INVESTIGATING THE MOLECULAR MECHANISMS OF BCL-2 AND BAX IN THE REGULATION OF APOPTOSIS

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ABSTRACT

The molecular mechanism by which the Bcl-2 family of proteins regulates the complex process of apoptosis is unknown. One insight into the function of these proteins is their requirement for membrane localization to regulate cell death. Bcl-2, the prototype of the family, is localized the endoplasmic reticulum (ER) and mitochondria. By examining the contribution of ER localized Bcl-2 in the regulation of apoptosis; two spatially distinct cell death pathways were identified. One pathway is characterized by the early loss of inner mitochondrial membrane potential and is inhibited by ER localized Bcl-2. The other pathway, not inhibited by ER localized Bcl-2, is characterized by the mitochondrial translocation of the cytoplasmic pro-apoptotic protein Bax. On mitochondria Bax oligomerizes inducing the release of cytochrome *c*. This mitochondrial pathway is influenced by the expression of the oncogene *Myc,* specifically, *Myc* is required for the transition from monomeric, membrane bound, Bax to oligomeric active Bax. This activation of Bax is not mediated by changes to its membrane topology as both monomeric and oligomeric membrane bound Bax adopts a multi-spanning membrane topology with helices α5, α6 and α9 partially inserted into the lipid bilayer. These studies have demonstrated that the function of Bcl-2 at the ER is dependent on apoptotic agonist; Bax activity at mitochondria is influenced by a *Myc* regulated factor and activation of Bax is not mediated by changes in this proteins membrane topology. Combined these studies have provided valuable insight into both the complexity and molecular mechanisms of Bcl-2 proteins in the regulation of apoptosis.

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ABBREVIATIONS USED IN THIS THESIS

Bcl-2	B-Cell lymphoma protein – 2
Bax	Bcl-2-associated X protein
BaxΔCT	Bax without the carboxyl-terminal 20 amino acids
Bid	BH3 interacting domain death agonist
Bak	Bcl-2 antagonistic Killer
Bad	Bcl-2 associated death promoter
ER	Endoplasmic reticulum
IASD	4-acetamido-4'-iodoacetyl-amino stilbene-2.2'-disulfonic acid
IEF	Isoelectric focusing
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate
SDS	Sodium dodecyl sulfate
IGEPAL-630	Nonylphenyl-polyethylenglycol (formally known as NP-40)
$\Delta\Psi_m$	Inner mitochondrial membrane potential
Caspase	Cysteinyl aspartate-specific protease
PARP	Poly (ADP-ribose) polymerase
IAP	Inhibitors of apoptosis protein
Cb5	Tail anchor of cytochrome b5
PTC	Permeability transition pore complex
GST	Glutathione *S*-transferase

for my family

what is death but a passage to life

-Travis M. Farnsworth

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Chapter One

General Introduction

Apoptosis (or programmed cell death) is a genetically encoded cell death program characterized by distinct biochemical and morphological changes (Wyllie et al., 1980). During development this process is critical at several stages from early cavitation of the developing mouse embryo to the removal of inter-digit webbing (reviewed in (Jacobson et al., 1997)). Beyond development, apoptosis is important for normal tissue homeostasis; aberrant programmed cell death is a contributing factor to tumour progression (Evan and Vousden, 2001) and neurodegenerative disorders (Liston et al., 2003).

The term apoptosis (pronounced "ap-o-TO-sis", meaning falling leaves from trees in autumn (Cohen, 1991)) was selected by John Kerr and colleagues to define the "active self destruction" or programmed cell death they observed in a variety of cell types (reviewed in (Wyllie et al., 1980)). These investigators were the first to characterize the morphological (plasma membrane blebbing and chromatin condensation, see Figure 1) and biochemical (DNA fragmentation) changes that are now considered hallmarks of apoptosis. Since these early investigations, a wealth of information about the process and regulation of cell death has emerged. Despite this vast knowledge the precise mechanisms of many key regulators of cell fate remain unresolved. For discussion, the regulation of cell death can be divided into three main phases: initiation, decision, and execution.

The initiation phase of cell death involves the recognition of a death stimulus. In general, there are two pathways to cell death: extrinsic and intrinsic. Activation of cell death machinery by an extracellular ligand initiates the extrinsic or receptor mediated cell

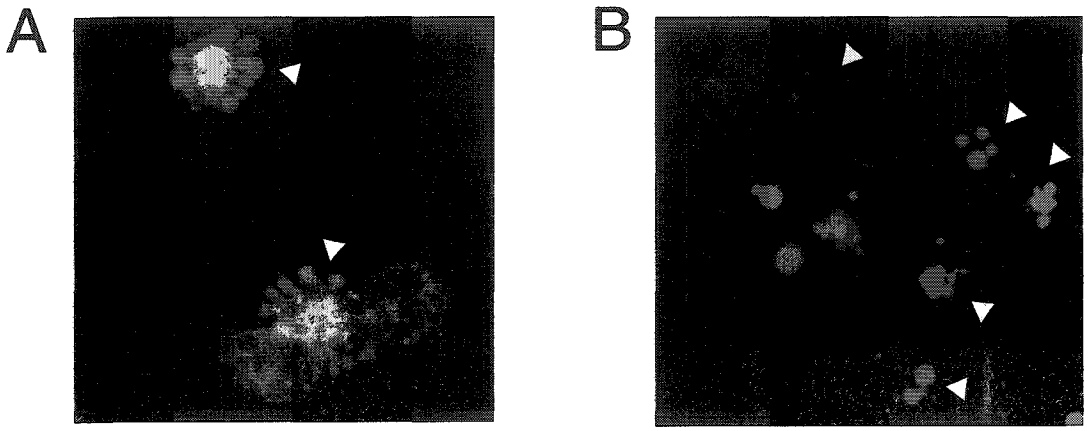

Figure 1. Morphological changes seen in apoptosis. A. Rat-1fibroblasts (HOmyc3) were treated with Taxol (0.5 μM for 12 hours) and stained for cytochrome c (red) and activated Bax (6A7, green). Cytochrome c released from mitochondria highlights the membrane blebbing that occurs during apoptosis (Membrane blebbing is indicated with arrowhead). This kind of experiment is described further in Chapters Three and Four. B. Rat-1 fibroblasts were treated with etoposide (6μM for 12 hours) and stained with the DNA binding dye Hoescht 33342 to observe chromatic condensation. Normal nuclear staining (yellow arrowhead) and chromatin condensation indicative of apoptotic cells is apparent (white arrowhead).

death pathway. Intrinsic cell death is initiated within the cell usually through the disruption of a cellular process; most chemotherapeutic agents initiate intrinsic cell death pathways.

Following the initiation of cell death a cell enters a critical decision phase. During this phase the Bcl-2 family of proteins are potent determinants of cell fate. This family is sub-divided into either pro- or anti-apoptotic members depending on function. This antagonistic relationship between family members determines whether a cell will live or commit suicide. Specific cell death signals activate pro-apoptotic Bcl-2 members which in turn initiate the disintegration of the cell by disrupting mitochondrial function. The prevailing theory for the mechanism by which anti-apoptotic proteins inhibit pro-apoptotic proteins involves sequestration by direct binding; however if an apoptotic agonist is successful the balance will shift and the execution phase of cell death is initiated.

Caspases (Cysteinyl ASPartate-specific proteASEs) are the main effectors of the execution phase of apoptosis. They exist as inactive zymogens until they are activated by multiple cleavage events (Degterev et al., 2003). Numerous proteins, involved in disparate cellular processes, are substrates of caspases (Fischer et al., 2003); most notable are the nuclear protein Poly (ADP-ribose) polymerase (PARP), involved in DNA repair and Inhibitor of Caspase-Activated DNase (ICAD), cleavage of which activates DNA fragmentation factor). Once activated, the execution phase represents the 'point of no return' for the cell as the activity of caspases is irreversible and leads to the biochemical and morphological changes that are hallmarks of apoptosis

Initiation, decision and execution represent the process of apoptosis; although emphasis is placed on the decision phase the other phases are not without regulation. The details of each of these phases are presented here with emphasis on the Bcl-2 family of proteins which are the focus of the investigations presented in this thesis.

Initiation of Apoptosis

Extrinsic Cell Death Pathway

Extrinsic pathways are considered "direct activators" of cell death as they engage the execution machinery upon receptor activation. Tumour Necrosis Factor (TNF), Fas, and TNF-Related Apoptosis-Inducing Ligand (TRAIL) are three members of the TNF receptor family. The intracellular portion of these death receptors contains a Death-Domain which binds to several adaptor proteins forming the Death-Inducing-Signaling-Complex (DISC). Upon ligand binding, TNF receptors trimerize inducing the activation of recruited pro-Caspase-8 by promoting dimerization. As a dimer pro-Caspase-8 is activated by self-cleavage and initiates cell death, in part, by the cleavage of two important substrates pro-Caspase-3 and the pro-apoptotic protein Bid (Li et al., 1998). Caspase-3 is an executioner caspase and, as such, has broad specificity and cleaves many substrates; thus the extrinsic pathway can engage the execution phase directly. Cleavage of the pro-apoptotic Bcl-2 protein, Bid, into t-Bid (truncated Bid) activates the intrinsic pathway by engaging mitochondrial apoptotic events (see below). Depending on the extent of Caspase-8 activity, determined by cellular context (see below), the extrinsic

pathway will either directly engage Caspase activation (through caspase-3 cleavage) or the execution phase will be initiated by t-Bid activity.

Although the extrinsic cell death pathway can directly initiate apoptosis; several mechanisms inhibiting this pathway are known including: decoy receptors (Pan et al., 1997); inactive caspase-8 decoy proteins (c-FLIP) (Irmler et al., 1997); expression of caspase-3 inhibitors (IAPs) (Roy et al., 1997) and expression of anti-apoptotic Bcl-2 proteins (Scaffidi et al., 1998). In addition, depending on the cellular context, TNF receptor signaling activates NF-KB which in turn increases the expression of anti-apoptotic proteins including IAPs (Stehlik et al., 1998). Thus despite the "direct activation" mechanism for the extrinsic pathway there are additional factors influencing the ability of this pathway to commit the cell to the execution phase of cell death.

Intrinsic Cell death Pathway

Unlike extrinsic cell death, initiation of the intrinsic pathway can occur by a variety of mechanisms. In the investigations presented in this thesis three apoptotic agonists are utilized: etoposide, ceramide, and serum starvation combined with deregulated growth signaling. The precise signaling mechanisms for each of the death agonists are unknown and the subject of the investigations presented herein.

Etoposide is a widely utilized chemotherapeutic that induces DNA damage by disrupting topo-isomerase II function (Laurie et al., 2004). In general, DNA damage is characterized by upregulation of p53 activity. Indeed, a functional requirement for p53 in etoposide induced cell death was demonstrated in mouse embryonic fibroblasts null for

p53 expression (Lowe et al., 1993; Villunger et al., 2003). Depending on the cellular context, DNA damage either leads to growth arrest or apoptosis. p53 is thought to induce cell death by increased transcription of pro-apoptotic Bcl-2 proteins including Bax (Miyashita et al., 1994), Noxa (Oda et al., 2000), and Puma (Nakano and Vousden, 2001). In addition, direct activation of Bax at mitochondria by p53 has recently been reported, suggesting the transcription functions of p53 may not be required for the induction of apoptosis (Mihara et al., 2003; Chipuk et al., 2004). Thus depending on the death stimulus, a combination of gene expression and mitochondrial localization of p53 induce the release of cytochrome *c* from mitochondria ultimately activating the execution phase of apoptosis.

Ceramide is a lipid second messenger generated through hydrolysis of the head group of sphingomyelin by sphingomyelinase (Kolesnick and Fuks, 2003). Ceramide production occurs in vivo with radiation induced cell damage (Chmura et al., 1997); *in vitro* a derivative of C2-ceramide is commonly used to mimic endogenous ceramide production. As a second messenger ceramide interacts with and regulates the activity of many targets in cell signaling (reviewed in (Kolesnick and Fuks, 2003)). Although the precise mechanism of ceramide induced cell death has remained elusive one target of ceramide activity appears to be the pro-apoptotic Bcl-2 protein Bad (Basu et al., 1998), whose activity is increased in the presence of ceramide. In addition, ceramide induced cell death is thought to be mediated by increased cytoplasmic levels of calcium (Pinton et al., 2001) leading to mitochondrial dysfunction (Smaili et al., 2000).

Growth factors (GF) provide survival signals by activating the protein kinase Akt/(PKB). Active Akt inhibits cell death by numerous mechanisms including: inactivating the pro-apoptotic Bcl-2 protein Bad through phosphorylation (Datta et al., 1997) increasing expression of IAPs by promoting NF-KB activity (Kane et al., 1999) and inhibiting Caspase-9 activation (Cardone et al., 1998). In the absence of GF (serum starvation) Akt is no longer activated resulting in the dephosphorylation of Bad and elimination of the survival signals outlined above. Deregulated expression of the proto-oncogene *c-myc* in the presence of low serum considerably enhances cell death and is often utilized as cell death stimulus (Evan et al., 1992). Expression of *c-myc* is thought to enhance cell death by sensitizing mitochondria to pro-apoptotic Bcl-2 proteins (Chapter Four and (Soucie et al., 2001; Juin et al., 2002)).

Despite the disparate mechanisms to initiate cell death the above stimuli all engage intrinsic cell death pathways by enlisting the activity of pro-apoptotic Bcl-2 proteins.

Decision Phase of Apoptosis

The Bcl-2 family of proteins

Intrinsic cell death and a side branch of the extrinsic cell pathway ultimately initiate the activity of Bcl-2 proteins. These proteins can be separated into two broad functional categories, either pro- or anti-apoptotic [recently reviewed in (Cory et al., 2003). To be a member, a protein must possess at least one of the four conserved Bcl-2 homology (BH) regions (important for intra-family interactions) and have a demonstrable effect on apoptosis (Figure 2). Currently, twenty Bcl-2 family members have been identified [reviewed in (Cory et al., 2003)].

Intra-family interactions are important to the function of Bcl-2 proteins, however when the structure for the anti-apoptotic protein Bcl-X_L (Muchmore et al., 1996) was solved in 1996, an additional function was proposed – channel formation. The structure of Bcl-X_L consists of two central hydrophobic helices surrounded by amphipathic helices in a structure that resembles a portion of the transmembrane domain of the pore forming bacterial protein diphtheria toxin. This basic structure is shared by Bid (Chou et al., 1999), Bax (Suzuki et al., 2000), Bcl-2 (Petros et al., 2001), and Bcl-w (Denisov et al., 2003; Hinds et al., 2003). Consistent with the known structures, there is good in vitro evidence that Bcl-2 (Schendel et al., 1997), Bax (Antonsson et al., 1997) and Bcl-X_L (Minn et al., 1997) can form pores or channels in lipid bilayers.

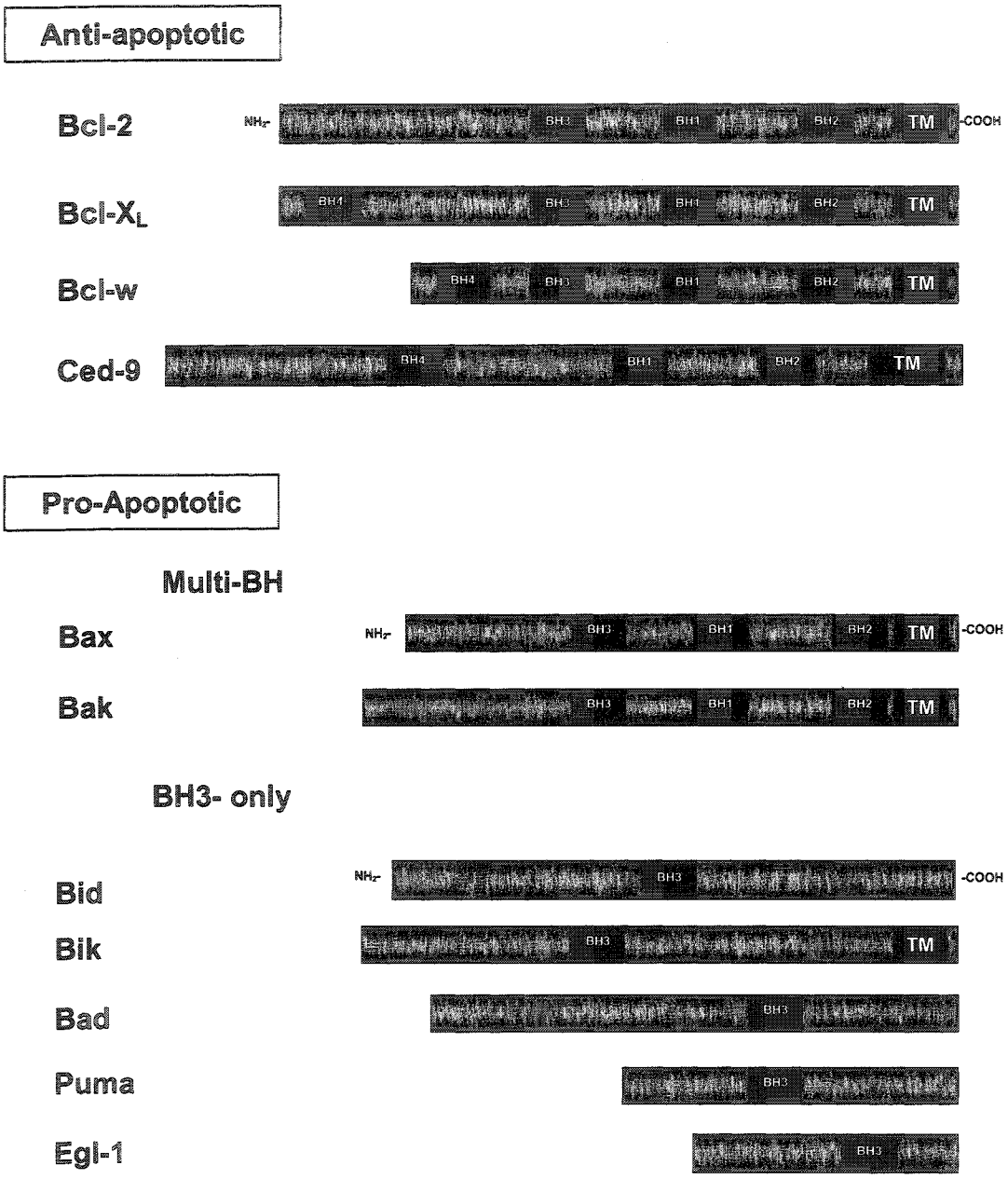

Figure 2. Linear schematics of select Bcl-2 proteins. This protein family is sub-divided into anti- and pro-apoptotic members. The 4 Bcl-2 Homology regions are indicated (BH1-4) as well as the (predicted for some members) transmembrane anchor (TM). The pro-apoptotic members are further sub-divided into either mutli-BH region members or BH3 only members (see text for description).

A combination of protein:protein interactions and pore/channel formation are postulated to constitute the main molecular mechanisms of Bcl-2 proteins.

