

Maintenance of Calcium Homeostasis in the Endoplasmic Reticulum by Bcl-2

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Abstract. The oncogene *bcl-2* encodes a 26-kD protein localized to intracellular membranes, including the ER, mitochondria, and perinuclear membrane, but its mechanism of action is unknown. We have been investigating the hypothesis that Bcl-2 regulates the movement of calcium ions (Ca^{2+}) through the ER membrane. Earlier findings in this laboratory indicated that Bcl-2 reduces Ca^{2+} efflux from the ER lumen in WEHI7.2 lymphoma cells treated with the Ca^{2+} -ATPase inhibitor thapsigargin (TG) but does not prevent capacitative entry of extracellular calcium. In this report, we show that sustained elevation of cytosolic Ca^{2+} due to capacitative entry is not required for induction of apoptosis by TG, suggesting that ER calcium pool

depletion may trigger apoptosis. Bcl-2 overexpression maintains Ca^{2+} uptake in the ER of TG-treated cells and prevents a TG-imposed delay in intraluminal processing of the endogenous glycoprotein cathepsin D. Also, Bcl-2 overexpression preserves the ER Ca^{2+} pool in untreated cells when extracellular Ca^{2+} is low. However, low extracellular Ca^{2+} reduces the antiapoptotic action of Bcl-2, suggesting that cytosolic Ca^{2+} elevation due to capacitative entry may be required for optimal ER pool filling and apoptosis inhibition by Bcl-2. In summary, the findings suggest that Bcl-2 maintains Ca^{2+} homeostasis within the ER, thereby inhibiting apoptosis induction by TG.

THE oncogene *bcl-2* encodes a 26-kD protein that localizes to the ER and perinuclear membrane, as well as the mitochondrial membrane (8, 48). *bcl-2* is a member of a family of apoptosis regulators, some of which inhibit apoptosis, including *bcl-2* and *bcl-x_L*, and some of which promote apoptosis. The Bcl-2 protein anchors to intracellular membranes through a hydrophobic region located near its carboxy terminus, while the major portion of the molecule is in the cytoplasm. Bcl-2's mechanism of action is unknown. Bcl-2 overexpression is associated with decreased formation of reactive oxygen species (19) and decreased oxygen radical-mediated lipid peroxidation (16), suggesting that Bcl-2 works in an antioxidant pathway to inhibit apoptosis. However, it has been suggested that Bcl-2 can also inhibit apoptosis in the absence of oxygen radicals (17, 35, 43).

Another theory is that Bcl-2 and Bcl-*x_L* may regulate ion fluxes. This concept is supported by evidence that *bcl-2* overexpression prevents Ca^{2+} redistribution from the ER to mitochondria following growth factor withdrawal (1) and that *bcl-2* overexpression inhibits apoptosis-associated Ca^{2+} waves (28) and nuclear Ca^{2+} uptake (29). Also, *bcl-2*

overexpression potentiates maximal uptake of Ca^{2+} by mitochondria (34) and preserves mitochondrial transmembrane potential (49). Two recent findings provide strong evidence that Bcl-2 family members regulate ion fluxes. First, the X-ray and NMR structure of Bcl-*x_L* resembles the physical structure of ion channel-forming bacterial toxins (33). Second, Bcl-*x_L* forms an ion channel in synthetic lipid membranes (32).

We have been investigating the hypothesis that Bcl-2 regulates the movement of Ca^{2+} through the ER membrane. A high concentration of Ca^{2+} is maintained in the ER lumen by a member of the sarcoplasmic endoplasmic reticulum calcium ATPase family of Ca^{2+} -ATPases that pump Ca^{2+} into the ER, counterbalancing a leak of Ca^{2+} ions through the ER membrane into the cytoplasm (27). Thapsigargin (TG),¹ a sesquiterpene lactone tumor promoter derived from the plant *Thapsia garganica*, is a selective inhibitor of the ER-associated Ca^{2+} -ATPase (45). Because the concentration of Ca^{2+} in the ER lumen is several orders of magnitude higher than the concentration of Ca^{2+} in the cytoplasm, TG-mediated Ca^{2+} -ATPase inhibition allows Ca^{2+} to flow from the ER lumen into the cytoplasm, producing a transient elevation of cytosolic Ca^{2+} concentration, followed by sustained elevation of cytosolic Ca^{2+} in response to capacitative entry (39, 45). We have

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1. Abbreviation used in this paper: TG, thapsigargin.

shown that in TG-treated WEHI7.2 mouse lymphoma cells, which lack Bcl-2, these disturbances of intracellular Ca^{2+} homeostasis precede cell death accompanied by the typical hallmarks of apoptosis, including CASPASE activation, cleavage of poly(ADP-ribose) polymerase, chromatin condensation, and DNA fragmentation (20; unpublished data). In stable transfectants of the WEHI7.2 line that express high levels of Bcl-2, TG treatment inhibits cell growth but does not induce apoptosis (20). Bcl-2 overexpression is associated with a reduction in the transient elevation of cytosolic Ca^{2+} induced by TG-mediated ER Ca^{2+} -ATPase inhibition (20). Based on this observation, we proposed that Bcl-2 inhibits apoptosis in TG-treated cells by inhibiting Ca^{2+} efflux from the ER. However, in subsequent studies we found that Bcl-2 does not prevent sustained cytosolic Ca^{2+} elevation due to capacitative entry of extracellular Ca^{2+} ; in fact, Bcl-2 prevents apoptosis even when cytosolic Ca^{2+} is markedly elevated following TG treatment (10). Therefore, based on the premise that sustained elevation of cytosolic Ca^{2+} triggers apoptosis in TG-treated cells (14, 18), we concluded that Bcl-2 acts downstream and independent of Ca^{2+} fluxes to inhibit apoptosis (10).

In the present report, we show that sustained elevation of cytosolic Ca^{2+} in TG-treated cells can be prevented by reducing extracellular Ca^{2+} concentration, which inhibits capacitative entry. These conditions do not prevent TG-induced apoptosis, indicating that sustained elevation of cytosolic Ca^{2+} secondary to capacitative entry is not required for induction of apoptosis by TG. Therefore, we focused on the hypothesis that Bcl-2 inhibits apoptosis by maintaining Ca^{2+} homeostasis in the ER. This hypothesis is supported by evidence that Bcl-2 maintains Ca^{2+} uptake and protein processing in the ER when the Ca^{2+} -ATPase is inhibited by TG. Also, Bcl-2 preserves the ER Ca^{2+} pool and thereby prevents growth inhibition when extracellular Ca^{2+} is withdrawn. Finally, we show that the antiapoptotic action of Bcl-2 is abrogated by a reduction in extracellular Ca^{2+} . Based on these findings, a model of how Bcl-2 preserves Ca^{2+} homeostasis in the ER and inhibits TG-induced apoptosis is proposed.

