a pro-apoptotic molecule. Bcl-2 is a membrane protein that is localized in the endoplasmic reticulum (ER) membrane, the outer mitochondrial membrane, and the nuclear envelope. Here, we demonstrate that transient expression of Bcl-2 at levels comparable to those found in stably transfected cells induces apoptosis in human embryonic kidney 293 cells and in the human breast cell line MDA-MB-468 cells. Furthermore, we have targeted Bcl-2 specifically to either the ER or the outer mitochondrial membrane to test whether induction of apoptosis by Bcl-2 is dependent upon its localization within either of these membranes. Our findings indicate that Bcl-2 specifically targeted to the mitochondria induces cell death, whereas Bcl-2 that is targeted to the ER does not. The expression of Bcl-2 does result in its cleavage to a 20-kDa protein; however, mutation of the caspase-3 cleavage site (D34A) does not inhibit its ability to induce cell death. Additionally, we find that transiently expressed ER-targeted Bcl-2 inhibits cell death induced by Bax overexpression. In conclusion, the ability of Bcl-2 to promote apoptosis is associated with its localization at the mitochondria. Furthermore, the ability of ER-targeted Bcl-2 to protect against Bax-induced apoptosis suggests that the ER localization of Bcl-2 may play an important role in its protective function.

Bcl-2 is the founding member of a family of proteins that regulate apoptosis. Originally isolated from the t(14;18) chromosomal breakpoint in human B cell lymphomas, bcl-2 has since been established to be a proto-oncogene that prolongs cell survival by inhibiting apoptosis (1–4). The protection from apoptosis conferred by Bcl-2 upon withdrawal of cytokine was found to be a consequence of its dysregulated overexpression in B cell lymphomas. The promotion of cell survival by Bcl-2 has been recapitulated in many cell line model systems in which Bcl-2 overexpression has been enforced by stable transfection. Although Bcl-2 has been found to inhibit cell death induced by a wide variety of apoptotic signals in many cell types, the mechanism of its protective action still remains unclear.

Bcl-2 family members are characterized by containing at least one of four Bcl-2 homology domains (BH1-BH4). Some of these proteins, such as Bax and Bak, function to promote apoptosis, whereas others like Bcl-2 and Bcl-XL inhibit apoptosis (5–7). The subcellular localization of these proteins provides important clues as to how these proteins function. Certain pro-apoptotic proteins, such as Bax, and anti-apoptotic proteins, such as Bcl-2, bear a C-terminal transmembrane-targeting domain that allows them to be inserted into the cytosolic face of intracellular membranes (8, 9). Bcl-2 is found in several intracellular membranes including the endoplasmic reticulum (ER),1 the outer mitochondrial membrane, and the nuclear envelope (10, 11). In contrast, Bax is not located in the ER or perinuclear membrane but remains in the cytoplasm until an apoptotic signal is generated and induces its translocation to mitochondria (12, 13). The oligomerization of Bax in the mitochondrial membrane has been shown to induce cytochrome c release and the subsequent steps in the execution phase of apoptosis (13, 14). The pro-apoptotic activity of other proteins, such as Bid and Bak, have been associated with their regulated targeting to the mitochondrial membrane (15, 16). Thus, mitochondria appear to be a target for mediators of apoptosis and occupy a central role in the apoptotic pathway (17).

The structures of both pro-apoptotic and anti-apoptotic proteins, Bax and Bcl-XL, respectively, have been determined and found to be strikingly similar (18, 19). Although pro-apoptotic and anti-apoptotic proteins share structural similarities and are both found in mitochondrial membranes, it is not understood how they can function in opposing ways to regulate apoptosis. Recent evidence has indicated, however, that Bcl-2 under certain conditions can function as a pro-apoptotic molecule. Bcl-2 and Bcl-XL can be cleaved by caspase-3 and thus be converted to a pro-apoptotic protein similar to Bax (20, 21).

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1 The abbreviations used are: ER, endoplasmic reticulum; HEK, human embryonic kidney; GFP, green fluorescence protein; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; PI, propidium iodide.
Conversely, Bax has also been shown to inhibit neuronal cell death when infected with Sindbis virus (22). These observations suggest that members of the Bcl-2 family have reversible roles in the regulation of apoptosis and have the potential to function in a pro-apoptotic or anti-apoptotic capacity.

Here, we demonstrate that acute Bcl-2 expression by transient transfection induces cell death in the human breast carcinoma cell line MDA-MB-468 and human embryonic kidney (HEK) 293 cells. By specifically targeting Bcl-2 either to the ER or the mitochondrial membrane, we find that this induction of apoptosis by Bcl-2 is associated with its localization at the mitochondrial membrane. Transient overexpression of either wild-type or mitochondrially targeted Bcl-2 is sufficient to induce cell death and is not inhibited by the mutation of the caspase-3 cleavage site (D34A). Furthermore, ER-targeted Bcl-2 was found to inhibit cell death induced by Bax overexpression, whereas wild-type and mitochondrially targeted Bcl-2 did not. Thus, ER-targeted Bcl-2 does confer protection against apoptosis by Bax overexpression, suggesting that the ER localization of Bcl-2 may play a significant role in its inhibition of cell death pathways mediated by Bax.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Cell Culture—**HEK 293 cells were cultured in Dulbecco's modified Eagle's medium buffered with 25 mM HEPES that was supplemented with 10% fetal bovine serum and 2 mM l-glutamine. African green monkey kidney cells (COS-7) were cultured in Dulbecco's modified Eagle's medium, and human breast epithelial cells MDA-MB-468 were cultured in improved minimum Eagle's medium; both were supplemented with 10% fetal bovine serum, 2 mM l-glutamine, and 1% penicillin/streptomycin sulfate. Cell lines were maintained at 37°C with 7% CO2.