Bcl-2

Bcl-2, the proto-type for the family, was identified due to a translocation event in human follicular lymphoma which linked Bcl-2 to the immunoglobulin heavy chain locus (Tsujimoto et al., 1984). Expression of Bcl-2, unlike proliferative oncogenes, promotes cell survival by inhibiting apoptosis (Vaux et al., 1988). Bcl-2 contains all four BH regions and a carboxyl terminal tail-anchor sequence that localizes it to the endoplasmic reticulum (ER), nuclear envelope and mitochondria (Krajewski et al., 1993). This broad localization allows Bcl-2 to function at spatially distinct regions of the cell, thereby enhancing its anti-apoptotic activity (Zhu et al., 1996; Lee et al., 1999; Annis et al., 2001; Thomenius et al., 2003).

The discovery that Bcl-2 targets via a tail-anchor sequence occurred at roughly the same time as the elucidation of the mechanisms that regulate targeting and integration of tail-anchor sequences in subcellular membranes (Kutay et al., 1993; Kim et al., 1997). These studies suggested that it would be possible to manipulate the location of Bcl-2 proteins by generating tail-anchor mutants. By using this approach, we demonstrated that Bcl-2 does not need to be located at both physiological sites (the ER and mitochondria) to function; in fact in stably transfected cells Bcl-2 prevents apoptosis from either organelle with overlapping but non-identical specificity (Zhu et al., 1996). The use of organelle

targeted mutants has been very important for studies analyzing the contribution of the ER to the regulation of apoptosis (reviewed in (Annis et al., 2004)).

Bax

A mechanism for Bcl-2 activity was proposed with the identification of the first pro-apoptotic binding partner, Bax (Oltvai et al., 1993). These authors proposed a rheostat model for cell fate determination; increased activity of pro-apoptotic members over anti-apoptotic members would tip the balance toward apoptosis and vice versa (Korsmeyer et al., 1993). Indeed, point mutations in Bcl-2 that disrupt the interaction between Bcl-2 and Bax (e.g. G145A of Bcl-2) also reduce the ability of Bcl-2 to inhibit certain apoptotic stimuli (Yin et al., 1994). However, mutants of Bax that inhibit binding to Bcl-2 do not inhibit Bax induced cell death suggesting Bax does not need to interact with Bcl-2 to induce apoptosis (Wang et al., 1998).

Similar to Bcl-2, Bax contains three BH regions and a putative carboxyl-terminal tail anchor sequence; however, unlike Bcl-2, in healthy cells Bax is located predominantly in the cytoplasm. Certain apoptotic stimuli trigger the translocation of Bax to mitochondria (Chapter Three and (Wolter et al., 1997; Annis et al., 2001)) and the ER (Nutt et al., 2002b; Scorrano et al., 2003). At mitochondria Bax induces the release of pro-apoptotic factors, including cytochrome *c* through perturbation of the outer mitochondrial membrane (Jurgensmeier et al., 1998). At the ER Bax activity is unclear as it has been reported to both decrease (Nutt et al., 2002b; Nutt et al., 2002a) and increase ER calcium stores (Scorrano et al., 2003). Although the functional

consequences for Bcl-2 molecules at the ER are now emerging (reviewed in (Annis et al., 2004) and see below), mitochondria remain an important central target for the activity of Bcl-2 proteins and mitochondria clearly contribute as a sensor for cell fate (reviewed in (Kuwana and Newmeyer, 2003)).

Despite containing a carboxyl-terminal tail-anchor sequence, Bax remains in the cytoplasm until after the induction of apoptosis. Targeting to mitochondria during cell death requires not only the tail-anchor sequence (Nechushtan et al., 1999), but the central α5 and α6 helices (Nouraini et al., 2000) as well as regions of the amino-terminus (Goping et al., 1998; Cartron et al., 2003). The "signal" to initiate Bax translocation to mitochondria is unknown; however in some cases the release of two Bax binding proteins, Ku70 (Sawada et al., 2003) and Humanin (Guo et al., 2003), that inhibit Bax translocation would be necessary. Once at mitochondria Bax oligomerizes and disrupts the outer-mitochondrial membrane allowing the release of apoptotic factors from the intermembrane space (Jurgensmeier et al., 1998; Antonsson et al., 2000). The change in Bax conformation due to oligomerization can be recognized by a monoclonal antibody, 6A7 (Hsu and Youle, 1997; Nechushtan et al., 2001). The 6A7 epitope is located in the amino-terminus of the Bax and is normally buried in inactive cytosolic Bax. However, upon translocation and oligomerization this epitope is exposed and recognized by the 6A7 antibody (Chapters Three and Four and (Soucie et al., 2001; Desagher et al., 1999)).

Expression of Bcl-2 inhibits both Bax activity prior to translocation (Murphy et al., 2000) and oligomerization on mitochondria (Mikhailov et al., 2001). In general, anti-

apoptotic Bcl-2 proteins function to inhibit the permeabilization of outer mitochondrial membrane by pro-apoptotic proteins.

Bid

Bid belongs to the BH3 only subclass of pro-apoptotic Bcl-2 proteins, Figure 2 (Wang et al., 1996). Members of this class function to activate multi-BH pro-apoptotic proteins (including Bax) and/or inhibit anti-apoptotic Bcl-2 proteins. During receptor mediated cell death Bid is cleaved into truncated-Bid (t-Bid) by the activity of Caspase-8 (Li et al., 1998). T-Bid induces the oligomerization and activation of Bax in mitochondria and liposomes (Eskes et al., 2000; Yethon et al., 2003). Unlike Bcl-2 or Bax, Bid has no membrane targeting sequences; however cleavage of Bid by Caspase-8 reveals an N-terminal glycine residue that can be myristoylated (Zha et al., 2000). Myristoylation of t-Bid enhances the targeting and activity of the protein (Zha et al., 2000).

Bak

Bak is a multi-BH domain containing pro-apoptotic Bcl-2 protein. Bak contains a carboxyl-terminal tail anchor sequence and it is constitutively localized to mitochondria and the endoplasmic reticulum (Griffiths et al., 1999; Nutt et al., 2002b; Scorrano et al., 2003; Chittenden et al., 1995). At mitochondria Bak, like Bax, undergoes oligomerization during apoptosis to induce the release of apoptotic factors (Wei et al., 2000). Similar to Bax, Bak is also thought contribute to Ca^{2+} regulation (Scorrano et al.,

2003; Nutt et al., 2002b; Zong et al., 2003). However, the precise role for Bcl-2 proteins in the regulation of ER calcium is unclear as both pro- and anti-apoptotic members have been demonstrated to alter ER calcium levels in either direction (reviewed in (Annis et al., 2004)). Nevertheless Bak and Bax are critical proteins involved in the progression of several apoptotic pathways (Wei et al., 2001a). Mouse embryonic fibroblasts deficient for both Bak and Bax expression are resistant to a disparate array of cell death agonists, including etoposide (Wei et al., 2001a). Mouse embryonic fibroblasts deficient for either Bak or Bax expression were partially resistant to etoposide induced cell death, thus these proteins appear to have a synergistic relationship in the induction of cell death.

Mitochondria

A role for mitochondria in the regulation of cell death was cemented with the identification of cytochrome *c* as an important inducer of apoptosis (Liu et al., 1996). Mitochondria undergo several dysfunction events during apoptosis including loss of inner mitochondrial membrane potential and release of pro-apoptotic factors including: cytochrome *c* (Liu et al., 1996), AIF (Susin et al., 1996), and Smac/Diablo (Du et al., 2000). Once released cytochrome *c* complexes with Apaf-1, Caspase-9 and dATP to form the apoptosome complex, that induces Caspase-9 activation and subsequent initiation of the execution phase of apoptosis (Li et al., 1997). Released AIF (Apoptosis Inducing Factor) is imported into the nucleus where it initiates DNA fragmentation by an undefined mechanism (Susin et al., 1999; Arnoult et al., 2003). Smac/Diablo (second mitochondria-derived activator of Caspase/direct IAP binding protein with low pI)

promotes apoptosis by inhibiting the ability of IAPs to bind to Caspases (Du et al., 2000; Verhagen et al., 2000). The mechanism underlying the release of these factors remains controversial; however two general mechanisms have been proposed: the formation pore/channel composed of pro-apoptotic Bcl-2 proteins or by the opening of the permeability transition pore complex leading to mitochondrial swelling and rupture of the outer mitochondrial membrane (Petit et al., 1998; Martinou and Green, 2001).

Detergent induced homo-oligomerization of Bax can induce the release of fluorescent dextrans approximately the size of cytochrome *c* from liposomes (Antonsson et al., 2000), suggesting that Bax alone can induce the release of cytochrome *c* from mitochondria. Indeed, detergent induced oligomerization of Bax does induce the release of cytochrome *c* from isolated mitochondria (Antonsson et al., 2000). However, oligomerization of Bax induced by a physiologically relevant stimulus may be regulated by additional unidentified mitochondrial associated proteins (Roucou et al., 2002; Kuwana et al., 2002). Although the ability of Bax to permeabilize membranes has been well documented, the molecular mechanism underlying this activity has not been determined. The Bcl-2 proteins are thought to form channels or pores by insertion of two central alpha-helices (α5 and α6) into the lipid bilayer; in Chapter Five of this thesis this hypothesis is investigated by determining the membrane topology of Bax in cells undergoing apoptosis and in cells in which apoptosis is impaired at the point of Bax activation.

Another proposed mechanism to induce the rupture of mitochondrial outer-membrane is through the permeability transition pore complex (PTC). The PTC is an

inner mitochondrial membrane channel that is opened in response to certain apoptotic stimuli (Marzo et al., 1998). Opening of the PTC leads to rapid loss of the inner mitochondrial membrane potential followed by osmotic swelling of the inner matrix compartment ultimately causing rupture of the outer mitochondrial membrane. This leads to the release of pro-apoptotic proteins from the mitochondria. The composition of the PTC and the mechanisms governing the opening of the pore are controversial and the subject of ongoing investigations (reviewed in (Newmeyer and Ferguson-Miller, 2003)). Although evidence for both proposed mechanisms exists, the contribution of either mechanism to the initiation of cell death is controversial.

Adding to the complexity of this process is the observation that under certain conditions AIF (~57kDa) is released prior to cytochrome *c* (~12 kDa), suggesting selective mechanisms, rather than large indiscriminate pores or breaks in the mitochondrial membrane, determine what is released (Susin et al., 2000; Cande et al., 2002; Arnoult et al., 2003; Martinou and Green, 2001). Indeed, selective mechanisms for protein release from mitochondria would also explain that cytochrome *c* can be released from mitochondria without the loss of the inner mitochondrial membrane potential ($\Delta\Psi_m$) ((Bossy-Wetzel et al., 1998) and Chapter Three of this thesis) and that cell death can be initiated in the absence of cytochrome *c* release ((Li et al., 2000; Achenbach et al., 2000) and Chapter Three of this thesis). These contrasting observations made the relative contributions of cytochrome *c* release and loss of $\Delta\Psi_m$, and hence opening of the mitochondria by Bcl-2 proteins or the PTC, controversial. For example, opening the PTC leads to the rapid loss of $\Delta\Psi_m$ and rupture of the mitochondrial outer membrane; however

this mechanism would not explain the release of cytochrome *c* in the absence of $\Delta\Psi_m$ loss. In Chapter Three of this thesis, a resolution to this controversy is presented. In each of the above proposed mechanisms for mitochondrial dysfunction one event, either the loss of $\Delta\Psi$m or cytochrome *c* release is indicative of the first change seen in opening of the PTC or pro-apoptotic Bcl-2 induced mitochondria dysfunction, respectively. By monitoring these early changes to mitochondrial function, initiated by disparate apoptotic agonists, two spatially distinct intrinsic cell death pathways were identified. Each pathway utilizes a different mechanism for the initiation of cell death and thus the contribution of either mechanism to mitochondrial dysfunction is determined by the apoptotic agonist.

Although a role for mitochondria in initiating cell death has been well documented (Desagher and Martinou, 2000), mitochondria are also the target of feedback amplification loops that are initiated by activated Caspases. Cleavage of the BH3 only protein Bid into t-Bid is a well documented mechanism of feedback amplification inducing the release of apoptotic factors from mitochondria. Indeed, the release of cytochrome *c* and AIF can proceed through Caspase-dependent and independent mechanisms (Arnoult et al., 2003; Cande et al., 2002; Breckenridge et al., 2003). Thus, early changes to mitochondrial physiology that are Caspase *independent* are therefore considered initiating events for apoptosis as these lead to the downstream activation of the execution phase of cell death. In contrast, changes to mitochondrial physiology that occur after Caspase activation are generally ascribed to the execution phase as they are considered to result from feedback amplification events.

Endoplasmic reticulum[1]

Recently, a number of studies have highlighted the ER as a key upstream signaling organelle for the regulation of apoptosis (Nutt et al., 2002b; Scorrano et al., 2003; Zong et al., 2003), and consistent with this, several members of the Bcl-2 family are also located at the ER (reviewed in (Annis et al., 2004)). While Bcl-2 family members regulate the release of apoptotic factors from mitochondria reviewed in (Newmeyer and Ferguson-Miller, 2003), it is less clear what processes Bcl-2 members regulate at the ER. One physiologically relevant candidate is control of ER Ca^{2+} levels.

The original observation that Bcl-2 regulates intracellular Ca^{2+} levels is now a decade old (Baffy et al., 1993). However the effect of Bcl-2 on intracellular and intra-organellar Ca^{2+} levels is still controversial and the literature contains conflicting data. Overexpression of Bcl-2 in human breast epithelial cells results in increased $[Ca^{2+}]_{ER}$ (Kuo et al., 1998), and in mouse lymphoma cells Bcl-2 increases Ca^{2+} uptake into the ER (He et al., 1997). Although the mechanism by which Bcl-2 increases $[Ca^{2+}]_{ER}$ is not clear, breast cells expressing Bcl-2 have increased sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) pump expression (Kuo et al., 1998). Increased $[Ca^{2+}]_{ER}$, or rather the maintenance of ER Ca^{2+} levels, promotes cell proliferation and growth, thus inhibiting cell death (He et al., 1997). However, other studies indicate that Bcl-2 expression causes decreased $[Ca^{2+}]_{ER}$ in HeLa cells (Pinton et al., 2000), human prostate cancer cells (Vanden Abeele et al., 2002), HEK-293 (human embryonic kidney) cells and R6

1 This subsection was published in the review Annis et al., 2004.

fibroblasts(Foyouzi-Youssefi et al., 2000). Here, decreases in $[Ca^{2+}]_{ER}$ by Bcl-2 are postulated to protect cells from apoptosis by limiting the amount of Ca^{2+} released into the cytoplasm following an apoptotic stimulus. Limiting the Ca^{2+} available for uptake into the mitochondria (Ferrari et al., 2002) by this mechanism can protect mitochondria from permeability transition and mitochondrial dysfunction [reviewed in (Smaili et al., 2003)]. In this case lower $[Ca^{2+}]_{ER}$ is due to a Bcl-2 mediated increase in leakage of Ca^{2+} from the ER by an unidentified mechanism (Pinton et al., 2000).

Consistent with the concept that pro- and anti-apoptotic Bcl-2 family members regulate similar aspects of cell physiology, recent investigation has demonstrated that Bax and Bak also modulate $[Ca^{2+}]_{ER}$ (Nutt et al., 2002b; Nutt et al., 2002a; Scorrano et al., 2003). And, similar to Bcl-2, Bak and Bax have also been reported to modulate $[Ca^{2+}]_{ER}$ in either direction.

In human PC-3 prostate adenocarcinoma cells, ectopically expressed Bax or Bak is located to both mitochondria and the ER. The expression of these proteins causes a decrease in $[Ca^{2+}]_{ER}$, which can be inhibited by Bcl-2. In addition, the Bak/Bax induced decrease in $[Ca^{2+}]_{ER}$ causes a corresponding increase uptake of Ca^{2+} into mitochondria. Furthermore, inhibition of mitochondrial Ca^{2+} uptake by treatment with RU360, an inhibitor of the Ca^{2+} uniporter, inhibits the release of cytochrome *c* and apoptosis induced by Bak/Bax overexpression (Nutt et al., 2002b). It is interesting to note that in these cells expression of Bcl-2 inhibits the release of ER Ca^{2+} induced by Bax expression. Whether this is the result of direct interaction between Bcl-2 and Bax or Bcl-2 regulating Ca^{2+} levels independently of Bax remains to be determined.

The influence of Bax and Bak on intracellular Ca^{2+} has also been examined in double knock-outs mouse embryonic fibroblasts (MEFs) where both the Bak and Bax alleles were deleted (Scorrano et al., 2003). Double knock-out cells have decreased $[Ca^{2+}]_{ER}$ compared to their wild-type counterparts. As a consequence, these cells are more resistant to the lipid second messenger ceramide, which induces cell death by increasing cytoplasmic Ca^{2+}(Pinton et al., 2001). Ectopic expression of HA-tagged-Bax restores $[Ca^{2+}]_{ER}$ to levels seen in wild-type MEFs and restores ceramide induced cell death. Thus, in contrast to experiments with prostate cancer cells, the lack of Bax expression causes a decrease $[Ca^{2+}]_{ER}$ in MEFs. Aside from the different cell types studied, the apparently contradictory results may be due to differences in the time course and level of Bax expression so that when expressed transiently at high levels Bax causes the release of $[Ca^{2+}]_{ER}$, however at low levels Bax appears to increase $[Ca^{2+}]_{ER}$.

Thus, the literature indicates that in different circumstances $[Ca^{2+}]_{ER}$ can be regulated in either direction by both pro- and anti-apoptotic Bcl-2 proteins. Bcl-2 can decrease $[Ca^{2+}]_{ER}$ (Ferrari et al., 2002; Foyouzi-Youssefi et al., 2000) yet, depletion of $[Ca^{2+}]_{ER}$ can have detrimental effects on the cell (Srivastava et al., 1999). Therefore, a delicate balance must exist between the activities of pro- and anti-apoptotic proteins in the regulation of $[Ca^{2+}]_{ER}$. The presence of a Ca^{2+} gradient between the ER [high] and the cytoplasm [low] indicates that regulation of $[Ca^{2+}]_{ER}$ by Bcl-2 proteins is unlikely to be explained solely by the ability of these proteins to function as ion selective pores: how would Bax/Bak 'pump' Ca^{2+} into the ER against a pre-existing concentration gradient?

Despite the controversy surrounding the regulation of Ca^{2+} at steady state, it is clear that changes in cytoplasmic Ca^{2+} levels can trigger apoptosis. Indeed blocking Ca^{2+} signaling with cytoplasmic chelators inhibits cell death (Dowd et al., 1992). Release of Ca^{2+} from the ER initiates cell death by activating the Ca^{2+} dependent protease Calpain, Caspases (Nakagawa and Yuan, 2000)) and/or directly causing mitochondrial dysfunction [reviewed in(Smaili et al., 2003)]. Crosstalk between the ER and mitochondria is a critical mechanism for determining cell fate (Hacki et al., 2000). Indeed, in Chapter Three of this thesis I demonstrate that ceramide, a lipid second messenger, triggers apoptosis through the early loss of mitochondrial membrane potential, a process that is believed to be mediated by increased cytoplasmic Ca^{2+} (Pinton et al., 2001). Consistent with a role for ER Ca^{2+} in this process, ceramide induced cell death is inhibited by ER localized Bcl-2 (Chapter Three and (Annis et al., 2001)).