Materials and Methods

Cell Lines and Culture Conditions

W.Hb12 and W.Hb15 cell lines were derived from the WEHI7.2 line by stable transfection with a cDNA encoding Bcl-2 (20). WEHI7.2-neo cells were derived by stable transfection with pSFFV-neo vector. The CaBP3 and CaBP20 cell lines were derived from the WEHI7.2 line by stable transfection of a cDNA encoding calbindin D (12). The S49-Bcl-2 line was derived by stably transfecting the S49 line with a cDNA encoding Bcl-2 (2). Cell lines were routinely cultured in DME supplemented with 2 mM glutamine, 50 U/ml penicillin, 50 $\mu\text{g}/\text{ml}$ streptomycin, 0.4 mM nonessential amino acids, and 10% (vol/vol) heat-inactivated horse serum at 37°C in a 7% CO_2 atmosphere. In certain experiments, cells were pelleted and resuspended in Ca^{2+} -free DME supplemented with 10% horse serum.

Cell Counting and Apoptosis Assay

Viable cells, defined as cells that exclude trypan blue dye, were counted using a hemocytometer. The percentage of cells with an apoptotic nuclear morphology was assessed by fluorescence microscopy (10). Five million cells were gently pelleted and resuspended in 0.2 ml tissue culture medium, and both ethidium bromide and acridine orange were added from

100 $\mu\text{g}/\text{ml}$ stock solutions to achieve 4 $\mu\text{g}/\text{ml}$ final concentrations of each. Cells were examined under a glass coverslip with ultraviolet illumination using a Nikon Optiphot microscope (Melville, NY).

Ca^{2+} Flux Measurements

Methods for measuring $^{45}\text{Ca}^{2+}$ uptake in the ER were described in detail (10, 20). Cells were pretreated with oxalate, which permeates the ER membrane and forms insoluble complexes with Ca^{2+} , reducing Ca^{2+} efflux and permitting unidirectional measurement of $^{45}\text{Ca}^{2+}$ uptake (6). Measurements were performed in the presence of azide at a free Ca^{2+} concentration buffered to 150 nM by EGTA, essentially eliminating the uptake of Ca^{2+} into low-affinity mitochondrial stores. Cells were washed twice with 0.9% NaCl, 50 μM EDTA solution at 4°C, and permeabilized by incubation for 5 min at 37°C in buffer containing 120 mM KCl, 30 mM imidazole, pH 6.8, 2 mM MgCl_2 , 1 mM ATP, 1 mM EGTA, and 0.0005% digitonin. Cells were then pelleted and resuspended at 4°C in buffer containing 140 mM KCl, 10 mM NaCl, 2.5 mM MgCl_2 , 0.0005% digitonin, 10 mM Hepes-KOH, pH 7.0, with 4 mM oxalic acid. To begin uptake measurements, 0.5-ml aliquots of the cell suspension were added at 37°C to 2.5 ml buffer containing 120 mM KCl, 30 mM imidazole, pH 6.8, 5 mM MgCl_2 , 5 mM ATP, 10 mM NaN_3 , 4 mM oxalic acid, and $^{45}\text{CaCl}_2$ buffered to 150 nM free $^{45}\text{Ca}^{2+}$ (8.9 mCi/mg) with EGTA as described (46). Immediately and at 1-min time intervals thereafter, 0.25-ml aliquots were removed from the continuously stirring cell suspension and placed into wash buffer containing 140 mM KCl, 10 mM NaCl, 2.5 mM MgCl_2 , 10 mM Hepes-KOH, pH 7.0, and 1 mM LaCl_3 . Cells were collected on glass fiber filters and washed three times, each with 4 ml of wash buffer. Filters were dried, dissolved in 10 ml 3a70 scintillation cocktail, and counted in a liquid scintillation counter (model LS7800; Beckman Instruments, Fullerton, CA).

Intracellular Ca^{2+} Measurements

Fluorometric measurements of intracellular Ca^{2+} were according to published methods (10, 20). Twenty million cells were pelleted by centrifugation at 150 g for 10 min and resuspended in 10 ml basal salt solution, CaBSS (130 mM NaCl, 5 mM KCl, 1.5 mM CaCl_2 , 1 mM MgCl_2 , 25 mM Hepes, pH 7.5, 5 mM glucose, and 1 mg/ml BSA). In certain experiments, CaCl_2 was deleted, yielding a buffer referred to as BSS. Free Ca^{2+} concentration of buffers and tissue culture medium was measured with a Ca^{2+} electrode. Cell suspensions were incubated for 45 min at 25°C with 1 μM Fura-2 AM from a 1 mM stock solution dissolved in DMSO. Cells were then pelleted and resuspended in 10 ml fresh CaBSS or BSS and maintained at 25°C for up to 60 min before fluorometry. For each measurement, a 1.5-ml aliquot of Fura-2-loaded cells was equilibrated to 37°C in a stirred quartz cuvette. Fura-2 fluorescence was continuously monitored at 339 nm excitation and 500 nm emission. Ca^{2+} -dependent Fura-2 fluorescence was calibrated after lysis of cells with 20 $\mu\text{g}/\text{ml}$ digitonin, and cytosolic free Ca^{2+} concentration was calculated, assuming a Fura-2 K_d of 220 nM.

Ca^{2+} Overlay and Western Blotting

Cells were lysed by suspension in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 10 mM iodoacetamide, 1% Triton X-100) for 15 min at 4°C and paired samples of 30 μg cytosolic protein were separated by SDS-gel electrophoresis under reducing and denaturing conditions. One half of the membrane corresponding to one set of samples was used for $^{45}\text{Ca}^{2+}$ overlay, and the other half of the membrane corresponding to the other identical set of samples was used for Western blotting. After electrophoresis, the proteins were transferred to a nitrocellulose membrane at a constant current of 100 mA for 1 h in Tris-glycine buffer. $^{45}\text{Ca}^{2+}$ overlay was performed essentially as described by Maruyama et al. (30). After transfer, the membrane was washed in IKM buffer (10 mM imidazole-HCl, pH 6.8, 5 mM MgCl_2 , and 60 mM KCl) for 1 h with three changes of buffer. The membrane was then incubated in the same buffer with 1 mCi/liter $^{45}\text{Ca}^{2+}$ for 10 min. After incubation, the membrane was rinsed with 50% ethanol for 5 min, three times. Excess ethanol was blotted away using Whatman filter paper No. 1, and the membrane was dried at room temperature for 3 h. After the membrane was completely dry, it was exposed to film for 2 d at -80°C.

For Western blotting, membranes were blocked by incubation for one h with 5% dry milk in TBS, pH 7.5, at 25°C or overnight at 4°C. After blocking, the membranes were overlaid with diluted primary antibody (mono-

clonal anti-calbindin D, Sigma Chemical Co. [St. Louis, MO]; or monoclonal anti-Bcl-2, PharMingen, San Diego, CA) and incubated for 1 h at 25°C. After washing, the membranes were incubated for 1 h at 25°C with horseradish peroxidase-conjugated goat anti-mouse whole immunoglobulin (Amersham Corp., Arlington Heights, IL) diluted 1:500 in TBS. After washing, the enhanced chemiluminescence Western blotting detection reagent (Amersham Corp.) was added, and the reaction was allowed to proceed according to the manufacturer. The membranes were exposed to film at room temperature for 30 s and then developed.