MDA-MB-468 cells stably expressing Bcl-2 were generated by transfection with the Bcl-2 expression vector (provided by Robert Mitchell, University of Arizona) with FuGENE6 (Roche Molecular Biochemicals). Transfected cells were subjected to selection in 0.8 mg/ml G418 (Life Technologies, Inc.).

**Construction of Bcl-2 Expression Vectors—**All GFP expression vectors described in this work were constructed with the pEmd-C1 expression vector. This expression vector uses the Emerald GFP variant in which GFP was enhanced for higher quantum efficiency (Packard Instrument Co., Meriden, CT). The ER-targeted GFP-Bcl-2 expression vector (pGFP-Bcl-2C5b) was constructed by the following cloning strategy. The coding region of Bcl-2 was PCR-amplified from the plasmid pB4, which contains the full-length human bcl-2 cDNA (ATCC, Manassas, VA). All PCR reactions were performed using Vent polymerase (New England Biolabs, Beverly, MA). The forward primer used in the PCR amplification contained a Bcl-2 restriction site, which ligated in frame with GFP. The resulting PCR fragment was digested with BglII and BbsI, which deletes the C-terminal 658–720 base pairs (corresponding to amino acids 210–239) and generates a BglII overhang at the 5' end. The nucleotides 297–401 of human cytochrome b5, which encode amino acids 100–134 (ITTDSSSSSVWTVPAISAVAVA LMYRILYMAED) were PCR-amplified from an expressed sequence tag obtained from ATCC containing a partial cDNA of human monoamine oxidase B. Oligonucleotides used to amplify this fragment generated BsfI and EcoRI restriction sites that were ligated in frame with Bcl-2 into pEmd-C1 and pCMV-Tag2B. The wild-type GFP-Bcl-2 expression vector was created by PCR amplification of the human Bcl-2-coding region from pB4 with oligonucleotides that generated a BglII at the 5' end and an EcoRI site at the 3' end. The PCR fragment was ligated in frame with GFP in the BglII/EcoRI-digested pEmd-C1 and BsmHI/EcoRI-digested pCMV-Tag2B expression vectors.

To generate the mitochondrially targeted GFP-Bcl-2 expression vector (pGFP-Bcl-2MAOB), Bcl-2 was PCR-amplified, and restriction was digested as described for pEmd-Bcl-2C5b. Nucleotides 1473–1562 that encode amino acids 492–520 (LLRLIGLTTFSTALGFLAHKRG- LLVRR) of monoamine oxidase B was PCR-amplified from an expressed sequence tag obtained from Genome Systems, Inc., which contains a partial cDNA for human B. Oligonucleotides used to amplify this fragment generated BsfI and EcoRI restriction sites that were ligated in frame with Bcl-2 into pEmd-C1 and pCMV-Tag2B.

Bcl-2, cytochrome b5, and monoamine oxidase B-targeting sequences were also fused in frame with GFP alone. The C-terminal domain of Bcl-2 used in this expression vector included amino acids 217–239. These constructs were made by PCR amplifying the targeting sequences using oligonucleotides containing a BglII restriction site, which enables in-frame ligation with GFP.

To generate the GFP-Bax, the full coding region of Bax was amplified from pSFFV-Bax, a plasmid containing Bax cDNA, with oligonucleotides, which created a 5' BglII site and a 3' EcoRI site. The BglII site of the Bax PCR fragment enabled an in-frame ligation with GFP into the pEmd-C1 expression vector digested with BglII and EcoRI. The resulting PCR fragment was digested with BglII and EcoRI digestion vector was generated by PCR amplification of Bcl-XI from pBS-Bcl-XI, which contains the Bcl-XI cDNA, to generate a PCR fragment with 5' BglII and 3' EcoRI restriction sites. This fragment was subsequently subcloned into pEmd-C1 as described above. All expression vectors were confirmed by sequence analysis.

**Flow Cytometry—**HEK 293 cells were plated on 35-mm coverslip dishes and fixed in 95% methanol. Cells were then blocked with 5% goat serum and 0.1% Triton X-100 in phosphate buffered saline (PBS) for 30 min. Bcl-2 was detected by incubating the cells with a dilution of 1:500 anti-human Bcl-2 antibody (6C8) (PharMingen) for 1 h. Cells were washed three times and then incubated with 1:500 dilution of anti-hamster IgG (PharMingen) for 1 h. Cells were then washed twice with PBS and then incubated for 30 min with 1:500 dilution of anti-mouse Alexa488 (Molecular Probes, Inc., Eugene, OR) for 15 min at 37°C.

**Fluorescence microscopy—**MDA-MB-468 cells stably expressing Bcl-2, Bcl-2, or GFP were grown on 35-mm coverslip dishes and fixed in 95% methanol. Cells were then blocked with 5% goat serum and 0.1% Triton X-100 in PBS for 30 min. Bcl-2 was detected by incubating the cells with a dilution of 1:500 anti-human Bcl-2 antibody (6C8) (PharMingen) for 1 h. Cells were washed three times and then incubated with 1:500 dilution of antimouse IgG (PharMingen) for 1 h. Cells were then washed twice with PBS and then incubated for 30 min with 1:500 dilution of anti-mouse Alexa488 (Molecular Probes, Inc.).

**Immunofluorescence microscopy—**MDA-MB-468 cells stably expressing Bcl-2, Bcl-2, or GFP were grown on 35-mm coverslip dishes and fixed in 95% methanol. Cells were then blocked with 5% goat serum and 0.1% Triton X-100 in PBS for 30 min. Bcl-2 was detected by incubating the cells with a dilution of 1:500 anti-human Bcl-2 antibody (6C8) (PharMingen) for 1 h. Cells were washed three times and then incubated with 1:500 dilution of antimouse IgG (PharMingen) for 1 h. Cells were then washed twice with PBS and then incubated for 30 min with 1:500 dilution of anti-mouse Alexa488 (Molecular Probes, Inc.).

