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Bcl-2 targeted to the endoplasmic reticulum can inhibit apoptosis induced by Myc but not etoposide in Rat-1 fibroblasts

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Bcl-2 is a key inhibitor of a broad range of apoptotic pathways, yet neither the mechanism of action nor the role of Bcl-2 subcellular localization are well understood. The subcellular localization of Bcl-2 includes the mitochondrial membrane as well as the contiguous membrane of the endoplasmic reticulum and nuclear envelope. Most studies suggest that the ability of Bcl-2 to confer cell survival is dependent upon its localization to the mitochondria. In this manuscript, we show that Bcl-2 targeted to the endoplasmic reticulum can inhibit Myc-, but not etoposide-induced apoptosis in the Rat-1 fibroblast cell line. By contrast, wild type Bcl-2 can inhibit apoptosis triggered by either death agonist. We further show both Myc and etoposide trigger disruption of mitochondrial membrane potential (MMP) and induce poly-ADP ribose polymerase (PARP) cleavage, but release of calcium was not evident. Bcl-2 abrogates apoptosis at or upstream of MMP depletion showing that Bcl-2 does not have to reside at the mitochondria to prevent apoptosis. These results further elucidate the biochemical events associated with Myc- and etoposide-induced apoptosis and significantly advance our understanding of Bcl-2 function.

Keywords: Myc; Bcl-2; apoptosis; etoposide; mitochondrial; membrane potential

Introduction

Programmed cell death or apoptosis plays a critical role in the prevention, genesis and eradication of cancer, yet the key regulatory processes of apoptosis remain unclear. Apoptosis is a genetic mechanism of cell death signified by a series of characteristic morphological and biochemical alterations (reviewed in Wyllie *et al*., 1980). Apoptotic agonists are thought to independently trigger specialized signal transduction pathways which ultimately converge and activate an irreversible core effector phase of cell destruction (for review see: Green and Martin, 1995). Bcl-2 has been shown to block apoptosis induced by a variety of agonists, suggesting that it plays an important role in controlling apoptosis. Understanding the biochemical and molecular mechanisms regulating apoptosis is essential to fully comprehend the process of cellular transformation and to design effective therapeutics to specifically kill tumor cells.

A wide variety of apoptotic agonists can trigger cells to die by diverse mechanisms. Many chemotherapeutic agents activate apoptosis by inflicting severe, irreparable damage to the cell. For example, etoposide antagonizes topoisomerase II activity which then leads to DNA-damage and cell death (Liu, 1989). Other apoptotic stimuli are less severe, yet are just as effective at killing transformed cells. For example, withdrawal of growth and survival factors can lead to growth arrest of non-transformed fibroblast cells; however, exposure of cells expressing an activated allele of the c-*myc* oncogene to such a growth-arrest signal can lead to apoptosis (Evan *et al*., 1992). The mechanism of Myc-induced death remains unclear, but likely differs from that of etoposide as DNA-damage is not involved. Despite the broad range of apoptotic agonists and their diverse individual signaling pathways, they are thought to ultimately activate a common effector phase of apoptosis which is largely centered and controlled at the mitochondria.

Mitochondrial function is highly associated with the apoptotic process, yet the precise role of mitochondria to the commitment phase of apoptosis, and the regulatory machinery controlling mitochondrial function remain unclear. The inner membrane of mitochondria possess electrochemical gradients which result in an asymmetric distribution of protons across the inner membrane leading to the formation of mitochondrial membrane potential (MMP). MMP disruption appears to be one of the universal events in the death process. It is disrupted in a wide-variety of cell types undergoing apoptosis in response to a diverse array of death agonists (Vayssiere *et al*., 1994; Zamzami *et al*., 1996). Moreover, kinetic analysis shows MMP disruption precedes the characteristic morphological features of apoptosis (Zamzami *et al*., 1995) and pharmacological agents such as bongkrekic acid are able to retain MMP and concomitantly suppress apoptosis (Marchetti *et al*., 1996). MMP disruption is believed to be a consequence of a process known as permeability transition (PT), which is thought to result from the opening of a megachannel spanning both mitochondrial inner and outer membranes (Kroemer, 1997). The collapse of MMP through PT has been proposed to be the rate limiting step prior to the irreversible degradation phase of apoptosis. This

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model suggests apoptotic signal transduction cascades must lead to MMP disruption and inhibitors of apoptosis must retain MMP to prevent apoptosis.

The potent anti-apoptotic activity of Bcl-2 is well established, yet its mechanism of action remains unclear. Many proposed mechanisms are related to the subcellular localization of Bcl-2. The carboxyl-terminal insertion sequence targets Bcl-2 to specific cytoplasmic membranes including the outer mitochondrial membrane, as well as the contiguous membrane of the nuclear envelope and endoplasmic reticulum (Akao *et al*., 1994; Chen-Levy and Cleary, 1990; Chen-Levy *et al*., 1989; Janiak *et al*., 1994; Krajewski *et al*., 1993; Monaghan *et al*., 1992; Nguyen *et al*., 1993; Riparbelli *et al*., 1995). For example, Bcl-2 has been proposed to function as an antioxidant and the subcellular localization of Bcl-2 overlaps with sites of reactive oxygen species generation (Hockenbery *et al*., 1993; Kane *et al*., 1993; Veis *et al*., 1993; Zhong *et al*., 1993). Moreover, Bcl-2 has been reported to regulate the release of calcium from intracellular stores primarily located at the endoplasmic reticulum (Baffy *et al*., 1993; He *et al*., 1997; Lam *et al*., 1994). Bcl-2 family members were also shown to form heterocomplexes with proteins such as Bax, Apaf-1 and Raf-1 showing Bcl-2 can function as a docking protein to directly or indirectly sequester these and other apoptotic regulatory molecules (Hu *et al*., 1998; Oltvai *et al*., 1993; Pan *et al*., 1998; Wang *et al*., 1996). Finally, another model suggests Bcl-2 directly suppresses mitochondrial PT through the direct formation of mitochondrial membrane channels (Mignotte and Vayssiere, 1998; Schendel *et al*., 1997). The latter model is supported by studies showing that Bcl-2 targeted to the mitochondrial membrane leads to the abrogation of apoptosis (Nguyen *et al*., 1994; Zhu *et al*., 1996). However, targeting Bcl-2 to other physiologically-relevant cellular membranes and determining the anti-apoptotic activity of Bcl-2 at these additional sites has not yet been thoroughly explored.

