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Endoplasmic reticulum localized Bcl-2 prevents apoptosis when redistribution of cytochrome *c* is a late event

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The disruption of mitochondrial function is a key component of apoptosis in most cell types. Localization of Bcl-2 to the outer mitochondrial and endoplasmic reticulum membranes is consistent with a role in the inhibition of many forms of apoptosis. In Rat-1 cells, a Bcl-2 mutant targeted exclusively to the endoplasmic reticulum (Bcl-cb5) was effective at inhibiting apoptosis induced by serum starvation/*myc*, or ceramide but not apoptosis induced by etoposide. The former conditions cause a decrease in mitochondrial transmembrane potential ($\Delta\psi_m$) as an early event that precedes the release of cytochrome *c* from mitochondria. By contrast, when cells are exposed to etoposide, a situation in which cytochrome *c* release and membrane localization of the pro-apoptotic protein Bax precede loss of $\Delta\psi_m$, wild type Bcl-2 but not Bcl-cb5 prevents apoptosis. Therefore, Bcl-2 functions in spatially distinct pathways of apoptosis distinguished by the order of cytochrome *c* release and loss of $\Delta\psi_m$. *Oncogene* (2001) **20,** 1939–1952.

Keywords: apoptosis; Bax; Bcl-2; cytochrome *c*; endoplasmic reticulum

Introduction

A large body of data supports the proposal that events localized at the mitochondria serve as sensors, regulators and amplifiers of apoptosis (reviewed in Green and Kroemer, 1998). Bcl-2 localization to the outer mitochondrial membrane is consistent with its role in regulating many forms of apoptosis upstream of the caspase effector sequence (Kroemer, 1997). However, Bcl-2 is also found at other intracellular membrane sites such as the endoplasmic reticulum (ER) and nuclear membrane (Zhu *et al*., 1996). To directly address the physiological relevance of the specific intracellular localization of Bcl-2, we created a mutant form of Bcl-2 in which the endogenous insertion sequence, responsible for membrane targeting and integration, was replaced by one from the ER specific isoform of cytochrome b5 (Zhu *et al*., 1996). This mutant, designated Bcl-cb5, is targeted exclusively to the ER, and is capable of inhibiting apoptosis induced by either serum starvation and *myc* overexpression in Rat-1 fibroblasts, or exposure to brefeldin A or tunicamycin in rat embryo fibroblasts, but incapable of preventing serum starvation induced apoptosis in the Madin Darby canine kidney epithelial cell line (Zhu *et al*., 1996; Haecki *et al*., 2000).

What determines whether Bcl-cb5 is effective? The difference may be due to a cell specific effect (epithelial cells versus fibroblasts), where some cell types activate an apoptotic pathway that can be inhibited at the ER. This distinction is analogous to the recent proposal by Krammer's group, where two types of cells were identified that differ in their dependence on the apoptogenic activity of mitochondria. The presence of mitochondrial dysfunction then determines whether Bcl-2/Bcl-XL can inhibit Fas mediated apoptosis in that cell type (Scaffidi *et al*., 1998). This model predicts that if any form of apoptosis in Rat-1 fibroblasts is blocked by Bcl-cb5 then all forms of apoptosis would be inhibited by Bcl-cb5. However, we have recently reported that Bcl-2, but not Bcl-cb5, can prevent etoposide induced apoptosis in Rat-1 cells (Lee *et al*., 1999). Thus, both ER-dependent and ER-independent apoptosis pathways are present within the same cell.

Are Bcl-cb5 and Bcl-2 regulating apoptosis via a similar mechanism? In many different cell types, the sequential reduction of the $\Delta\psi_m$ and generation of reactive oxygen species are early changes in mitochondrial function that may or may not directly mediate the later manifestations of apoptosis, including alterations in the plasma membrane and fragmentation of DNA (Zamzami *et al*., 1995, 1996; Marchetti *et al*., 1996; Susin *et al*., 1996). Many metabolic alterations can affect mitochondrial membrane potential. However, pharmacologic inhibition of the disruption of $\Delta\psi_m$ prevents later manifestations of apoptosis in many experimental systems, suggesting that it may function as a critical coordinating step (Zamzami *et al*., 1996). The release to the cytoplasm of cytochrome *c* from the mitochondrial intermembrane space is another key event in activating the downstream effector phase of apoptosis (Zou *et al*.,

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1997). The relative role played by cytochrome *c* release as compared to the loss of $\Delta\psi_m$ in activating the irreversible effector phase of apoptosis has yielded conflicting results in different cell systems (Bossy-Wetzel *et al*., 1998; Petit *et al*., 1998; Finucane *et al*., 1999). Our data provide a possible resolution of the controversy regarding the order of loss of $\Delta\psi_m$ and cytochrome *c* release. By determining whether apoptosis can be inhibited by Bcl-cb5 we have identified two spatially distinct pathways for apoptosis present in Rat-1 cells. Apoptosis elicited by stimuli that lead to a decrease in $\Delta\psi_m$, that precedes cytochrome *c* release is effectively inhibited by Bcl-cb5. By contrast, in the same cell type, Bcl-cb5 is not effective at inhibiting apoptosis induced by stimuli that cause mitochondrial translocation of Bax and redistribution of cytochrome *c* at a time when decreased $\Delta\psi_m$ is not yet apparent. Thus two pathways, distinguishable by the order of early changes at the mitochondria, can be activated in the same cell, but only one pathway is regulated by events at the ER.

Results

Bcl-cb5 prevents mitochondrial changes induced by serum starvation/myc

Serum starvation and constitutive c-*myc* overexpression induces apoptosis efficiently in Rat-1 fibroblasts (Evan *et al*., 1992). A mutant Bcl-2 targeted exclusively to the endoplasmic reticulum (Bcl-cb5) is as active as wild type Bcl-2 in preventing this form of cell death, as measured by changes in nuclear morphology and clonal survival (Zhu *et al*., 1996). To determine whether Bcl-cb5 prevented apoptosis upstream or downstream of mitochondrial changes we measured the decrease in $\Delta\psi_m$ and generation of reactive oxygen species during apoptosis in Rat-1 cells. In vector-transfected control cells, inducing apoptosis by serum starvation and c-*myc* overexpression lead to decreased $\Delta\psi_m$ in 46% of cells as measured by $DIOC_6(3)$ fluorescent intensity, and the generation of reactive oxygen species in 10% of cells after 18 h. In contrast, these values were 10 and 3% in untreated cells, respectively (Figure 1a, control). Apoptosis induced by serum starvation/*myc* is also associated with other features such as the presence of phosphatidyl serine on the cell surface (PS, Figure 1b) and fragmentation of nuclear DNA (Figure 1c). Both of these features are characteristic of programmed cell death as a result of mitochondrial dysfunction (Zamzami *et al*., 1995; Marchetti *et al*., 1996). As expected from previous observations (Susin *et al*., 1996; Zhu *et al*., 1996), expression of Bcl-2 prevented all of these manifestations of apoptosis (Figure 1a–c). Significantly, Bcl-cb5 was as effective as Bcl-2 in preventing these features of apoptosis, both mitochondrial (Figure 1a) and post-mitochondrial (Figure 1b,c). Thus, Bcl-2 does not have to be localized to mitochondria to prevent the loss of $\Delta\psi_m$ associated with the early phase of apoptosis caused by serum starvation/*myc*.

Bcl-cb5 inhibits the mitochondrial changes associated with ceramide and staurosporine induced apoptosis

We previously reported that other agents including etoposide and tert-butyl hydroperoxide induce apoptosis that is not blocked by Bcl-cb5 (Zamzami *et al*., 1998; Lee *et al*., 1999). Therefore, the question arose as to whether apoptosis that is blocked by Bcl-cb5 resulted from activation of an unique pathway induced by serum starvation/*myc* but not other agents. As part of a screen of agents that induce apoptosis that can be blocked by Bcl-2, we discovered that Bcl-cb5 protects Rat-1 fibroblasts from apoptosis after a 6 h exposure to either ceramide or the kinase inhibitor staurosporine. Thus, Bcl-cb5 prevented loss of $\Delta\psi_m$ (Figure 2a), externalization of phosphatidyl serine (PS, Figure 2b), and fragmentation of DNA (Figure 2c) during apoptosis induced by either of these agents. The identification of multiple stimuli that induce a program of apoptosis that can be blocked by Bcl-cb5 argues that the pathway blocked by Bcl-cb5 may be a relevant response to many agents and in other cell types.