Cahepsin D Immunoprecipitation

Wild-type S49 cells and Bcl-2 transfectants (40 million cells each) were suspended in 2 ml methionine-free medium supplemented with 10% horse serum, 1 mM glutamine, and nonessential amino acids and incubated for 30 min before adding 150 μ Ci [35 S]methionine. Either 200 nM TG or DMSO vehicle were added to duplicate sets of cells at the beginning of the pulse label. The cells were incubated for 2 h until the chase was initiated by adding 1 mg/ml unlabeled methionine. The chase was ended by washing cells twice with PBS at 4°C. Cell pellets were suspended in an equal volume of lysis buffer (25 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM EDTA, 10% glycerol, 0.8% deoxycholic acid, 0.8% NP-40). At 30–120 min into the chase, the cell suspensions were frozen and then thawed, followed by centrifugation at 14,000 g for 20 min at 4°C. The clear supernatant was collected and, based on TCA precipitation analysis, equal amounts of [35 S]methionine-labeled protein were incubated with rabbit anti-human cathepsin D antiserum (1:30 dilution) (13) for 1 h at 4°C and then incubated with protein A-Sepharose for 30 min at 4°C. Protein A-Sepharose pellets were then washed four times with lysis buffer at 4°C. Bound proteins were extracted by resuspending protein A-Sepharose pellets in sample buffer for gel electrophoresis and analyzed by SDS-PAGE under reducing and denaturing conditions as described previously (21).

Results

Bcl-2 Mediates ER Ca²⁺ Uptake

We compared WEHI7.2 cells, which do not express Bcl-2, and W.Hb12 cells, which were derived from WEHI7.2 by stable transfection with the human Bcl-2 cDNA and therefore express a high level of Bcl-2 mRNA and protein (20). Findings have been confirmed in an additional high Bcl-2 clone and WEHI7.2 cells stably transfected with pSFFV-neo vector. Because TG is an irreversible Ca²⁺-ATPase inhibitor, TG treatment inhibits Ca²⁺ uptake in the ER, thereby producing sustained ER Ca²⁺ pool depletion (45). Based on this information, one would predict that TG treatment should cause sustained ER calcium pool depletion in both WEHI7.2 and W.Hb12 cells, unless Bcl-2 mediates ER Ca²⁺ uptake. To test this hypothesis, the uptake of ⁴⁵Ca²⁺ in the ER of digitonin-permeabilized cells was measured in the presence of azide at a free Ca²⁺ concentration buffered to 150 nM to eliminate Ca²⁺ uptake into low-affinity mitochondrial stores. Uptake was measured in the presence and absence of oxalate, which enters the ER through nonselective anion channels and binds Ca²⁺ to form insoluble Ca²⁺-oxalate complexes, thus inhibiting Ca²⁺ efflux (47). Oxalate permeability is a property specific to the ER (47 and references therein). In the absence of oxalate, ⁴⁵Ca²⁺ uptake was detected in W.Hb12 cells, but not in WEHI7.2 cells (Fig. 1 A). In the presence of oxalate, ⁴⁵Ca²⁺ uptake was detected in WEHI7.2 cells, but the rate of uptake was consistently greater in W.Hb12 cells than in WEHI7.2 cells (Fig. 1 B). These findings indicate that Bcl-2 overexpression enhances calcium uptake and retention by the ER of untreated cells. ⁴⁵Ca²⁺ uptake was markedly diminished in both WEHI7.2 and W.Hb12 cells when the ER calcium-ATPase was inhibited by 5–100 nM

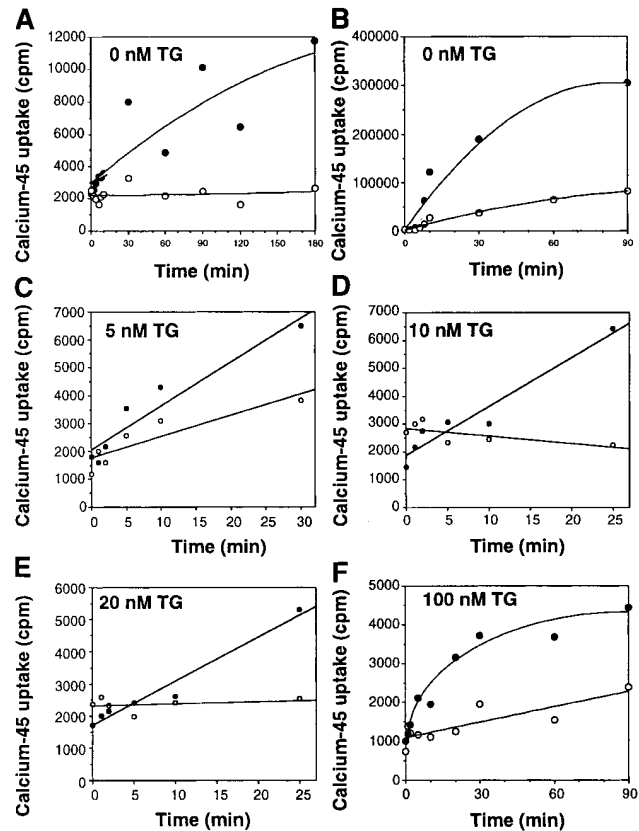


Figure 1. Bcl-2 mediates ER Ca²⁺ uptake. Uptake of ⁴⁵Ca²⁺ was measured in digitonin-permeabilized cells in the absence (A) or presence of oxalate (B–F), and in the absence (A and B) or presence of 5 nM TG (C), 10 nM TG (D), 20 nM TG (E), or 100 nM TG (F). Open symbols, WEHI7.2 cells; closed symbols, W.Hb12 cells. Symbols represent the mean of duplicate determinations in a single experiment that was repeated at least twice with a similar result.

TG (Fig. 1, C–F), indicating that ⁴⁵Ca²⁺ uptake by the ER of untreated cells was primarily mediated by the ER-associated calcium-ATPase. However, residual ⁴⁵Ca²⁺ uptake was detected in TG-treated W.Hb12 cells, whereas ⁴⁵Ca²⁺ uptake was barely detected in TG-treated WEHI7.2 cells. Thus, Bcl-2 enhances Ca²⁺ accumulation in the ER of untreated cells and maintains a low but detectable level of Ca²⁺ accumulation in the ER of TG-treated cells.