**Flow Cytometry—**HEK 293 cells were plated onto 60-mm culture dishes and were used for transient transfection the following day. Cells were transfected as described previously for fluorescence microscopy. 24 h after transfection, cells were harvested for analysis by flow cytometry. Typically, transfection efficiencies ranged from 30–50%. Cells were washed twice with PBS and subsequently fixed and permeabilized in 95% methanol. Cells were washed twice with PBS after fixation and
resuspended in PBS, 20 μg/ml RNaseA, and 1 mM EDTA for 15 min at 37 °C. Cells were then stained with 50 μg/ml propidium iodide (PI) and 0.05% Nonidet P-40. Cells transfected with GFP-Bcl-2 or FLAG-Bcl-2 expression vectors were analyzed for GFP or AlexaFluor488 and PI fluorescence on a Coulter XL flow cytometer with a 15-milliwatt air-cooled argon laser (excitation = 488 nm). GFP fluorescence was measured using a 525-nm band pass filter, and PI fluorescence was measured using a 620-nm band pass filter.

For comparison of Bcl-2 expression levels in transient and stably transfected MDA-MB-468 cells, cell were grown in 100-mm Petri dishes. The parental cells were transiently transfected with 5.6 μg of pGFP-Bcl-2 using FuGENE6 transfection reagent. After 24 h, both transiently transfected and stably transfected cells were harvested and fixed in 95% methanol. Cells were blocked with 5% goat serum and 0.1% Triton X-100 in PBS. Cells were bound with 1:500 dilution of anti-mouse Alexa488 and then stained with 5 μg/ml propidium iodide.

In the Bax/Bcl-2 co-transfection experiments, HEK 293 cells were co-transfected with 0.5 μg of pGFP-Bax and 1.5 μg of pFLAG-Bcl-2 expression vectors. After 24 h, cells were fixed and permeabilized in 95% methanol. Cells that were positive for GFP fluorescence were gated that were positive or negative for GFP fluorescence were gated for DNA content analysis.

Data analysis of results obtained by fluorescence-activated cell sorter was performed using WinMDI 2.7 software program (written by Joseph Trotter, Scripps Research Institute, La Jolla, CA). Histograms of un-transfected and transfected cells were plotted by gating cells that were negative for GFP and/or AlexaFluor488 fluorescence, respectively.

**Western Blot**—HEK 293 cells were transfected as described previously with either GFP-Bcl-2 or FLAG-Bcl-2 expression vectors and harvested after 24 h. For GFP-Bax/FLAG-Bcl-2 co-transfection experiments, cells were transfected with 0.5 μg of pGFP-Bax and 1.5 μg of pFL-Bcl-2 plasmid DNA. Cells were lysed in radioimmune precipitation buffer, and 50 μg of protein of each lysate was separated by SDS-polyacrylamide gel electrophoresis (14% acrylamide). The protein was then electrophoretically blotted to a polyvinylidene difluoride membrane (Millipore) and incubated with a 1:1000 dilution of anti-human Bcl-2 antibody (6C8) followed by 1:2000 dilution of an anti-rabbit horseradish peroxidase conjugate (Pharmingen). Bax was detected by an anti-Bax polyclonal antibody (N-20) (Santa Cruz Biotechnologies) diluted 1:1000 followed by an anti-rabbit horseradish peroxidase conjugate (Amersham Pharmacia Biotech). Detection of Bcl-2 or Bax on the membrane was performed using ECL (Amersham Pharmacia Biotech).

**RESULTS**

**Immunolocalization of Bcl-2 in MDA-MB-468 Cells**—To study the localization of Bcl-2 in cells that stably overexpress Bcl-2, we transfected a human breast epithelial cell line MDA-MB-468 with a Bcl-2 expression vector and selected for stable expression by growing the cells in G418. These cells do not endogenously express detectable levels of Bcl-2 and previously have been shown to be protected against apoptosis when Bcl-2 endogenously express detectable levels of Bcl-2 and previously have been shown to be protected against apoptosis when Bcl-2 is stably transfected (24). The subcellular localization of Bcl-2 in these cells was determined by immunofluorescence microscopy (Fig. 1). To visualize mitochondria, cells were loaded with MitoTracker Red, a fluorescent dye that binds specifically to mitochondrial membranes. The pattern of Bcl-2 was found to colocalize with mitochondria as well as with a more extensive reticular network resembling the ER (Fig. 1, A–C). The localization of ER membranes was determined by immunofluorescent detection of ER resident proteins using an anti-KDEL antibody (Fig. 1, D–F). Bcl-2 immunolocalization studies were also performed on control cells transfected with empty vector; however, there was no detectable immunofluorescence signal above background, indicating that there is little, if any, endogenous Bcl-2 in the parental cell line. The pattern of Bcl-2 localization that we observe in the stably transfected MDA-MB-468 cells is thus consistent with previous reports that Bcl-2 is localized to both mitochondrial and ER membranes (10, 11).