Caspases are critical and conserved components of the effector phase of apoptosis that directly degrade many cellular macromolecules, as well as activating other enzymes that degrade DNA (Thornberry and Lazebnik, 1998). As a consequence, inhibition of caspase activity prevents many, but not all, of the features of programmed cell death (Xiang *et al*., 1996 McCarthy *et al*., 1997). The role of caspases in the earlier recognition and regulatory phases of apoptosis is more variable, and depends upon the cell type and specific stimulus for apoptosis (Green and Kroemer, 1998). Moreover, the decrease in $\Delta\psi_m$ during apoptosis can occur by both caspase-dependent, and -independent pathways (Susin *et al*., 1997). If the decrease in $\Delta\psi_m$ caused by serum starvation/*myc*, ceramide or staurosporine depends on prior activation of caspases this may explain why Bcl-2 localized to the endoplasmic reticulum can prevent this mitochondrial dysfunction. To investigate whether the stabilization of the mitochondrial transmembrane potential by Bcl-cb5 was due to a caspase dependent event we determined whether the decrease in $\Delta\psi_m$ caused by staurosporine or ceramide in Rat-1 cells could be prevented by the general caspase inhibitor zVAD-fmk. In both cases, addition of zVAD-fmk had no effect on the decrease in $\Delta\psi_m$ in vector-transfected cells (Figure 2a). However, addition of zVAD-fmk resulted in partial inhibition of externalization of phosphatidyl serine after exposure of vector-transfected cells to either staurosporine or ceramide (Figure 2b). By contrast, zVAD-fmk completely prevented DNA fragmentation after exposure to either drug (Figure 2c), consistent with the caspase dependent activation of a DNAse during apoptosis (Enari *et al*., 1998; Sakahira *et al*., 1998). Thus, staurosporine and ceramide elicit a multistep apoptotic pathway, which includes components that are both caspase dependent and independent. However, the early step that results in the decrease in $\Delta\psi_m$, that is inhibited by Bcl-cb5, is

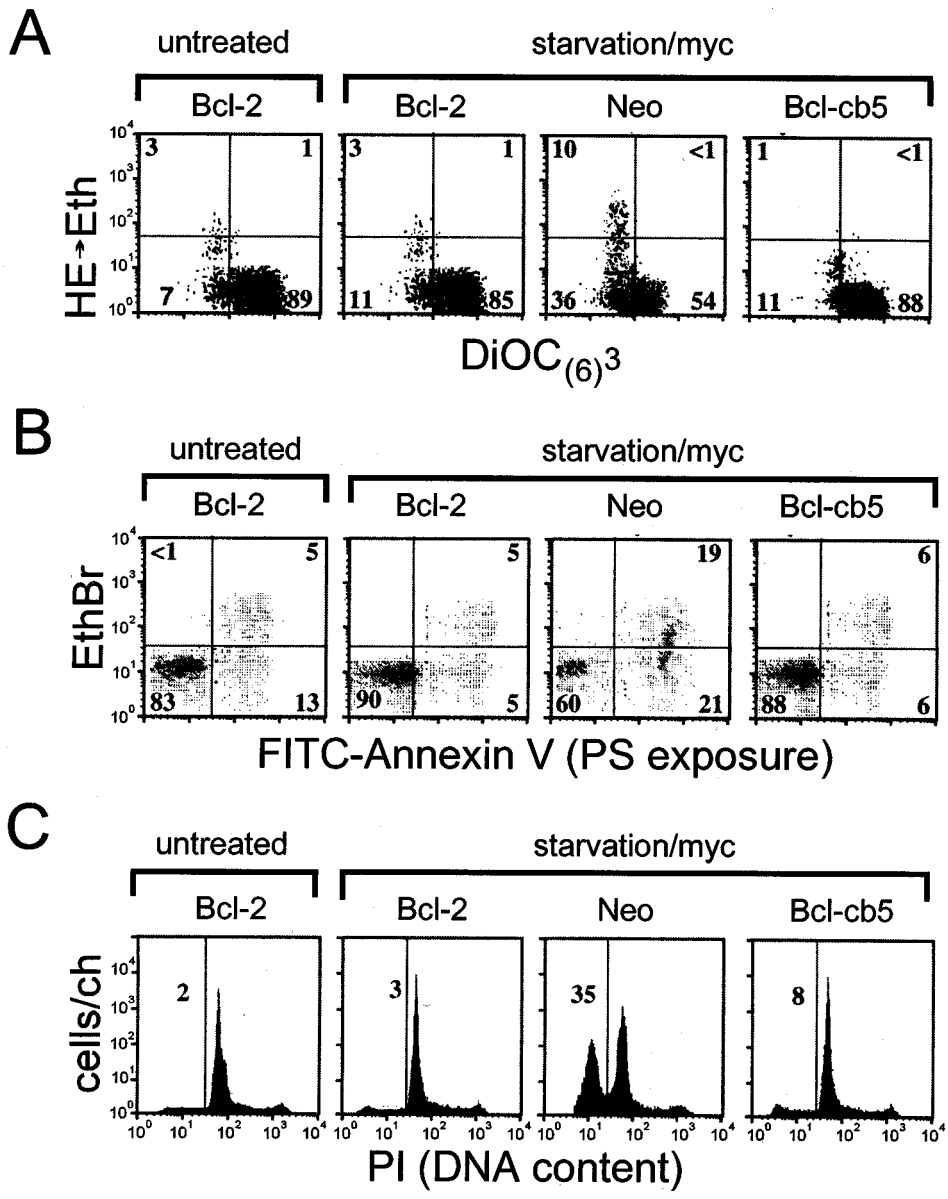

Figure 1 Bcl-2 and the endoplasmic reticulum targeted Bcl-cb5 prevent apoptosis due to serum starvation/*myc*. Cytofluorometric analysis of apoptosis associated parameters in Rat-1 cells expressing Bcl-2, Bcl-cb5 or in cells transfected with a control vector (Neo) after culture for 24 h in 0.1% FCS, 2 μM estradiol (starvation/*myc*) or 5% FCS (untreated). (**a**) Loss of $\Delta\psi_m$ determined by the potential sensitive dye $DIOC_6(3)$, ROS generation leading to oxidation of (hydroethidine) HE to (ethidium) Eth. (**b**) Exposure of phosphatidylserine (PS) on the plasma membrane detected by Annexin V-FITC conjugate and loss of ethidium bromide (EthBr) exclusion. (**c**) Propidium iodide (PI) detection of loss of nuclear DNA (sub-diploidy). Results are representative of three independent experiments

not dependent on caspase activation. Thus, Bcl-cb5 does not prevent the loss of $\Delta\psi_m$ by inhibiting cytoplasmic or endoplasmic reticulum associated caspases (Nakagawa *et al*., 2000).

Decrease in $\Delta\psi_m$ precedes cytochrome c *release in serum starvation*/myc *and ceramide induced apoptosis*

The release of cytochrome *c* to the cytoplasm from the intermembrane space of mitochondria can activate the downstream effector phase of apoptosis by binding to apaf-1 and caspase 9 (Zou *et al*., 1997). In some systems, cytochrome *c* release is believed to precede loss of $\Delta\psi_m$ (Kluck *et al*., 1997; Yang *et al*., 1997). However, in other circumstances, decreased $\Delta\psi_m$ is the prior event (Petit *et al*., 1998). To determine if the temporal order of cytochrome *c* release and decrease in $\Delta\psi_m$ is a distinguishing feature of the ability of Bcl-cb5 to block apoptosis, individual cells undergoing apoptosis were examined. We used laser scanning confocal microscopy to monitor changes to $\Delta\psi_m$, using the potentiometric dye Mitotracker, and the localization of