In summary, the decision phase of cell death is centrally regulated by the Bcl-2 family of proteins. This family functions at both the ER and mitochondria to determine cell fate. If a death signal is 'strong' enough to overcome the inhibitory activity of the anti-apoptotic Bcl-2 proteins then execution phase of cell death is initiated.

Execution Phase – Caspase activation and cell disintegration

The ultimate target of any apoptotic pathway is the activation of Caspases. These cellular proteases exist as inactive zymogens until activated either by proteolytic cleavage, in the case of executioner caspases, or dimerization as seen with initiator Caspases

(reviewed in (Boatright and Salvesen, 2003)). Eleven human Caspases have been identified and are subdivided into either initiator or executioner subfamilies (reviewed in (Degterev et al., 2003)). Initiator caspases include the apical Caspase of the extrinsic pathway, Caspase-8, Caspase-9 and the murine ER-stress activated Caspase-12. These initiator Caspases then cleave and activate the executioner or effector Caspases including Caspase-3, -6, and -7.

Caspases have very strict substrate specificity; however targets for their activity have been identified in almost all known cellular processes including cell adhesion; RNA synthesis, protein translation, DNA repair and cell signaling (reviewed in (Fischer et al., 2003)). Most Caspases are considered terminal activators of cell death as the cleavage of their substrates is irreversible. The nuclear protein Poly (ADP-ribose) polymerase (PARP) is commonly utilized substrate to monitor the activity of Caspases-3 and -7 and hence the execution phase of cell death. PARP has been implicated in the regulation of numerous cellular processes including DNA replication, transcription, DNA repair, apoptosis, and genome stability (Bouchard et al., 2003). Cleavage by Caspases separates the catalytic polymerase function of PARP from its DNA binding motifs thus preventing the repair of DNA strand breaks (Tewari et al., 1995). Since the execution phase is common to both intrinsic and extrinsic cell death pathways, cleavage of PARP indicates the execution phase has been initiated.

Another important substrate for Caspase activity is Inhibitor of Caspase-Activated DNase (ICAD or DFF45) (Enari et al., 1998; Liu et al., 1997). ICAD is bound to Caspase-Activated DNase (CAD/DFF40) in the cytoplasm until Caspase-3 cleaves ICAD

liberating it from CAD. Once free of ICAD, CAD is imported into the nucleus where it cleaves DNA into internucleosomal fragments, a hallmark of apoptosis (Sakahira et al., 1998).

Although the activation of effector caspases is often considered the "point of no return" for apoptosis there are several Caspase inhibitors (IAPs) present in the cell. Seven mammalian IAP proteins have been identified, six of which inhibit the activity of effector caspase-3 and -7 (reviewed in (Liston et al., 2003)). IAPs bind to and inactivate caspases by altering the conformation of the Caspase or sterically preventing the substrate binding (Shiozaki et al., 2003). Interestingly the mitochondrial protein Smac, which is released during apoptosis, disrupts the binding of IAPs to caspases and thus promotes apoptosis (Du et al., 2000). Cleavage of Caspase substrates results in the morphological and biochemical changes that occur during cell death; and thus, is the final regulated phase of cell death.

Determining cell fate – The influence of Cellular 'Context'

As outlined above, cell death is regulated at all stages from the initial recognition of a death stimulus to the cleavage of substrates by activated Caspases. The susceptibility of a given cell to any apoptotic agonist will depend on its cellular 'context' (Fridman and Lowe, 2003), that is gene expression, presence of growth factors etc. A good example of this is seen in the response of different lymphoid cell lines to Fas induced cell death. As outlined above, Fas initiates the extrinsic cell death pathway via activation of Caspase-8 which in turn activates effector Caspases as well as the pro-apoptotic protein Bid (cleaved

into t-Bid). However, depending on the cellular 'context', Fas treatment of certain lymphoid cell lines (referred to as type II cells) will not lead to the direct activation of effector Caspases; rather these cells die due to the activity of t-Bid. In contrast, type I cells respond to Fas by activation of Caspase-8 and then direct engagement of effector Caspases. Thus even with the same apoptotic stimulus in similar cell types individual cells lines will behave differently. Interestingly, expression of the anti-apoptotic proteins Bcl-2 or Bcl-X_L will inhibit Fas induced cell death in type II, but not type I cells (Scaffidi et al., 1998). Thus the cellular context also determines the influence of pro- and anti-apoptotic Bcl-2 proteins on cell fate determination.

Another important factor influencing cell fate is the expression of the proto-oncogene c-Myc (referred to as Myc in this thesis). Myc is a basic helix-loop-helix leucine zipper transcription factor that has roles in both cell proliferation and apoptosis (Mateyak et al., 1997; Evan et al., 1992; Bissonnette et al., 1992). In quiescent cells Myc is not expressed, however following growth factor stimulation, expression is rapidly induced and maintained in cycling cells. Ectopic expression of Myc can promote cell cycle progression in reduced growth factor conditions (Cavalieri and Goldfarb, 1987). Although an exact role in cell cycle progression is unknown, deletion of Myc in Rat-1 fibroblasts results in extension of the cell cycle (Mateyak et al., 1997).

A role for Myc in apoptosis was first proposed when ectopic Myc expression was shown to sensitize Rat-1 fibroblasts to serum starvation induced cell death (Evan et al., 1992). Since this initial observation Myc expression has been shown to sensitize cells to a variety of apoptotic stimuli (Soucie et al., 2001; Juin et al., 2002), suggesting Myc

activity may influence a central target in cell death. In Chapter Four of this thesis I demonstrate that Myc expression is required for the activation and oligomerization of the pro-apoptotic protein Bax as well as the release of cytochrome *c* from mitochondria.

In summary, the response of a given cell to specific apoptotic agonists is dependent on gene expression and environmental cues. This reality makes generalizations about the details of any apoptotic pathway difficult, as the details are frequently both cell and 'context' specific. Despite this limitation, the molecular functions of the main apoptotic players appear universal; however the precise molecular mechanisms remain unknown for many of these proteins.

Model for cell fate regulation

The first molecular details of the regulation of apoptosis were discovered in the nematode *C. elegans*. This worm is used as a model organism because of its size, genetics and well characterized maturation; during development precisely 131 of 1090 cells undergo apoptosis (Ellis and Horvitz, 1986). Investigations performed by Nobel Laureate Robert Horvitz and colleagues identified fifteen genes involved in the process of programmed cell death (Horvitz, 2003). Central to the regulation of cell fate are four genes: *ced-9*, *ced-4*, *ced-3*, and *egl-1*. In a simplified model (Figure 3), *ced-9* encodes a cell survival protein (Hengartner and Horvitz, 1994) that inhibits cell death by sequestering the adaptor protein Ced-4 (Chinnaiyan et al., 1997a) and the cellular protease Ced-3 (Xue and Horvitz, 1995). To induce cell death this ternary

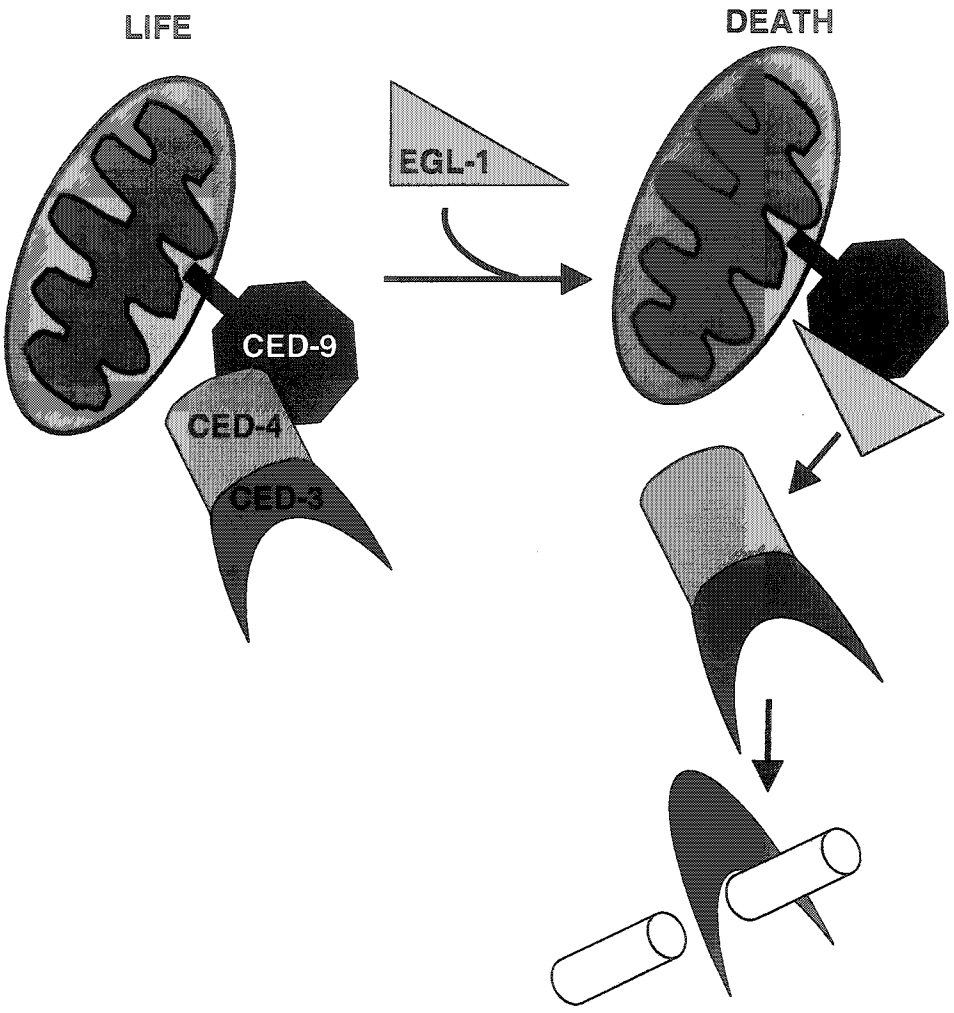

Figure 3. Model for the regulation of cell death in *C. elegans*. In healthy cells Ced-9 tethers Ced-4 and Ced-3 to membranes (in this example, mitochondria). Expressed Egl-1 binds to Ced-9 disrupting its interaction with Ced-4. The release of Ced-4/Ced-3 leads to the activation of Ced-3 (a cellular protease), cleavage of substrates and the execution phase of cell death.

complex is disrupted by the pro-death protein Egl-1 binding to Ced-9 displacing Ced-4 leading to Ced-3 activation and downstream apoptotic events (Chinnaiyan et al., 1997b). Homologues for these proteins exist in mammalian cells: Bcl-2 (Ced-9), BH3 only proteins (Egl-1), Caspases (Ced-3), and Apaf-1 (Ced-4) (Horvitz, 2003). However, the regulation of cell death in mammalian cells is not as direct as that of the nematode. For example, unlike in *C. elegans* interactions between Bcl-2 and BH3 proteins does not induce the direct activation of Caspases; rather BH3 proteins promote the disruption of mitochondrial membrane releasing 'second messengers' such as cytochrome *c* that trigger the activation of Caspases through the apoptosome complex (Figure 4). This increased complexity in mammalian cells provides additional regulatory mechanisms to determine cell fate.

In these models the direct interaction with pro-apoptotic proteins is critical to the function of the anti-apoptotic proteins; however hetero-dimerization independent activity has also been reported for these proteins. Disrupting the ability of Bcl-2 to bind to the pro-apoptotic protein Bax, as in the BH1 mutant G145A, impairs Bcl-2 activity in serum starvation, gamma-irradiation and Bad induced cell death (Yin et al., 1994; Thomenius et al., 2003). However, expression of this Bcl-2 mutant confers resistance to spontaneous and glucocorticoid induced cell death in thymocytes (St Clair et al., 1997). Similar observations have been made for the anti-apoptotic protein Bcl-X_L(Minn et al., 1999; Cheng et al., 1996). However, it is important to note that these "hetero-dimerization deficient"

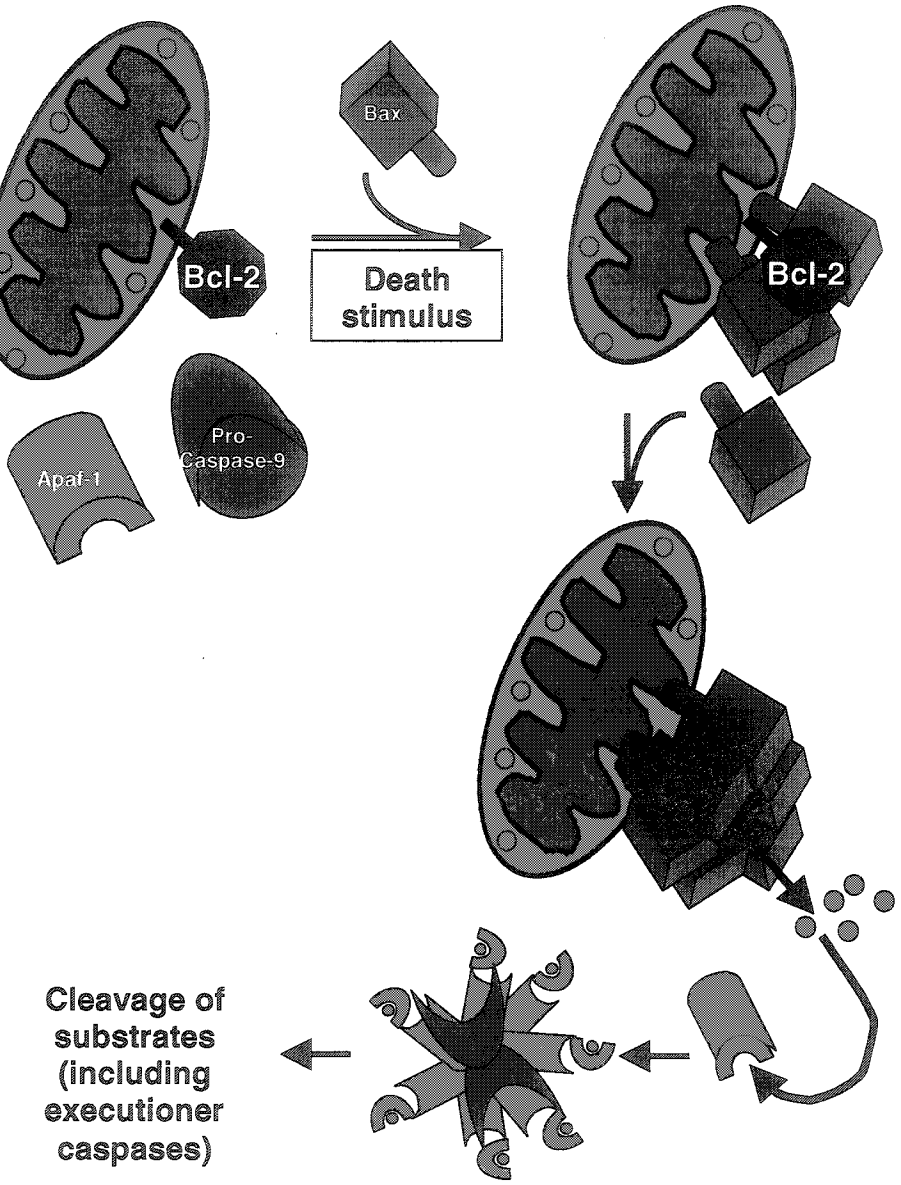

Figure 4. A Model for Caspase activation at mitochondria. In healthy cells Bcl-2 is anchored the ER and mitochondria (mitochondria in this example). In these cells Apaf-1 and Caspase-9 are cytoplasmic and inactive. Stimulation of cells with a death agonist induces the translocation of Bax to mitochondria. If Bcl-2 is present then Bax oligomerization is delayed in these cells. However, prolonged exposure to a death agonist leads to increased Bax on the mitochondria membrane eventually overwhelming the Bcl-2 and triggering the release of cytochrome *c*. Once released cytochrome c interacts with apaf-1, and in the presence of dATP (not shown) the apoptosome is formed. Pro-Caspase-9 is recruited to the complex and activated, resulting in initiation of the execution phase of apoptosis (This model is based on published and experimental evidence presented in this thesis, see text for further details).

mutants disrupt interactions with specific pro-apoptotic proteins and thus interactions with other Bcl-2 family members are still possible.

Investigations presented in this thesis

Expression of Bcl-2 confers resistance to a variety of apoptotic stimuli (Vaux et al., 1988; Zhu et al., 1996; Annis et al., 2001; Soucie et al., 2001). In part, resistance to these disparate apoptotic agonists is thought to be mediated by the multiple locations for Bcl-2 within the cell (ER, mitochondria and nuclear envelope). To address the relative contribution of Bcl-2 at these organelles mutants of Bcl-2 which localized exclusively to either the endoplasmic reticulum or mitochondria were generated (Zhu et al., 1996; Lee et al., 1999). By using this approach, we demonstrated that Bcl-2 does not need to be located at both physiological sites (the ER and mitochondria) to function; however ER localized Bcl-2 can only inhibit certain apoptotic death stimuli. In Chapter Three of this thesis, the characteristics of cell death pathways inhibited by ER localized Bcl-2 are compared to pathways where ER localized Bcl-2 does not function. In this analysis two spatially distinct intrinsic cell death pathways are presented and related to the activity of Bcl-2. Cell death stimuli where Bcl-2-cb5 is functional are characterized by the early loss of $\Delta\Psi_m$; whereas death stimuli that are not inhibited by Bcl-2-cb5 expression are characterized by the early release of cytochrome *c*. Thus by examining early changes to mitochondrial physiology the upstream signalling pathways for a specific cell death agonist can be determined.

The ability of Bcl-2 expression to inhibit cell death has been linked to the expression of the proto-oncogene Myc (Vaux et al., 1988; Fanidi et al., 1992; Soucie et al., 2001). This observation suggests Myc expression sensitizes cells to apoptosis by activating pro-apoptotic Bcl-2 proteins; however the target has remained elusive. In Chapter Four of this thesis, the contribution of Myc in the progression of cell death is investigated by examining the response to c-Myc null Rat-1 fibroblasts to apoptotic stimuli. In the absence of Myc, Bax translocates to mitochondria but remains monomeric and cytochrome *c* is not released. In cells expressing Myc, apoptotic stimuli trigger Bax translocation, oligomerization and cytochrome *c* is release. These investigations revealed that Myc sensitizes cells to apoptotic stimuli by augmenting mitochondrial dysfunction through the activity of Bax. In addition, I demonstrate that membrane integration and activation of Bax are separable steps and suggest Bax activation proceeds through multiple regulatory stages: translocation; integration into membranes, activation and oligomerization.