**Transient Expression of Bcl-2 Induces Cell Death**—It was observed that transfection of Bcl-2 in MDA-MB-468 cells initially resulted in a large number of dead cells relative to those that were transfected with a control vector. This was quantified by transfecting these cells with a GFP-Bcl-2 fusion protein and analyzing GFP fluorescent cells by flow cytometry (Fig. 2). After 24 h, cells were harvested, fixed, and stained with PI, a fluorescent dye that binds to DNA. A hallmark of apoptotic cell death is the enzymatic cleavage of chromosomal DNA by endonucleases activated by the caspase cascade. This degradation of chromosomal DNA results in a decrease in total DNA content to a level that is lower than the DNA content of a cell in G1 phase (25). Consequently, the DNA content of apoptotic cells appears in a DNA content profile as a peak below the DNA content of cells in G1 phase. By gating GFP-positive and GFP-negative cells, we determined that 22.9% of cells expressing GFP-Bcl-2 were apoptotic compared with 7.7% apoptotic cells in the population that did not express GFP-Bcl-2 (Fig. 2A). Because MDA-MB-468 cells demonstrate low transfection efficiency, we chose to test whether this effect is observed in other cell types. Thus, we repeated this transient transfection assay in HEK 293 cells and compared the effect of expression of several Bcl-2 family members including Bax, a known pro-apoptotic protein, and another anti-apoptotic protein Bcl-XL. Bax, Bcl-2, and Bcl-XL were expressed as GFP fusion proteins, and their pattern of expression was observed by fluorescence.
microscopy in MCF-7 cells, which are well suited for imaging (Fig. 2B). GFP-Bax was found to be diffusely localized throughout the cytoplasm, and in some cells, it exhibited a punctate pattern, which probably reflects its translocation to the mitochondrial membrane as described previously (12). GFP-Bcl-2 was found to be localized both in mitochondria, which surround the nucleus, as well as the ER, which resembles an extensive network throughout the cell. Unlike Bcl-2, GFP-Bcl-X₇ was found to be diffused throughout the cell but was also found in the mitochondrial membranes that surround the nucleus. These expression vectors were subsequently transiently transfected in HEK 293 cells. After 24 h, the cells were harvested and analyzed by flow cytometry. The results indicate that the expression of Bax and Bcl-2 induces apoptosis, whereas Bcl-X₇ expression did not induce apoptosis significantly above levels observed for GFP transfection alone (Fig. 2C). These results are also consistent with previous findings in which infection of glioblastoma cells with an adenoviral Bcl-2 expression vector induced apoptosis, whereas infection with a Bcl-X₇ expression vector did not (26). It should also be noted that we obtained similar results in transient transfections of MCF-7 cells, although cell death was found to occur more slowly possibly because of the deficiency of caspase-3 in MCF-7 cells.

**Stably Overexpressed Bcl-2 Protects MDA-MB-468 Cells from Apoptosis**—To test whether Bcl-2 can block apoptosis in MDA-MB-468 cells, cells stably overexpressing Bcl-2 and cells containing a control vector were treated with 1 μM staurosporine or 100 nM thapsigargin (Fig. 3). After 48 h, control cells treated with either staurosporine or thapsigargin were found to exhibit typical morphological features of apoptosis, such as lifting from the surface, condensed chromatin, and fragmentation into apoptotic bodies. However, apoptosis was significantly inhibited in Bcl-2 expressing cells treated with either staurosporine or thapsigargin. Thus, as expected, Bcl-2 expressed stably results in the protection of MDA-MB-468 cells from programmed cell death.

The discrepancy between the cytotoxic effect of transient Bcl-2 expression and its protective effect in stably expressing cells may be attributed to a difference in expression levels. This view was also shared by others who have previously reported...
the toxic effect of transient Bcl-2 expression (26, 27). Thus, we examined whether the toxicity of transient transfection of Bcl-2 required expression at much higher levels than that observed in stably transfected cells. To compare Bcl-2 expression levels between transiently and stably transfected MDA-MB-468 cells, Bcl-2 protein was detected on a single cell basis by using an anti-human Bcl-2 monoclonal antibody followed by a secondary antibody-Alexa488 conjugate and measuring fluorescence intensity by flow cytometry (Fig. 4A). It has been previously demonstrated that antibody detection of Bcl-2 protein levels by flow cytometry correlates well with results obtained by Western blot (28). Analysis of Bcl-2 expression level by this method revealed that 80% of transiently transfected cells expressed Bcl-2 within the same range of Bcl-2 levels detected in stably overexpressing cells (Fig. 4B). Expression levels attained in transiently transfected cells appear to vary over a wider range compared with stably transfected cells, however, only 20% of transiently transfected cells attain Bcl-2 expression levels higher than what is observed in stably transfected cells. When transient Bcl-2-expressing cells that express the same levels as stably expressing cells were analyzed for cell death (Fig. 4C), we found that ~20% of these cells were apoptotic, indicating that most of the apoptotic cells expressed the same levels of Bcl-2 as stably expressing cells.

**Targeting of Bcl-2 to ER and Mitochondria**—The cell death-inducing effect of many pro-apoptotic Bcl-2 family proteins has been found to be a result of their association with the mitochondrial membrane. Bax, a cytosolic protein, translocates to the mitochondrial membrane when the cell receives an apoptotic signal (12, 13). Also, Bid, another pro-apoptotic Bcl-2 family member, has been found to translocate to mitochondria after being cleaved by caspase-8 (15, 16). The translocation of both Bid and Bax to mitochondria has been demonstrated to induce the release of cytochrome c and subsequent cell death (29, 30). Bcl-2, unlike Bax and other pro-apoptotic proteins, is localized to both the ER and mitochondrial membranes, and its localization does not appear to be regulated by apoptotic stimuli. It is not known whether the pro-apoptotic effect of Bcl-2 expression requires its localization at either of these membranes or both. To test this, we generated a series of Bcl-2 expression vectors in which Bcl-2 is targeted specifically to the ER or both. To target Bcl-2 specifically to the ER membrane, the ER-targeting domain of cytochrome b₅ (amino acids 100–134 of cytochrome b₅) was used to replace amino acids 492–520 of monoamine oxidase B (Bcl-2A5). To target Bcl-2 specifically to the outer mitochondrial membrane, these amino acids were replaced by the mitochondrial-targeting domain (amino acids 492–520) of monoamine oxidase B (Bcl-2M1A5B). These targeted versions of Bcl-2 were expressed either as fusion proteins with GFP or with the FLAG epitope tag at the N terminus.