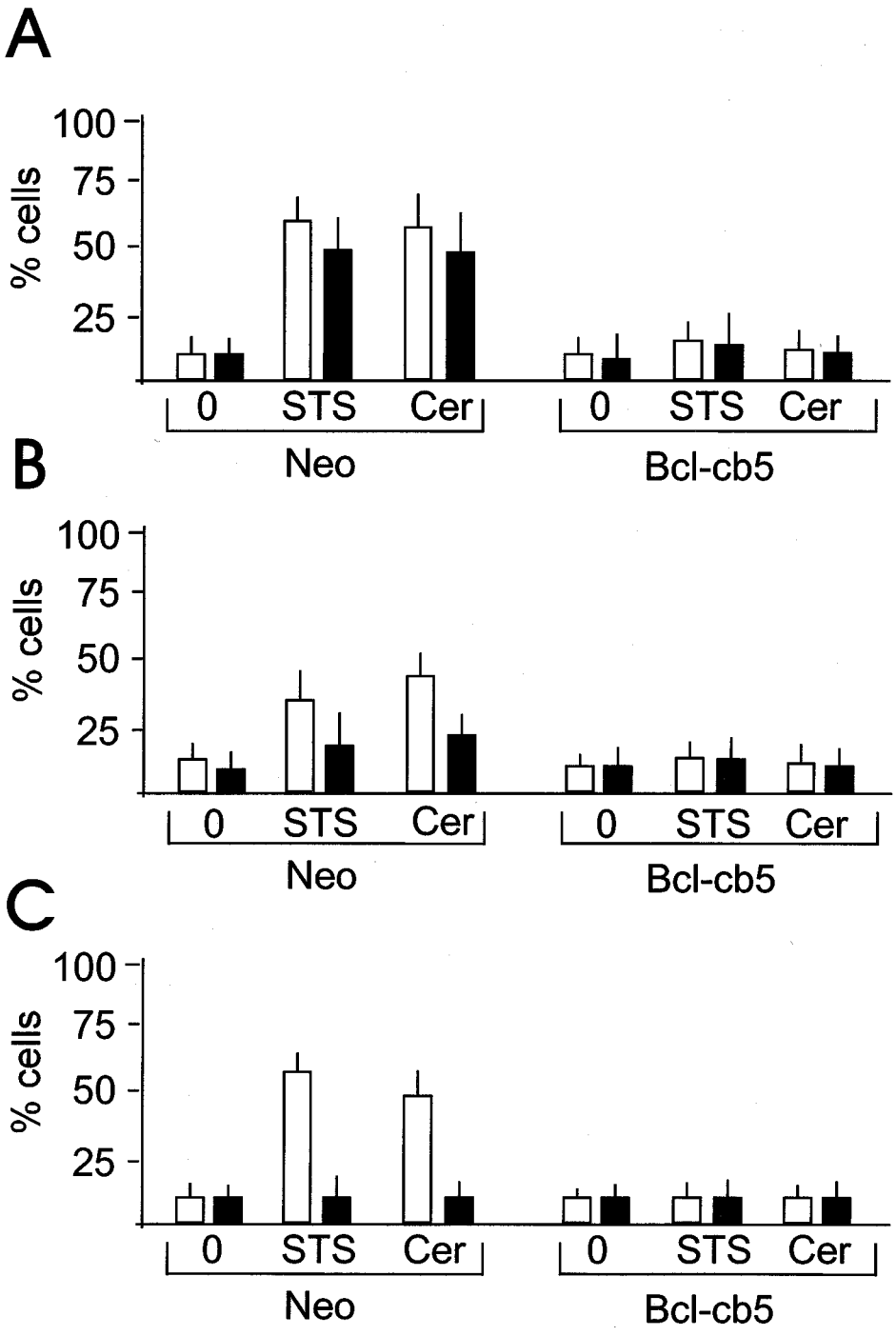

Figure 2 Bcl-cb5 prevents induction of apoptosis by ceramide (Cer) or staurosporine (STS) in the presence or absence of zVAD-fmk. Rat-1 fibroblasts cultured with 50 μM ceramide or 100 nM staurosporine in the presence (solid bars) or absence (open bars) of 50 μM zVAD-fmk for 12 h followed by cytofluorometric analysis of (**a**) $\Delta\psi_m$; (**b**) phosphatidylserine exposure on the plasma membrane; (**c**) sub-diploidy. Experiments were repeated three times with similar results

cytochrome *c*, using affinity purified antibodies to cytochrome *c*, in cells exposed to either serum starvation/*myc*, ceramide or etoposide (Figures 3 and 4 and data not shown). In a separate experiment, cells were stained with either Mitotracker or with affinity purified antibodies to cytochrome *c* and co-stained with a monoclonal antibody that recognizes the mitochondrial matrix chaperone Hsp-60 (Figure 3 and data not shown).

In vector-transfected cells growing in normal serum, the mitochondria retain cytochrome *c* and $\Delta\psi_m$ as evidenced by cytochrome *c* staining and the accumulation of Mitotracker dye, respectively (Figures 3 and 4). When these cells were exposed to the protonophore m-

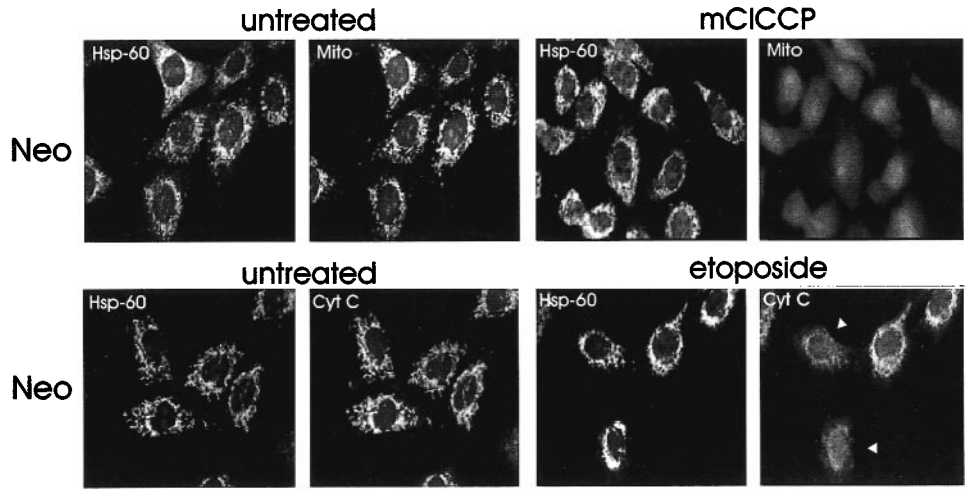

Figure 3 Disruption of $\Delta\psi_m$ or release of cytochrome *c* does not alter the localization of the mitochondrial matrix protein Hsp-60. Cells were costained either with antibodies to Hsp-60 (Hsp-60) and cytochrome *c* (Cyt C) to visualize cytochrome *c* release or with antibodies to Hsp-60 and with Mitotracker (Mito) to visualize $\Delta\psi_m$. Compared to untreated controls the protonophore m-CICCP (50 μM for 15 min) caused complete loss of Mitotracker staining in vector transfected cells (Neo) indicating that staining intensity is proportional to $\Delta\psi_m$. Incubation in 40 μM etoposide for 6 h led to release of cytochrome *c* in 20% of vector transfected cells (Neo) co-stained for Hsp-60 and cytochrome *c* (arrowheads)

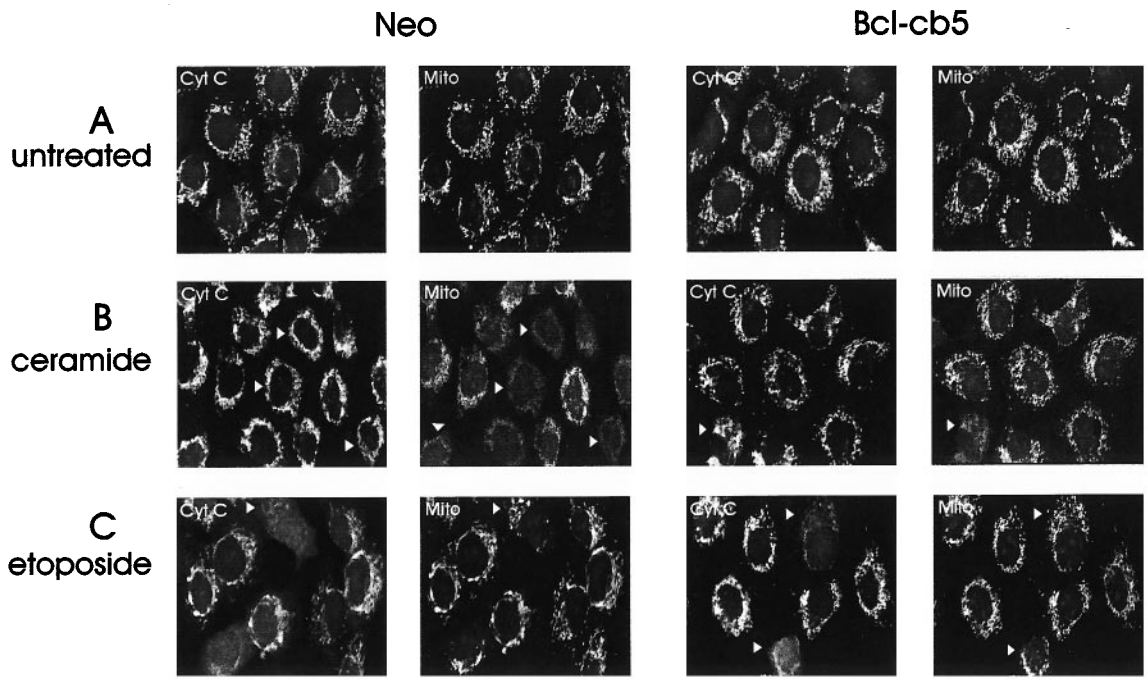

Figure 4 Cytochrome *c* (Cyt C) release precedes loss of $\Delta\psi_m$ (Mito) in etoposide induced apoptosis. (**a**) In untreated, vector transfected (Neo) and Bcl-cb5 expressing Rat-1 cells grown in normal medium, in co-stained cells the staining patterns for cytochrome *c* and Mitotracker, were identical. (**b**) Exposure to 50 μM ceramide for 6 h resulted in decreased Mitotracker staining (arrowheads) of vector transfected cells without release of cytochrome *c*. Loss of Mitotracker staining was dramatically reduced by expression of Bcl-cb5. (**c**) Incubation in 40 μM etoposide for 6 h resulted in diffuse mitochondrial staining for cytochrome *c* but no loss in Mitotracker staining (arrowheads) in an equal proportion of vector control (Neo) and Bcl-cb5 cells. The width of each image is 144 μm

CICCP to induce a loss of mitochondrial membrane potential in all of the cells, the Mitotracker was no longer localized to mitochondria but was found throughout the cell. In contrast there is no change in Hsp-60 or cytochrome *c* staining (Figure 3 and data not shown).