The increased accumulation of ⁴⁵Ca²⁺ in the ER of W.Hb12 cells could be due to a direct effect of Bcl-2 on Ca²⁺ movement through the ER membrane or to an indirect effect of Bcl-2 on the Ca²⁺-binding capacity in the ER lumen. The levels of three major resident ER Ca²⁺-binding proteins, GRP78 (BiP), GRP94, and calreticulin, are unaffected by Bcl-2 overexpression (31; McColl, K., and C. Distelhorst, unpublished data). To exclude the possibility that Bcl-2 increases the expression of an unidentified Ca²⁺-binding protein, we compared Ca²⁺-binding proteins in WEHI7.2 and W.Hb12 cells by ⁴⁵Ca²⁺ overlay (Fig. 2). Cellular proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, renatured, and incubated with ⁴⁵Ca²⁺. An autoradiogram reveals a number of radioactive bands corresponding to proteins that bound

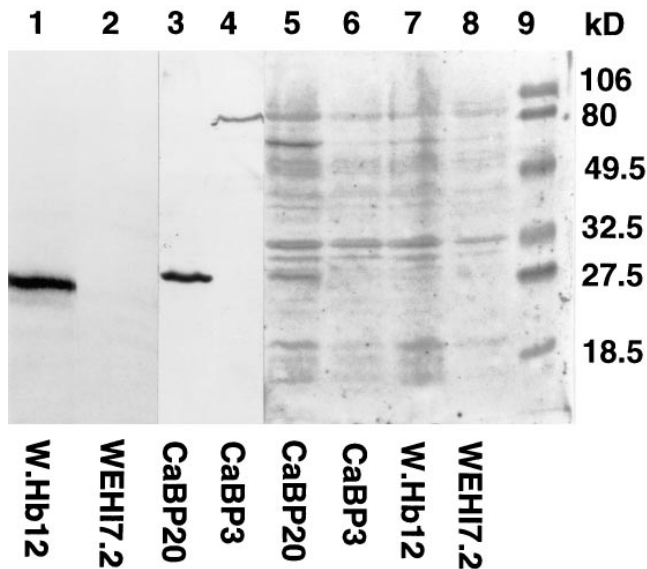


Figure 2. Bcl-2 does not increase expression of Ca²⁺-binding proteins. Extracts from untreated cells listed on the horizontal axis were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Lanes 1–4 were subjected to Western blotting using anti-Bcl-2 antibody (lanes 1 and 2) and anticalsestron antibody (lanes 3 and 4) as probes. Lanes 5–8 were incubated with ⁴⁵Ca²⁺ and then exposed to x-ray film. Molecular mass standards are in lane 9. An unidentified protein that immunoreacts with anticalsestron antibody is detected at ~80-kD in lane 4, and an unidentified Ca²⁺-binding protein was detected in CaBP20 cells (lane 5) midway between the 49.5- and 80-kD markers. The appearance of these proteins was variable among experiments and therefore not considered significant.

⁴⁵Ca²⁺. As a positive control, extracts from CaBP3 and CaBP20 cells were included. These cells were derived previously by stably transfecting WEHI7.2 cells with a cDNA encoding the Ca²⁺-binding protein, calbindin (12). As shown by the Western blot in Fig. 2 (lanes 3 and 4), calbindin protein is detected in CaBP20 but not in CaBP3 cells. A ⁴⁵Ca²⁺-labeled protein corresponding to calbindin is detected in CaBP20 cells (Fig. 2, lane 5), documenting the detection of Ca²⁺-binding proteins by the ⁴⁵Ca²⁺ overlay technique. Significantly, the pattern of ⁴⁵Ca²⁺-binding proteins is the same in WEHI7.2 and W.Hb12 cells. Therefore, Bcl-2-mediated Ca²⁺ uptake in the ER does not appear to be secondary to altered expression of Ca²⁺-binding proteins.

BCL-2 Prevents a TG-imposed Delay in Protein Processing

Maintenance of Ca²⁺ homeostasis within the ER is required for normal protein processing and transport (15, 25, 42). Depletion of the ER Ca²⁺ pool by TG inhibits glycoprotein processing and maturation in the ER lumen (25). Therefore, to investigate the effect of Bcl-2 on Ca²⁺-dependent ER function, we examined the effect of TG treatment on the processing of cathepsin D, an endosomal cysteine protease that has been implicated as a mediator of apoptosis (9). The 48-kD cathepsin D protein is glycosylated within the ER, forming a 53-kD precursor that is

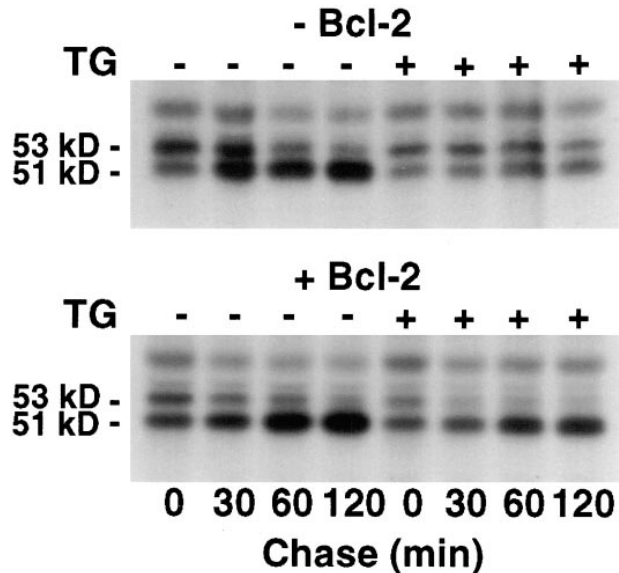


Figure 3. Bcl-2 prevents TG-induced delay in cathepsin D processing. Pulse-chase analysis of cathepsin D processing in wild-type S49 cells (*-Bcl-2*) and Bcl-2 transfectants (*+Bcl-2*) in the presence or absence of 200 nM TG. [³⁵S]methionine-labeled cathepsin D was immunoprecipitated, resolved by PAGE under reducing and denaturing conditions, and analyzed by autoradiography.

modified to a 51-kD mature form after transfer to the Golgi (13). In earlier studies, we observed a similar pattern of cathepsin D maturation in the S49 mouse lymphoma cell line, a T-cell line similar to WEHI7.2 (21). Therefore, in the present study, the processing of cathepsin D was assessed by pulse-chase analysis in S49 cells, which do not express Bcl-2, and stable transfectants that are resistant to TG-induced apoptosis (2). The processing of cathepsin D is relatively rapid in untreated wild-type S49 cells and Bcl-2 transfectants, such that up to half of the cathepsin D synthesized during the 2 h pulse-labeling period was converted to the more mature 51-kD form by the beginning of the chase (Fig. 3). (Labeling of cathepsin D was suboptimal for immunoprecipitation analysis when the labeling period was shortened.) During the chase, the labeled 53-kD form matured to the 51-kD form almost completely in both wild-type S49 cells and Bcl-2 transfectants. Examination of the labeling pattern at 30 min into the chase suggests that processing of cathepsin D may be somewhat more rapid in the Bcl-2 transfectants compared to the wild-type cells. After TG treatment, however, there was a major difference between the wild-type cells and the Bcl-2 transfectants. In wild-type cells, TG treatment completely inhibited cathepsin D processing, whereas cathepsin D processing was unaffected by TG treatment in the Bcl-2 transfectants. Similar findings were observed in comparisons of WEHI7.2 and W.Hb12 cells (data not shown). In summary, these observations indicate that Bcl-2 overexpression maintains calcium-dependent protein processing, providing additional evidence that Bcl-2 preserves calcium homeostasis within the ER lumen when the ER Ca²⁺-ATPase is inhibited by TG.