In our previous work, we compared the ability of Bcl-2 to suppress apoptosis when targeted to the endoplasmic reticulum as well as the mitochondria (Zhu *et al*., 1996). Targeting Bcl-2 to these specific organelles was achieved by replacing the native insertion sequence of Bcl-2 with the mitochondrial-specific insertion sequence of Act A or the endoplasmic reticulum-specific insertion sequence derived from cytochrome b5 (cb5). We showed wild type Bcl-2 and Bcl-2 targeted to the mitochondria could abrogate apoptosis induced by serum-deprivation in the MDCK epithelial cell line; whereas Bcl-2 targeted to the endoplasmic reticulum was unable to confer cell survival in this system. However, the endoplasmic reticulum form of Bcl-2 was able to inhibit Myc-induced apoptosis in the Rat-1 fibroblast cell line. It is unclear whether the differential anti-apoptotic activity of Bcl-2 targeted to the endoplasmic reticulum was dependent upon the cell-type (epithelial or fibroblast) or the apoptotic agonist (serum-withdrawal alone or Myc-activation and serum-withdrawal). In this study we address the latter by assessing the role of subcellular localization to Bcl-2 function when a single cell-type, fibroblasts, are challenged with two different apoptotic agonists; Myc-activation under low serum conditions or exposure to etoposide. We also investigate the mechanism of action of the Myc-induced and etoposide-triggered apoptosis pathways and evaluate the point at which Bcl-2 can inhibit these cascades.

Results

MMP disruption is evident in Rat-1 cells undergoing Myc- or etoposide-induced apoptosis

Our previous work showed Myc-induced apoptosis could be inhibited by a mutant form of Bcl-2 targeted to the endoplasmic reticulum (Bcl-cb5). These results suggested that Myc may trigger apoptosis by a unique mitochondria-independent mechanism of action. To determine if mitochondria are involved in Myc-triggered apoptosis, we assayed MMP during apoptosis triggered by either etoposide, as a positive control (Nguyen *et al*., 1993), or Myc-activation in serum-deprived Rat-1MycER cells. The inducible c-*myc* system was employed in these studies to allow the mechanism of apoptosis triggered by either etoposide or Myc to be directly compared within the same cell line. Rat-1MycER cells constitutively express an inactive fusion protein consisting of human c-Myc linked in-frame to the ligand-binding domain of the estrogen receptor (Eilers *et al*., 1989; Evan *et al*., 1992). The inactive MycER fusion protein is constitutively expressed and activated within minutes of exposure of cells to 4-hydroxytamoxifen (OH-T). MycER-activation in the presence of growth and survival factors stimulates cell proliferation, while under low serum conditions MycER-activation triggers Rat-1 cells to undergo apoptosis. The activity of MycER following OH-T activation is directly comparable to that of the wild type c-*myc* allele (Eilers *et al*., 1989; Evan *et al*., 1992; Facchini *et al*., 1997; Marhin *et al*., 1997).

To determine the MMP of Rat-1MycER cells undergoing apoptosis, a cationic amphipathic fluorochrome DilCl(5) and flow cytometry were employed (Backway *et al*., 1997). When MMP is intact, the dye concentrates in the mitochondrial matrix and the fluorescence intensity is measured using flow cytometry. Disruption of MMP significantly compromises DilCl(5) accumulation which is detected as a decrease in fluorescence intensity. As a positive control for the detection of MMP disruption, Rat-1MycER cells were exposed to the MMP-disrupting agent CCCP and then incubated with DilCl(5) as well as propidium iodide (PI). The latter is used to distinguish live from dead cells by the retention or loss of cell surface membrane integrity, respectively. To characterize the biochemical and molecular events occurring as cells are undergoing apoptosis, the MMP of PI-excluding cells was assayed by flow cytometry. As shown in Figure 1a, MMP disruption was evident in response to CCCP exposure, as indicated by the decrease in relative fluorescence intensity in CCCP-exposed cells (low MMP cells are indicated by an arrow) compared to untreated control cells. Superimposing the untreated and CCCP fluorescence profiles clearly shows the shift in fluorescence intensity which corresponds to MMP disruption (Figure 1a, third panel).

To determine whether MMP was disrupted in Rat-1MycER cells in response to etoposide, asynchronously growing cells were exposed to DMSO as a solvent

control (0 μM) or etoposide at 10, 20, 40 and 60 μM for 18 h. In these experiments MycER is inactive as the cells were not exposed to OH-T. Following exposure to etoposide, cells were harvested and incubated with DilCl(5) as well as PI as above. As shown in Figure 1b, overnight exposure to DMSO solvent control (0 μM) showed a unimodal population of cells with narrow distribution of MMP which is similar to untreated cells (Figure 1a). By contrast, overnight exposure to 10 μM etoposide induces MMP disruption, as indicated by the emergence of a sub-population of cells with a decrease in relative fluorescence intensity (indicated by arrow). The fraction of cells found in the low MMP sub-population increases with the dose of etoposide (Figure 1b) and positively correlates with the degree of cell death as detected by microscopy as well as acridine orange/ethidium bromide staining as previously conducted (data not shown, Zhu *et al*., 1996). Thus etoposide-induced apoptosis results in the disruption of MMP in Rat-1MycER cells.

To determine whether MMP was disrupted in the Myc-induced apoptosis pathway, subconfluent asynchronous cells were exposed to OH-T in serum-depleted medium for 0, 12, 24 and 40 h. Cells were then harvested and analysed by flow cytometry as described above. As shown in Figure 1c, control untreated cells (0 h) showed a single population of cells with narrow distribution of MMP. Following 12 h of treatment, a population of cells with low MMP was observed (indicated by arrow). This low MMP cell population increased with time of treatment (24 and 40 h) which was proportional to the degree of cell death evident in the population (Evan *et al*., 1992; Zhu *et al*., 1996). Clearly, MMP disruption is a feature of the Myc-induced apoptosis pathway in Rat-1 cells.

Bcl-2 inhibits MMP disruption in both etoposide- and Myc-induced apoptotic pathways

Anti-apoptotic protein Bcl-2 has been shown to inhibit apoptosis triggered by either Myc- or etoposide (Fanidi *et al*., 1992; Nguyen *et al*., 1993). We next determined whether Bcl-2 inhibits apoptosis upstream or downstream of MMP disruption. Rat-1MycER cells expressing the puromycin-resistance gene alone (control) or the human *bcl-2* cDNA as well as the