These studies reinforced the observation that Bax oligomerization is required for pore formation as measured by cytochrome *c* release (Antonsson et al., 2000). Having the ability to examine cytosolic, translocated and oligomeric forms of Bax individually, provided the unique opportunity to address the importance of membrane topology changes in Bax activity. Bcl-2 proteins are thought to form pores or channels in membranes by the insertion of their putative pore forming domains into the lipid bilayer. To test this hypothesis, cysteine specific chemical labelling was used to determine the membrane topology of Bax in the presence and absence of Myc expression. As Bax has

the general structure of a tail anchor protein we predicted that translocated but monomeric Bax would adopt the topology of a typical tail anchor protein with only the tail inserted into the lipid bilayer; however when Bax oligomerizes (and thus promotes the release of cytochrome *c*) the pore forming domain (helices α5 and α6) would also be inserted into the lipid bilayer. Interestingly, the results from this chemical labelling analysis suggest that after translocation Bax adopts a multiple membrane spanning topology in both the monomeric and oligomeric state. Thus, it would appear that insertion of helices α5 and α6 is not sufficient for the release of cytochrome *c* from mitochondria.

The investigations presented in this thesis address several controversial aspects of cell death regulation namely: the contribution of the ER to the regulation of cell death; the target of Myc in sensitizing cells to apoptosis; and the contribution of Bax membrane topology and oligomerization to pro-apoptotic activity. I show that the ER regulates a cell death pathway spatially distinct from pathways that rely on Bax mediated permeabiliztion of mitochondria. Bax activity at mitochondria depends on myc expression and sensitizes cells to apoptosis induced by a variety of stimuli by promoting Bax oligomerization rather than a topology change at mitochondria. Combined these studies provide valuable insight into both the complexity of the regulation and the molecular mechanisms of Bcl-2 proteins in the regulation of apoptosis.

Chapter Two

Materials and Methods

Cell culture:

Rat-1-myc fibroblasts constitutively expressing human Bcl-2 and Bcl-2-cb5 (Zhu et al., 1996) were maintained in phenol red minus, alpha-minimal essential medium supplemented with 10% charcoal-filtered treated fetal bovine serum (Gibco-BRL). Rat-1-mycERTM fibroblasts constitutively expressing human Bcl-2 and Bcl-2-G145A were maintained in alpha-minimal essential medium (Gibco-BRL) supplemented with 10 % fetal bovine serum (Hyclone Inc). Rat HO15.19 and HOmyc3 cells were maintained in Dulbecco's Modified Eagle Media supplemented with 10 % Calf Serum (Gibco BRL).

Plasmid construction:

Single Cysteine Bax mutants: Mutations were made to the cDNA sequence for human Bax (pMac-1529) using Quick-Change™ mutagenesis (Stratagene) according to the manufactures' protocol with the following exceptions: dNTP final concentration 0.5 mM, PFU DNA polymerase (MBI Fermentas), oligonucleotides 0.4 μM, and an extension time of 15 minutes (for 18 cycles). The sequences for the oligonucleotides utilized to generate the mutations are in Table I. After mutagenesis all constructs were sequenced and the Bax cDNA excised using Bgl II/EcoR I restriction enzymes, the resulting fragment was gel purified and sub-cloned into the BamH 1 and EcoR I sites of pBABE-mnIRES-GFP (pMac-1248). These constructs were sequenced using primers MAC-781 and MAC-782. The resulting constructs were then package and the virus used to infect the *Myc* null (HO15.19) and *Myc* reconstituted cells (HOmyc3) by Erinn L. Soucie a graduate student in Dr. Linda Z. Penn's laboratory at the University of Toronto.

Cells expressing green fluorescence protein (GFP) were selected using a fluorescence activated cell sorter and pooled populations were established.

Bcl-2-G145A: This mutation was made to the cDNA sequence for human Bcl-2 (pMac-656) using Quick-Change™ mutagenesis (Stratagene) according to the manufactures' protocol with the following exceptions: dNTP final concentration 0.5 mM, PFU DNA polymerase (MBI Fermentas), oligonucleotides 0.4 μM, and an extension time of 15 minutes (for 18 cycles). After mutagenesis this construct was sequenced and the Bcl-2 cDNA excised using Bgl II and Sal I restriction enzymes, the resulting fragment was gel purified and sub-cloned into the BamH I and Xba I sites of pBABE-Hygro (pMac-1742). The resulting construct was transfected into Rat-1-mycERTM fibroblasts using ExGen500 transfection reagent (MBI Fermentas) according to the manufacture's protocol. A pooled population of cells was established after selection in 150μg/mL hygromycin B (Invitrogen).

Preparation of Cellular Extracts by Nitrogen Cavitation

For the Rat-1-myc cells five 100 mm Petri dishes of cultured cells (approximately 60-70% confluent) were harvested in their respective medium using a rubber policeman. For the HOmyc3 and HO15.19 cells ten and twenty 100 mm Petri dishes of cultured cells, respectively, were harvested. The cells were then pelleted by centrifugation in a clinical centrifuge (setting 6) for 3 minutes 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 phenylmethylsulfonyl fluoride, 1 mM Dithiothreitol and 2 X PIN (0.2 μg/mL of

chymostatin, antipain, leupeptin and pepstatin and 0.4 μg/mL of aprotinin)). The final pellet was resuspended in an equal volume of cell buffer. This suspension was held at 150 psi for 15 minutes 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 500x g for 2 minutes in a microcentrifuge (Eppendorf). The resulting supernatant, termed the whole cell lysate (WCL), was then split into a post-heavy membrane supernatant (S^1) and pellet (P^1) fraction by centrifugation at 1,000 x g for 25 minutes in a Beckman TLA100 rotor at 4 °C. Pellet fractions were resuspended in cell buffer. Alternatively, for the analysis of Bax localization (Chapter Three), the WCL was subjected to centrifugation at 100,000 x g for 1 hour to generate supernatant (S^{100}) and pellet (P^{100}). Carbonate extraction of membrane fractions were performed in 0.1 sodium carbonate on ice for 30 minutes and then separated into soluble (SC) and pellet fractions by centrifugation through a 0.5 M sucrose cushion at 100,000 x g for 1 hour at 4°C in the TLA100 rotor (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).

Cell Death Assays and Immunofluorescence

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 40 μM etoposide (McMaster Pharmacy) (for 5 hours), 50 μM C2-ceramide (Sigma) (for 6 hours) or 0.1 % fetal bovine serum + 2 μM estradiol (for 24 hours), as specified. The pan

Caspase inhibitor zVAD-fmk (Enzyme System Products) was added (200 μM) 30 minutes prior to treatment with etoposide (40 μM, 6hrs). The HO15.19 and HOmyc3 cells were treated with 6 μM etoposide or 0.5 μM Taxol (Sigma) for the indicated times. 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 minutes in the dark, the cells were incubated in culture medium at 37 °C for 15 minutes 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 3-{3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate (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 of antibody used for immunostaining, 1:50). For competition experiments, purified 6A7 antibody was incubated in 3% BSA bovine serum albumin–phosphate buffered saline containing either 5 μg of purified Bax glutathione *S*-transferase (GST), 5 μg of GST–Bcl-2, or an equal volume of buffer at room temperature for 1 h

prior to staining. This antibody was then used for immunofluorescence as described above.

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. Antibodies to cytochrome *c* were affinity purified according to on-line Andrews' Lab manual protocol using an Affigel-10 (Bio-Rad) coupled to cytochrome *c* (Rabbit, Sigma) matrix. The antibody to poly-ADP ribose polymerase (PARP) was purchased from BioMol and used according to the manufacturer's instructions. Mouse anti-human Bax (2D2) was obtained from Exalpha-Biologicals. Purified Bax protein was prepared by Dr. Jeremy Yethon (Yethon et al., 2003). For standard SDS-PAGE, protein samples were separated on a 10% polyacrylamide tricene gel, followed by transfer to a PVDF membrane for PARP, Bcl-2 and Bax immunostaining, or to nitrocellulose membrane for use with other antibodies. The membranes were blocked and then incubated with either affinity purified Sheep anti-cytochrome *c* (dilution 1:5,000), Rabbit anti-Hsp60 (dilution 1:10,000), Mouse anti-PARP (1:10,000), Mouse anti-rat Bax (dilution 1:5,000), Mouse anti-human Bax (1:5000) and Rabbit anti-Bcl-2 (1:10000) antibodies and developed after incubation with peroxidase-conjugated donkey anti-sheep, mouse or rabbit (Jackson Laboratories) using an enhanced chemiluminescence detection system (NEN-Life Science).

Gel filtration chromatography

One hundred micrograms of membrane protein (P^1) was solubilized in cell buffer + 300 mM NaCl and 2% CHAPS for 30 minutes on ice. Samples were applied to a

Superdex 200 HR 10/30 column (Amersham Biosciences) equilibrated in 20 mM Na HEPES pH 7.5, 300 mM NaCl, 0.2 mM DTT, and 2% (w/v) CHAPS. Fractions (400 µL) were collected starting at the column void volume; proteins were precipitated with TCA, and analyzed after SDS-PAGE by immunoblotting. The elution profile for known standards was determined by Dr. Jeremy Yethon using commercial protein standards.

IASD labeling reactions:

Ten 100 mm dishes of ~70 % confluent HO15.19 cells expressing the Bax mutants were treated with etoposide for 48 hours and then subjected to nitrogen cavitation and subcellular fractionation to separated soluble and membrane bound Bax (See Nitrogen Cavitation). One hundred microgram aliquots of protein from both the supernatant and membrane fraction were labeled with 150 nmol of 4-acetamido-4'-((iodoacetyl)amino)stilbene-2,2'-disulfonic acid (IASD) (Molecular Probes). Samples were removed 5 and 15 minutes after the addition of IASD. Control samples were either left unlabeled or detergent solubilized (2% CHAPS/4% IGEPAL-630) prior to being labeled with IASD. Reactions were halted with 5000 nmols of DTT (100x excess). Samples were resolved on 4-6 IEF gel (1.5% 4-6 ampholytes (Bio-Rad), 0.5 % 3-10 ampholytes (Bio-Rad), 5% acrylamide (40%T/3%C BioShop), 8M urea (BioShop), 4% IGEPAL-630 (Sigma) , 2% CHAPS (BioShop) then transferred to PVDF in 1 % acetic acid/4% SDS using a semi-dry transfer apparatus (50 mA per gel for 1 hour with ice pack). Membranes were then decorated with the appropriate antibodies.

Quantification of band intensities:

To determine the extent of labeling, immunoblots were developed directly on the Typhoon9200 imaging station and data was collected in Chemiluminescence mode at 800 V Medium sensitivity. Band intensities were quantified using ImageQuant 5.2 Software subtracting background using a local average. The percent protected from IASD is simply the intensity of unmodified Bax divided by the sum of unmodified Bax and modified Bax at 15 minutes. Three separate gels were used in the determination of protection from IASD labeling, except for positions 142 and 126 where only two images were used as the band intensities were too weak in the other images to be quantified.

Mutant	Sense	Anti-Sense
G3C	5'cagatctaccatggactgttccggggagcagcc3'	5'ggctgctccccggaacagtccatggtagatctg3'
G40C	5'gggcgaatggggtgtgaggcacccgag3'	5'ctcgggtgcctcacaccccattcgccc3'
62C (C126A)	5' gaagctgagcgaggctctcaagcgcatcg 3'	5' cgatgcgcttgagagcctcgctcagcttc 3'
R109C	5'gcaacttcaactggggctgtgttgtcgccctt3'	5'aagggcgacaacacagccccagttgaagttgc3'
V111C	5'ctggggccgggtttgcgccctttctac3'	5'gtagaaaagggcgcaaacccggccccag3'
S118C	5'tctactttgcctgcaaactggtgctcaag3'	5'cttgagcaccagtttgcaggcaaagtaga3'
L122C	5'gccagcaaactggtgtgcaaggccctggcc3'	5'ggccagggccttgcacaccagtttgctggc3'
A124C	5'ctggtgctcaagtgcctggccaccaagg3'	5'ccttggtggccaggcacttgagcaccag3'
126C (C62A)	5'gctcaaggccctggccaccaaggtgccgg 3'	5'ccggcaccttggtggccagggccttgagc 3'
R134C	5'ggtgccggaactgatctgtaccatcatgggctgg3'	5'ccagcccatgatggtacagatcagttccggcacc3'
I136C	5'ccggaactgatcagaacctgcatgggctggacattgg3'	5'ccaatgtccagcccatgcaggttctgatcagttccgg3'
G138C	5'atcagaaccatcatgtgctggacattggacttc3'	5'gaagtccaatgtccagcacatgatggttctgat3'
D142C	5'tgggctggacattgtgcttcctccgggag3'	5'ctcccggaggaagcacaatgtccagccca3'
L144C	5'ggacattggacttctgccgggagcggctg3'	5'cagccgctcccggcagaagtccaatgtcc3'
I175C	5'cagaccgtgacctgctttgtggcg3'	5'cgccacaaagcaggtcacggtctg3'
G179C	5'catctttgtggcgtggctcaccg3'	5'cggtgagcacacacgccacaaagatg3'
S184C	5'gctcaccgcctgtctcaccatct3'	5'agatggtgagacaggcggtgagc3'
G192C	5'ccatctggaagaagatgtgctgaggcccgaattcat3'	5'atgaattcgggcctcagcacatcttcttccagatgg3'

Table 1. Oligonucleotide sequences for single cysteine mutants of human Bax.

Chapter Three

Identification and Characterization of two organelle specific apoptotic pathways in Rat-1 fibroblasts

Data in this chapter were published in:

Annis MG, Zamzami N, Zhu W, Penn LZ, Kroemer G, Leber B, Andrews DW. (2001) Endoplasmic reticulum localized Bcl 2 prevents apoptosis when redistribution of cytochrome c is a late event. Oncogene. 20(16):1939 52.

Introduction:

There are two general cell death pathways termed extrinsic and intrinsic depending on the source of the initial stimulus (Wajant, 2002). Extra-cellular, receptor mediated, death signals activate the extrinsic pathway whereas most chemotherapeutic agents target and disrupt essential cell processes and thus activate intrinsic cell death pathways. Although each pathway has alternative initiation events, both pathways converge on mitochondria, which function as central sensors, regulators and amplifiers of apoptosis (Kroemer, 1999).

Although mitochondria function as an important target for the intrinsic pathway; other organelles are involved in the regulation of cell death (Ferri and Kroemer, 2001). Indeed, many Bcl-2 family members function not only at mitochondria (Kuwana and Newmeyer, 2003), but the endoplasmic reticulum (ER) as well (reviewed in (Annis et al., 2004)). Although Bcl-2 was first shown to function at the ER in 1996 (Zhu et al., 1996), the importance of Bcl-2 at the ER has only recently been appreciated. To determine the contribution of the ER in cell death regulation, we examined apoptosis in cells in which the anti-apoptotic protein Bcl-2 was located exclusively at the ER.

To address the physiological relevance of Bcl-2 at the ER, a mutant form of Bcl-2 was created in which the endogenous insertion sequence, responsible for membrane targeting and integration, was replaced by one from the ER specific isoform of cytochrome b5 (Figure 1) (Zhu et al., 1996). This mutant, designated Bcl-2-cb5, is targeted exclusively to the ER, and inhibits apoptosis induced by serum

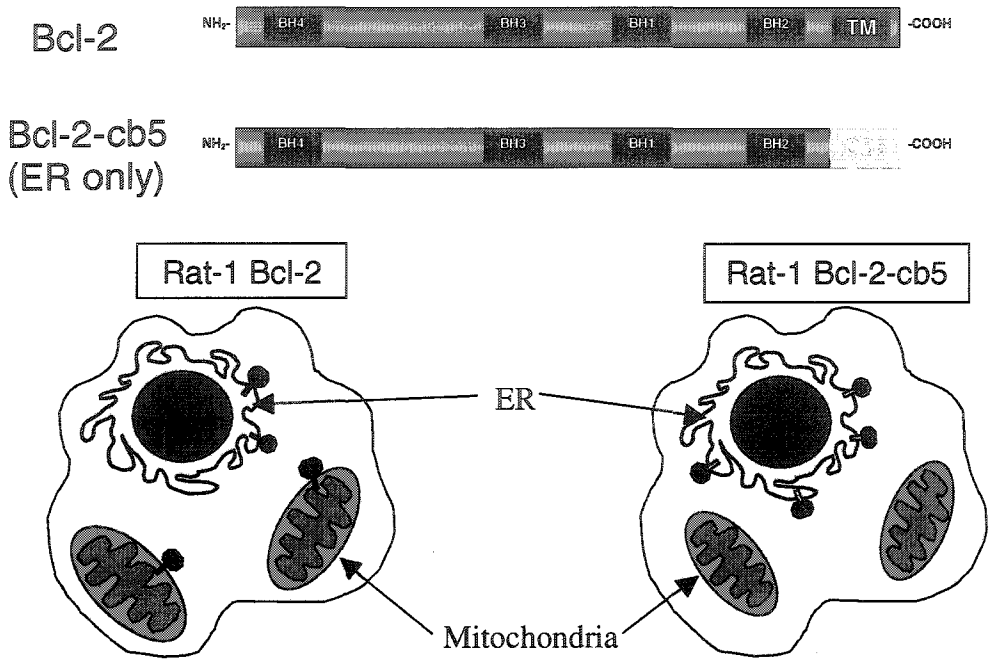

Figure 1. Schematic structure of Bcl-2 and Bcl-2-cb5. The linear structure of Bcl-2 with the Bcl-2 homology domains (BH1 – 4) are indicated along with the carboxyl terminal targeting sequence (TM). To generate ER localized Bcl-2 the targeting sequence of wildtype Bcl-2 was exchanged for the targeting sequence for the ER isoform of cytochrome b5 (CB5). Wildtype Bcl-2 is localized to both the ER and mitochondria, whereas Bcl-2-cb5 targets exclusively to the endoplasmic reticulum.

starvation/deregulated myc expression in Rat-1 fibroblasts (Zhu et al., 1996), brefeldinA and tunicamycin treatment in rat embryo fibroblasts (Hacki et al., 2000), DNA damage in Jurkat cells (Rudner et al., 2001), and Bad expression in MDA-MB-468 (Thomenius et al., 2003). However, Bcl-2-cb5 is not effective at inhibiting cell death stimulated by etoposide treatment (Lee et al., 1999).

What determines whether Bcl-2-cb5 inhibits apoptosis? Although certain death stimuli are known to cause ER stress (tunicamycin or brefeldinA); DNA damage, Bad expression and serum starvation cannot be directly linked to ER function. To determine if there is a common signaling pathway for these death stimuli, we monitored changes to the central target of cell death - mitochondria. During apoptosis mitochondria can undergo several dysfunction events including; reduction of the inner membrane potential ($\Delta\Psi_m$) (Zamzami et al., 1995b), generation of reactive oxygen species (Zamzami et al., 1995a), and the release of apoptotic regulators including cytochrome *c* (Liu et al., 1996) from the intermembrane space. These changes to mitochondrial function lead to the activation of downstream effectors of apoptosis and constitute some of the earliest detectable events. However, there are conflicting reports as to whether the release of cytochrome *c* or the loss of $\Delta\Psi_m$ is **the** initiating event of cell death (von Ahsen et al., 2000) (Petit et al., 1998) (Finucane et al., 1999). By examining Bcl-2-cb5 inhibition of cell death, we identified two spatially distinct intrinsic pathways to apoptosis and a possible resolution to this controversy. Apoptosis elicited by stimuli that lead to a decrease in $\Delta\Psi_m$, that precedes cytochrome *c* release is effectively inhibited by Bcl-2-cb5. By contrast, in the same cell, Bcl-2-cb5 is not effective at inhibiting apoptosis induced by

stimuli that cause redistribution of cytochrome *c* at a time when decreased $\Delta\Psi_m$ is not yet apparent. Thus two intrinsic pathways, distinguishable by the order of early changes to mitochondria, can be activated in the same cell, but only one pathway is regulated by Bcl-2-cb5 and hence, events at the ER.