In vector-transfected cells exposed to serum starvation/*myc* for 24 h, there is no evidence of cytochrome *c* release from mitochondria (data not shown), however at this time point 26% of the cells showed decreased $\Delta\psi_m$ (Table 1). Both Bcl-2 and Bcl-cb5 prevented loss of $\Delta\psi_m$ with equal efficiency in these cells as measured

Table 1 Loss of mitochondrial membrane potential assessed by confocal microscopy

Plasmid	*Serum starvation/myc* (24 h)	*Ceramide* (6 h)
Neo	26%, $n=129$	64%, $n=94$
Bcl-cb5	8%, $n=131$	16%, $n=112$
Bcl-2	13%, $n=156$	18%, $n=95$

Mitochondrial membrane potential was assessed visually by confocal microscopy of cells labeled with Mitotracker and antibodies to Hsp-60. In each of the conditions cells were counted from eight fields of view accumulated from at least two separate coverslips. The fraction of cells with reduced mitochondrial membrane potential is expressed as a percentage, *n* is the total number of cells counted

by Mitotracker staining (Table 1) and cytofluorometry with $DiOC_6 3$ (Figure 1a). The fact that both dyes give qualitatively similar results even though in one case cells are detached from the growth support argues that these measurements accurately reflect membrane potential changes.

Similar to serum starvation/*myc*, exposure of vector-transfected cells to ceramide for 6 h resulted in a significant proportion of cells demonstrating a loss of $\Delta\psi_m$ (Figures 2a and 4b, arrowheads). Assay of mitochondrial transmembrane potential at 1.5, 3 and 6 h revealed that the total proportion of cells with reduced $\Delta\psi_m$ after exposure to ceramide increased over time (data not shown). After incubation in ceramide for 6 h, 64% of the cells had reduced $\Delta\psi_m$ (Table 1). By contrast, less than 20% of Bcl-2 or Bcl-cb5 expressing cells exhibited reduced $\Delta\psi_m$ after 6 h exposure to ceramide (Table 1 and Figure 4b, arrowheads). Similar to serum starvation/*myc*, when cells were treated with ceramide, cytochrome *c* release was not observed in either vector control or Bcl-cb5 cells, even in cells with reduced $\Delta\psi_m$ (Figure 4b). Despite the lack of cytochrome *c* release, cleavage of the caspase substrate PARP is evident in vector control cells subjected to serum starvation/*myc* or ceramide at time points where we observe loss of $\Delta\psi_m$ (Figure 5). Expression of Bcl-cb5 delayed PARP cleavage with both apoptotic stimuli.

If loss of $\Delta\psi_m$ is due to permeability transition then exposure to the permeability transition inhibitor cyclosporin A should be delay the loss $\Delta\psi_m$ by approximately 60 min (Zamzami *et al*., 1995). When Rat-1 cells were treated with cyclosporin A and ceramide we were unable to detect a delay in loss of $\Delta\psi_m$ as measured by Mitotracker staining (data not shown). Therefore within the temporal resolution of our experimental system the loss of $\Delta\psi_m$ induced by ceramide in adherent cells appears to be mechanistically distinct from permeability transition.

In contrast to cells subjected to serum starvation/*myc* or ceramide, treatment with etoposide for 6 h, resulted in diffuse cytochrome *c* staining in 20% of the adherent cells, a result consistent with release of mitochondrial cytochrome *c* into the cytoplasm (Figures 3 and 4c, arrowheads). Even though cytochrome *c* staining was diffuse (cytoplasmic), staining

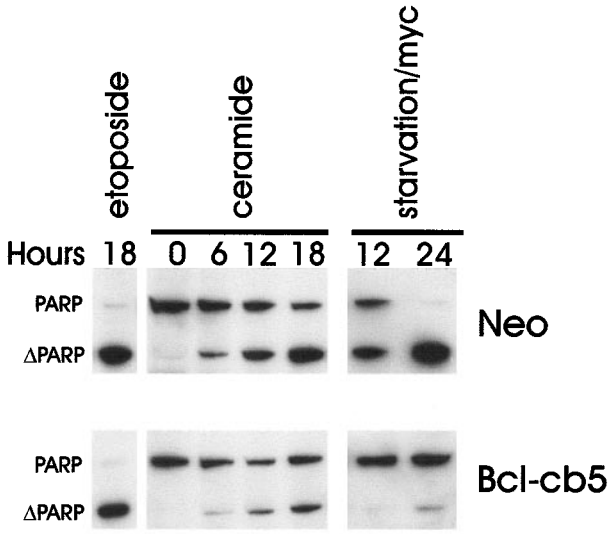

Figure 5 Bcl-cb5 inhibits PARP cleavage during apoptosis induced by ceramide or serum starvation/*myc* (starvation/*myc*), but not etoposide. Whole cell extracts were prepared from Neo or Bcl-cb5 cells (as indicated to the right of the panels) growing in regular medium (0 h), or exposed to 50 μM ceramide, serum starvation/*myc* (starvation/*myc*), or 40 μM etoposide for the number of hours indicated. The migration positions of PARP and its major cleavage product (ΔPARP) are indicated to the left and the cell line (Neo or Bcl-cb5 expressing) is indicated to the right of the panels

for Hsp60 remained mitochondrial (Figure 3). This result is consistent with loss of integrity of the outer but not inner mitochondrial membrane. However, it is evident from the micrographs that even in the cells with diffuse staining, a proportion of the cytochrome *c* was still found to co-localize with mitochondria (Figures 3 and 4c). PARP cleavage was evident in the cells by 6 h after adding etoposide, confirming that apoptosis was well underway (data not shown). However, at this time point none of the cells had decreased $\Delta\psi_m$, including those with diffuse staining for cytochrome *c* (Figure 4c, arrowheads). Consistent with the inability of Bcl-cb5 to prevent etoposide induced apoptosis, the proportion of Bcl-cb5 expressing cells with diffuse cytochrome *c* staining was the same as that for vector control (Figure 4c).

Bcl-cb5 does not prevent etoposide mediated release of cytochrome c

Selective release of cytochrome *c* is most often assayed by immunoblotting of cell fractions. It is possible that cytochrome *c* release would appear earlier when assayed by blotting than it does in micrographs of adherent cells, because both adherent and non-adherent cells are assayed. Furthermore, fragmentation of mitochondria during cell breakage and fractionation (perhaps symptomatic of the initial swelling of mitochondria reported to precede rupture of the outer membrane (Petit *et al*., 1998; Vander Heiden *et al*., 1997)) may also increase the apparent release of cytochrome *c*. Therefore, we assayed cell fractions by

immunoblotting with affinity purified antibodies to cytochrome *c*. The sub-cellular fractions were also probed with antibodies to Hsp60 as a mitochondrial marker. Unlike the commonly used marker for mitochondria (the integral membrane protein CoxIV) Hsp60 is a soluble protein located in the mitochondrial matrix therefore, its distribution is also an indicator of the integrity of the inner mitochondrial membrane. In order to break cells without exposing them to hypotonic solutions (known to compromise the outer mitochondrial membrane) we used low pressure nitrogen cavitation. The use of nitrogen cavitation for cell fractionation offers substantial benefits as cells are broken in iso-osmotic buffer and cavitation results in decreased rupture of outer mitochondrial membranes as compared to mechanical homogenization (Adachi *et al*., 1998). Furthermore, cell lysis is more efficient with this technique than with Dounce homogenization in iso-osmotic solutions.

Mitochondria isolated from normal cells ruptured by nitrogen cavitation can be pelleted at lower *g* forces (1000 *g*, P^1) than mitochondria isolated by conventional means (typically 10–15 000 *g* depending on cell type or tissue source). This result indicates that mitochondria, which are large elongated structures in these cells, are relatively intact when recovered compared to other homogenization protocols (Adachi *et al*., 1998). Therefore, after removal of nuclei and cell debris from whole cell lysates by centrifugation at ~480 *g* for 2 min, the distribution of cytochrome *c* was examined by subjecting 100 microliter aliquots of lysate to centrifugation at 1000 *g* for 25 min. Centrifugation over a short distance at low *g* force permitted quantitative recovery of mitochondria (as judged by blotting for Hsp60). Immunoblot analysis demonstrated that on average 95% of the cytochrome *c* and 96% of the Hsp-60 in untreated (vector-transfected, Bcl-cb5 transfected and Bcl-2 transfected) cells pelleted at 1000 *g* after homogenization by nitrogen cavitation (Figure 6a). The variation in total protein recovered in the fractions was approximately 20% for cytochrome *c* and 15% for Hsp-60 (data not shown). In contrast other attempts to assay cytochrome *c* release in Rat-1 cells reported significant amounts of cytochrome *c* in the cytosol of untreated cells (Juin *et al*., 1999). We interpret our results as indicating that the mitochondria in Rat-1 cells remain largely intact during processing by low pressure nitrogen cavitation although cell breakage was very efficient as assessed by direct visualization and by immunoblotting for a cytosolic protein (data not shown).