Bcl-2 is a tail-anchored membrane protein that is inserted post-translationally into the cytoplasmic face of intracellular membranes by its C-terminal transmembrane domain (8, 31). To target Bcl-2 to the cytosolic face of the ER membrane, the C-terminal targeting domain of Bcl-2 was replaced by that of the ER-specific protein cytochrome b₅ (Fig. 5). Rat cytochrome b₅ is an ER-specific tail-anchored membrane protein that also has its membrane-targeting domain at its C terminus. This targeting signal has been demonstrated to be sufficient to target proteins exclusively to the ER membrane (23). This signal has also been used in previous work by others to target Bcl-2 specifically to the ER membrane (32, 33). We constructed a chimeric protein in which amino acids 210–239 of Bcl-2 were replaced by the C-terminal amino acids 100–134 of cytochrome b₅, the domain that was found to be sufficient for targeting to the ER membrane, and designated this chimeric protein as Bcl-2C2b5. To target Bcl-2 specifically to the outer mitochondrial membrane, we used the targeting sequence of monoamine oxidase B, a tail-anchored membrane protein that is inserted into the cytosolic face of the outer mitochondrial membrane (34). The C-terminal amino acids 493–520 of monoamine oxidase B were used to replace the Bcl-2-targeting signal to gen-

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**Fig. 4. Comparison of stable versus transient expression of Bcl-2.** A, MDA-MB-468 cells stably or transiently expressing Bcl-2 were harvested and fixed. Cells were treated with anti-Bcl-2 antibody followed by an anti-mouse-Alexa488 conjugate. Before analysis by flow cytometry, cells were stained with propidium iodide. Gate R4 represents Bcl-2 expression levels achieved in stably transfected cells. The range of Bcl-2 expression of stably transfected cells is marked by the region M1. C, stable and transient Bcl-2-expressing cells within the R4 gate were analyzed for cell death by sub-G1 DNA content.

**Fig. 5. Construction of Bcl-2 expression vectors.** To target Bcl-2 specifically to the ER membrane, the ER-targeting domain of cytochrome b₅ (amino acids 100–134) was used to replace amino acids 217–239 of Bcl-2 (Bcl-2A5B). To target Bcl-2 to the outer mitochondrial membrane, these amino acids were replaced by the mitochondrial-targeting domain (amino acids 492–520) of monoamine oxidase B (Bcl-2M1A5B). These targeted versions of Bcl-2 along with wild-type Bcl-2 were expressed either as fusion proteins with GFP or with the FLAG epitope tag at the N terminus.
Transgenic expression of Bcl-2 as a fusion protein with GFP: which Bcl-2 is expressed as a fusion protein with the following expression vectors in COS-7 cells were transiently transfected and mitochondrially targeted Bcl-2. Wild-type Bcl-2, ER-targeted Bcl-2, and mitochondrial Bcl-2 were also generated in which the targeted Bcl-2 molecules were expressed with an N-terminal FLAG epitope tag, which enables detection by indirect immunofluorescence.

To determine whether Bcl-2Cb5 and Bcl-2MAOB were targeted to the appropriate organelles, COS-7 cells were transiently transfected with these GFP fusion expression vectors and observed in living cells by fluorescence microscopy (Fig. 6). Transfection of wild-type Bcl-2 fused to GFP revealed that Bcl-2 is located in the nuclear envelope and in an extensive reticular pattern that is typical for ER localization (Fig. 6, A–C). Bcl-2 is also present in structures surrounding the nucleus that resemble mitochondria. These structures were identified to be mitochondria by loading the cells with the dye MitoTracker Red (Fig. 6, D–I). Bcl-2Cb5 was also found in the nuclear envelope, ER, and mitochondria. The relative amounts of Bcl-2 between ER and mitochondria varied from cell to cell. In most transfected COS-7 cells, a higher proportion of wild-type Bcl-2 was located in mitochondria than in ER. However, there were some cells in which wild-type Bcl-2 was only found in the reticular ER pattern. We have also observed that the proportion of Bcl-2 localized in the ER versus the mitochondria varies between cell types (data not shown). The subcellular localization pattern of GFP-Bcl2Cb5 displayed the reticular pattern that does not co-localize with that of MitoTracker Red (Fig. 6, D–F). GFP-Bcl2Cb5 was also found in the nuclear envelope, which is known to be contiguous with the ER. These results verify that GFP-Bcl2Cb5 is excluded from mitochondria and co-localizes with the pattern observed for MitoTracker Red (Fig. 6, G–I). Localization studies were also done in cells transfected with the FLAG epitope-tagged Bcl-2 expression vectors by indirect immunofluorescence and reflected similar results to what was observed with GFP-Bcl2 fusion proteins (data not shown).

Wild-type and Mitochondrially Targeted Bcl-2 Expression Induces Cell Death—The targeted Bcl-2 expression vectors were then used in the transient transfection assay in HEK 293 cells to determine whether their expression could induce apoptosis. Using flow cytometric analysis, the percentage of cells containing sub-G1 levels of DNA was determined in both transfected and untransfected cells by comparing cells that were either positive or negative for GFP fluorescence, respectively. The results from these experiments indicate that transfection of wild-type GFP-Bcl-2 induced cell death (32.8% apoptotic cells) compared with untransfected cells within the same sample (1.8% apoptotic cells) (Fig. 7, A and C). However, transfection with ER-targeted GFP-Bcl-2 (Bcl-2Cb5) induced little apoptosis (5.2% apoptotic cells in transfected versus 1.6% apoptotic cells in untransfected). Interestingly, GFP-Bcl-2-targeted to mitochondria (Bcl-2MAOB) was found to be equally or more severely cytotoxic compared with wild-type Bcl-2 transfection (50.8% apoptotic). These results were also verified by using FLAG epitope-tagged Bcl-2 expression vectors (Fig. 7B) and confirmed that the toxicity by Bcl-2 and Bcl-2MAOB occurs in the absence of GFP. Additionally, the number of apoptotic cells after transient transfection was measured over 72 h to assess whether the toxicity by GFP-Bcl-2Cb5 expression may arise at a later time point. Apoptosis in cells expressing either wild-type GFP-Bcl-2 or GFP-Bcl-2MAOB increased over 72 h, whereas apoptosis in cells expressing GFP-Bcl-2Cb5 did not increase dramatically (Fig. 7D). To rule out the possible toxic effects of the transfection reagent, we also repeated these experiments using a different transfection reagent ExGen500 (MBI Fermentas), a cationic polymer polyethyleneimine, and compared the results obtained by transfection with the cationic...
lipid FuGENE6. The same results were obtained using either ExGen500 or FuGENE6 (data not shown), therefore, the cell death induced by wild-type or mitochondrially targeted Bcl-2 expression is unlikely to be because of cytotoxicity of the lipid-DNA complexes created by the transfection reagent.