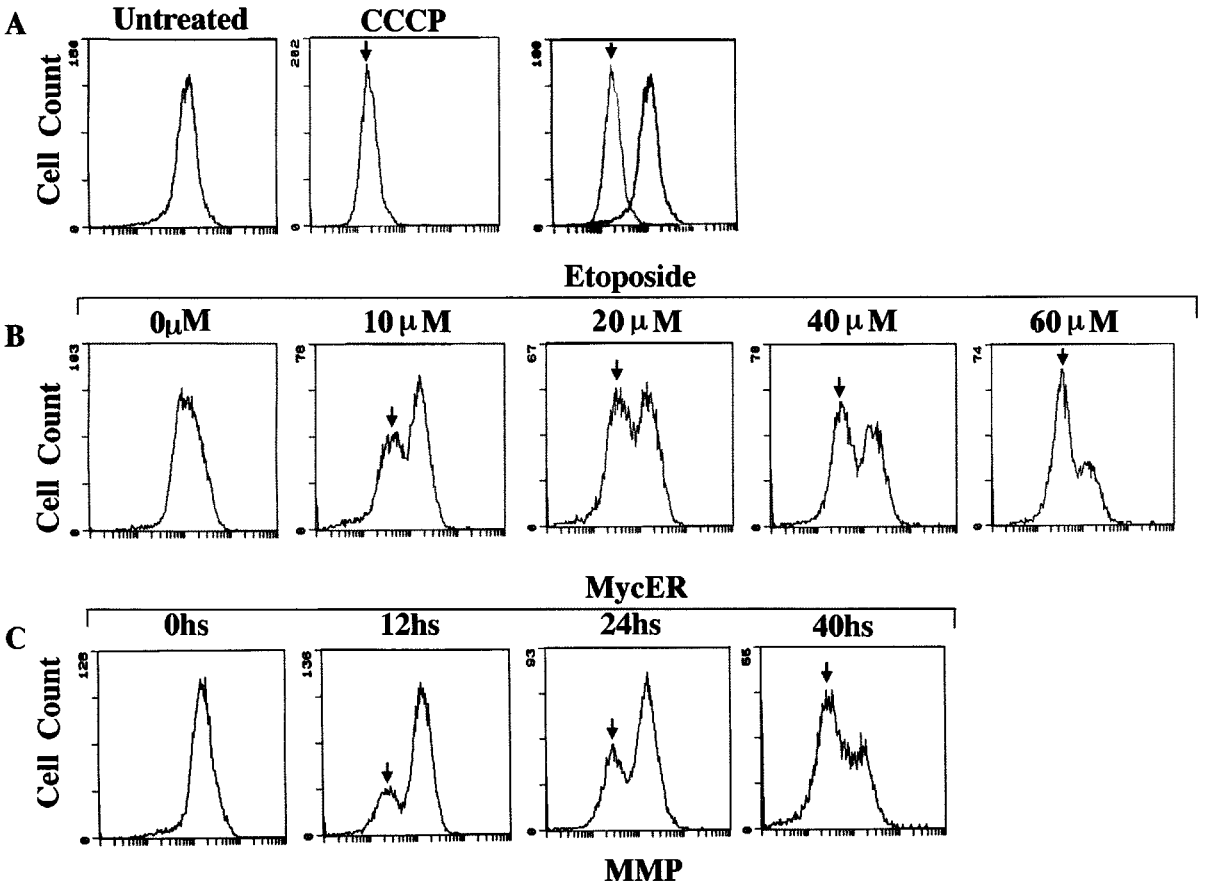

Figure 1 Disruption of mitochondrial membrane potential (MMP) occurs during etoposide and Myc-induced apoptosis in Rat-1MycER cells. Rat-1MycER cells were incubated with the fluorogenic dye DilCl(5), a PT-dependent mitochondrial stain, and fluorescence intensity was then measured by flow cytometric analysis. (**a**) As a positive control for the detection of MMP disruption, Rat-1MycER cells were untreated or exposed to the MMP-disrupting agent CCCP and the fluorescence intensity measured. In untreated cells MMP is intact (untreated), whereas CCCP exposure disrupts MMP (CCCP) leading to altered fluorescence intensity (indicated by arrow). By superimposing the untreated and CCCP fluorescence profiles, the shift in fluorescence intensity corresponding to MMP disruption is clearly evident (see the third panel). (**b**) Etoposide induces MMP disruption of Rat-1MycER cells. Asynchronously growing Rat-1MycER cells were exposed to DMSO (0 μM), or 10, 20, 40, and 60 μM etoposide for 18 h, then harvested and stained with DilCl(5), and analysed by flow cytometry as above. (**c**) Myc-induced apoptosis triggers MMP disruption in Rat-1MycER cells. Cells were exposed to 4-hydroxytamoxifen under low serum conditions to activate the MycER fusion protein and induce apoptosis for 0, 12, 24 and 40 h and then analysed by flow cytometry as above. In both (**b**) and (**c**) the population of cells with fluorescence intensity indicative of MMP disruption is marked by an arrow

puromycin-resistance gene were previously derived by retroviral infection and puromycin selection (Zhu *et al*., 1996) Stable populations of puromycin-resistant cells were seeded subconfluently and either exposed to etoposide (0, 10, 20, 40, 60 μM) or to OH-T in serum-depleted medium for increasing periods of time (0, 12, 24, 40 h). Cell death was measured by microscopy (data not shown) while MMP was assayed using DilCl(5) as described earlier. Cells expressing exogenous Bcl-2 consistently demonstrated a unimodal population of cells with narrow distribution of MMP at an intensity indicative of an intact MMP (Figure 2). Unlike control cells (Figure 1), MMP disruption was not evident in cells expressing ectopic Bcl-2 in response to etoposide, at a range of 0 to 60 μM, nor Myc-induced death for up to 40 h. Thus ectopic Bcl-2 expression inhibited Myc and etoposide-induced apoptosis at or upstream of MMP-disruption in Rat-1 fibroblasts.

Another feature of apoptosis that may or may not be independent of MMP is activation of caspases. To assay caspase activation and Bcl-2-dependent inhibition of this process, cells were assayed for the cleavage of endogenous poly-ADP ribose polymerase (PARP). PARP is specifically cleaved by caspases, including caspase 3 which is activated during both Myc and etoposide induced apoptosis (Kangas *et al*., 1998; Widmann *et al*., 1998). Asynchronously growing control and Bcl-2 cells were either left untreated, exposed to 40 μM etoposide for 18 h or to OH-T under low serum conditions for 40 h, then protein extracts prepared and assayed by immunoblot (Figure 3). Under these conditions Myc and etoposide triggered 39% and 30% cell death in control cells, respectively; and 11% and 16% cell death in cells expressing Bcl-2, respectively, as assayed by PI-exclusion using flow cytometry. Immunoblot analysis shows intact PARP migrates at 116 kDa in control cells, whereas in cells undergoing either Myc or etoposide induced death, the 85 kDa cleavage product is evident, showing that one or more caspases is activated in these cells (Figure 3). This cleavage product was not detectable in cells expressing ectopic Bcl-2. Thus, Bcl-2-mediated abrogation of apoptosis triggered by either Myc or etoposide occurs prior to activation of caspases.