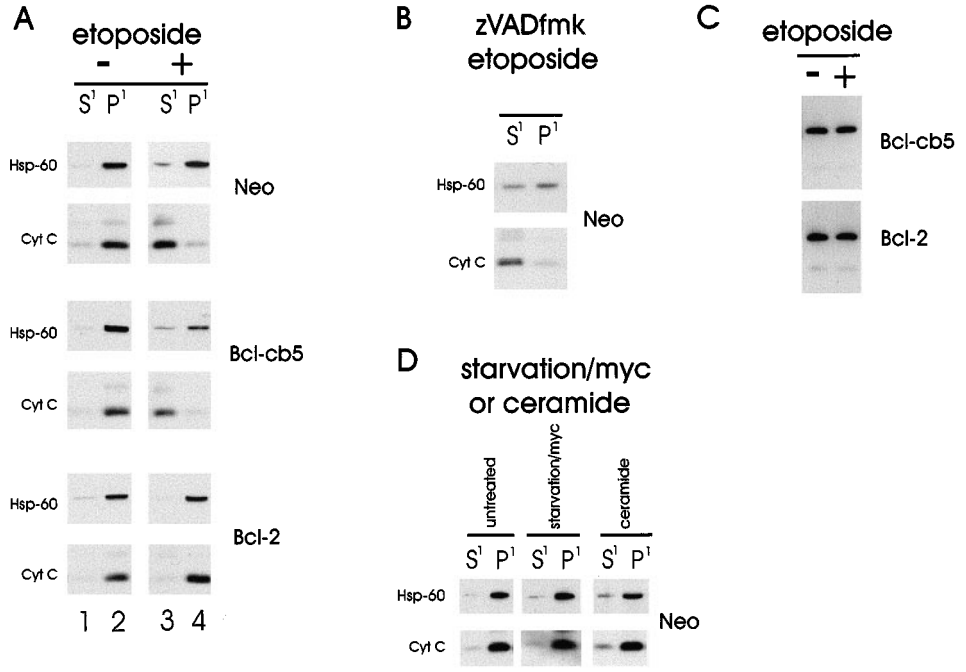

Figure 6 Etoposide induced release of cytochrome *c* is caspase independent. Whole cell extracts were prepared from control or etoposide treated cells (40 μM, 6 h) by low pressure nitrogen cavitation, nuclei and cell debris was removed by centrifugation and the resulting whole cell lysate was fractionated into a low speed supernatant (S^1) and pellet (P^1) by centrifugation at 1000 *g* for 25 min. (**a**) Vector transfected Rat-1 (Neo), Bcl-cb5 expressing (Bcl-cb5), and Bcl-2 (Bcl-2) expressing cells (as indicated to the right of the panels) were treated with etoposide (+) or left untreated (−). Equal cell volumes of the supernatant and pellet were immunoblotted with affinity purified anti-cytochrome *c* (Cyt C) or anti-Hsp60 (Hsp-60) antibodies as indicated to the left of the panels. (**b**) Vector transfected Rat-1 cells (Neo) were incubated with 200 μM zVAD-fmk for 30 min and then treated with etoposide. (**c**) Five micrograms of whole cell lysate of control (−) and etoposide (+) treated Bcl-cb5 (Bcl-cb5) or Bcl-2 (Bcl-2) expressing cells (as indicated to the right of the panels) were immunoblotted with anti-Bcl-2 antibodies. (**d**) Low speed supernatant (S^1) and pellet (P^1) fractions from vector transfected Rat-1 cells (Neo) exposed to serum starvation/*myc* for 24 h or 50 μM ceramide for 6 h were analysed by immunoblotting for Hsp-60 and cytochrome *c* (Cyt C) as indicated to the left of the panels

We have previously reported that exposure of Rat-1 cells to 40 micromolar etoposide for 18 h is associated with extensive apoptosis, loss of $\Delta\psi_m$ in more than 50% of cells, and loss of ability to exclude propidium iodide in 30% of cells (Lee *et al*., 1999). In this situation Bcl-2, but not Bcl-cb5, prevented both loss of $\Delta\psi_m$ and cleavage of poly-ADP ribose polymerase (PARP), a substrate for caspase-3 (Figure 5, and Lee *et al*., 1999). Unlike Bcl-cb5, which is located exclusively on the cytoplasmic face of the endoplasmic reticulum (Zhu *et al*., 1996), Bcl-2 is located on both the outer mitochondrial membrane and the cytoplasmic face of the ER (Janiak *et al*., 1994). Therefore, our results indicate that Bcl-2 localized to the mitochondrial membrane prevented apoptosis due to etoposide. To determine when cytochrome *c* is released during apoptosis via a mechanism that cannot be blocked by Bcl-cb5, we assessed the sub-cellular distribution of cytochrome *c* by confocal microscopy (Figure 4) and by immunoblotting of cell fractions prepared from etoposide treated cells (Figure 6a).

When we examined the fractionation of cytochrome *c* in cells treated with etoposide the majority of cytochrome *c* (88%) was found in the 1000 *g* supernatant. A small amount of Hsp-60 was also found in the 1000 *g* supernatant fraction but most of it (87%) was found in the 1000 *g* pellet, confirming that after exposure to etoposide, mitochondrial matrix proteins remain associated with very large structures. This result also demonstrates that the cytochrome *c* recovered in the 1000 *g* supernatant fraction is not due to fragmentation of the mitochondria.

Bcl-cb5 does not prevent apoptosis due to etoposide, therefore, it was not surprising that Bcl-cb5 does not prevent etoposide induced release of cytochrome *c* into the 1000 *g* supernatant (Figure 6a) or the cleavage of PARP that reflects the activation of caspases by cytochrome *c* (Figure 5). In etoposide treated cells expressing Bcl-cb5, 88% of the cytochrome *c* was recovered in the 1000 *g* supernatant. In contrast, less than 3% of the cytochrome *c* was found in the 1000 *g* supernatant in similarly treated Bcl-2 expressing cells. Thus, in etoposide treated cells, release of cytochrome *c* into the 1000 *g* supernatant correlates with apoptosis. We assume that the main reason that more cytochrome *c* release is detected by fractionation than by confocal microscopy is because both adherent and non-adherent cells are analysed by fractionation.

To determine whether or not redistribution of cytochrome *c* from the 1000 *g* pellet to the 1000 *g* supernatant was dependent on activated caspases we treated cells with the broad spectrum caspase inhibitor zVAD-fmk. This inhibitor abolishes many of the features of apoptosis in etoposide treated, vector-transfected, Rat-1/*myc* cells. However, redistribution of cytochrome *c* was not blocked by zVAD-fmk, demonstrating that activated caspases are not essential for this process (Figure 6b). To determine if the inability of Bcl-cb5 to prevent apoptosis in etoposide treated cells was due to selective caspase cleavage or degradation of Bcl-cb5, cell lysates were analysed by immunoblotting. Comparison of untreated controls with lysates from cell exposed to etoposide for 6 h revealed no significant change in the amount or integrity of either Bcl-2 or Bcl-cb5 (Figure 6c).

In contrast to etoposide treated cells, at early time-points during apoptosis due to serum starvation/*myc* or exposure to ceramide both cytochrome *c* and Hsp-60 were found almost exclusively in the 1000 *g* pellet (Figure 6c). Thus early 'release' of cytochrome *c* is not observed when apoptosis is induced by a mechanism that can be blocked by Bcl-cb5.

Bcl-cb5 does not prevent the etoposide induced conformational change in or membrane translocation of Bax

Previous work suggests that the pro-apoptotic family member Bax can induce the release of mitochondrial cytochrome *c* (Eskes *et al*., 1998). Furthermore, during apoptosis, Bax undergoes a conformational change that allows it to integrate into mitochondrial membranes. This change in Bax structure results in the exposure of a normally inaccessible epitope that is recognized by the monoclonal antibody 6A7. To determine if etoposide induced release of cytochome *c* is associated with these changes in Bax, we examined the subcellular localization and conformation of Bax by cell fractionation and confocal microscopy in Rat-1 cells exposed to etoposide or ceramide for 6 h.

Cells were disrupted by nitrogen cavitation and the lysates subjected to centrifugation to separate cytosolic and membrane bound fractions. To determine if proteins found in the pellet were integrated into any cellular membranes, a 100 000 *g* pellet was incubated with sodium carbonate (pH 11.5) and then separated into soluble and membrane fractions by centrifugation. Consistent with other published results, in untreated cells Bax was not membrane bound in either Bcl-cb5 or vector transfected control cells (Figure 7). Exposure to etoposide does not change the total amount of Bax in cells (Figure 7), but rather causes a re-distribution of approximately 25% (average of two independent experiments) of Bax to a membrane bound fraction in both vector and Bcl-cb5 cells (Figure 7). Relocalization of Bax to membranes is not observed in either vector or Bcl-cb5 cells exposed to ceramide for 6 h (Figure 7).