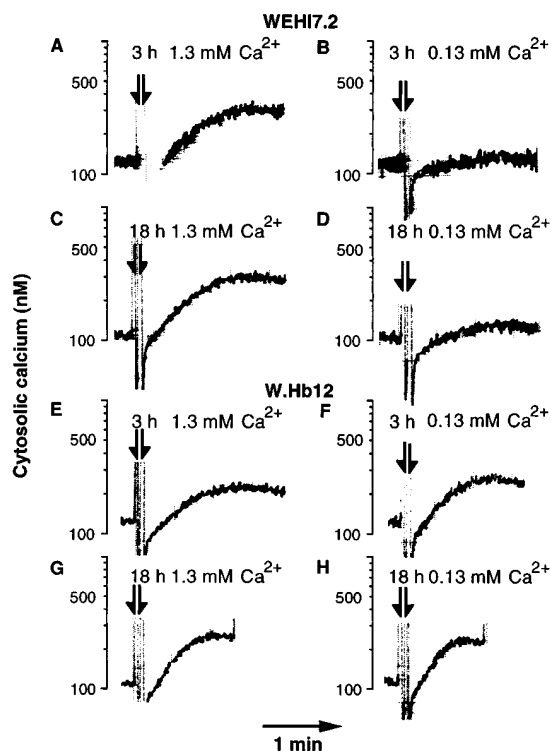


Figure 4. Bcl-2 prevents ER Ca^{2+} pool depletion. WEHI7.2 cells (A–D) and W.Hb12 cells (E–H) were incubated for 3 h (A, B, E, and F) or 18 h (C, D, G, and H) in medium with a Ca^{2+} concentration of 1.3 mM (A, C, E, and G) or medium with a Ca^{2+} concentration of 0.13 mM (B, D, F, and H) and then loaded with Fura2-AM. Shown are continuous fluorometric tracings in which addition of 100 nM TG (right hand arrow) induces an increase in cytosolic Ca^{2+} concentration. Note that EGTA (left hand arrow) was added immediately before TG to chelate extracellular Ca^{2+} ; hence, the increase in cytosolic Ca^{2+} that follows TG addition represents release of Ca^{2+} from the ER pool. Large vertical oscillations in fluorescence near the beginning of each tracing correspond to brief light exposure during EGTA and TG additions.

Bcl-2 Preserves the ER Ca^{2+} Pool

The preceding experiments examined the effect of Bcl-2 on ER Ca^{2+} homeostasis when the ER Ca^{2+} -ATPase was inhibited by TG. Another condition that compromises ER Ca^{2+} pool filling is a reduction in extracellular Ca^{2+} . Therefore, we compared the effect of low extracellular Ca^{2+} on the ER Ca^{2+} pool of WEHI7.2 and W.Hb12 cells. These cells are routinely cultured in DME supplemented with 10% serum, providing a physiologic extracellular Ca^{2+} concentration of 1.3 mM. In preliminary experiments, we incubated cells under completely Ca^{2+} -free conditions by adding EDTA or EGTA to the tissue culture medium; however, rapid loss of viability was observed in both WEHI7.2 and W.Hb12 cells (Lam, M., and C. Distelhorst, unpublished observations). Moreover, incubating cells in Ca^{2+} -free DME supplemented with either no serum or only 1% serum produced rapid loss of viability. Therefore, in the following experiments cells were incubated in Ca^{2+} -free DME supplemented with 10% serum; under these conditions, the extracellular Ca^{2+} concentration, measured with a Ca^{2+} electrode, is 0.13 mM, ~ 10 -fold below the normal extracellular Ca^{2+} concentration.

The effect of low extracellular Ca^{2+} on free cytosolic Ca^{2+} concentration and the TG-mobilizable Ca^{2+} pool was measured using Fura-2 AM, a Ca^{2+} -responsive fluorescent indicator that localizes to the cytoplasm of intact cells (Fig. 4). Cells were loaded with Fura-2 AM at 3 and 18 h after being placed in either normal or low extracellular Ca^{2+} medium. In the presence of a physiologic concentration of extracellular Ca^{2+} , the free cytoplasmic Ca^{2+} concentration in WEHI7.2 and W.Hb12 cells is 184 ± 10 and 141 ± 8 nM, respectively, whereas the free cytoplasmic Ca^{2+} concentration decreases to 118 ± 4 and 111 ± 9 nM, respectively, when the extracellular Ca^{2+} concentration is decreased to 0.13 mM. The mobilizable ER Ca^{2+} pool was estimated by measuring the transient increase in cytosolic Ca^{2+} produced by adding 100 nM TG to Fura-2 AM-loaded cells. Note that EGTA was added immediately before adding TG to chelate extracellular Ca^{2+} ; hence, the increase in Fura-2 fluorescence is due to Ca^{2+} efflux from the ER, rather than uptake of extracellular Ca^{2+} . The increase in cytosolic Ca^{2+} concentration induced by TG was markedly reduced when WEHI7.2 cells were incubated for either 3 or 18 h in low extracellular Ca^{2+} . In contrast, the TG-mobilizable Ca^{2+} pool was essentially unchanged when W.Hb12 cells were incubated in low extracellular Ca^{2+} . Thus, Bcl-2 overexpression preserves the ER Ca^{2+} pool when extracellular Ca^{2+} is withdrawn.

Effect of Low Extracellular Ca^{2+} on Cell Growth and Viability

When extracellular Ca^{2+} is reduced, the growth of WEHI7.2 cells is inhibited (Fig. 5 A), whereas the growth of W.Hb12 cells is maintained (Fig. 5 C). These findings are consistent with earlier evidence that the ER Ca^{2+} pool is required for cell division and that a decrease in this pool induces growth arrest (44). It appears that Bcl-2 maintains cell growth by preserving the ER Ca^{2+} pool when extracellular Ca^{2+} is reduced. Apoptosis was not induced by incubating untreated WEHI7.2 cells in low extracellular Ca^{2+} , indicating that incomplete reduction of the ER Ca^{2+} pool is compatible with survival (Fig. 5 B).

The alterations in intracellular Ca^{2+} homeostasis induced by TG are modified considerably by low extracellular Ca^{2+} . Thus, the transient elevation of cytosolic Ca^{2+} induced by TG-mediated ER Ca^{2+} -ATPase inhibition is reduced by incubating WEHI7.2 cells in low extracellular Ca^{2+} (Fig. 4, B and D). Also, the marked elevation of cytosolic Ca^{2+} secondary to capacitative entry is markedly reduced when either WEHI7.2 or W.Hb12 cells are treated with TG in low extracellular Ca^{2+} (Fig. 6). Nevertheless, low extracellular Ca^{2+} does not prevent the induction of apoptosis by TG in WEHI7.2 cells (Fig. 5 F), indicating that marked elevation of cytosolic Ca^{2+} due to capacitative entry is not necessary for apoptosis induction by TG.