Bcl-2 targeted to the endoplasmic reticulum inhibits Myc-, but not etoposide-induced apoptosis and prevents MMP disruption

Our previous studies showed that a Bcl-2 mutant targeted to the endoplasmic reticulum (Bcl-cb5) protected cells from Myc-induced death as efficiently

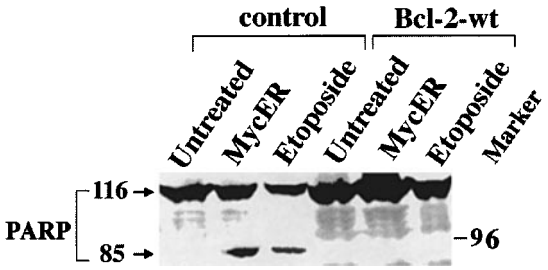

Figure 3 Immunoblot analysis of PARP in Rat-1MycER cells. Asynchronously growing Rat-1MycER cells expressing control vector (control) or exogenous wild type Bcl-2 (Bcl-2) were left untreated or exposed to either 40 μM etoposide (Etoposide) for 18 h or to OH-T in low serum for 40 h (MycER). Cells were harvested and assayed by Western blot to detect endogenous PARP protein. Intact PARP (116 kDa) and a cleavage product (85 kDa) are indicated. The 96 kDa molecular weight marker is also shown

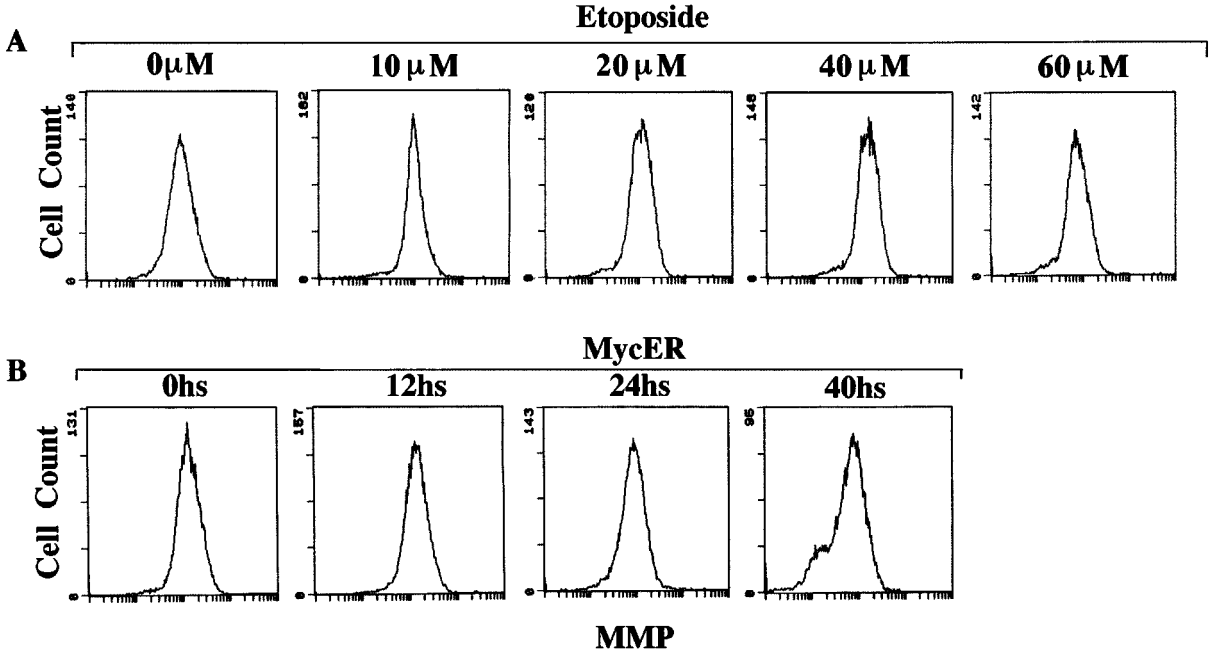

Figure 2 Bcl-2 inhibits MMP disruption and apoptosis triggered by etoposide- or Myc-induced apoptosis in Rat-1MycER cells. Asynchronously growing Rat-1MycER cells were (**a**) exposed to DMSO (0 μM) or 10, 20, 40 and 60 μM etoposide for 18 h or (**b**) exposed to OH-T under low serum conditions for 0, 12, 24 and 40 h to trigger Myc-induced apoptosis, then harvested and stained with DilCl(5), and analysed by flow cytometry as above

as wild type Bcl-2; however it remained unclear whether Bcl-cb5 could protect Rat-1 cells from other apoptotic agonists. To determine whether Bcl-2 targeted to the endoplasmic reticulum could inhibit etoposide- as well as Myc-triggered apoptosis, control, Bcl-2 and Bcl-cb5 expressing cells described previously (Zhu *et al*., 1996), were triggered to undergo apoptosis in response to etoposide or Myc-activation and analysed by microscopy. Importantly, levels of ectopic Bcl-2 and Bcl-cb5 were comparable in these cells (Zhu *et al*., 1996). Microscopic examination showed evidence of apoptosis as detected by cell rounding and lifting off from the cell monolayer below (Figure 4). Rat-1MycER cells ectopically expressing the puromycin-resistance gene alone (control; Figure 4a, d and g), wild type Bcl-2 (Bcl-2; Figure 4b, e and h), Bcl-2 targeted to the endoplasmic reticulum (Bcl-cb5; Figure 4c, f and i), were plated under subconfluent conditions. Cells were then left untreated (Figure 4a–c), exposed to 40 μM etoposide for 18 h (Figure 4d–f) or exposed to OH-T in serum-deprived medium for 40 h (Figure 4g–i). Untreated cell cultures do not undergo apoptosis and show similar growth and morphological characteristics (Figure 4a–c). Exposure to etoposide triggers apoptosis in control cells (Figure 4d) which is not evident in cells expressing Bcl-2 (Figure 4e), but clearly detectable in Bcl-cb5 expressing cells (Figure 4f). Myc-induced apoptosis is evident in the control cell population (Figure 4g), but not evident in cells expressing Bcl-2 (Figure 4h), or Bcl-cb5 (Figure 4i). Thus, microscopic evidence shows Bcl-2 targeted to the endoplasmic reticulum can inhibit Myc- but not etoposide-triggered apoptosis.