Results

Bcl-2-cb5 prevents apoptosis induced by serum starvation/myc and ceramide, but not etoposide

Serum starvation and constitutive c-myc expression induces apoptosis efficiently in Rat-1 fibroblasts (Evan et al., 1992). A mutant Bcl-2 targeted exclusively to the ER (Bcl-2-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-2-cb5 prevented apoptosis initiated by other death stimuli, Rat-1 fibroblasts expressing Bcl-2-cb5 or control empty vector (Neo) were treated with ceramide or etoposide. As a positive control for Bcl-2-cb5 function cells were serum starved in the presence of deregulated c-myc expression (serum starved/myc), Cleavage of the Caspase-3/-7 substrate, poly (ADP-ribose) polymerase (PARP), was used to monitor the execution phase of cell death (Figure 2). PARP is a 116 kDa protein (PARP, Figure 2) that is cleaved once by either caspase-3 or -7 into 85 kDa (ΔPARP, Figure 2) and 31 kDa fragments (not detected with this monoclonal antibody).

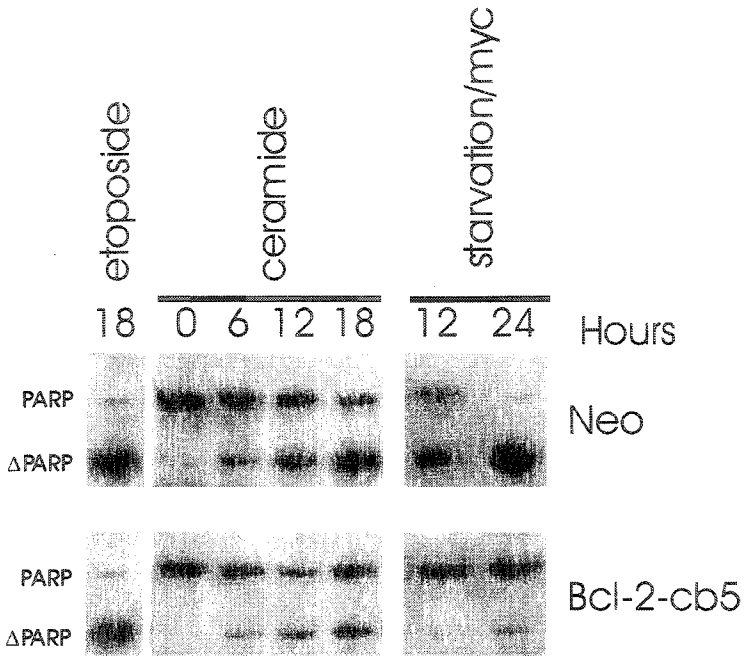

Figure 2. Bcl-2-cb5 inhibits PARP cleavage during apoptosis induced by ceramide or serum starvation/myc (starvation/myc), but not etoposide. Whole cell extracts were prepared from Neo or Bcl-2-cb5 cells (as indicated to the right of the panels) growing in regular medium (0 hours), or exposed to 50 μM 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-2-cb5 expressing) are indicated to the right of the panels.

Treatment of vector transfected Rat-1 fibroblasts (Neo) by serum starvation/myc, ceramide or etoposide results in the activation of these caspases as noted by the relative increase in the 85 kDa ΔPARP fragment over the duration of treatment. Expression of Bcl-2-cb5 delayed Caspase activity in serum starved/myc and ceramide, but not etoposide treated Rat-1 fibroblasts. Thus Bcl-2-cb5 expression effectively delays apoptosis initiated by serum starvation/myc and ceramide, but not etoposide.

Bcl-2-cb5 functions upstream of mitochondrial dysfunction

To determine if Bcl-2-cb5 inhibits apoptosis upstream of the mitochondria we monitored the loss of $\Delta\Psi_m$ and cytochrome *c* release by laser scanning confocal microscopy (Figure 3). Loss of $\Delta\Psi_m$ was monitored by staining cells with the potentiometric dye Mitotracker. Mitotracker is taken up by cells and concentrates in mitochondria due to the electrochemical gradient across the inner mitochondrial membrane. If mitochondria have lost their membrane potential then Mitotracker fails to accumulate in these mitochondria (Scorrano et al., 1999). To determine the order of mitochondrial dysfunction events, cells were co-stained with an affinity purified antibody to cytochrome *c*. Co-staining cells for both Mitotracker and cytochrome *c* allows for the temporal ordering of mitochondrial changes on an individual cell basis. As expected, in untreated, healthy, Rat-1 fibroblasts cytochrome *c* and Mitotracker staining are co-localized (Figure 3, untreated). Treatment of vector control Rat-1 fibroblasts with ceramide resulted in the loss of Mitotracker staining; however these same cells consistently retained mitochondrial staining for cytochrome *c* (Figure 3,

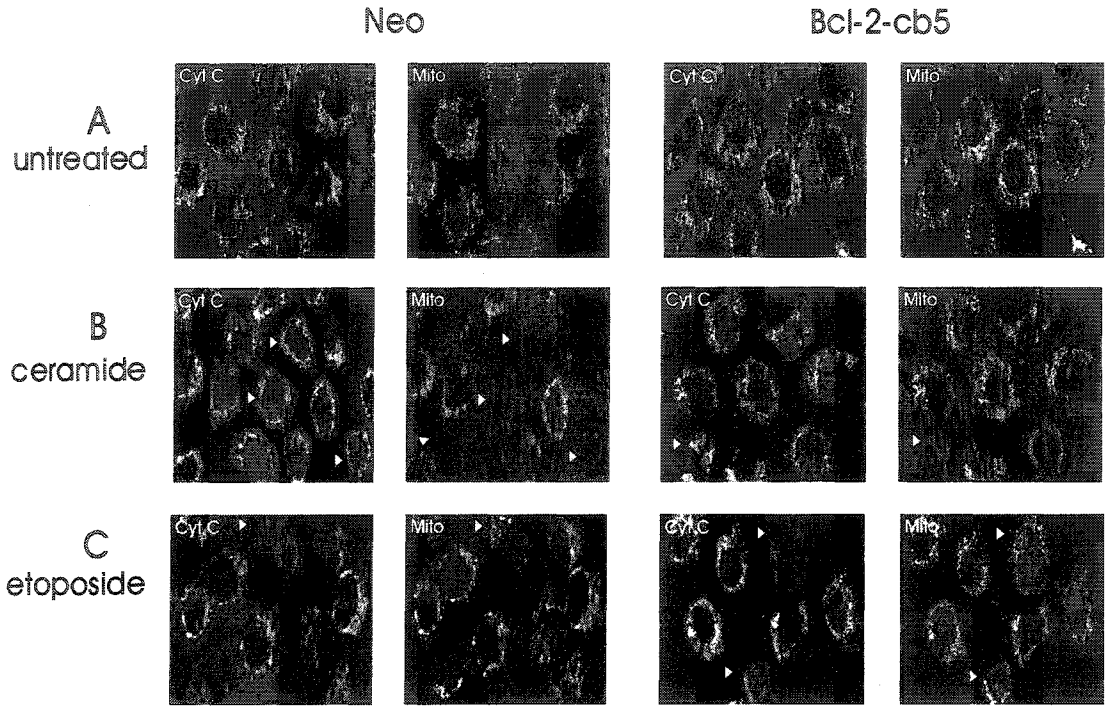

Figure 3. Cytochrome *c* release precedes loss of $\Delta\Psi_m$ in etoposide induced apoptosis. **(A)** In untreated, vector transfected (Neo) and Bcl-2-cb5 expressing Rat-1 cells grown in normal medium, in co-stained cells the staining patterns for cytochrome *c* and Mitotracker, were identical. **(B)** Exposure to 50 μM ceramide for 6 hours 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-2-cb5. **(C)** Incubation in 40 μM etoposide for 6 hours 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-2-cb5 cells. The width of each image is 144 μm.

ceramide arrowheads). Thus, the loss of $\Delta\Psi_m$ precedes the redistribution of cytochrome *c* in ceramide treated cells. From these same micrographs it is clear that apoptosis occurs in a cell autonomous manner as not all cells have decreased $\Delta\Psi_m$ at this time point.

Expression of Bcl-2-cb5 delayed the loss of Mitotracker staining in ceramide treated cells (Figure 3, ceramide arrowheads). Images were collected and the number of cells with decreased $\Delta\Psi_m$ was scored against those that retain mitochondrial membrane potential (Table 1). Expression of Bcl-2-cb5 or Bcl-2 inhibits ceramide induced loss of $\Delta\Psi_m$ as only 16% and 18% of these cells (respectively) have decreased Mitotracker staining, compared to 64% in the vector transfected Rat-1 fibroblasts (Table 1).

Interestingly, etoposide treated vector control cells retained mitochondrial staining for Mitotracker; however some of these same cells had diffuse staining for cytochrome *c*. In addition, diffuse cytochrome *c* staining could also be seen in etoposide treated Bcl-2-cb5 expressing Rat-1 cells. Consistent with Bcl-2-cb5 expression being ineffective at inhibiting etoposide induced PARP cleavage; however expression of wild type Bcl-2 prevented etoposide induced cytochrome *c* redistribution (Figure 4 and data not shown). Thus etoposide treatment induces the early redistribution of cytochrome *c* leaving mitochondrial membrane potential unaffected.

Cytochrome *c* redistribution is an early event in etoposide, but not ceramide or serum starvation/myc induced apoptosis

While micrographs permit analysis of individual cells, the extent of apoptosis is underestimated as this technique only examines adherent cells, thus if cytochrome *c*

release occurred after loss of adherence then it would not be observed in this analysis. Therefore, to obtain a more accurate indication of the extent of apoptosis cytochrome *c* release was also assayed by immunoblotting of cell fractions collected from both adherent and non-adherent cells (Figure 4). Cells were disrupted by low pressure nitrogen cavitation in an iso-osmotic sucrose buffer followed by centrifugation to generate cytosolic (post-heavy membrane supernatant) and membrane fractions. Breaking cells by nitrogen cavitation has two major advantages: the use of an iso-osmotic buffer avoids exposure to hypotonic solutions, which are known to compromise the outer mitochondrial membrane and cavitation results in decreased rupture of outer mitochondrial membranes as compared to mechanical homogenization (Adachi et al., 1998). Cell fractions from untreated and etoposide treated Rat-1 fibroblasts stably expressing Bcl-2, Bcl-2-cb5 or empty vector were immunoblotted with affinity purified anti-cytochrome *c* and anti-Hsp-60 antibodies. Hsp-60 is heat shock protein localized predominantly to the matrix of the mitochondria and thus serves as a control for the integrity of isolated mitochondria (Soltys and Gupta, 1996). In untreated cells the majority of cytochrome *c* and Hsp-60 are found in the pellet (membrane) fraction, indicating that mitochondria are not significantly fragmented or ruptured during nitrogen cavitation (Figure 4A and B). Etoposide treatment of vector and Bcl-2-cb5 expressing cells resulted in the redistribution of cytochrome *c* to the supernatant (cytosolic) fraction,

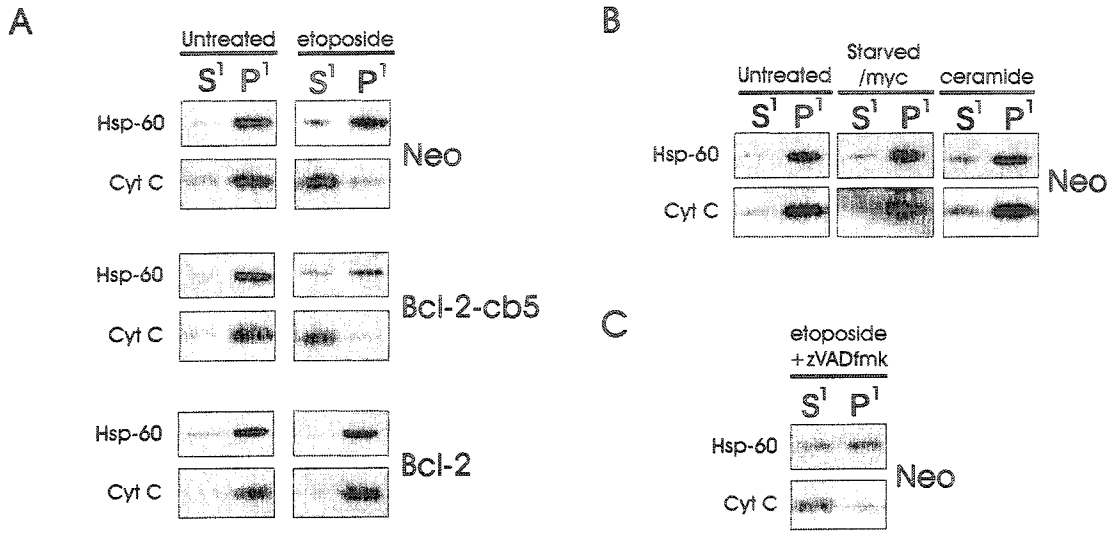

Figure 4. Etoposide, but not ceramide or serum starvation/myc. induces caspase-independent release of cytochrome c. Whole cell extracts were prepared from control or treated cells by low pressure nitrogen cavitation, nuclei and cell debris was removed by centrifugation and the resulting whole cell lysate was fractionated into a low speed supernatant (S1) and pellet (P1) by centrifugation at 1,000 x g for 25 min. 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. Vector transfected Rat-1 (Neo), Bcl-2-cb5 expressing (Bcl-2-cb5), and Bcl-2 (Bcl-2) expressing cells are indicated to the right of the panels. A. Cell fractions from Rat-1 cells untreated or exposed to etoposide (40 μM for 6 hours). B. Cell fractions from Rat-1 cells untreated or exposed to either ceramide (50 μM for 6 hours) or serum starved/myc (24 hours). C. Vector transfected Rat-1 cells (Neo) were incubated with 200 μM zVAD-fmk for 30 minutes and then treated with etoposide (40 μM for 6 hours).

while Hsp-60 was retained in the membrane fraction, indicating the redistribution of cytochrome is not an artifact of complete mitochondrial rupture during nitrogen cavitation (Figure 4A, Neo and Bcl-2-cb5). Expression of Bcl-2 inhibited cytochrome *c* redistribution in etoposide treated cells (Figure 4A, Bcl-2).

Similar to the micrograph analysis, ceramide treated or serum starved/myc Rat-1 fibroblasts retain cytochrome *c* and Hsp-60 in the membrane fraction (Figure 4B).

Cytochrome *c* release is coincident with Bax translocation and is Caspase independent

Cytochrome *c* release is thought to be mediated by the activity of multi-domain pro-apoptotic members Bax or Bak (Wei et al., 2001a). Bax is located in the cytoplasm or loosely associated with the mitochondria of healthy cells; however induction of apoptosis causes the translocation of Bax to mitochondria where it induces the release of cytochrome *c* (Wolter et al., 1997; Jurgensmeier et al., 1998). To determine if either redistribution of cytochrome *c* or loss of $\Delta\Psi_m$ was coincident with the translocation of Bax, cell fractions were immunoblotted with a monoclonal antibody specific for rat Bax (Figure 5). As Bax has been reported to be loosely associated with the mitochondria, carbonate extractions, which remove peripherally associated proteins, were performed on total membrane fractions, to determine if translocated Bax was membrane integrated.

In untreated Rat-1 fibroblasts Bax is predominately found in the cytosolic fraction (Figure 5, untreated). Treatment of Rat-1 cells expressing Bcl-2-cb5 or empty vector

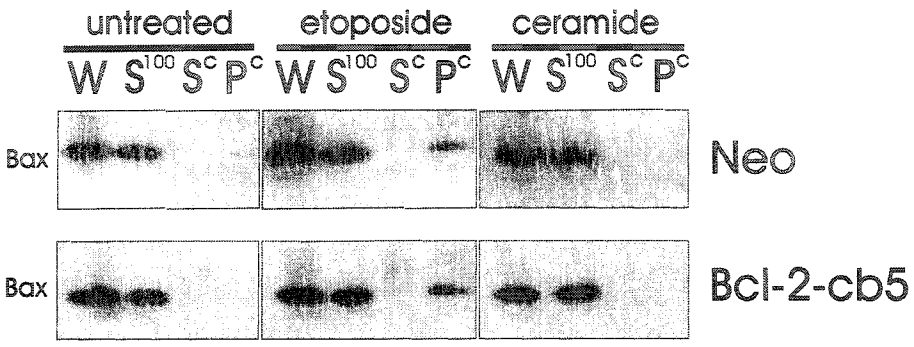

Figure 5. Etoposide, but not ceramide induces Bax membrane binding. Whole cell extracts (W) were prepared by low pressure nitrogen cavitation and then separated into supernatant (S^{100}) and pellet (P^{100}) fractions by centrifugation at 100,000 x g for one hour. The pellet fraction was resuspended and then incubated in 0.1 M sodium carbonate, pH 11.5 for 30 minutes at 4°C. Membrane bound proteins (P^C) were separated from peripheral proteins (S^C) by centrifugation at 100,000 x g 30 minutes. 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-2-cb5 (Bcl-2-cb5) were untreated or exposed to either 40 μM etoposide or 50 μM ceramide for 6 hours.

with etoposide resulted in the translocation of a fraction of the total cellular Bax to the carbonate resistant membrane fraction. However ceramide treatment did not induce membrane translocation of Bax. Thus Bax translocation is coincident with redistribution of cytochrome *c* but not the loss of $\Delta\Psi_m$. There are several proposed mechanisms leading to the activation Bax, one of these involves the cleavage of the BH3 only protein, Bid, by caspases. To determine if the release of cytochrome *c* was dependent on activated caspases cells were treated with the broad spectrum caspase inhibitor zVAD-fmk (Wu and Fritz, 1999). This inhibitor did not prevent the redistribution of cytochrome *c* in etoposide treated, vector transfected, Rat-1 fibroblasts (Figure 4C). Thus, this early change to mitochondrial physiology represents an initiating event for cell death as it occurs prior to the activation of the execution phase of apoptosis.