It is possible that the differences in cell death induction by transient Bcl-2 expression may be attributed to differing levels of expression rather than differences in membrane localization. Expression levels between the transfected samples were determined by examining GFP fluorescence intensity of positively transfected cells (Fig. 8A). The results indicate that expression levels achieved by transfection of each GFP-Bcl-2 expression vector or GFP-Bcl-XL do not differ greatly. In fact, the expression level of GFP-Bcl-2MAOB is significantly lower than that of GFP-Bcl-2 and GFP-Bcl-2Cb5, and thus the induction of cell death by GFP-Bcl-2 or GFP-Bcl-2MAOB is not because of higher levels of expression compared with GFP-Bcl-2Cb5. It also should be noted that both FL-Bcl-2Cb5 and FL-Bcl-2.
2MAOB stably expressed in MDA-MB-468 cells do provide protection against thapsigargin- and staurosporine-induced apoptosis, indicating that both of these Bcl-2 variants have retained the protective function of wild-type Bcl-2.

An additional possibility is that the toxicity induced by Bcl-2 or Bcl-2MAOB may be a consequence of overexpression of their C-terminal targeting sequences alone at the mitochondrial membrane. This was illustrated by the fact that the expression of the MAOB-targeting signal fused to GFP was sufficient to induce cell death although at a lower level than GFP-Bcl-2MAOB expression (Fig. 7, B and C). Transient expression of GFP-Bcl-X₅, which also localizes to the mitochondrial membrane, does not induce apoptosis and suggests that the toxicity of GFP-MAOB or GFP-Bcl-2MAOB expression is not a general effect of overexpressing a protein at the outer mitochondrial membrane, but most probably it is attributed to a specific effect of the MAOB-targeting signal itself. Further experiments in which the C-terminal targeting domain of Bcl-2 (amino acids 217–239) was fused to GFP showed that the expression of this domain was sufficient to induce apoptosis (Fig. 8. A and B). Although the numbers of apoptotic cells were slightly lower than what was observed for the expression of full-length Bcl-2, this difference was not significant (p = 0.07). The finding that the expression of GFP-Bcl-2 and GFP-MAOB-targeting do-

![Fig. 8. Expression of the C-terminal targeting domain of Bcl-2 is sufficient to induce apoptosis. A, HEK 293 cells were transiently transfected with the full-length GFP-Bcl-2 expression vectors (left column) or with the GFP C-terminal-targeting domains of Bcl-2, cytochrome oxidase b₅, or monoamine oxidase B (right column). 24 h after transfection, cells were harvested, fixed, and stained with propidium iodide and analyzed by flow cytometry. Cells are represented in a dot plot where PI fluorescence intensity is plotted versus GFP fluorescence intensity. Gates were outlined to include cells expressing GFP. B, the percentage of cells with sub-diploid DNA content was determined from the transfected cell population thresholded in the indicated gates in A. Values plotted are the average of three independent experiments. Statistical significance was determined by the Student’s paired t test.](http://www.jbc.org/)

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mains can induce cell death suggests that these domains themselves contribute to the cytotoxicity of Bcl-2 expression, and that the C-terminal targeting domain of Bcl-2 may be partially responsible for the toxicity that is observed when it is transiently expressed.

Cells Transfected with Wild-type or Mitochondrially Targeted Bcl-2 Exhibit Apoptotic Nuclear Morphology—To confirm that Bcl-2 expression induces apoptotic cell death, transfected cells were examined for the nuclear morphological changes that are typical for cells undergoing apoptosis (35). HEK 293 cells were transfected with each GFP-Bcl-2 expression vector, loaded with the DNA binding dye Hoechst 33342 after 24 h, and then examined by fluorescence microscopy. The nuclear morphology of transfected cells was determined by comparing fluorescence images of both GFP and Hoechst (Fig. 9). Cells transfected with wild-type Bcl-2, identified by green fluorescence, had begun to shrink and lift off from the culture dish and were usually not in the same focal plane as the untransfected cells, which were still adherent. The transfected cells also had condensed nuclear chromatin, and apoptotic bodies were clearly visible. Nuclear degradation was also observed in cells transfected with GFP-Bcl-2MAOB, whereas the nuclei of the surrounding untransfected cells remained intact. In contrast, cells transfected with GFP-Bcl-2Cb5 did not induce changes in nuclear morphology and remained indistinguishable from neighboring untransfected cells. These observations confirm that the expression of wild-type and mitochondrially targeted Bcl-2 induces cell death, and that these cells demonstrate the morphological changes typical of cells undergoing apoptosis.

Cleavage of Bcl-2 Is Not Required for Its Induction of Cell Death—It has been previously reported that Bcl-2 can be converted to a Bax-like death-inducing molecule upon cleavage by caspase-3 (20). Bcl-2 is cleaved by caspase-3 upon the withdrawal of interleukin-3 or Fas receptor activation. The cleavage fragment is then converted to a pro-apoptotic molecule, which can proceed to induce cytochrome c release (36). The toxicity of transient expression of Bcl-2 that we observe may thus be a consequence of its cleavage by caspase-3 and, thereby, function as an amplification step in the cell death pathway. The site of caspase cleavage was previously determined to be aspartate 34 (20), resulting in the truncation of the BH4 domain. To test whether apoptosis induced by transient Bcl-2 expression requires this cleavage step, we generated expression vectors in which aspartate 34 of Bcl-2 was changed to alanine. This mutation, which renders Bcl-2 resistant to cleavage by caspase-3, was also generated in each of the targeted Bcl-2 expression vectors.