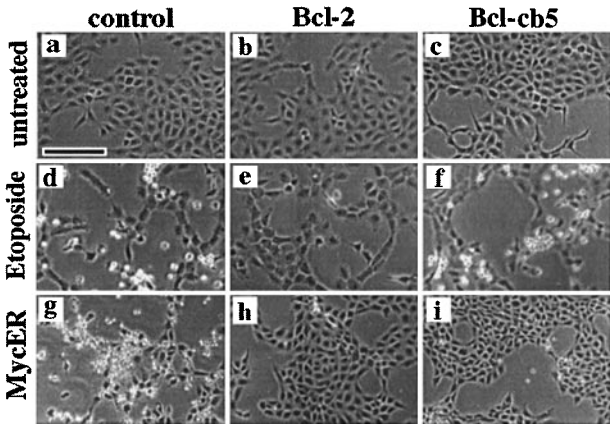

Figure 4 Bcl-2 targeted to endoplasmic reticulum can inhibit Myc but not etoposide-induced apoptosis. Microscopic examination shows apoptosis of Rat-1 cells as rounded refractile cells which lift off the cell monolayer. Asynchronously growing Rat-1MycER cells expressing control vector (**a,d,g**), exogenous wild type Bcl-2 (**b,e,h**), Bcl-2 targeted to the endoplasmic reticulum (**c,f,i**) were left untreated (**a,b,c**) or exposed to 40 μM etoposide for 18 h (Etoposide; **d,e,f**) or exposed to OH-T under low serum conditions for 40 h (MycER; **g,h,i**). Photomicrographs show cells at 1000-fold magnification (bar is equivalent to 15 μm)

We next addressed whether the mechanism of the anti-apoptotic effect of Bcl-cb5 was associated with MMP disruption of the cell. To this end, experiments were conducted as described earlier using DilCl(5) and PI staining with flow cytometry. As expected, MMP is intact in untreated Rat-1MycER cells (Figure 5a and b). Exposure to 40 μM etoposide for 18 h lead to the characteristic disruption of MMP in control cells which was significantly abrogated in cells expressing ectopic Bcl-2, as shown by the unimodal population of cells with narrow distribution (Figure 5a). MMP in cells expressing Bcl-cb5 resembled that of control cells (Figure 5a). Disruption of MMP in control cells was detectable following 40 h Myc-induced apoptosis (Figure 5b). In contrast to etoposide, Myc-activated cells expressing Bcl-2 or Bcl-cb5 showed little to no

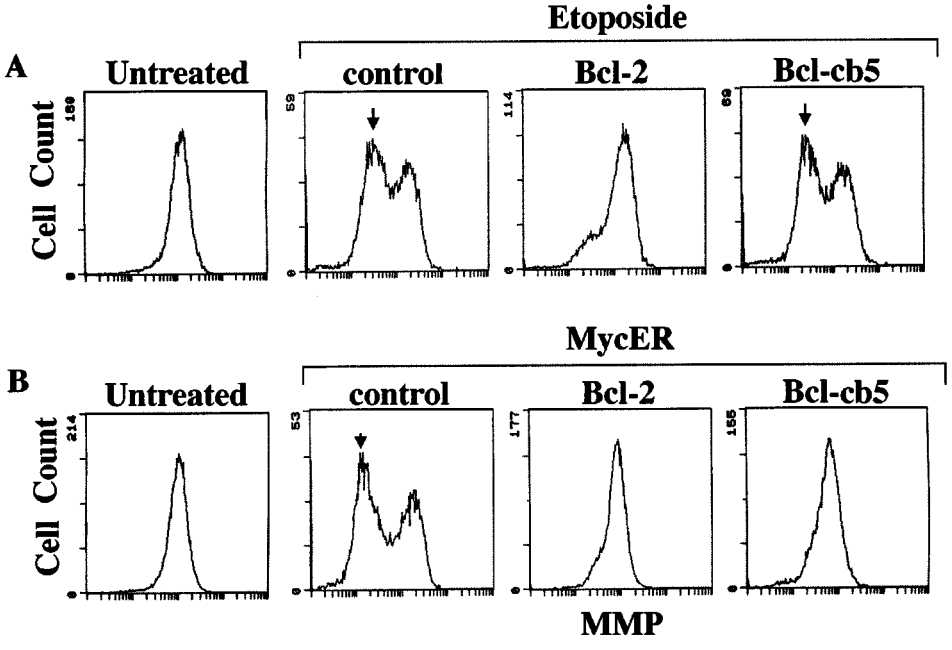

Figure 5 Bcl-2 targeted to the endoplasmic reticulum can inhibit MMP disruption during Myc- but not etoposide-induced apoptosis in Rat-1MycER cells. Asynchronous growing Rat-1MycER control, Bcl-2 and Bcl-cb5 cells were (**a**) left untreated or exposed to 40 μM etoposide (Etoposide) for 18 h or (**b**) left untreated or exposed to OH-T under low serum conditions for 40 h (MycER). The disruption of MMP is indicated by the changes of the fluorescence intensity (marked by the arrow)

evidence of MMP disruption (Figure 5b). The degree of MMP disruption was proportional to the cell death triggered in these cells as detected by PI-exclusion and flow cytometry (Figure 6). Taken together, our results show that wild type Bcl-2 is able to inhibit apoptosis and MMP disruption induced by two different apoptotic agonists. However, Bcl-2 targeted to the endoplasmic reticulum antagonizes Myc-induced MMP disruption and apoptosis, but is unable to abrogate etoposide-induced apoptosis or its associated disruption of MMP.

Intracellular calcium levels are not elevated during Myc- or etoposide-induced apoptosis

Bcl-2 has been suggested to inhibit apoptosis by regulating endogenous calcium in various cell types (Baffy *et al*., 1993; Lam *et al*., 1994). To determine whether calcium levels were altered in either the etoposide- or Myc-induced apoptosis pathways, the level of intracellular calcium was measured by flow cytometry. Asynchronously growing Rat-1MycER cells were left untreated or, as a positive control, exposed to the calcium ionophore ionomycin. Cells were then harvested and stained with the calcium-binding dye indo-1 to measure intracellular calcium. A clear shift in fluorescence is detected in cells exposed to ionomycin (Figure 7a) showing release of calcium can be readily measured by this approach in Rat-1MycER cells. To determine the concentration of intracellular calcium as cells are undergoing apoptosis, asynchronously growing Rat-1MycER cells were left untreated or exposed to either 40 μM etoposide for 18 h (Figure 7b) or to OH-T in low-serum containing medium for 40 h (Figure 7c). The cells were harvested and analysed as above. Calcium levels did not increase in control cells undergoing apoptosis in response to either Myc or etoposide nor did the intracellular calcium concentration change significantly in cells expressing either Bcl-2 or Bcl-cb5. Elevation of cytosolic calcium does not play a key regulatory role in etoposide-or Myc-induced apoptosis in Rat-1 cells. Thus, Bcl-2 does not inhibit apoptosis triggered by these stimuli by controlling calcium levels in these cells.

Discussion

We have shown that Bcl-2 targeted to the endoplasmic reticulum can inhibit Myc-, but not etoposide-induced apoptosis, whereas wild type Bcl-2 can abrogate apoptosis triggered by either stimulus in Rat-1 fibroblasts. Mechanistically, both Myc- and etoposide-triggered apoptosis pathways are associated with MMP disruption, and conversely, the anti-apoptotic activity of Bcl-2 was strongly associated with an intact MMP. These data suggest Bcl-2 inhibits apoptosis at or upstream of MMP disruption and further supports the notion that mitochondrial function plays an important role in the commitment of a cell to enter the irreversible degradation phase of apoptosis. As Bcl-2 targeted to the endoplasmic reticulum inhibited MMP disruption as well as Myc-induced apoptosis, our data demonstrates Bcl-2 does not have to reside at the mitochondria to control mitochondrial PT and abrogate apoptosis. Thus, in Rat-1 cells there are at least two Bcl-2-inhibitable, spatially-distinct apoptosis pathways leading to MMP disruption.