Bcl-2-cb5 does not prevent the etoposide induced conformational change in Bax

During apoptosis, Bax undergoes a conformational change coincident with the release of cytochrome *c* from mitochondria (Nechushtan et al., 1999). This conformational change in Bax structure results in the exposure of a normally inaccessible epitope that is recognized by the monoclonal antibody 6A7 (Nechushtan et al., 1999). The cell fractionation analysis presented (Figure 4 and 5) provides a correlation between Bax translocation and the release of cytochorme c; however to determine if Bax activation correlates with cytochrome *c* release on a single cell basis we examined the exposure of the 6A7 epitope by immuno-fluorescence in Rat-1 cells exposed to etoposide

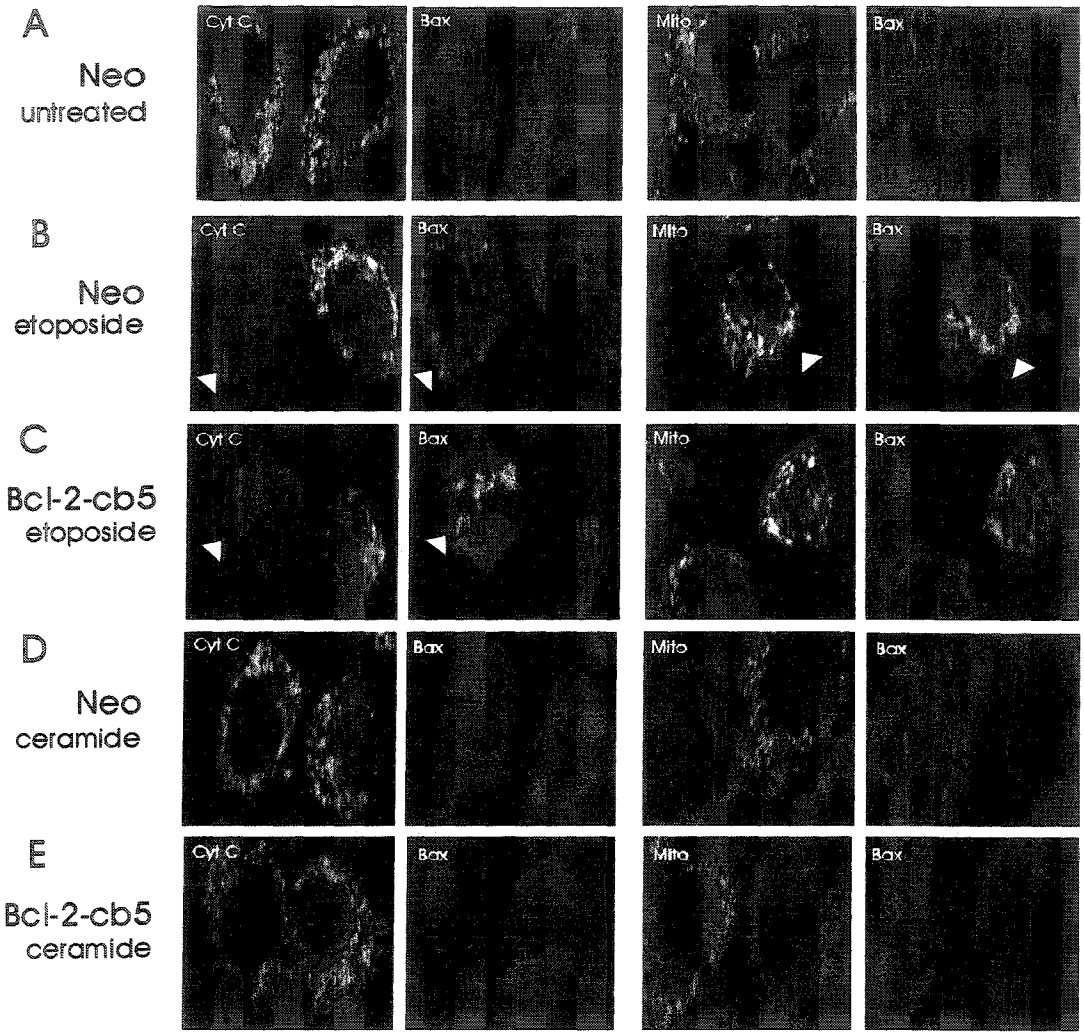

Figure 6. Etoposide induced conformational change and relocalization of Bax correlates with release of mitochondrial cytochrome c. Cells were stained with the conformation specific Bax antibody 6A7 (Bax) and with either antibodies to cytochrome *c* (Cyt C) or Mitotracker (Mito) to visualize $\Delta\Psi_m$ with. **(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-2-cb5 cells treated with etoposide exhibit 6A7 positive Bax that co-localizes with Mitotracker. 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-2-cb5 cells treated with ceramide retain Mitotracker and cytochrome c, but did not stain with the 6A7 antibody.

or ceramide for 6 hours. To confirm Bax activation was co-localized to mitochondria, cells were co-stained with Mitotracker (Figure 6).

As expected, the Bax conformation specific epitope recognized by 6A7 was not exposed in untreated cells (Figure 6A). However, etoposide treated cells showed staining with 6A7 that co-localized with Mitotracker staining, indicating that activated Bax is located on mitochondria (Figure 6B). Similarly, cells that stained for 6A7 also demonstrated diffuse staining for cytochrome *c*. This result is congruent with biochemical studies suggesting that translocation of Bax to mitochondria is sufficient to provoke cytochrome *c* release in vitro (Antonsson et al., 2000). Thus Bax localization at mitochondria is coincident with cytochrome *c* release and precedes loss of $\Delta\Psi_m$. Expression of Bcl-2, but not Bcl-2-cb5, prevented exposure of the 6A7 epitope and diffuse cytochrome *c* staining in etoposide treated cells (Figure 6C and data not shown). In ceramide treated cells activated Bax is not detected in cells with decreased $\Delta\Psi_m$ (Figure 6D and E), indicated Bax activation is not required for ceramide induced loss of $\Delta\Psi_m$.

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, cells exposed to ceramide retained cytochrome *c* staining and did not stain with 6A7, even in cells with decreased $\Delta\Psi_m$.

Bcl-2 expression delays the oligomerization of Bax during etoposide induced cell death.

Treatment of Rat-1 fibroblasts with etoposide results in Bax translocation, activation and cytochrome *c* release. Expression of Bcl-2, but not Bcl-2-cb5 inhibits Bax translocation and activation and thereby delays the release of cytochrome *c* (Figure 4) and cleavage of PARP (Figure 7 and data not shown) caused by etoposide treatment. The requirement for Bcl-2 to be located at mitochondria to prevent these changes suggests the direct involvement of Bcl-2 in the inhibition of Bax induced permeabilization of mitochondria.

Once at mitochondria Bax oligomerizes, a process that is thought to form a channel/pore allowing the release of cytochrome *c* from the mitochondria (Antonsson et al., 2000). Because Bcl-2 has been shown to bind directly to Bax under some circumstances (Oltvai et al., 1993) it is possible that Bcl-2 interferes with the oligomerization of Bax directly. To determine the oligomeric status of Bax and Bcl-2, membrane fractions from etoposide treated cells were solubilized in 2% CHAPS (higher molecular weight complexes of Bax have been shown to be stable at this detergent concentration (Antonsson et al., 2000)) followed by gel filtration chromatography. Eluted fractions were immunoblotted for Bcl-2 and Bax. Larger molecular weight complexes, due to their size, are excluded from the matrix of the column and hence elute in the earlier fractions whereas smaller complexes elute in later fractions. In untreated cells, Bcl-2 elutes predominately in fractions 16/18 and the small amount of membrane associated Bax elutes in fractions 22/24 (Figure 8, untreated). Bcl-2 expression delays the mitochondrial translocation of Bax in etoposide treated cells (data not shown), thus to

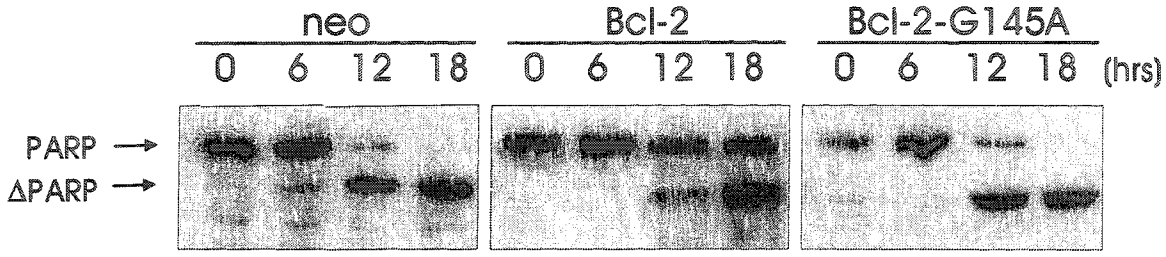

Figure 7. Expression of Bcl-2, but not Bcl-2-G145A inhibits etoposide induced PARP cleavage. Whole cell extracts were prepared from Neo, Bcl-2 and Bcl-2-G145A expressing cells (as indicated on top of the panels) growing in regular medium (0 hours), or exposed to 6 μM etoposide for the number of hours indicated. Whole cell lysates were immunoblotted for PARP. The migration positions of PARP and its major cleavage product (ΔPARP) are indicated to the left of the panels.

determine if Bcl-2 also prevented the oligomerization of Bax, extended incubation with etoposide was required (12 and 18 hours). The extent of cell death for these extended time points was monitored by immunoblotting cell lysates for the nuclear protein PARP (Figure 7)

Etoposide treatment of Rat-1 fibroblasts induces mitochondrial translocation of Bax (Figure 4). Higher molecular weight complexes can be detected in membrane fractions 12 hours and 18 hours after treatment (Figure 8, neo). In contrast, after 12 hours exposure to etoposide, Bax is primarily found in low molecular weight complexes in Bcl-2 expressing cells (Figure 8, Bcl-2). After 18 hours, higher molecular weight complexes of Bax are detected. In etoposide treated cells a significant fraction of the Bax co-fractionates with Bcl-2 and these complexes co-migrate at both 12 and 18 hour treatment time points suggesting that Bcl-2 and Bax are in a complex together. Thus, it is likely that Bcl-2 delays the oligomerization of Bax by binding directly to the membrane bound form of Bax in etoposide treated cells.

To determine if a direct interaction between Bcl-2 and Bax was required for Bcl-2 to inhibit Bax oligomerization a mutant of Bcl-2, Bcl-2-G145A was expressed in Rat-1 fibroblasts. This single point mutant of Bcl-2, located in the BH1 region, abolishes the interaction between Bcl-2 and Bax (Yin et al., 1994). Unlike Bcl-2, expression of Bcl-2-G145A does not confer resistance to etoposide induced cell death (Figure 7). In addition, expression of Bcl-2-G145A does not inhibit the oligomerization of Bax during etoposide induced cell death (Figure 8). Thus the ability to directly interact with Bax is required for

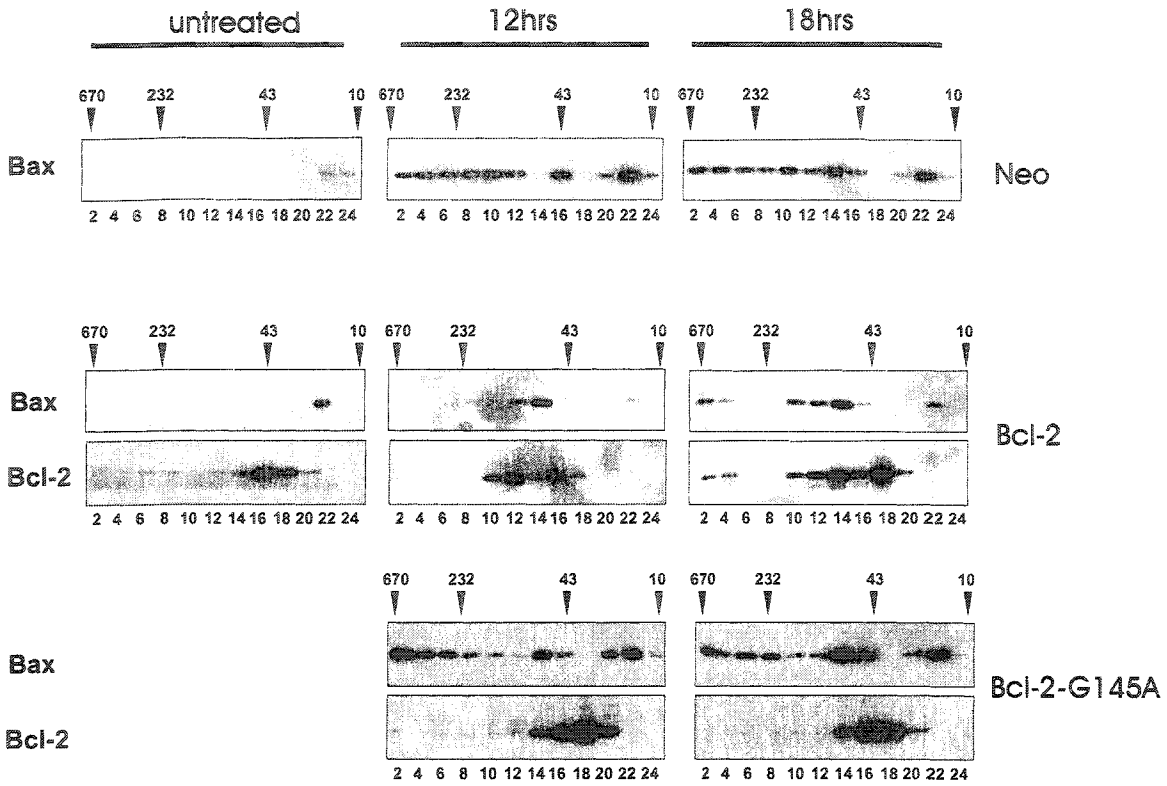

Figure 8. Bcl-2 expression inhibits etoposide induced Bax oligomerization. Membrane fractions from untreated and etoposide treated Rat-1 cells were solubized in 2% CHAPS and subjected to gel filtration chromatography. Fractions were collected after the void volume (frac. 1) and proteins in every other fraction were precipitated with trichloroacetic acid and immunoblotted for Bcl-2 and/or Bax. In untreated cells, Bcl-2 elutes in fractions 16/18 whereas Bax elutes in fractions 22/24. Treatment of vector expressing Rat-1 cells (Neo) for 12 and 18 hours induces the oligomerization of Bax. Cells expressing Bcl-2 inhibit etoposide induced oligomerization of Bax at 12 hours; however by 18 hours larger molecular weight complexes containing Bax are apparent. Treatment of Rat-1 cells expressing Bcl-2-G145A with etoposide induces the oligomerization of Bax in the absence of Bcl-2 oligomerization. Approximate molecular weights are indicated above the immunoblots and fraction numbers indicated below. Cell lines are indicated to the right of the immunoblots.

Bcl-2 to inhibit the oligomerization of Bax and cell death.

Discussion

Examining the temporal order of $\Delta\Psi_m$ loss and release of cytochrome *c* in response to different apoptotic stimuli revealed two spatially distinct pathways of apoptosis in Rat-1 fibroblasts. One pathway is characterized the early loss of $\Delta\Psi_m$ and resulted in the activation of caspases without significant cytochrome *c* release from mitochondria. Expression of Bcl-2 or Bcl-2-cb5 inhibits this form of cell death, indicating the involvement of the endoplasmic reticulum in the initiation of this death pathway. An additional pathway characterized by the early translocation and activation of Bax is blocked by the expression of Bcl-2, but not Bcl-2-cb5. Thus in this death pathway events at the mitochondria are the critical point regulated by Bcl-2. Because the site of Bcl-2 regulation is located on distinct subcellular organelles our results suggest that these two pathways to apoptosis in Rat-1 fibroblasts are spatially distinct (Figure 9).

It has been shown previously in isolated mitochondria, that Bcl-2 can prevent the loss of $\Delta\Psi_m$ (Susin et al., 1996). My results demonstrate that in living cells expression of Bcl-2-cb5 inhibits the loss of $\Delta\Psi_m$ induced by ceramide, staurosporine, and serum starvation/myc (Figures 3 and 7 and also published in (Annis et al., 2001)). Thus, there at least two mechanisms to prevent the loss of $\Delta\Psi_m$: one involving direct intervention in organelle function (Shimizu et al., 1999), and another which must be indirect since it prevents mitochondrial dysfunction from a distance.

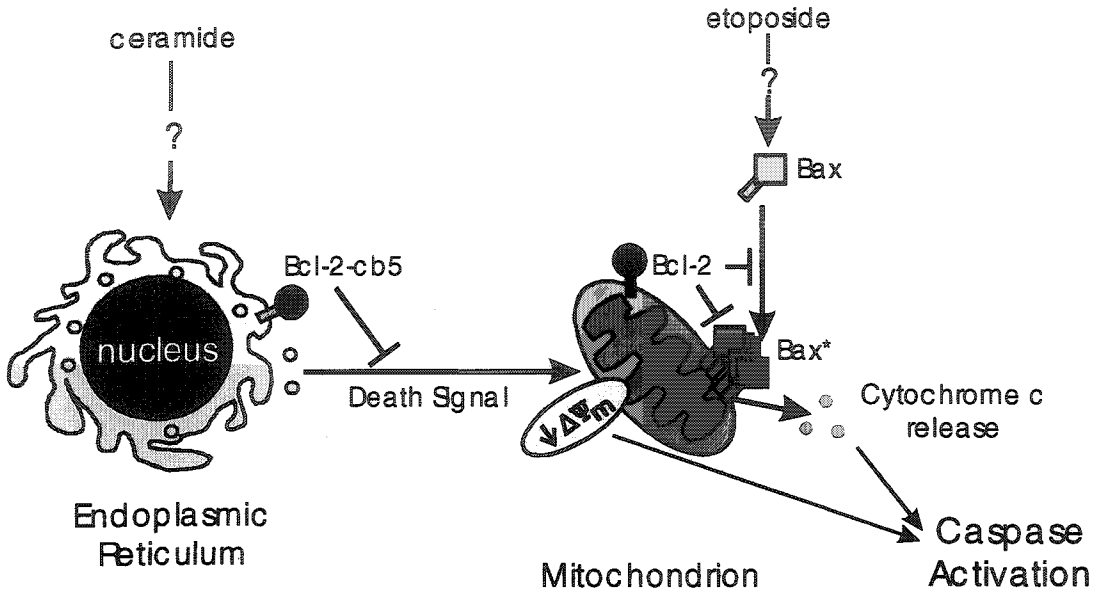

Figure 9. Initiation of apoptosis by ER and mitochondrial specific pathways. ER initiated apoptosis, induced by serum starvation or ceramide treatment, involves the release of a signal from the ER that causes the early loss of mitochondria membrane potential ($\downarrow\Delta\Theta_m$). The mitochondrial pathway, initiated by etoposide treatment, involves the early translocation, oligomerization and activation of Bax (Bax*) causing the release of cytochrome *c*. Expression of Bcl-2-cb5 can inhibit ER initiated apoptosis, but not the mitochondrial pathway. In the mitochondrial pathway wild-type Bcl-2 can inhibit the translocation and oligomerization of Bax at mitochondria. Loss of $\Delta\Theta_m$ and release of cytochrome *c* initiate cell death through caspase activation.

How can Bcl-2-cb5 prevent the loss of $\Delta\Psi_m$ when it is localized to the ER? The most obvious ER candidate is calcium (Ca^{2+}). Indeed, ceramide treatment (40 μM for 5 minutes) of permeabilized HepG2 cells followed by the addition of 50 μM Ca^{2+} induced the loss of $\Delta\Psi_m$ without inducing the release of cytochrome *c* (Hajnoczky et al., 2003). In this analysis, the addition of a permeability transition pore complex (PTC) inhibitor, cyclosporin A prevented the loss of $\Delta\Psi_m$. However, as cyclosporin A is reported to function for less than one hour (Zamzami et al., 1995a), we cannot determine the contribution of the PTC in ceramide induced cell death due to the temporal scale of our experiments. In addition, opening of the PTC triggers the loss of $\Delta\Psi$m and swelling of the matrix compartment leading to the rupture of the outer mitochondrial membrane. However, we do not observe the release of cytochrome *c* in cells with decreased $\Delta\Psi_m$, suggesting that if the PTC is involved in ceramide induced cell death then opening of the PTC must not always lead to rupture of the outer mitochondrial membrane.