When W.Hb12 cells are suspended in medium containing a physiologic concentration of Ca^{2+} , TG treatment inhibits cell growth (Fig. 5 G) but does not induce apoptosis (Fig. 5 H). However, when W.Hb12 cells are suspended in low Ca^{2+} medium, TG induces not only growth arrest (Fig. 5 G), but also apoptosis (Fig. 5 H). These observations suggest that TG-induced ER Ca^{2+} pool depletion is sufficient to inhibit cell growth, but not to induce apoptosis,

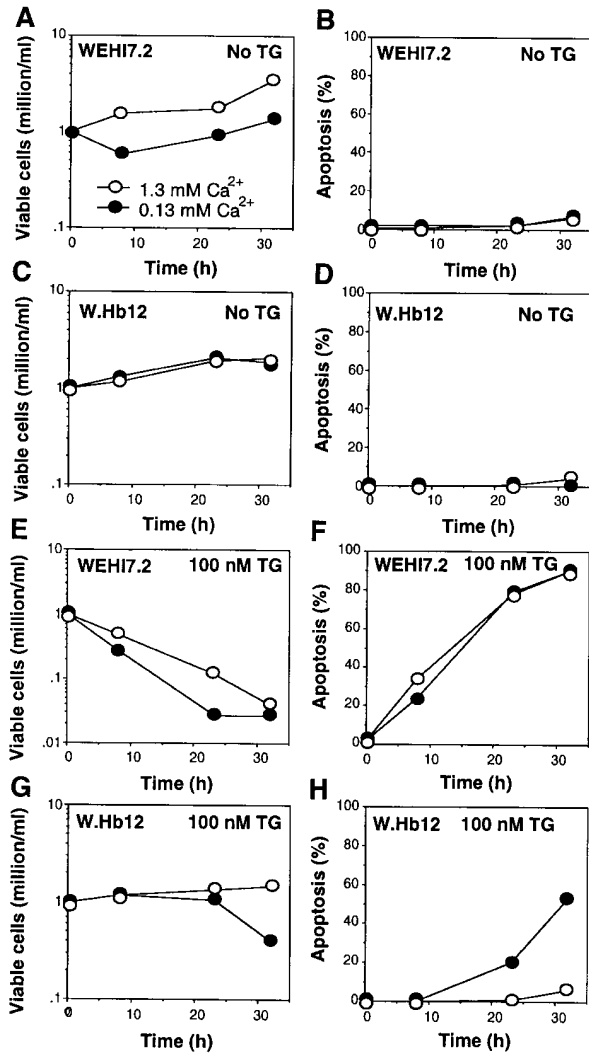


Figure 5. Effect of Bcl-2 on cell growth and viability. WEHI7.2 cells (A, B, E, and F) and W.Hb12 cells (C, D, G, and H) were incubated in medium containing 1.3 mM Ca^{2+} (open symbols) or 0.13 mM Ca^{2+} (closed symbols), in either the absence of TG (A–D) or presence of 100 nM TG (E–H), for 32 h. At various time points, the number of viable cells, defined as cells that exclude trypan blue dye, and the percentage of apoptotic cells, based on nuclear apoptotic changes detected on fluorescence microscopy of cells stained with acridine orange, were measured. Symbols represent the mean of duplicate determinations in a single experiment that was repeated three times with a similar result.

when W.Hb12 cells are suspended in medium containing a physiologic concentration of Ca^{2+} . Low extracellular Ca^{2+} may reduce the ability of Bcl-2 to maintain ER Ca^{2+} uptake when the ER Ca^{2+} -ATPase is inhibited by TG, allowing the concentration of Ca^{2+} within the ER to fall below a critical level necessary for cell survival.

Discussion

Evidence that ER Ca^{2+} Pool Depletion Induces Apoptosis

Inhibition of the ER Ca^{2+} -ATPase by TG induces three

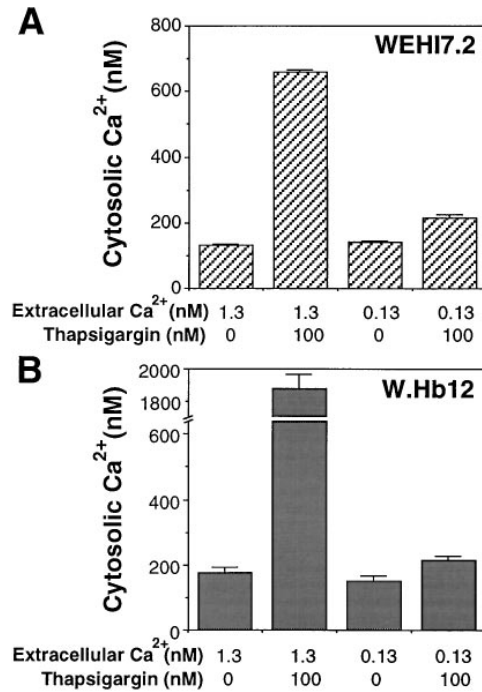


Figure 6. Low extracellular Ca^{2+} inhibits cytosolic Ca^{2+} elevation in TG-treated cells. WEHI7.2 cells (A) and W.Hb12 cells (B) were incubated in medium containing 1.3 mM Ca^{2+} or 0.13 mM Ca^{2+} , in the presence or absence of 100 nM TG, for 4 h before loading with Fura-2 AM. Cytosolic Ca^{2+} concentration was measured according to Fura-2 fluorescence. Symbols represent the mean \pm SE of multiple determinations in three separate experiments.

well-defined changes in intracellular Ca^{2+} homeostasis: transient elevation of cytosolic Ca^{2+} due to efflux of Ca^{2+} through the ER membrane, ER Ca^{2+} pool depletion, and sustained elevation of cytosolic Ca^{2+} secondary to capacitative entry of extracellular Ca^{2+} . In this report, we show that both transient and sustained elevations of cytosolic Ca^{2+} are inhibited when WEHI7.2 cells are treated with TG in the presence of low extracellular Ca^{2+} (Figs. 4 and 6), without inhibiting TG-induced apoptosis (Fig. 5). Thus, it appears that ER Ca^{2+} pool depletion can trigger apoptosis in TG-treated WEHI7.2 cells. The concept that ER Ca^{2+} pool depletion induces cell death is consistent with the findings of others (2, 22, 23, 41). However, in thymocytes and prostate cancer cells, TG-induced apoptosis is inhibited by preincubating cells with the intracellular Ca^{2+} indicator, BAPTA-AM, or overexpressing the cytosolic Ca^{2+} -binding protein, calbindin, suggesting that cytosolic Ca^{2+} elevation may also be a factor in inducing apoptosis (14, 18). However, TG-induced apoptosis in WEHI7.2 cells is not inhibited by pretreatment with BAPTA-AM or overexpression of calbindin (Lam, M., and C. Distelhorst, unpublished observations).