We speculated that Bcl-2 targeted to endoplasmic reticulum may inhibit apoptosis through the regulation of the calcium signaling pathway (Zhu *et al*., 1996). The endoplasmic reticulum is the main storage compartment of intracellular calcium (Carafoli, 1987) and calcium release can trigger apoptosis (Shibasaki

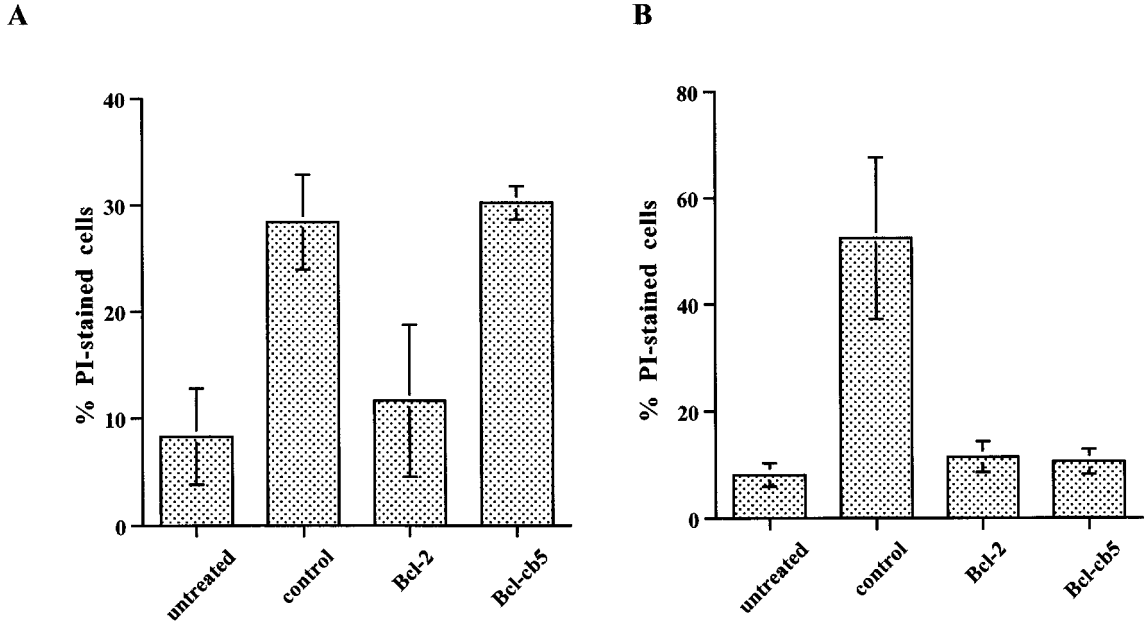

Figure 6 Flow cytometric analysis of cell death of Rat-1MycER cells. Asynchronous growing Rat-1MycER control, Bcl-2 and Bcl-cb5 cells were (**a**) left untreated or exposed to 40 μM etoposide (Etoposide) for 18 h or (**b**) left untreated or exposed to OH-T under low serum conditions for 40 h (MycER) and then incubated with propidium iodide (PI). The percentage of PI-stained cells was quantitated by flow cytometry

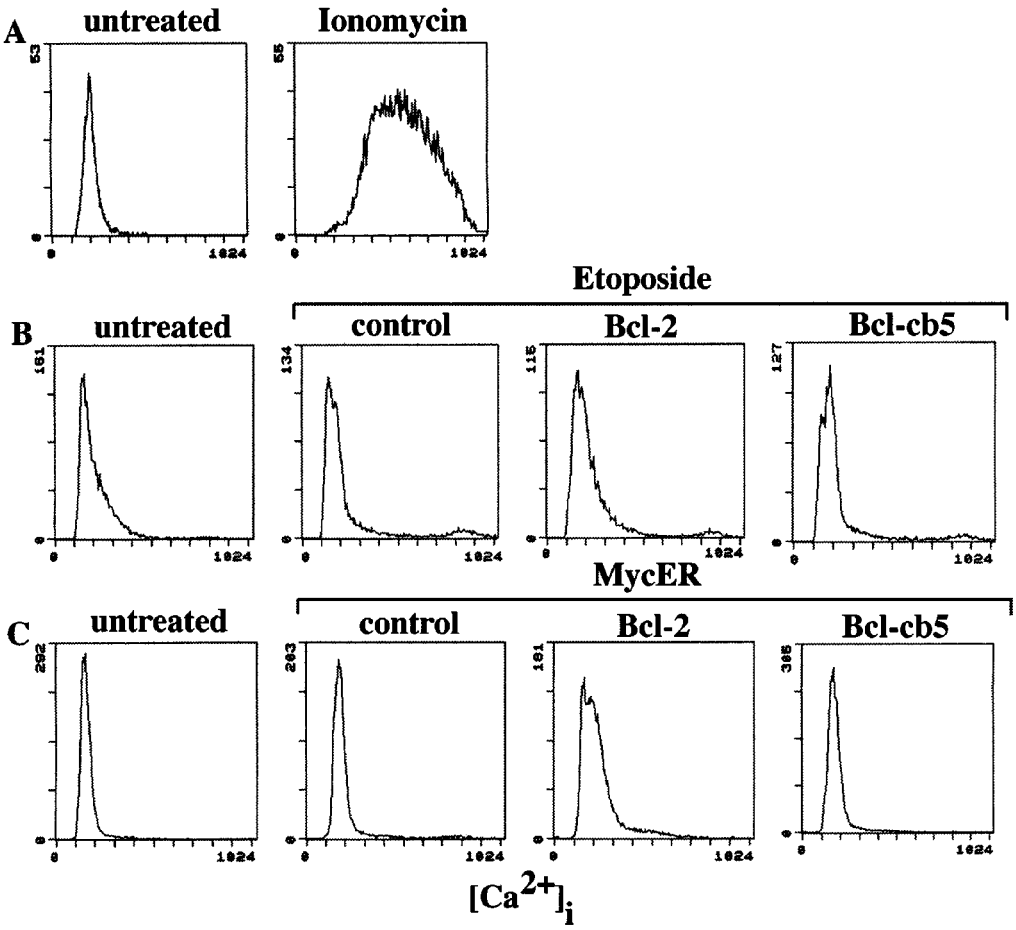

Figure 7 Intracellular calcium concentration is not elevated during etoposide or Myc-induced apoptosis in Rat-1MycER cells. Asynchronous growing Rat-1MycER cells were (**a**) left untreated or exposed to 5 μM ionomycin, (**b**) Rat-1MycER control, Bcl-2 and Bcl-cb5 cells were left untreated or exposed to 40 μM etoposide (Etoposide) for 18 h or (**c**) left untreated or exposed to OH-T under low serum conditions for 40 h (MycER). To examine intracellular calcium concentration in cells undergoing apoptosis, cells were harvested, incubated with calcium-binding dye indo-1 and then analysed by flow cytometry