The role of Bcl-2 in Ca^{2+} regulation is unclear. Bcl-2 expression has been linked with decreased ER Ca^{2+} levels (Pinton et al., 2000), however there are conflicting reports (Kuo et al., 1998). At the mitochondria Bcl-2 expression increases both the tolerance for spikes in cytoslic $Ca^{2+}$and total mitochondria Ca^{2+} loading (Zhu et al., 1999). Thus, wildtype Bcl-2 may function to both decrease the releasable ER Ca^{2+} stores and limit the effect of cytosolic Ca^{2+} increases by increasing the tolerance of mitochondria. It remains to be determined if Bcl-2-cb5 regulates ER Ca^{2+}.

Although Ca^{2+} is the most likely mediator of ceramide induced loss of $\Delta\Psi_m$, a role for other Bcl-2 family members or ER associated proteins in the response is also possible (reviewed in (Annis et al., 2004)). One proposed mechanism for Bcl-2 activity is to bind and inhibit the activity of pro-apoptotic proteins and this function may be independent of specific membrane localization. Although we did not detect translocation of Bax in ceramide treated cells, expression of Bcl-2-cb5 inhibited Bad induced cell death by sequestering Bad away from mitochondria (Thomenius et al., 2003). A point mutation in Bcl-2-cb5, G145E that inhibits binding to Bad, also eliminated Bcl-2-cb5 inhibitory activity (Thomenius et al., 2003). As ceramide induced cell death may involve the activity of Bad (Basu et al., 1998), generation of selective point mutants in the BH domains of Bcl-2-cb5 that disrupt binding to pro-apoptotic Bcl-2 members, may provide insight as to whether additional Bcl-2 members are involved in the ER death pathway.

Etoposide treatment of Rat-1 fibroblasts triggers Bax translocation, oligomerization and activation leading to the release of cytochrome *c* from mitochondria. The inability of Bcl-2-cb5 to prevent etoposide induced mitochondrial Bax translocation and cytochrome *c* release suggests that Bcl-2 prevents mitochondrial Bax activity by direct sequestration. Consistent with this, expression of wild-type Bcl-2 inhibits Bax oligomerization and Bax/Bcl-2 complexes co-migrate in the gel filtration analysis (Figure 8). In addition, expression of a Bax binding deficient Bcl-2 mutant, Bcl-2-G145A, did not inhibit the oligomerization of Bax (Figure 8) or apoptosis (Figure 7). Taken together, these results suggest that Bcl-2 must be co-localized with Bax where it directly inhibits Bax oligomerization.

Bcl-2 expression inhibits cell death, however eventually incubation with etoposide will induce the activation of the execution phase of cell death, as monitored with the cleavage of PARP (Figure 7). Caspase activity can be seen in the Rat-1 fibroblasts expressing Bcl-2 after exposure to etoposide for 18 hours. At this same time point larger Bax/Bcl-2 complexes can be detected in the gel filtration analysis and cytochrome c release can be detected by sub-cellular fractionation. Consistent with the rheostat model of cell death, cytochrome *c* release may occur when the amount of Bax in the membrane exceeds the binding capacity of Bcl-2. Thus, perhaps when the Bax and Bcl-2 hetero-oligomers are large enough - pores form, irrespective of the fact that the oligomers include molecules of Bcl-2. Since apoptosis is cell autonomous it is difficult to determine if this model is correct, however it is consistent with the migration of Bcl-2 into larger molecular weight complexes coincident with the execution phase of cell death.

Recently functions for Bax at the ER have been demonstrated (Nutt et al., 2002a; Nutt et al., 2002b; Scorrano et al., 2003). Interestingly, Scorrano *et al.* demonstrated that Bax activity was required at both the ER and mitochondria for etoposide induced cell death (Scorrano et al., 2003). In the investigations presented here expression of Bcl-2-cb5 was not effective at inhibiting etoposide induced cytochrome *c* release or cell death – suggesting that either Bcl-2-cb5 is not able to prevent ER Ca^{2+} release by etoposide, or that inhibition of Ca^{2+} release is not sufficient to inhibit etoposide induced cell death. Clearly, determining if Bcl-2-cb5 functions to regulate ER Ca^{2+} is important in determining the contribution of Ca^{2+} in cell death.

In summary, 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. Although it was clear at the onset of this study that different cell types respond to specific death stimuli differently (Zhu et al., 1996), it was not known that in an individual cell there exist at least two intrinsic pathways to cell suicide. These pathways are differentially sensitive to inhibition by Bcl-2-cb5 and can be identified by which of the two manifestations of mitochondrial dysfunction occurs first: release of cytochrome *c* or loss of $\Delta\Psi_m$.

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

Table I Loss of Mitochondrial Membrane Potential Assessed by Confocal Microscopy 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 8 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.

Chapter Four

Myc expression affects Bax activity

The data shown in figures 2, 5, and 6 were included in Soucie EL, Annis MG, Sedivy J, Filmus J, Leber B, Andrews DW, Penn LZ. (2001) Myc potentates apoptosis by stimulating Bax activity at the mitochondria.

Mol Cell Biol. 21(14):4725 36.

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I have included only the data that I generated in this chapter.

Introduction

Gerard Evan and Karen Vousden have proposed that only two critical events are required for neoplastic progression, uncontrolled cell proliferation and suppression of apoptosis (Evan and Vousden, 2001). Determining how these two events are regulated is critical to our understanding of cancer development. Intriguingly, expression of the proto-oncogene Myc influences both cell proliferation and apoptosis (Evan et al., 1992).

Myc is a member of the basic helix-loop-helix leucine zipper transcription factor family and has a central role in the proliferation of normal cells (O'Brien et al.,). Although an exact role in cell cycle progression is unknown, deletion of Myc in Rat-1 fibroblasts results in extensions of G_1 and G_2 phases of the cell cycle (Mateyak et al., 1997). In addition, Myc knockout mice die during development due to multiple organ failures (Davis et al., 1993).

A role for Myc in apoptosis was revealed when cells were incubated in the absence of survival factors (Evan et al., 1992). In normal cells, serum starvation results in decreased Myc expression and exit from the cell cycle resulting in quiescent cells; however when Myc expression is deregulated these cells undergo apoptosis (Evan et al., 1992).

How does Myc expression affect apoptosis? As a transcription factor, Myc is thought to alter gene expression; however the addition of protein synthesis inhibitors along with an apoptotic agonist does not block Myc mediated apoptosis (Wagner et al., 1994). Thus Myc pre-sensitizes cells to apoptosis. Indeed, Myc expression sensitizes

cells to a variety of apoptotic agonists (Soucie et al., 2001), suggesting a common apoptotic pathway is the target of Myc 'activity'. As presented in Chapter Three, mitochondria are central targets of many apoptotic pathways. Thus it is possible that Myc expression may sensitize mitochondria to apoptotic agonists.

The activity of Myc has been examined using a chimeric fusion of Myc to the estrogen receptor (mycER^{TM}) (Littlewood et al., 1995). This fusion protein has minimal transcription activity, as monitored by gene expression of characterized Myc targets. Addition of tamoxifen alters the conformation of the estrogen receptor resulting in the activation of Myc protein. Although the mycER^{TM} fusion protein has provided insight into the influence of Myc on apoptosis it is a 'leaky' system. The endogenous Myc expression remains and the mycER^{TM} fusion is not completely inactive (Mateyak et al., 1997).

To determine the influence of Myc expression on apoptosis we have utilized an immortalized Rat-1 fibroblast cell line (HO15.19) in which both Myc alleles have been deleted through homologous recombination (Mateyak et al., 1997). In order to maximize the difference between the presence and absence of Myc expression, another cell line was utilized in which Myc is expressed constitutively by retroviral infection of the Myc null cells (HOmyc3) (Mateyak et al., 1997; Bush et al., 1998). The expression of Myc in these cells is deregulated but the average expression level is not inconsistent with what is seen in cycling cells (EL Soucie and LZ Penn, unpublished data). The HO15.19 cells have a slower growth rate, larger cell size and lengthened cell cycle compared to either the parental line or HOmyc3 cells (Mateyak et al., 1997; Bush et al., 1998).

The studies reported in this chapter were performed in collaboration with Erinn L. Soucie, a graduate student in Dr. Linda Z. Penn laboratory at the University of Toronto and form the basis of the publication Soucie *et al.*, 2001. Erinn tested the response of the HO15.19 and HOmyc3 fibroblasts to a variety of apoptotic agonists including: etoposide, doxorubicin, Taxol (paclitaxol), ceramide and serum starvation. Dose response curves were generated by measuring the amount of DNA fragmentation that occurred after exposure to each of these death agonists for a fixed amount of time corresponding to approximately one cell cycle (18 hours and 54 hours for the HOmyc3 and HO15.19 cells, respectively). Although treatment of HOmyc3 cells with increasing amounts of apoptotic agonist correlated with increased DNA fragmentation; the HO15.19 cells did not respond in a dose dependent manner. Extended exposure of the HO15.19 cells to either Taxol or Etoposide resulted in increased DNA fragmentation eventually matching that observed for the HOmyc3 cells. Thus, Myc expression accelerates or sensitizes cell to apoptosis. Interestingly, expression of Bcl-2 delayed apoptosis in the HOmyc3, but not the HO15.19 cells suggesting Myc regulates the expression of a protein required for Bcl-2 function and/or that Myc sensitizes cells via the upregulation of a Bcl-2 inhibitable apoptosis pathway (Soucie et al., 2001).

In Chapter Three I demonstrated that etoposide induced cell death proceeds through the translocation, oligomerization and activation of Bax followed by the release of cytochrome *c* (Figure 1). Here I have extended this observation by examining the

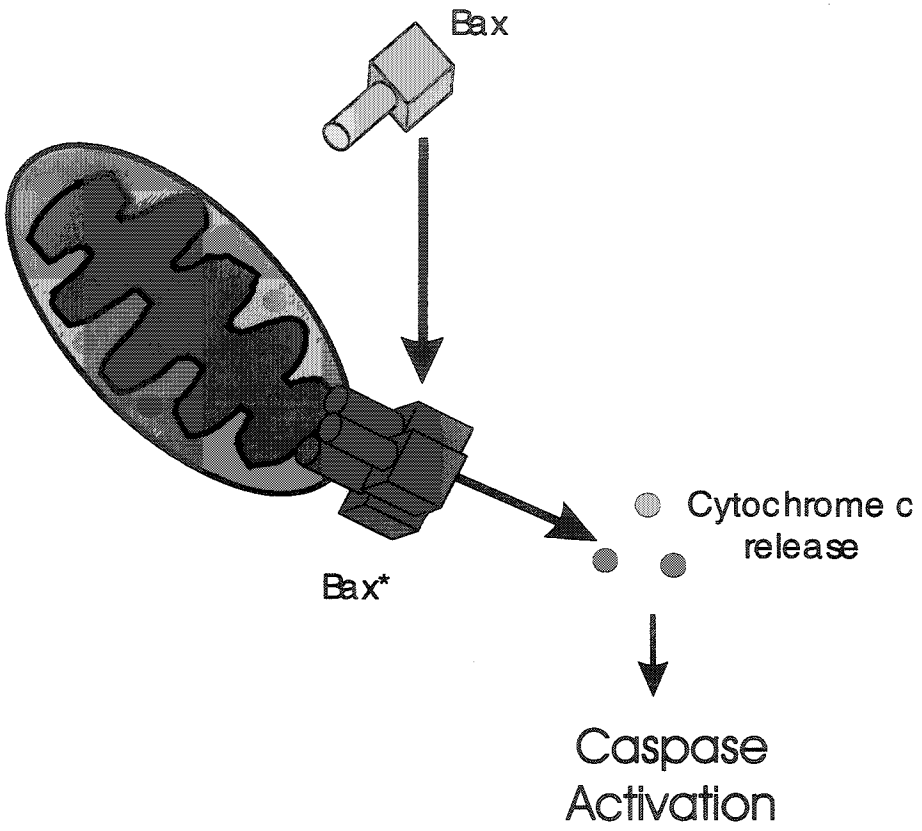


Figure 1. Bax induced cell death. Treatment of Rat-1 cells with etoposide induces the translocation of cytoplasmic Bax (green), to mitochondria. At mitochondria Bax is activated (Bax*) and oligomerizes (red) to induce the release of apoptotic factors including cytochrome *c*. Once in the cytoplasm cytochrome c promotes the activation of Caspases through complex with Caspase-9 and Apaf-1.

progression of etoposide (and Taxol) induced apoptosis in presence and absence of Myc. In this chapter I report the identification of a post-translocation conformational change as a critical, Myc regulated, stage in the activation of Bax.

Results

Expression of Myc accelerates apoptosis

Expression of Myc has been shown to accelerate cell death stimulated by serum starvation (Evan et al., 1992). Having demonstrated in the HO15.19 and HOmyc3 cell system that Myc expression sensitized cells to a wide variety of apoptotic stimuli we selected two chemotherapeutics, etoposide and Taxol for further study. HO15.19 and HOmyc3 cells were incubated with either drug and PARP cleavage assayed by immunoblotting whole cell lysates as an indicator of the execution phase of apoptosis (Figure 2). In the cells expressing Myc, PARP cleavage was detected as early as 12 hours after treatment of etoposide or taxol. In the HO15.19 cells, PARP cleavage was detected in taxol treated cells at 48 hours and in etoposide treated cells as early as 24 hours. These results are in agreement with the DNA fragmentation analysis performed by Erinn Soucie. Thus, the defect in the HO15.19 cells is not Caspase activation as two Caspase dependent events, PARP cleavage (Figure 2) and DNA fragmentation, still occur in theses cells. However, Myc expression accelerated the activation of Caspases in both Taxol and etoposide induced apoptosis suggesting Myc may enhance Caspase initiation signals. [For clarity, only the results of etoposide treated cells are presented here;

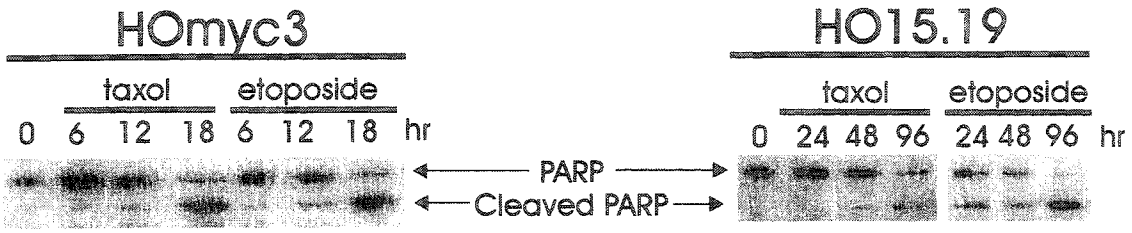


Figure 2. Expression of Myc accelerates etoposide and taxol induced cell death. Ten micrograms of whole cell lysate, generated from untreated, 6 μM etoposide and 0.5 μM taxol treated HOmyc3 and HO15.19 cells was immunoblotted for PARP. Cells were treated for the indicated times. The migration positions of full length PARP and cleaved PARP are indicated.

however the results from Taxol treated cells were similar to the etoposide treated cells and are published in (Soucie et al., 2001)].

Expression of Myc is required for cytochrome *c* release in etoposide treated cells

Etoposide treatment of Rat-1 fibroblasts results in the translocation, oligomerization, and activation of Bax as well as the redistribution of cytochrome *c* (Chapter Three). To determine if this mitochondrial pathway is functional in HO15.19 cells, cytochrome *c* release was assayed by immunoblotting cellular fractions prepared from etoposide treated cells (Figure 3). As a control for integrity of the mitochondrial inner membrane the same cell fractions were immunoblotted for the mitochondrial matrix protein, Hsp-60 (Figure 3).

As expected, in untreated HO15.19 and HOmyc3 cell lines, cytochrome *c* and Hsp-60 both co-fractionate in the pellet (membrane) fraction (Figure 3, untreated). In the HOmyc3 cells etoposide treatment results in the redistribution of cytochrome *c* into the supernatant fraction (12 hrs). Over the time course of exposure to etoposide the relative amount of cytochrome *c* in the supernatant fraction increases. This is consistent with the PARP cleavage data demonstrating that more cells are undergoing apoptosis with extended exposure to etoposide. Interestingly in the absence of Myc expression, etoposide treatment does not cause the redistribution of cytochrome *c*. Even after 48 hours exposure to death agonist, a time point when Caspase activity is evident (Figure 2), cytochrome *c* is retained in the membrane fraction. This indicates the deficiency in the HO15.19 cells occurs prior to or at the level of cytochrome *c* release.

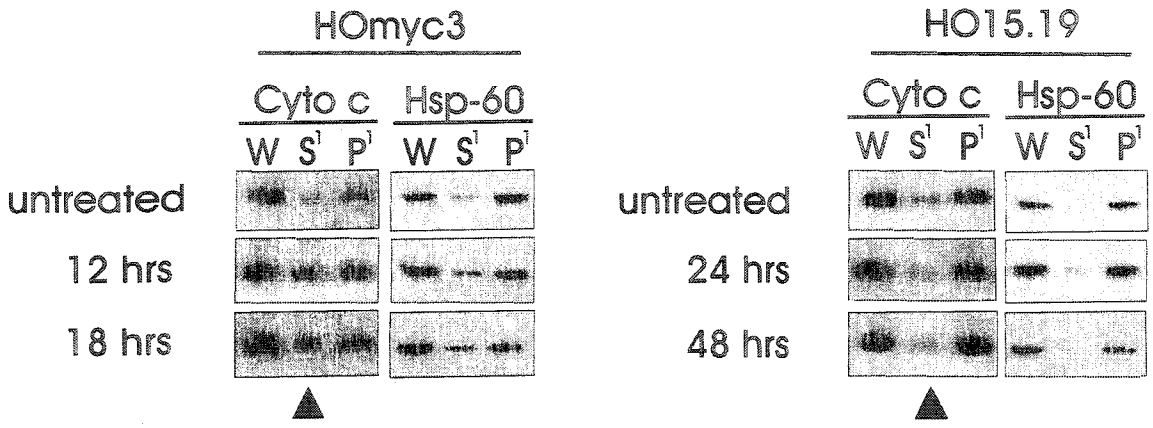


Figure 3. Expression of Myc is required for etoposide induced release of cytochrome *c*. HOmyc3 and HO15.19 cells were untreated or incubated with etoposide for the times indicated; cells were then disrupted by nitrogen cavitation, cell debris removed to yield whole cell lysate (W). Membrane (P) and supernatant (S) fractions were generated by the centrifugation of the whole cell lysate at 1 000 x g for 30 minutes. Ten micrograms of whole cell lysate and equivalent volumes of subcellular fractions were immunoblotted with antibodies towards cytochrome *c* (Cyto c) and Hsp-60 (indicated above each column). Incubation time with etoposide is indicated to the left of each row.