To confirm that a major site of Bax membrane integration was mitochondria, and to determine whether or not Bax localization in mitochondria was co-incident with cytochrome *c* release, localization of 6A7 positive Bax was monitored by confocal microscopy. As expected, the Bax conformation specific epitope recognized by 6A7 is not exposed in untreated cells (Figure 8a). However, etoposide treated cells showed staining of mitochondria with 6A7 yet retained Mitotracker stain (Figure 8b,c, right two panels). Thus Bax localization at mitochondria precedes loss of $\Delta\psi_m$.

The fraction of cells staining with 6A7 did not differ between vector and Bcl-cb5 expressing cells at three different time points (data not shown). Moreover,

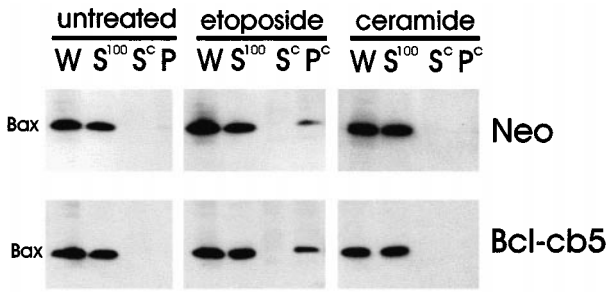

Figure 7 Etoposide, but not ceramide induces Bax membrane binding. Whole cell extracts (W) were prepared by low pressure nitrogen cavitation and then 100 microliters was separated into supernatant (S^{100}) and pellet (P^{100}) fractions by centrifugation at 100 000 *g* for 1 h. The pellet fraction was resuspended and then incubated in 0.1 M sodium carbonate, pH 11.5 for 30 min at 4°C. Membrane bound proteins (P^c) were separated from peripheral proteins (S^c) by centrifugation at 100 000 *g* for 30 min. Ten micrograms of whole cell lysate and equivalent cell volumes of the supernatant and pellet were immunoblotted with 1D1 anti-Bax antibody. The migration position of Bax is indicated to the left of the panels. Vector transfected Rat-1 cells (Neo) or cells expressing Bcl-cb5 (Bcl-cb5) were untreated or exposed to either 40 μM etoposide or 50 μM ceramide for 6 h

those cells that stained for 6A7 had lost the mitochondrial staining pattern for cytochrome *c*. Conversely, cells that retained cytochrome *c* within mitochondria did not stain with 6A7 above background (Figure 8b,c). This result is congruent with biochemical studies suggesting that translocation of Bax to mitochondria is sufficient to provoke cytochrome *c* release *in vitro* (Eskes *et al*., 1998). Unlike Bcl-cb5, expression of Bcl-2 in these cells was sufficient to block the conformation change in Bax that exposes the epitope recognized by 6A7 (data not shown). Taken together, these results suggest that Bcl-2 must be localized to mitochondria to prevent the etoposide induced conformational change in Bax and release of cytochrome *c*. In contrast to cells treated with etoposide, cells exposed to ceramide retained cytochrome *c* (Figure 8d,e, left two panels) and did not stain with 6A7 even in those cells in which loss of $\Delta\psi_m$ abolished Mitotracker staining (Figure 8d,e, right two panels).

Discussion

Examination of the temporal ordering of loss of $\Delta\psi_m$ and release of cytochrome *c* in response to different apoptotic stimuli revealed two spatially distinct pathways of apoptosis in Rat-1 fibroblasts. In the pathway in which cytochrome *c* release precedes loss of $\Delta\psi_m$ (e.g. due to etoposide), apoptosis is characterized by an induced structural change in Bax and relocalization of Bax to mitochondrial membranes. Both of these changes can be blocked by Bcl-2 molecules located at the mitochondria. In contrast, when loss of $\Delta\psi_m$ (measured by cytofluorometry using potential sensitive dye $DIOC_6(3)$ (Figures 1 and 2) or DilCl(5) (Figures 1, 2 and 5 in Lee *et al*., 1999) or by confocal microscopy using Mitotracker (Figure 4) precedes release of cytochrome *c*, there is no change in the conformation of Bax, nor is it relocated to mitochondria (Figures 7 and 8d,e). Nevertheless, this form of cell death is apoptosis, as measured by changes in nuclear morphology (Zhu *et al*., 1996), exposure of PS on the outer leaflet of the plasma membrane (Figure 2b), fragmentation of nuclear DNA (Figure 2c), and cleavage of PARP (Figure 5). This form of apoptosis was inhibited by an isoform of Bcl-2 located exclusively at the endoplasmic reticulum (Bcl-cb5). Thus, the two pathways are spatially distinct in Rat-1 cells.

To determine how Bcl-cb5 selectively blocks apoptosis due to serum starvation/*myc* or ceramide but not apoptosis induced by etoposide, we examined several of the known steps in apoptosis. When we examined serum starvation/*myc* induced apoptosis by cytofluorometry in Rat-1 cells, we observed the well characterized sequences of dysfunction reported in other cell types: abolition of the $\Delta\psi_m$ with concomitant generation of reactive oxygen species, and the activation of effector mechanisms of apoptosis involving changes in the plasma membrane and DNA fragmentation (Figure 1). Thus it appears that similar to the effects of Bcl-cb5 on ceramide induced apoptosis (Figure 4), Bcl-cb5 inhibits serum starvation/*myc* induced apoptosis in part by preventing the loss of $\Delta\psi_m$ (Figure 1 and Table 1). Previously serum starvation/*myc* was shown to lead to cytochrome *c* release in Rat-1 cells and to be required for the late stage events (morphology changes) in apoptosis (Juin *et al*., 1999). While we also observed cytochrome *c* release after serum starvation/*myc* it was clearly preceded by loss in $\Delta\psi_m$. Previous studies have not examined the temporal order of these two events in Rat-1 cells. However, the confocal micrographs of the distribution of cytochrome *c* after serum starvation/*myc* (Juin *et al*., 1999) are entirely congruent with our observations.

Bcl-2 can prevent the loss of $\Delta\psi_m$, in isolated intact mitochondria and in an *in vitro* system with a reconstituted permeability transition pore complex, and inhibits the cell death and decreased $\Delta\psi_m$ in cells derived from Apaf-1 knock-out mice (Susin *et al*., 1996; Marzo *et al*., 1998; Haraguchi *et al*., 2000). Our current results are therefore surprising in that they demonstrate that in living cells Bcl-2 does not have to be localized to the mitochondria to prevent loss of $\Delta\psi_m$, and suggest that Bcl-2 can prevent the loss of $\Delta\psi_m$ by at least two mechanisms: one involving direct intervention in organelle function (Shimizu *et al*., 1999), and another which must be indirect to prevent mitochondrial dysfunction from a distance.

How can Bcl-cb5 prevent the loss of $\Delta\psi_m$ when it is localized to the ER? The loss of $\Delta\psi_m$ is a consequence of many different types of apoptotic stimuli, which may be either caspase dependent or independent. It is therefore plausible that Bcl-2 at the ER, a site distant from the mitochondria, may prevent loss of $\Delta\psi_m$ by either inhibiting cytosolic caspase activation, inhibiting an ER-localized caspase (Nakagawa *et al*., 2000), or protecting a critical caspase substrate that is located at the ER (Ng *et al*., 1997). However, our results argue