and McKeon, 1995). A possible explanation for an endoplasmic reticulum-specific pathway regulated by Bcl-2 is control of calcium flux (He *et al*., 1997; Lam *et al*., 1994). To examine whether calcium elevation was associated with Myc- or etoposide-induced apoptosis we used a cytofluorometric approach. As our results show, calcium elevation is unlikely to play a major role in apoptosis induced by either Myc or etoposide in Rat-1 cells. Thus, we speculate that Bcl-2 targeted to the endoplasmic reticulum inhibits apoptosis via a mechanism independent of direct regulation of intracellular calcium homeostasis.

The mechanism of Bcl-cb5 inhibition of MMP disruption and Myc-induced apoptosis remain unclear; however, numerous models can be proposed. When targeted to the mitochondria, Bcl-2 is thought to directly regulate mitochondrial homeostasis through a channel-forming property. Consistent with this model, Bcl-2 forms ion channels *in vitro* and is thought to function like Bcl-X_L, another Bcl-2 family member, whose three-dimensional structure is similar to bacterial toxins with pore-forming activity (Minn *et al*., 1997; Muchmore *et al*., 1996; Schendel *et al*., 1997). Bcl-cb5 may inhibit Myc-induced apoptosis by forming a channel in the membrane of the endoplasmic reticulum to allow the release of ionic, proteinaceous or other small molecular-weight constituents of this unique organelle. These molecules may in turn inhibit apoptosis by directly regulating mitochondrial PT or by indirectly curtailing the apoptotic signals at or upstream of the mitochondria. In addition to its channel-forming properties, Bcl-2 has also been shown to function as a docking protein to sequester apoptotic regulators (Reed, 1997). Bcl-cb5 may bind to rate-limiting proteins of the apoptotic process and thereby prevent the cell from undergoing apoptosis. These interacting molecules would not likely be those that have been previously reported, such as Bax, Apaf-1 or Raf-1, as binding these universal regulators of apoptosis would potentially allow Bcl-cb5 to inhibit both the Myc and etoposide-induced death pathways. As Myc-, but not etoposide-induced death is abrogated by Bcl-cb5, the binding protein is predicted to be unique to the Myc-activated apoptosis pathway and function at or upstream of mitochondrial PT. Such a protein could be cytosolic or targeted to the endoplasmic reticulum (Ng *et al*., 1997). Further evidence that the Myc-induced apoptosis pathway differs from that of etoposide or other death agonists is provided by Kagaya *et al*. (1997) showing that serine protease inhibitors can abrogate Myc but not other apoptotic pathways. Understanding the mechanism(s)

of Bcl-cb5 inhibition of MMP disruption has important therapeutic implications and requires further investigation.

To further understand the mechanism of Bcl-2 inhibition of both apoptosis and MMP disruption, it would be informative to determine which domain of Bcl-2 is required for the inhibition of Myc-triggered apoptosis when localized at the endoplasmic reticulum. It would also be instructive to learn which other death pathways can be inhibited by Bcl-cb5 in Rat-1 fibroblasts, and to know whether Bcl-cb5 can inhibit Myc-triggered apoptosis in other cell types. Clearly, further investigation into the molecular mechanism of the protective effect of Bcl-cb5 will add insight into both the Myc-induced apoptosis pathway and Bcl-2 function. To this end, the targeted Bcl-2 molecules can serve as valuable tools.

Materials and methods

Cell lines and apoptosis induction

The cell lines used in this study have been previously described (Zhu *et al*., 1996). For etoposide-induced apoptosis experiments, cells were exposed to DMSO as a solvent control (40 μl) or etoposide (Sigma) was added to the medium at a final concentration of 10–60 μM and incubated for 18 h. For Myc-induced apoptosis experiments, cells were exposed to ethanol as a solvent control or 100 nM OH-T (Research Biochemical International) in 0.03% serum-containing medium for 12–48 h.

Flow cytometry

For flow cytometric analysis, both floating and adherent cells were harvested, and cell pellets were resuspended at 10^6 cells/ml 10%FBS/αMEM and kept on ice. All fluorogenic dyes were obtained from Molecular Probes, (Eugene, OR, USA). To assay cells for mitochondrial membrane potential (MMP), 5×10^5 cells were incubated at 37°C for 30 min with 20 nM of DilCl(5) prior to flow analysis (Backway *et al*., 1997). Intracellular ionized calcium was examined by incubating 5×10^5 cells with 3 μM indo-1 acetoxymethyl ester at 37°C for 30 min prior to analysis. In addition, to distinguish cells with intact surface membrane integrity, 5 μg/ml PI was added to all samples prior to flow analysis. To stimulate MMP disruption or calcium release, cells were incubated with 200 μM of the uncoupling agent CCCP (Sigma) and 5 μM ionomycin (Calbiochem), respectively.

Flow cytometry was performed using an Epics Elite cell sorter (Coulter), fitted with three air cooled lasers emitting at 325 nm, 488 nm, and 633 nm. PI was excited at 488 nm, and collected at 640 nm. Indo-1 was excited by a 325 nm Helium/Cadium laser, with a 440 nm bandpass filter used for collection. Intracellular calcium ion concentration was obtained by electronic ratio measurements of the calcium bound (405 nm) and calcium free (525 nm) emission of indo-1. DilCl(5) was excited at 633 nm and fluorescence collected at 675 nm.

PARP immunoblot

Following apoptosis induction, both floating and adherent cells were harvested and suspended in extraction buffer containing loading dye (100 μl of 50 mM glucose, 25 mM Tris-HCl pH 8, 10 mM EDTA, 1 mM PMSF mixed with 50 μl of 50 mM Tris-HCl pH 6.8, 6 M urea, 6% 2-mercaptoethanol, 3% SDS, 0.003% bromophenol blue). The cell samples were then sonicated and incubated for 15 min at 65°C prior to fractionated by 6% SDS-polyacrylamide gel electrophoresis and blotting onto PVDF membrane (NEN Life Science). The resultant Western blot was probed with a polyclonal antibody against full-length recombinant PARP (Boehringer Mannheim) at 0.5 μg/ml. The blot was developed using an enhanced chemiluminescence system according to the manufacturer's instructions (NEN Life Science Products).