After transfecting HEK 293 cells with each Bcl-2 expression vector, cells were harvested, and lysates were analyzed by Western blot. In cells expressing wild-type GFP-Bcl-2 or FLAG-Bcl-2, Bcl-2 was cleaved to a 20-kDa fragment as described by Cheng et al. (20) (Fig. 10A). However, this cleavage fragment was not present in cells expressing GFP-Bcl-2Cb5 or GFP-Bcl-2MAOB. Evidently, cell death induced by Bcl-2-MAOB can occur without Bcl-2 being cleaved at the D34 caspase-3 cleavage site. When the Bcl-2 D34A mutant was expressed, the 20-kDa cleavage fragment was no longer detected. However, expression of this caspase-3-resistant mutant Bcl-2 was still able to induce cell death to the same extent as wild-type Bcl-2 (Fig. 10B). Thus, our findings indicate that although Bcl-2 cleavage does occur upon overexpression in HEK 293 cells, cleavage at the N-terminal aspartate 34 is not required for its ability to induce apoptosis. This is also confirmed by the observation that Bcl-2MAob expression, which also is pro-apoptotic, does not undergo cleavage by caspases.

ER-targeted Bcl-2 Protects Cells from Cell Death Induced by Bax Overexpression—Although wild-type Bcl-2 was shown to inhibit cell death when stably transfected in MDA-MD-468 cells, it was not clear whether transient expression of either wild-type or Bcl-2 targeted to ER or mitochondria is able to confer any protection against apoptosis. In our transient expression model, overexpression of Bax in HEK 293 cells induces cell death without the requirement of any additional apoptotic stimuli. This is similar to previous findings that showed that Bax overexpression is sufficient to induce apoptotic cell death (29, 37). To test the protective action of transient expression of wild-type, ER, or mitochondrial Bcl-2, we transiently co-transfected Bax and Bcl-2 in HEK 293 cells. We generated an expression vector in which Bax is expressed as a fusion protein with GFP. This construct was transiently co-transfected with an excess of FLAG epitope tagged-wild-type or targeted Bcl-2 into HEK 293 cells, and the cells were subsequently analyzed by flow cytometry after 24 h (Fig. 11A). Overexpression of Bax alone induced cell death, consistent with previously reported results (37). Cell death was enhanced when wild-type or mitochondrial targeted Bcl-2 was co-transfected with GFP-Bax. However, when GFP-Bax was co-transfected with ER-targeted Bcl-2, we observed a marked decrease in the number of apoptotic cells. Expression of GFP-Bax and FLAG-Bcl-2 was confirmed by Western blot (Fig. 11B).

**DISCUSSION**

The work presented here reveals that acute Bcl-2 overexpression by transient transfection can induce apoptosis in HEK 293 and MDA-MB-468 cells. This effect was not observed when Bcl-2 was selectively targeted to the ER membrane and suggests that the toxicity of Bcl-2 expression is a result of Bcl-2 on the mitochondrial membrane. Pro-apoptotic activity of Bcl-2 upon transient expression has been previously reported by others (26, 27). The authors concluded that this effect resulted from high levels of Bcl-2 expression. However, we find that...
Transient Bcl-2 expression at levels that are comparable with those achieved in stable overexpression is sufficient to induce apoptosis. The possibility that Bcl-2 induces cell death when localized to mitochondria may provide an alternative explanation for its toxicity in transient transfections.

Some models of Bcl-2 function assign its anti-apoptotic activity to its ability to block events occurring at the mitochondria during apoptosis (38, 39). Mitochondria have been found to be a critical site of action in some apoptotic pathways (17). Several pro-apoptotic proteins, such as Bax, Bak, and Bid, translocate to the mitochondrial membrane, and this localization is associated with their pro-apoptotic activity. A loss of mitochondrial integrity and function has been found to occur during apoptosis as evidenced by the loss of mitochondrial membrane potential, generation of reactive oxygen species, and release of proteins such as cytochrome c, which activate downstream components of the apoptotic pathway. Whether transient expression of Bcl-2 at mitochondria causes these indicators of mitochondrial damage is currently being investigated.

The expression of Bcl-2 does not result in an increase in the levels of endogenous Bax protein (data not shown), however, it is not known whether Bcl-2 expression influences the localization of endogenous Bax to mitochondria in these cells.

The GFP-targeting data have several implications regarding our interpretations of Bcl-2 function. Because the expression of
GFP-MAOB- and GFP-Bcl-2-targeting domains are both capable of inducing apoptosis, we cannot exclude the possibility that the toxicity of Bcl-2 expression may also be caused by its presence at the ER membrane. However, we believe that Bcl-2 toxicity at the ER membrane is unlikely because expression of Bcl-2C5, which has been shown to have protective function as wild-type Bcl-2 in a stably expressing cell line, does not induce apoptosis. Another implication of the targeting data suggest that the toxic effect of Bcl-2 expression occurs primarily because of its C-terminal domain. However, the expression of GFP-MAOB was found to be less toxic in comparison with GFP-Bcl-2MAOB expression, suggesting that the domains of Bcl-2 other than the MAOB-targeting signal contribute to the observed cytotoxicity when localized at the mitochondrial membrane. Therefore, it was unexpected that the difference in induction of apoptosis between the expression of GFP-Bcl-2 versus expression of its C-terminal domain was not as great as the differences that were observed between expression of GFP-Bcl-2MAOB and the MAOB-targeting tail alone. One factor that may explain this finding could be that because wild-type Bcl-2 is found at both the ER and mitochondria, Bcl-2 found at the ER membrane may have anti-apoptotic activity when transiently expressed and protect against toxicity caused by Bcl-2 at the mitochondrial membrane. This would then result in underestimation of apoptosis induced by mitochondrially localized wild-type Bcl-2. Using experimental methods described in this study, we are unable to distinguish between these possibilities.