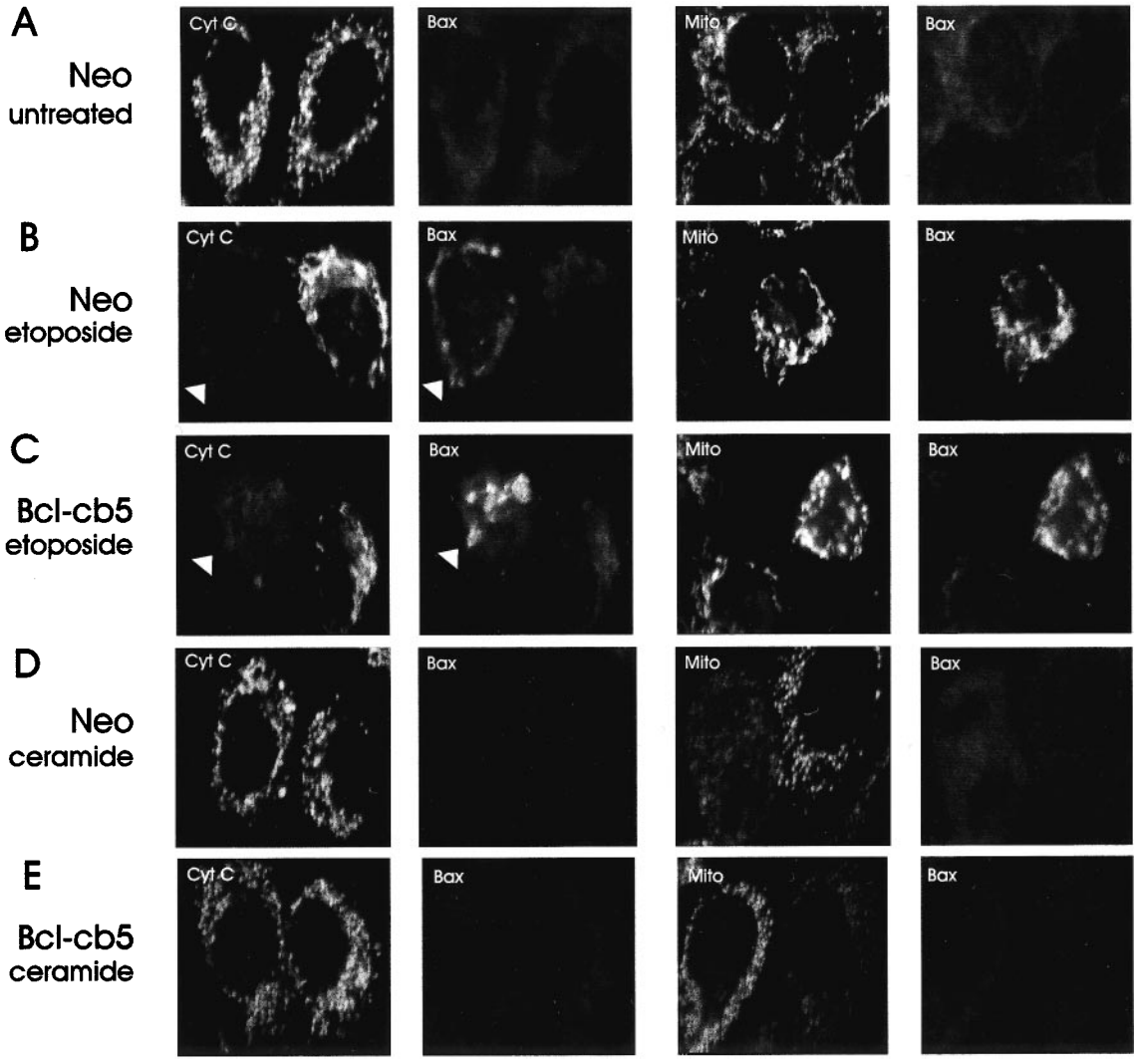

Figure 8 Etoposide, but not ceramide induced conformational change and relocalization of Bax correlates with release of mitochondrial cytochrome *c*. Cells were stained with the conformation specific Bax antibody 6A7 (Bax) and with either antibodies to cytochrome *c* (Cyt C) or to visualize $\Delta\psi_m$ with Mitotracker (Mito). (**a**) Untreated vector transfected cells (Neo) stained with antibodies to cytochrome *c* and with Mitotracker but did not stain with the conformation specific Bax antibody 6A7. (**b**) Vector transfected (Neo) and (**c**) Bcl-cb5 cells treated with etoposide exhibit mitochondrial localization of 6A7 positive Bax. Cells that stain with 6A7 have released mitochondrial cytochrome *c* (arrowheads). Conversely, cells that retain cytochrome *c* in mitochondria do not stain with 6A7. (**d**) Vector transfected (Neo) and (**e**) Bcl-cb5 cells treated with ceramide retain Mitotracker and cytochrome *c*, but did not stain with the 6A7 antibody. The width of each image is 580 μm

against these models as loss of $\Delta\psi_m$ induced by staurosporine or ceramide was not prevented by the general caspase inhibitor zVAD-fmk (Figure 2) yet was inhibited by Bcl-cb5. While ceramide induced loss of $\Delta\psi_m$ precedes cytochrome *c* release by several hours our results do not establish a direct causal relationship between these two events.

Bcl-2 has been reported to have other membrane sequestering activities that might be independent of a specific membrane site. Bcl-2 and other anti-apoptotic family members such as Bcl-XL have been reported to bind directly to pro-apoptotic family members such as Bax, Bik and Bad (Sedlak *et al*., 1995; Boyd *et al*., 1995; Zha *et al*., 1996). Thus Bcl-cb5 might prevent apoptosis by sequestering Bax away from mitochondria. However, if this were the case Bax bound to Bcl-cb5 would have been recovered in the supernatant from the carbonate extraction because pelleted membranes containing Bcl-cb5 were used for this analysis. The membranes were pelleted at 100 000 *g* in this experiment to ensure that if Bax was localized at any cellular membrane it would be detected. Moreover, if rather than binding to Bcl-cb5, Bax inserts into membrane bilayer because of an interaction with Bcl-cb5, then it would be recovered in the carbonate pellet. As we did not observe Bax in either fraction when Rat 1 cells were treated with ceramide, yet Bax translocation was readily detected in response to etoposide treatment (Figure 7), we conclude that Bcl-cb5 does not prevent apoptosis by sequestering Bax.

Finally, the amino terminal domain of Bcl-2 binds to calcineurin *in vivo* and *in vitro* (Shibasaki *et al*., 1997), and thereby inhibits its enzymatic activity. While we have not directly addressed the issue of the binding of Bcl-cb5 to calcineurin, our recent results indicate that this interaction may not be relevant in the context of serum starvation/*myc* induced apoptosis, as there is no detectable increase in cytosolic calcium in these cells as would be necessary for calcineurin activation (Lee *et al*., 1999).

Thus, although the apoptosis pathways induced by ceramide and etoposide eventually converge, inhibition of an early step in the ceramide induced pathway by Bcl-cb5 reveals that ceramide induces an apoptosis pathway spatially distinct from Bax mediated apoptosis and for which the early steps are at present largely unknown.

Etoposide perturbs mitochondrial structure

To determine whether Bcl-cb5 prevents cytochrome *c* release we developed a technique to prepare subcellular fractions that largely preserves the integrity of entire mitochondria, as evidenced by the efficient pelleting of Hsp60 but not cytoplasmic markers at $1000 \times g$. Comparison of the results obtained by sub-cellular fractionation and confocal microscopy in Rat-1 cells suggests that the release of cytochrome *c* from the intermembrane space is an early event in etoposide induced apoptosis. This result is not unique to etoposide early cytochrome c release was also observed when cells were treated with Taxol (Soucie *et al*., submitted) When cytochrome *c* is found in the supernatant from the low *g* force centrifugation, the bulk of the matrix protein Hsp-60 is associated with the low *g* force pellet. It is possible that the small amount of Hsp-60 found in the low *g* force supernatant (Figure 6a) is the same fraction previously reported to contribute to activation of caspase 3 and to be released from the intermembrane space of mitochondria along with cytochrome *c* during apoptosis (Samadi *et al*., 1999; Xanthoudakis *et al*., 1999). Alternatively, it may represent release of Hsp-60 from the relatively small fraction of mitochondria in which the integrity of the inner membrane was compromised during homogenization and fractionation.

Our data do not permit an unambiguous determination of cause and effect, however in etoposide treated cells the change in Bax conformation and membrane binding associated with the early redistribution of cytochrome *c* occurs prior to loss in $\Delta\psi_m$ (Figures 3, 4, 6, 7 and 8). This result suggests that activation of Bax is a critical event regulating etoposide induced apoptosis. As Bcl-2, but not Bcl-cb5, prevented these changes, it appears that Bcl-2 must be targeted to mitochondria to prevent the change in Bax conformation that initiates the rest of this pathway of apoptosis. Bcl-cb5 may be ineffective at inhibiting etoposide induced apoptosis because when located at the ER, it is unable to prevent translocation of Bax to mitochondria (Figure 7) or the change in Bax conformation that results in 6A7 positivity (Figure 8). Both Bcl-cb5 and Bcl-2 bind the detergent induced 6A7 positive conformer of Bax (Zhu *et al*., 1996). This result indicates that substitution of the carboxyl terminus of Bcl-2 does not alter the structure of the protein such that it is no longer able to bind to Bax. Instead, we do not detect relocalization of Bax to endoplasmic reticulum membranes biochemically (Figure 7) nor do we detect the 6A7 positive conformer of Bax at endoplasmic reticulum (Figure 8 and data not shown). Therefore, it appears that Bcl-cb5 is unable to prevent activation and translocation of Bax because when located at the ER it no longer comes into contact with Bax (Figure 9). We speculate that Bcl-cb5 functions by blocking an as yet unknown, caspase independent signal that eminates from ER and triggers loss of $\Delta\psi_m$ at mitochondria (Figure 9).