Maintenance of Ca^{2+} homeostasis within the ER is essential for a number of vital cellular functions, including signal transduction (7), translation (4), protein processing and transport (15, 25, 42), and cell division (44). Therefore, ER Ca^{2+} pool depletion might affect cells adversely in multiple ways. Three potential mechanisms by which

ER Ca^{2+} pool depletion might contribute to apoptosis induction are considered here. First, depletion of the ER Ca^{2+} pool might destabilize the Ca^{2+} -protein gel and its associated membrane (3, 42), leading to vesiculation and formation of apoptotic blebs. This concept is intriguing in view of evidence that apoptotic blebs are composed of ER membrane and resident ER Ca^{2+} -binding proteins, including calreticulin, GRP78 (BiP), and GRP94 (5). Second, disruption of protein processing and transport within the ER may contribute to TG-induced apoptosis. This concept is consistent with evidence that induction of the genes encoding the ER proteins GRP78, GRP94, and calreticulin, in response to ER Ca^{2+} pool depletion, protects against TG-induced cell death (24). These proteins bind Ca^{2+} with low affinity and, therefore, facilitate Ca^{2+} sequestration in the ER lumen (3, 42). Moreover, these proteins function as chaperones, aiding in the folding and processing of nascent proteins in the ER lumen. Accordingly, the failure of WEHI7.2 cells to induce the transcription of these stress genes may account for the marked susceptibility of WEHI7.2 cells to TG-induced apoptosis (31). Disruption of normal protein processing secondary to TG-induced ER Ca^{2+} pool depletion and failure to induce GRP78 (BiP), GRP94, and calreticulin may cause a proteolytically active cathepsin D precursor, or other protease precursor, to accumulate within the ER lumen, initiating a proteolytic cascade that leads to apoptosis. This theory is based on recent evidence implicating increased cathepsin D expression and delayed processing in apoptosis induction by tumor necrosis factor- α , interferon- γ , and Fas/APO-1 (9). A third theory is that TG-induced ER Ca^{2+} pool depletion releases an endonuclease into the nucleus responsible for DNA fragmentation. This theory is based on evidence that a deoxyribonuclease involved in nuclear DNA fragmentation is confined to the ER until it gains access to the nucleus during apoptosis (37). Although these hypotheses have not been formally tested in TG-treated cells, they illustrate potential mechanisms by which TG-induced ER Ca^{2+} pool depletion might trigger apoptosis.

Evidence that Bcl-2 Maintains Ca^{2+} Homeostasis in the ER

The findings of this report, summarized in the form of a model (Fig. 7), suggest that Bcl-2 maintains ER Ca^{2+} homeostasis when the Ca^{2+} -ATPase is inhibited by TG or extracellular Ca^{2+} is withdrawn. In untreated WEHI7.2 cells cultured in the presence of a physiologic extracellular Ca^{2+} concentration, ER Ca^{2+} pool filling is maintained by the ER-associated Ca^{2+} -ATPase, which pumps Ca^{2+} from the cytoplasm into the ER against a steep concentration gradient (Fig. 7, A and B). When the ER Ca^{2+} -ATPase is inhibited by TG, the ER Ca^{2+} pool is reduced, signaling capacitative entry and thereby producing sustained elevation of cytosolic Ca^{2+} (Fig. 7, C and D). In cells that lack Bcl-2, TG treatment inhibits Ca^{2+} uptake, and the ER Ca^{2+} pool is severely depleted (Fig. 7 C). In the model, we propose that Bcl-2 overexpression maintains partial ER pool filling by mediating Ca^{2+} uptake when the Ca^{2+} -ATPase is inhibited by TG (Fig. 7 D). However, Bcl-2 does not appear to completely fill the ER Ca^{2+} pool under these conditions; this conclusion is based on evidence that TG

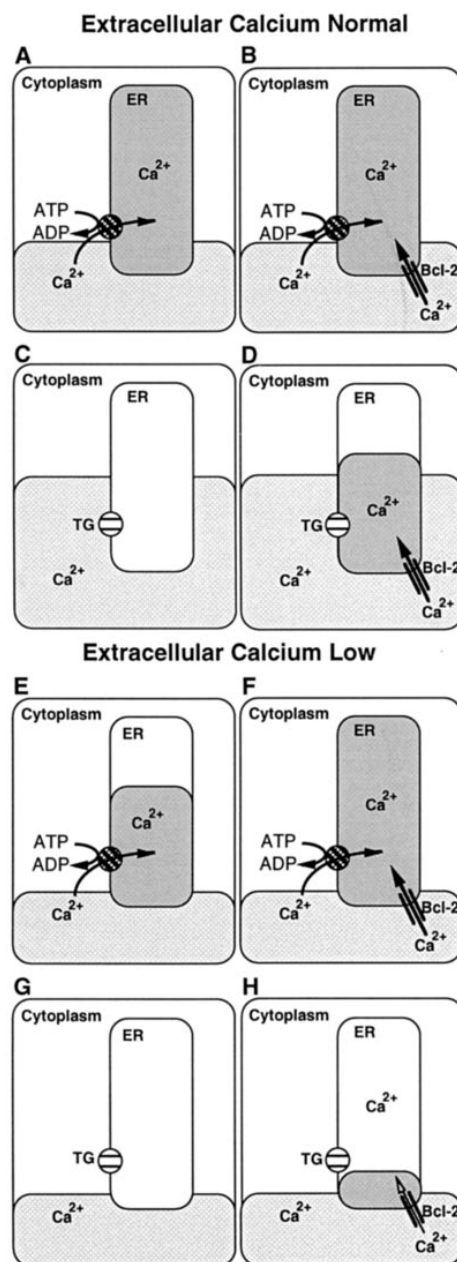


Figure 7. Model: Bcl-2 regulation of Ca^{2+} homeostasis in the ER. The relationship between Ca^{2+} pools in the cytoplasm and ER is illustrated. A–D represent cells cultured in medium containing a physiologic concentration of Ca^{2+} (1.3 mM), and E–H represent cells cultured in medium containing a low concentration of Ca^{2+} (0.13 mM). A, C, E, and G represent cells that lack Bcl-2, and B, D, F, and H represent cells that express Bcl-2. A, B, E, and F represent untreated cells in which Ca^{2+} is pumped into the ER by the Ca^{2+} -ATPase, and C, D, G, and H represent cells in which the Ca^{2+} -ATPase pump is inhibited by TG. Bcl-2 is represented by two parallel lines anchored in the ER membrane.

treatment induces capacitative entry of extracellular Ca^{2+} , the signal for which is a reduction in the ER calcium pool (40). The concept that Bcl-2 prevents complete ER Ca^{2+} pool depletion is based primarily on $^{45}\text{Ca}^{2+}$ uptake studies (Fig. 1) and evidence that Bcl-2 overexpression prevents a TG-imposed delay in protein processing (Fig. 3).