Bel-XL is a Bcl-2 family protein that has been shown to demonstrate anti-apoptotic function equivalent to that of Bcl-2 (40). The observation that Bel-XL did not induce apoptosis in our transient expression assay indicates that the pro-apoptotic activity of Bcl-2 is specific and not a common feature of all anti-apoptotic Bcl-2 family members. However, Bel-XL has been demonstrated to localize to mitochondrial membranes (41) and thus raises the question of why its transient expression does not induce apoptosis as observed for Bcl-2. Our observations of the subcellular localization of Bel-XL (Fig. 2B) in MCF-7 cells indicate that although Bcl-XL is found in mitochondrial membranes, a significant proportion of the protein remains in the cytoplasm. This is also consistent with previous reports of Bel-XL localization in the cytosol as measured by subcellular fraction (42). The pro-apoptotic effect of Bcl-2 expression may thus be dependent upon a certain threshold level of protein to be localized at the mitochondrial membrane, and thus, when expressed transiently, the proportion of Bcl-2 found in the mitochondrial membrane is sufficient to induce apoptosis. It is possible then that Bel-XL expression does not direct a sufficient level of Bel-XL protein to the mitochondrial membrane to induce apoptosis. This possibility may also explain the observation that the potency of Bcl-2-induced apoptosis varies among cell types and, thus, may reflect differences in the proportion of protein levels that are found in the ER and mitochondrial membranes.

Cell death induction by Bcl-2 expression has been reported in previous work and was found to occur as a result of the cleavage of the Bcl-2 protein, which converts it into a pro-apoptotic molecule (20). Both Bcl-2 and Bel-XL have subsequently been shown to be substrates for caspase-3 (20, 21). The cleavage of Bcl-2 or Bel-XL occurs in cells induced to undergo apoptosis by activation of Fas or withdrawal of interleukin-3. Conversion of Bcl-2 to a pro-apoptotic protein then serves to amplify the apoptotic pathway after its initiation via Fas activation. The results of our studies indicate that the cleavage of Bcl-2 at aspartate 34 is not obligatory for its induction of apoptosis upon transient expression. Although we observed cleavage of wild-type Bcl-2 to a 20-kDa truncated protein, mitochondrially targeted Bcl-2, which also induces cell death, was not cleaved. Additionally, the D34A caspase-3-resistant mutant was able to induce apoptosis when transiently expressed, indicating that the expression of Bcl-2 is sufficient to induce apoptosis, and that the amplification by Bcl-2 cleavage is not required. Thus, the apoptotic cell death we observe in this system occurs by a different mechanism from the Bcl-2 amplification of the Fas-induced pathway.

The paradoxical finding that Bcl-2 expressed transiently induces cell death but is protective when stably expressed can be interpreted to reflect a functional difference in the effect of Bcl-2 overexpression between these conditions. Although acute expression of wild-type Bcl-2 induces apoptosis in many cells, it does not induce cell death in all cells that express Bcl-2, illustrated by the fact that stable Bcl-2 overexpression has been established in many cell types. Additionally, stable overexpression of ER and mitochondrially targeted Bcl-2 as well as wild-type Bcl-2 was established in Rat-1/myc fibroblasts and Madin-Darby canine kidney cells (32). Because the pro-apoptotic effect of Bcl-2 is likely to be associated with its location at the mitochondrial membrane and its overexpression at the mitochondria may cause mitochondrial dysfunction, an interesting possibility could be that the cells not killed by Bcl-2 expression at the mitochondria possess increased intracellular defenses against mitochondrial damage. The development of resistance to the toxicity of transient Bcl-2 expression could thus play a role in the mechanism of protection against apoptosis by Bcl-2 when its stable expression is established.

Another mechanism of protection by Bcl-2 may be because of its location at the ER membrane. Our observation that ER-targeted Bcl-2 protects against cell death induced by Bax overexpression suggests that Bcl-2 is not required at the mitochondria to inhibit apoptosis mediated by Bax. Preliminary data suggest that Bax remains cytosolic and does not translocate to mitochondria when ER-targeted Bcl-2 is co-expressed (data not shown). Thus, ER-targeted Bcl-2 may inhibit a step upstream of Bax activation. One current model proposes that Bax undergoes a conformational change induced by binding the BH3 only protein, Bid, resulting in its activation at the mitochondrial membrane and eventually leading to cytochrome c release (43, 44). At present, it is not known whether pro-caspase-8 activation or Bid processing occurs during cell death induced by transient Bax overexpression, and these elements of the pathway are being investigated. Nevertheless, there is an accumulating body of evidence that Bcl-2 localized at the ER can protect against apoptosis. ER-targeted Bcl-2 was found to inhibit disruption of mitochondrial membrane potential and cell death by myc overexpression in Rat-1/myc cells (32, 45, 46). Additionally, ER-localized Bcl-2 has been shown to inhibit cytochrome c release and eventual cell death by brefeldin A and tunicamycin treatment (33). These findings strongly suggest that Bcl-2 at the ER membrane can act to preserve the integrity of the mitochondria after an apoptotic stimulus, and our results further indicate that ER-localized Bcl-2 may play a significant role in inhibiting cell death induced by apoptotic pathways mediated by Bax activation.

In summary, our findings show that transient expression of Bcl-2 can induce apoptosis, and that this effect is probably because of its localization at the mitochondrial membrane. We also find that ER-targeted Bcl-2 is capable of inhibiting cell death induced by Bax overexpression, suggesting that Bcl-2 can inhibit apoptosis from non-mitochondrial sites, and that

\[ \text{N. S. Wang, M. T. Unkila, E. Z. Reineks, and C.W. Distelhorst, unpublished observations.} \]
the ER may be an important site of action with respect to the regulatory role of Bcl-2 in apoptosis.

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Transient Expression of Wild-type or Mitochondrially Targeted Bcl-2 Induces Apoptosis, whereas Transient Expression of Endoplasmic Reticulum-targeted Bcl-2 Is Protective against Bax-induced Cell Death

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