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References

Akao Y, Otsuki Y, Kataoka S, Ito Y and Tsujimoto Y. (1994). *Cancer Res.,* **54,** 2468–2471.

Backway KL, McCulloch EA, Chow S and Hedley DW. (1997). *Cancer Res.,* **57,** 2446–2451.

Baffy G, Miyashita T, Williamson JR and Reed JC. (1993). *J. Biol. Chem.,* **268,** 6511–6519.

Carafoli E. (1987). *Annu. Rev. Biochem.,* **56,** 395–433.

Chen-Levy Z and Cleary ML. (1990). *J. Biol. Chem.,* **265,** 4929–4933.

Chen-Levy Z, Nourse J and Cleary ML. (1989). *Mol. Cell. Biol.,* **9,** 701–710.

Eilers M, Picard D, Yamamoto KR and Bishop JM. (1989). *Nature,* **340,** 66–68.

Evan GI, Wyllie AH, Gilbert CS, Littlewood TD, Land H, Brooks M, Waters CM, Penn LZ and Hancock DC. (1992). *Cell,* **69,** 119–128.

Facchini LM, Chen S, Marhin WW, Lear JN and Penn LZ. (1997). *Mol. Cell. Biol.,* **17,** 100–114.

Fanidi A, Harrington EA and Evan GI. (1992). *Nature,* **359,** 554–556.

Green DR and Martin SJ. (1995). *Curr. Opin. Immunol.,* **7,** 694–703.

He H, Lam M, McCormick TS and Distelhorst CW. (1997). *J. Cell. Biol.,* **138,** 1219–1228.

Hockenbery DM, Oltvai ZN, Yin XM, Milliman CL and Korsmeyer SJ. (1993). *Cell,* **75,** 241–251.

Hu Y, Benedict MA, Wu D, Inohara N and Nunez G. (1998). *Proc. Natl. Acad. Sci. USA,* **95,** 4386–4391.

Janiak F, Leber B and Andrews DW. (1994). *J. Biol. Chem.,* **269,** 9842–9849.

Kagaya S, Kitanaka C, Noguchi K, Mochizuki T, Sugiyama A, Asai A, Yasuhara N, Eguchi Y, Tsujimoto Y and Kuchino Y. (1997). *Mol. Cell. Biol.,* **17,** 6736–6745.

Kane DJ, Sarafian TA, Anton R, Hahn H, Gralla EB, Valentine JS, Ord T and Bredesen DE. (1993). *Science,* **262,** 1274–1277.

Kangas A, Nicholson DW and Hottla E. (1998). *Oncogene,* **16,** 387–398.

Krajewski S, Tanaka S, Takayama S, Schibler MJ, Fenton W and Reed JC. (1993). *Cancer Res.,* **53,** 4701–4714.

Kroemer G. (1997). *Cell. Death. Diff.,* **4,** 443–457.

Lam M, Dubyak G, Chen L, Nunez G, Miesfeld RL and Distelhorst CW. (1994). *Proc. Natl. Acad. Sci. USA,* **91,** 6569–6573.

Liu LF. (1989). *Annu. Rev. Biochem.,* **58,** 351–375.
Marchetti P, Hirsch T, Zamzami N, Castedo M, Decaudin D, Susin SA, Masse B and Kroemer G. (1996). *J. Immunol.,* **157,** 4830–4836.
Marhin WW, Chen S, Facchini LM, Fornace Jr AJ and Penn LZ. (1997). *Oncogene,* **14,** 2825–2834.
Mignotte B and Vayssiere JL. (1998). *Eur. J. Biochem.,* **252,** 1–15.
Minn AJ, Velez P, Schendel SL, Liang H, Muchmore SW, Fesik SW, Fill M and Thompson CB. (1997). *Nature,* **385,** 353–357.
Monaghan P, Robertson D, Amos TA, Dyer MJ, Mason DY and Greaves MF. (1992). *J. Histochem. Cytochem.,* **40,** 1819–1825.
Muchmore SW, Sattler M, Liang H, Meadows RP, Harlan JE, Yoon HS, Nettesheim D, Chang BS, Thompson CB, Wong SL, Ng SL and Fesik SW. (1996). *Nature,* **381,** 335–341.
Ng FW, Nguyen M, Kwan T, Branton PE, Nicholson DW, Cromlish JA and Shore GC. (1997). *J. Cell. Biol.,* **139,** 327–338.
Nguyen M, Branton PE, Walton PA, Oltvai ZN, Korsmeyer SJ and Shore GC. (1994). *J. Biol. Chem.,* **269,** 16521–16524.
Nguyen M, Millar DG, Yong VW, Korsmeyer SJ and Shore GC. (1993). *J. Biol. Chem.,* **268,** 25265–25268.
Oltvai ZN, Milliman CL and Korsmeyer SJ. (1993). *Cell,* **74,** 609–619.
Pan G, O'Rourke K and Dixit VM. (1998). *J. Biol. Chem.,* **273,** 5841–5845.
Reed JC. (1997). *Nature,* **387,** 773–776.
Riparbelli MG, Callaini G, Tripodi SA, Cintorino M, Tosi P and Dallai R. (1995). *Exp. Cell. Res.,* **221,** 363–369.
Schendel SL, Xie Z, Montal MO, Matsuyama S, Montal M and Reed JC. (1997). *Proc. Natl. Acad. Sci. USA,* **94,** 5113–5118.
Shibasaki F and McKeon F. (1995). *J. Cell. Biol.,* **131,** 735–743.
Vayssiere JL, Petit PX, Risler Y and Mignotte B. (1994). *Proc. Natl. Acad. Sci. USA,* **91,** 11752–11756.
Veis DJ, Sorenson CM, Shutter JR and Korsmeyer SJ. (1993). *Cell,* **75,** 229–240.
Wang HG, Rapp UR and Reed JC. (1996). *Cell,* **87,** 629–638.
Widmann C, Gibson S and Johnson GL. (1998). *J. Biol. Chem.,* **273,** 7141–7147.
Wyllie AH, Kerr JF and Currie AR. (1980). *Int. Rev. Cytol.,* **68,** 251–306.
Zamzami N, Marchetti P, Castedo M, Decaudin D, Macho A, Hirsch T, Susin SA, Petit PX, Mignotte B and Kroemer G. (1995). *J. Exp. Med.,* **182,** 367–377.
Zamzami N, Susin SA, Marchetti P, Hirsch T, Gomez-Monterrey I, Castedo M and Kroemer G. (1996). *J. Exp. Med.,* **183,** 1533–1544.
Zhong LT, Sarafian T, Kane DJ, Charles AC, Mah SP, Edwards RH and Bredesen DE. (1993). *Proc. Natl. Acad. Sci. USA,* **90,** 4533–4537.
Zhu W, Cowie A, Wasfy GW, Penn LZ, Leber B and Andrews DW. (1996). *Embo J.,* **15,** 4130–4141.