It should be noted that $^{45}\text{Ca}^{2+}$ uptake measurements may underestimate Bcl-2-mediated Ca^{2+} entry because in the assay, free Ca^{2+} concentration is buffered to 150 nM to prevent uptake of Ca^{2+} by low-affinity pools (i.e., mitochondria). When cells are treated with TG in the presence of a physiologic concentration of extracellular Ca^{2+} , cytosolic concentration is much higher than 150 nM because of capacitative entry of extracellular Ca^{2+} (Fig. 6). An elevated concentration of Ca^{2+} in the cytoplasm appears to be required for inhibition of TG-induced apoptosis by Bcl-2. This conclusion is based on evidence that TG induces apoptosis in cells that overexpress Bcl-2 if extracellular calcium is low, thereby preventing sustained elevation of cytosolic Ca^{2+} in response to capacitative entry. Thus, in the model Bcl-2 maintains at least partial ER Ca^{2+} pool filling when cells are treated with TG in the presence of a physiologic extracellular calcium concentration (Fig. 7 D) but is unable to maintain a level of Ca^{2+} in the ER necessary to inhibit apoptosis when cells are treated with TG in the presence of low extracellular calcium (Fig. 7 H).

The concentration of Ca^{2+} in the ER is difficult to quantify directly. We have attempted to measure the ER Ca^{2+} pool with high-affinity fluorescent dyes (i.e., Mag-Fura-2 AM) and by electron probe microanalysis without success. Therefore, in the absence of a facile method for directly quantitating ER Ca^{2+} concentration, we have resorted to a functional assay to indirectly assess ER Ca^{2+} pool filling, based on previous evidence that protein processing within the ER lumen is inhibited by TG-induced ER Ca^{2+} pool depletion (25). Consistent with the hypothesis that Bcl-2 maintains Ca^{2+} homeostasis in the ER, we find that Bcl-2 overexpression prevents the TG-imposed delay in cathepsin D processing in the ER (Fig. 3).

ER Ca^{2+} pool filling is often estimated according to the ability of TG to dump Ca^{2+} from the ER lumen into the cytoplasm, producing a measurable elevation of cytosolic Ca^{2+} . This method was used in Fig. 4 to measure the effect of extracellular Ca^{2+} withdrawal on the ER Ca^{2+} pool of untreated cells, indicating that the ER Ca^{2+} pool is diminished in cells that lack Bcl-2 (Fig. 7 E) but maintained in cells that overexpress Bcl-2 (Fig. 7 F). A recent study has reported that TG fails to induce a detectable elevation of cytosolic Ca^{2+} in Bcl-2-expressing cells that were treated with the nonselective Ca^{2+} ionophore ionomycin in the absence of extracellular Ca^{2+} (41). Based on this observation, the authors proposed that intracellular Ca^{2+} stores are not required for inhibition of apoptosis by Bcl-2 (41). However, ionomycin does not inhibit the Ca^{2+} -ATPase pump and the failure to detect a mobilizable Ca^{2+} pool does not exclude the possibility that Ca^{2+} -ATPase-mediated and/or Bcl-2-mediated Ca^{2+} uptake maintains a concentration of Ca^{2+} within the ER lumen necessary for cell survival. Moreover, the concept that Bcl-2 maintains cell survival in the absence of intracellular Ca^{2+} stores is improbable, in view of the requirement for Ca^{2+} to maintain the structural and functional integrity of the ER (3, 42).

The proposed effect of Bcl-2 on ER Ca^{2+} homeostasis, as conceptualized in Fig. 7, appears to correlate with maintenance of cell growth and viability in cells that overexpress Bcl-2. For example, the diminution in the ER Ca^{2+} pool induced by extracellular Ca^{2+} withdrawal (Fig. 4) is associated with growth inhibition in cells that lack Bcl-2

(Fig. 5 A), consistent with evidence that ER Ca^{2+} pool depletion inhibits cell division (44). In cells that overexpress Bcl-2, the ER Ca^{2+} pool is preserved when extracellular Ca^{2+} is withdrawn (Fig. 4), consistent with evidence that cell growth is maintained under these conditions (Fig. 5 C). In cells that overexpress Bcl-2, TG treatment in the presence of a physiologic extracellular Ca^{2+} concentration induces growth inhibition (Fig. 5 G), consistent with the hypothesis that Bcl-2 maintains partial filling of the ER Ca^{2+} pool (Fig. 7 G). However, as discussed above, TG treatment induces apoptosis when cells that overexpress Bcl-2 are suspended in low Ca^{2+} medium (Fig. 5 H), suggesting that extracellular Ca^{2+} is required for maintenance of ER Ca^{2+} homeostasis by Bcl-2 when the ER Ca^{2+} -ATPase is inhibited by TG.

Mechanism of Bcl-2-mediated Ca^{2+} Uptake

The mechanism by which Bcl-2 mediates Ca^{2+} uptake in the ER is unknown. Bcl-2 does not appear to prevent Ca^{2+} -ATPase inhibition (Fig. 1; reference 20) or to increase the level of Ca^{2+} -binding proteins in the ER (Fig. 2). One theory is that Bcl-2 decreases oxidative damage to the ER membrane, thereby decreasing the leak of Ca^{2+} through the ER and increasing the Ca^{2+} -sequestering capacity of the ER. This theory is consistent with evidence that the ER is a prime target of oxidative damage that depletes the ER Ca^{2+} pool (36) and with evidence that Bcl-2 overexpression reduces oxidative damage to membranes (16). Also, this hypothesis is consistent with evidence that Bcl-2 overexpression preserves ER Ca^{2+} pool filling when cells are treated with hydrogen peroxide (11). A second theory is that Bcl-2 functions as a cation channel that transports Ca^{2+} into the ER lumen, or alternatively as an anion channel that draws Ca^{2+} into the ER by producing an electrochemical potential across the ER membrane. This concept is supported by recent evidence of a structural similarity between Bcl- X_L and bacterial toxins that form ion channels in cell membranes (33). One such toxin, the diphtheria toxin, appears to undergo a pH- and temperature-dependent conformational change by which it becomes hydrophobic and inserts into the cell membrane (26). It is possible that Bcl- X_L might undergo a similar conformational change and insert through the ER membrane, the perinuclear membrane, or the mitochondrial membrane, thereby regulating ion homeostasis within these organelles. A recent study indicates that Bcl- X_L forms an ion channel in synthetic lipid membranes (32), providing direct evidence that Bcl-2 family members are indeed involved in ion homeostasis.

General Conclusion

Bcl-2 inhibits apoptosis in response to a wide variety of signals. Furthermore, Bcl-2 is localized not only to the ER but also to the perinuclear membrane and the outer mitochondrial membrane. Each of these locations plays a role in inhibiting apoptosis, depending on the apoptosis-inducing signal (50). By regulating ion fluxes, Bcl-2 could act at different locations to regulate diverse cell death signals. Thus, while an effect of Bcl-2 on Ca^{2+} uptake at the level of the ER might inhibit TG-induced apoptosis, regulating ion fluxes in mitochondria, or both the ER and mitochon-

dria, might inhibit apoptosis in response to other signals. The possibility that Bcl-2 might affect diverse cellular functions by regulating ion fluxes is illustrated by evidence that perinuclear Ca^{2+} concentration regulates nuclear pore activity (38). Hence, the ability of Bcl-2 to regulate ion fluxes through the ER membrane and contiguous perinuclear membrane might regulate nuclear translocation of transcription factors that control cell growth and viability.

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