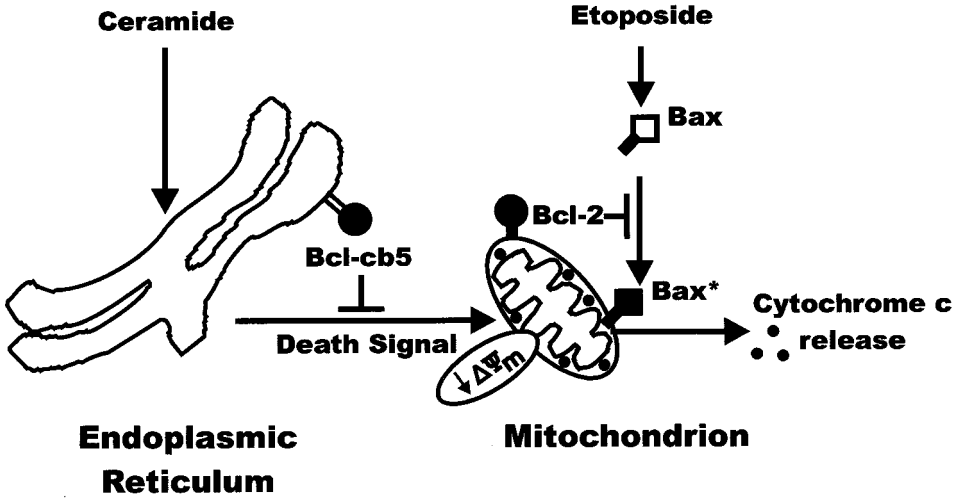

Figure 9 Initiation of apoptosis by ER or mitochondrial specific pathways. $\downarrow\Delta\psi_m$ indicates loss of mitochondrial membrane potential. Bax* indicates 6A7 positive Bax

One hypothesis to account for cytochrome *c* release in the absence of a decrease in $\Delta\psi_m$ that is compatible with our data is formation or opening of an outer membrane channel by Bax (Figure 9 and Eskes *et al*., 1999). Bcl-2 located at the mitochondria prevents this mode of activating apoptosis (Figure 9). Because the decrease in $\Delta\psi_m$ occurs relatively late (16–18 h (Lee *et al*., 1999), and data not shown) compared to cytochrome *c* release (6 h, Figures 3, 4 and 6) our results indicate that for etoposide induced apoptosis, loss of $\Delta\psi_m$ is a late amplification event.

Interestingly, redistribution of cytochrome *c* is a later event than loss of $\Delta\psi_m$ in those forms of apoptosis for which Bcl-cb5 was an effective inhibitor. However, at time points where there was substantial loss of $\Delta\psi_m$, other features of apoptosis such as externalization of PS, DNA fragmentation and cleavage of PARP (Figures 1, 2 and 5) are also observed. Therefore, in apoptosis induced by ceramide, it appears that cytochrome *c* redistribution acts as a late amplifier. Our results indicate, but do not prove, that loss of $\Delta\psi_m$ is critical for commitment to the execution phase of apoptosis in ceramide treated cells.

Taken together, our findings suggest that the debate about the relative importance of mitochondrial transmembrane potential versus cytochrome *c* release based on data from *in vitro* model systems can be reconciled by noting that multiple, spatially distinct apoptosis pathways exist in the same cell. These pathways are differentially sensitive to inhibition by Bcl-cb5 and can be identified by which of the two manifestations of mitochondrial dysfunction occurs first: release of cytochrome *c* or loss of $\Delta\psi_m$.

Materials and methods

Preparation of cellular extracts by nitrogen cavitation

Four 100 mm Petri dishes of cultured cells were harvested in their respective medium using a rubber policeman. The cells were then pelleted by centrifugation in a clinical centrifuge for 3 min at 4°C. The cell pellet was washed twice with cell buffer (250 mM Sucrose, 20 mM HEPES pH 7.5, 2 mM $MgCl_2$, 1 mM NaEDTA, 1 mM PMSF, 1 mM DTT and a cocktail of protease inhibitors). The final pellet was resuspended in an equal volume of cell buffer. This suspension was held at 150 p.s.i. for 15 min on ice in a 45 ml Nitrogen Bomb (Parr Instruments) and cells were disrupted by releasing the pressure. The nuclei and cell debris in the expelled lysate were removed by centrifugation at ~480 *g* for 2 min at 4°C in a microcentrifuge (Eppendorf). The resulting supernatant, termed the whole cell lysate (WCL), was then split into aliquots of 100 microliters and cytosolic (S^1) and pellet (P^1) fractions were isolated by centrifugation at 1000 *g* for 25 min in a Beckman TLA100 rotor. Due to the small volume used in our differential centrifugation, the distance required to pellet intact mitochondria is small and therefore, in lysates of untreated cells sedimentation of cytochrome *c* and Hsp-60 in the P^1 fraction is very efficient. Alternatively, for the analysis of Bax localization, the WCL was subjected to centrifugation at 100 000 *g* for 1 h to generate supernatant (S^{100}) and pellet (P^{100}). Carbonate extraction of membrane fractions was performed as previously described (Janiak *et al*., 1994). The fractions were then snap frozen in liquid nitrogen and stored at −80°C. Protein in samples was quantified using a standard Bradford assay (Bio-Rad).

Immunoblotting

Antibodies to Hsp-60 and the 1D1 monoclonal antibody directed against Bax were generous gifts of Dr R Gupta, McMaster University and Dr R Youle, NIH. Sheep antiserum and affinity purified antibodies to cytochrome *c* were obtained from Exalpha Biologicals, Roxbury, MA, USA. The antibody to poly-ADP ribose polymerase (PARP) was purchased from Biomol and used according to the manufacturer's instructions. Rabbit antiserum to Bcl-2 has been described (Zhu *et al*., 1996). Protein samples were separated on a 10% polyacrylamide tricene gel, followed by transfer to a PVDF membrane for PARP immunostaining, or to nitrocellulose membrane for other antibodies. The membranes were blocked and then incubated with either affinity purified anti-cytochrome *c* (dilution 1 : 5000), anti-Hsp60 (dilution 1 : 10 000), anti-PARP (1 : 10 000), anti-Bcl-2 (1 : 10 000) or 1D1 anti-bax (dilution 1 : 5000) antibodies and developed after incubation with peroxidase-conjugated donkey anti-sheep or donkey anti-mouse antibodies (Jackson Laboratories) using an enhanced chemiluminescent detection system (NEN-Life Science). Band intensities were quantified using a densitometer and the data was analysed using ImageQuant software. The variation in recovery from subcellular fractionation was calculated by measuring the total protein recovered from all fractions from duplicate samples processed in parallel for six separate experiments.

Cell death assays

Rat-1/*myc* cells were grown to ~75% confluence in a 100 mm Petri dish. After washing twice with PBS, the media was replaced with medium containing either 40 μM Etoposide (for 5 h), 50 μM C2-Ceramide (for 6 h) or 0.1% foetal bovine serum + 2 μM estradiol (for 24 h). Cytochrome *c* release was monitored either by sub-cellular fractionation or immunofluorescence microscopy (dilution of affinity purified antibodies was 1 : 750). Change in the conformation of Bax was assessed by microscopy using the conformation specific antibody 6A7 (dilution 1 : 100). To monitor mitochondrial transmembrane potential by direct visualization, cells were grown on coverslips under the experimental conditions described above. At the end of the exposure to the apoptotic stimulus, Mitotracker dye (Molecular Probes) was added to a concentration of 150 nM. After incubation for 15 min in the dark, the cells were incubated in culture medium at 37°C for 15 min and then washed with PBS, fixed with 4% paraformaldehyde, and with the exception of 6A7 staining, permeabilized and processed conventionally for immunofluorescence. For 6A7 immunolocalization cells were permeabilized using CHAPS (0.2%) in PBS. Mitochondrial transmembrane potential was monitored by direct visualization of the accumulation of the dye by comparison with the distribution of the mitochondrial matrix protein Hsp-60 (dilution 1 : 50). Exposure of Mitotracker-treated cells to the protonophore carbamoyl cyanide m-clorophenylhydrazone (m-CICCP) for 15 min resulted in no detectable mitochondrial fluorescent signal compared to untreated cells, indicating that Mitotracker alone did not substantially affect mitochondrial membrane potential under these conditions (Scorrano *et al*., 1999). Control experiments demonstrated

that the decreased intensity of Mitotracker staining in cells undergoing apoptosis was not due to variations in stain delivery as staining did not vary significantly in different areas of the coverslips (data not shown) and thus is an accurate indicator of the mitochondrial transmembrane potential in the conditions used here. Cytofluorometric analysis of mitochondrial transmembrane potentials with 3,3′ dihexyloxacarbocyanine iodide ($DiOC_6(3)$) and reactive oxygen species were performed as described (Marchetti *et al*., 1996). Analysis of externalized phosphatidyl serine (PS) by annexin V labelling and nuclear DNA loss by propidium iodine (PI) staining of cells were as described (Marchetti *et al*., 1996).

Cell culture

Rat-1/myc fibroblasts constitutively expressing human Bcl-2 and Bcl-cb5 (Zhu *et al*., 1996) were maintained in phenol red minus, alpha-minimal essential medium supplemented with 10% charcoal-filtered treated foetal bovine serum.

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