Bax translocation occurs independently of Myc expression

In Chapter Three, I demonstrated that etoposide induced cytochrome *c* release correlated with the translocation and activation of Bax on mitochondria. Thus, the absence of cytochrome *c* release in the HO15.19 cells could be caused by either a defect in Bax translocation or activation. To determine if the deficiency was due to a block in Bax translocation, cell fractions from etoposide treated HO15.19 and HOmyc3 cells were immunoblotted for Bax (Figure 4).

In untreated cells Bax appears in the supernatant fraction of both the HO15.19 and HOmyc3 cell lines. As expected etoposide treatment of HOmyc3 cells induces translocation of Bax to the membrane fraction at 12 hours and the amount in the membrane fraction increases at the 18 hour time point. Accumulation of Bax in the membrane fraction correlated with increased cytochrome *c* in the supernatant fraction (Figure 3); suggesting Bax is responsible for cytochrome *c* release.

Interestingly, in the HO15.19 cells etoposide treatment also induced Bax translocation. Although somewhat delayed, Bax appears in the membrane fraction after 48 hours exposure to etoposide (Figure 4, arrowhead). Thus, the deficiency in the HO15.19 cells occurs after Bax translocation.

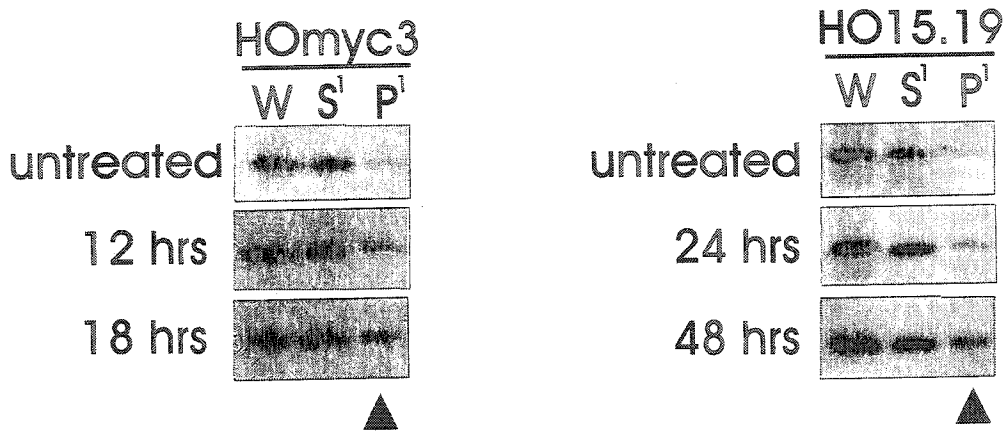


Figure 4. Expression of Myc is not required for the translocation of Bax. HOmyc3 and HO15.19 cells were untreated or incubated with etoposide for the times indicated before cells were disrupted by nitrogen cavitation and cell debris was removed to yield whole cell lysate (W). Membrane (P) and supernatant (S) fraction was generated by centrifugation of the whole cell lysate at 1 000 x g for 30 minutes. Ten micrograms of whole cell lysate and equivalent volumes of subcellular fractions were immunoblotted with antibodies towards Bax. Incubation time with etoposide is indicated to the left of each row.

Expression of Myc is required for Bax activation

Translocation of Bax to membranes in the absence of cytochrome *c* release is not consistent with the prevailing proposed mechanism for Bax activity (Figure 1), in which activation, translocation and the release of cytochrome *c* occur in one concerted step (Jurgensmeier et al., 1998; Desagher et al., 1999). Since etoposide treatment induces a conformational change in Bax that correlates with activation and cytochrome c release (Chapter Three, Figure 6) the question arises as to whether or not the translocated Bax in the Myc null cells has undergone this conformational change.

To determine whether Bax is 'activated' in cells lacking Myc expression, HO15.19 and HOmyc3 cells were treated with etoposide (controls were untreated) fixed and stained for cytochrome *c* (red) and 'activated' Bax (green) using affinity purified antibodies to cytochrome *c* and the 6A7 monoclonal antibody, respectively (Figure 5A).

In untreated HOmyc3 and HO15.19 cells, cytochrome *c* staining appears localized to long tubular structures indicative of mitochondria. Exposing the HOmyc3 cells to etoposide for 12 hours induces the redistribution of cytochrome *c* and the conformational change in Bax detected by 6A7 antibody staining is clearly evident. However, in the absence of Myc expression, cytochrome *c* remains associated with mitochondria after the cells were exposed to etoposide for 48 hours, confirming the results obtained from cellular fractionation. Importantly, microscopic examination using the 6A7 antibody demonstrates that there is no Bax detected by the 6A7 monoclonal

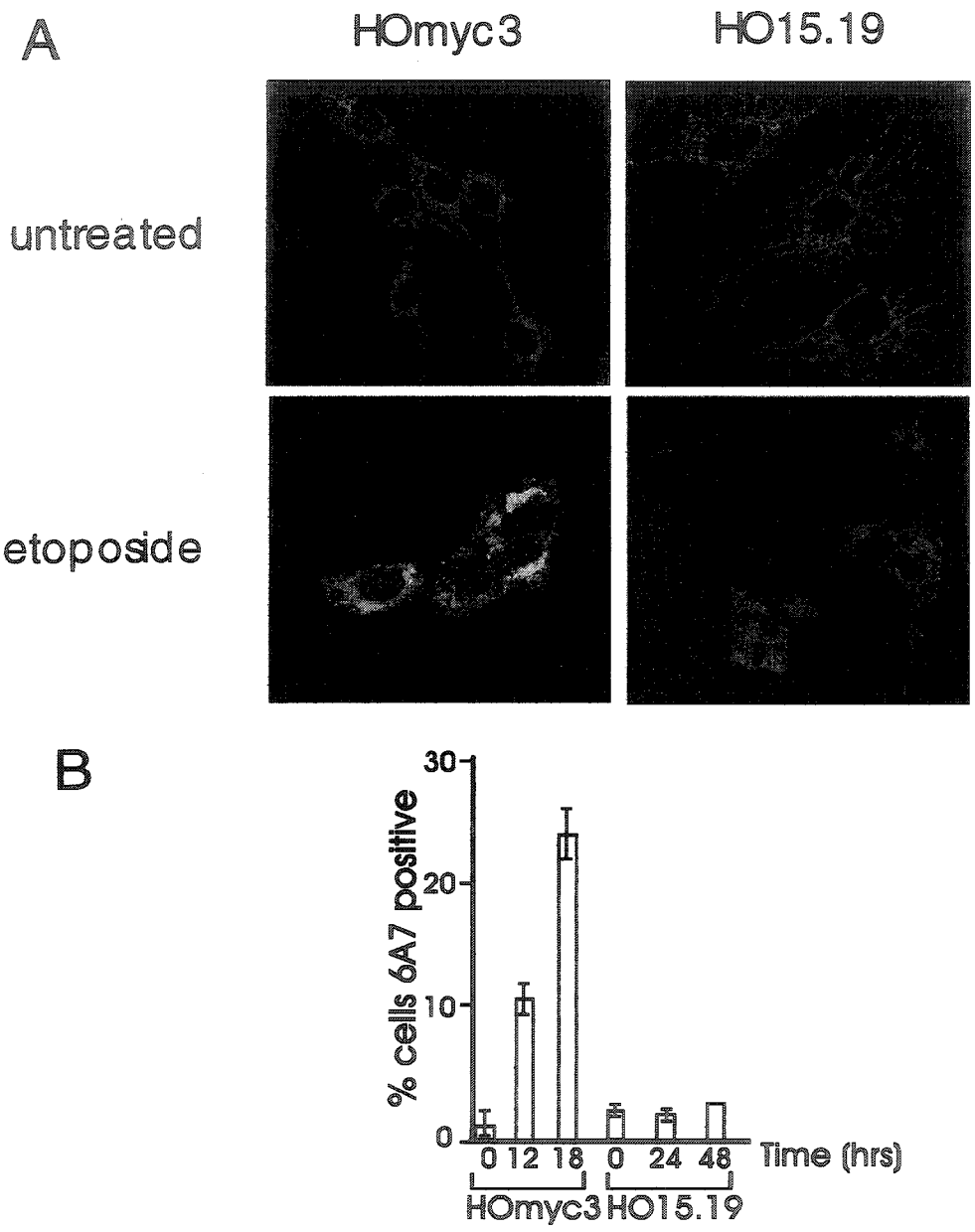


Figure 5. Expression of Myc is required for the activation of Bax. (A) HO15.19 and HOmyc3 left untreated or incubated with 6 μM etoposide for 48 and 18 hours respectively. Cells were then fixed and co-stained for activated Bax (6A7-green) and cytochrome *c* (Red). Images of cells were recorded using a Ziess LSM510 confocal microscope. The width of each image is ~ 140 μm. (B) A minimum of three fields of 40 cells each were counted independently by two different observers for each of the indicated conditions and time points to determine the percentage of cells stained by 6A7. Error bars indicate the range between the two observers.

antibody in these cells. Since apoptosis is an asynchronous process (Messam and Pittman, 1998) several images were collected under a lower magnification and the number of cells containing activated Bax and cytochrome *c* redistribution were counted (Figure 5B). Consistent with the absence of cytochrome *c* release there was no appreciable change in the number of cells that stained with the 6A7 Bax antibody in the treated HO15.19 cells. However, treatment of the HOmyc3 cells with etoposide caused an increase in the number of 6A7 positive cells after 12 hours continuing to 18 hours. This confirms that 6A7 staining correlates activation of Bax to release cytochrome c. Thus, the block in Myc null cells is between the translocation and activation of Bax.

To confirm that the activated Bax was localized to mitochondria, cells were co-stained with Mitotracker (red) and the 6A7 antibody (green, Figure 6). In untreated HOmyc3 cells, mitochondria are Mitotracker positive and 6A7 negative. After 12 hours exposure to etoposide 6A7 positive cells are apparent and activated Bax co-localizes with Mitotracker (yellow mitochondria). Etoposide treatment causes changes to the cellular morphology and altered mitochondrial distribution; however the membrane potential is maintained in these mitochondria (Chapter Three, Figure 3 and 6). To address the specificity of the 6A7 antibody, purified Bax-GST fusion protein was pre-incubated with the antibody prior to staining the cells. This effectively blocks the ability of this antibody to bind to activated Bax (Figure 6, arrowheads indicate dying cells).

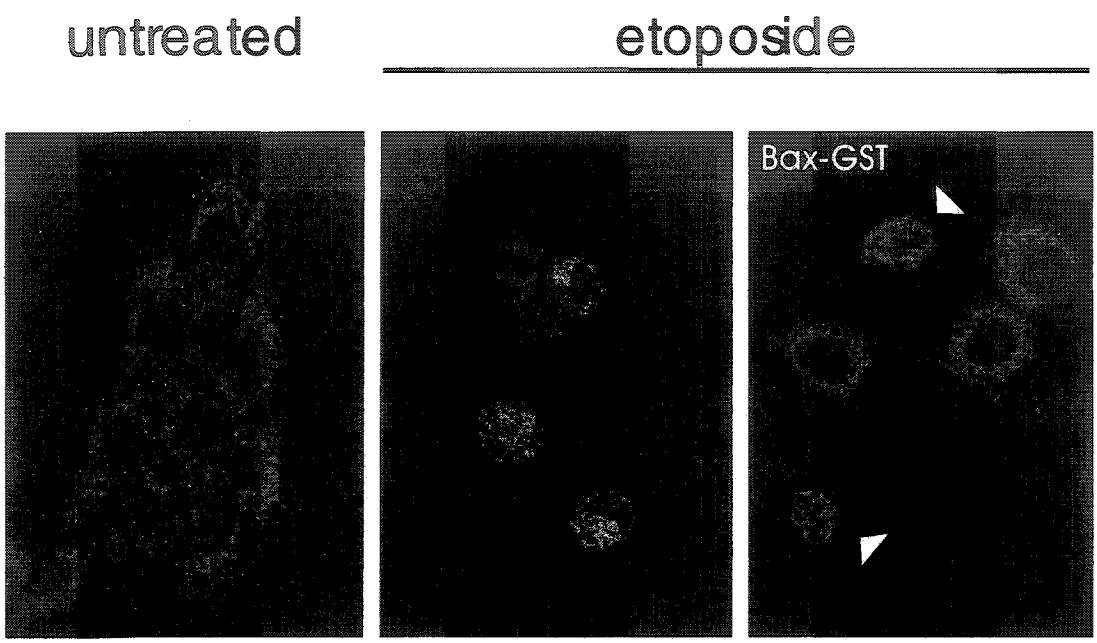


Figure 6. Activated Bax co-localizes with mitochondria. HOmyc3 cells were left untreated or exposed to 6 μM etoposide for 12 hours then stained with Mitotracker red. Cells were co-stained for activated Bax using 6A7 antibody or Bax-GST blocked 6A7 antibody (Bax-GST). Arrowhead mark apoptotic cells (membrane blebbing is evident). Images were collected using the Ziess LSM510 confocal microscope. The width of each image is ~ 140 μm.

Expression of Myc is required for the oligomerization of Bax

The release of apoptotic factors from mitochondria is facilitated by the oligomerization of Bax (Antonsson et al., 2000). The above results demonstrate that in the absence of Myc expression Bax translocates to mitochondria, however it does not change conformation or induce the release of cytochrome *c*. Thus it follows that the block in Bax activity in the Myc null cells may either be the absence of Bax oligomerization or a conformational change that triggers the opening of the Bax oligomeric pore.

To determine the oligomeric status of Bax the membrane fraction from etoposide treated HOmyc3 and HO15.19 cells were isolated, solubilized in 2 % CHAPS and the constituent protein complexes were analyzed by gel filtration chromatography. The eluted fractions were immunoblotted for Bax (Figure 7). Similar to Rat-1 fibroblasts (Chapter Three, Figure 8), treatment of HOmyc3 cells with etoposide induced the oligomerization of Bax. However, after 48 hours exposure to etoposide Bax appears as a monomer in the HO15.19 cells. Thus, Myc expression is required for the oligomerization and activation of Bax in etoposide induced cell death. Whether or not oligomerization and activation are different steps remains to be determined.

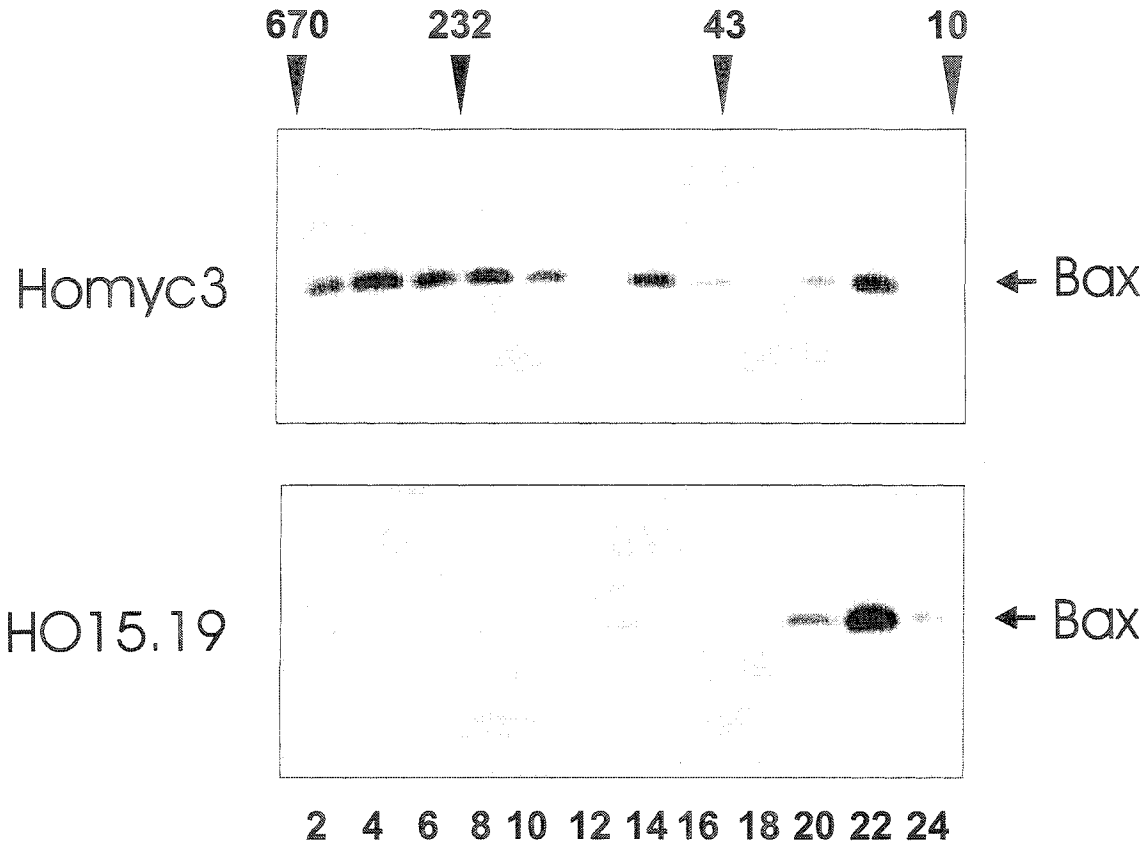


Figure 7. Determination of the oligomeric status of Bax. Heavy membranes (P^1 fraction) from etoposide treated HOmyc3 (18 hours) or HO15.19 (48 hrs) cells were solublized in 2% CHAPS and subjected to gel filtration chromatography on a Superdex S-200 column (in 20 mM HEPES pH 7.4, 300 mM NaCl, 2% CHAPS, 0.2 mM DTT). Proteins were precipitated from individual fractions using TCA, separated by SDS-PAGE and immunoblotted for Bax. Fraction numbers are indicated at the bottom of the images. The elution positions of calibration standards are indicated by their corresponding molecular weights on the top of the figure.

Discussion:

Myc sensitizes cells to a variety of apoptotic stimuli (Soucie et al., 2001). Examining the effect of Myc expression on etoposide (and taxol) treated cells revealed that Myc influences the mitochondrial death pathway by promoting a critical step in Bax induced death.

Bax was first identified as a Bcl-2 binding partner with pro-apoptotic activity (Oltvai et al., 1993). Bax is a predominantly cytoplasmic protein that induces cell death by translocating to mitochondria where it oligomerizes and induces the release of apoptotic factors, including cytochrome *c*. For the most part, Bax translocation and activation were thought to occur in one concerted step. However, by examining apoptosis in myc null cells we have identified a membrane 'docked' version of Bax that exists prior to activation/oligomerization (Figure 9). Thus, Bax docks onto the mitochondrial membrane first as a monomer then oligomerization is triggered by the influence of one or more of the genes regulated by Myc.

Recently, two proteins have been described that regulate Bax translocation to membranes, Humanin and Ku70 (Guo et al., 2003) (Sawada et al., 2003), and here I have shown evidence for a Myc regulated factor that mediates Bax activation/oligomerization. Thus, Bax activity is a tightly regulated.

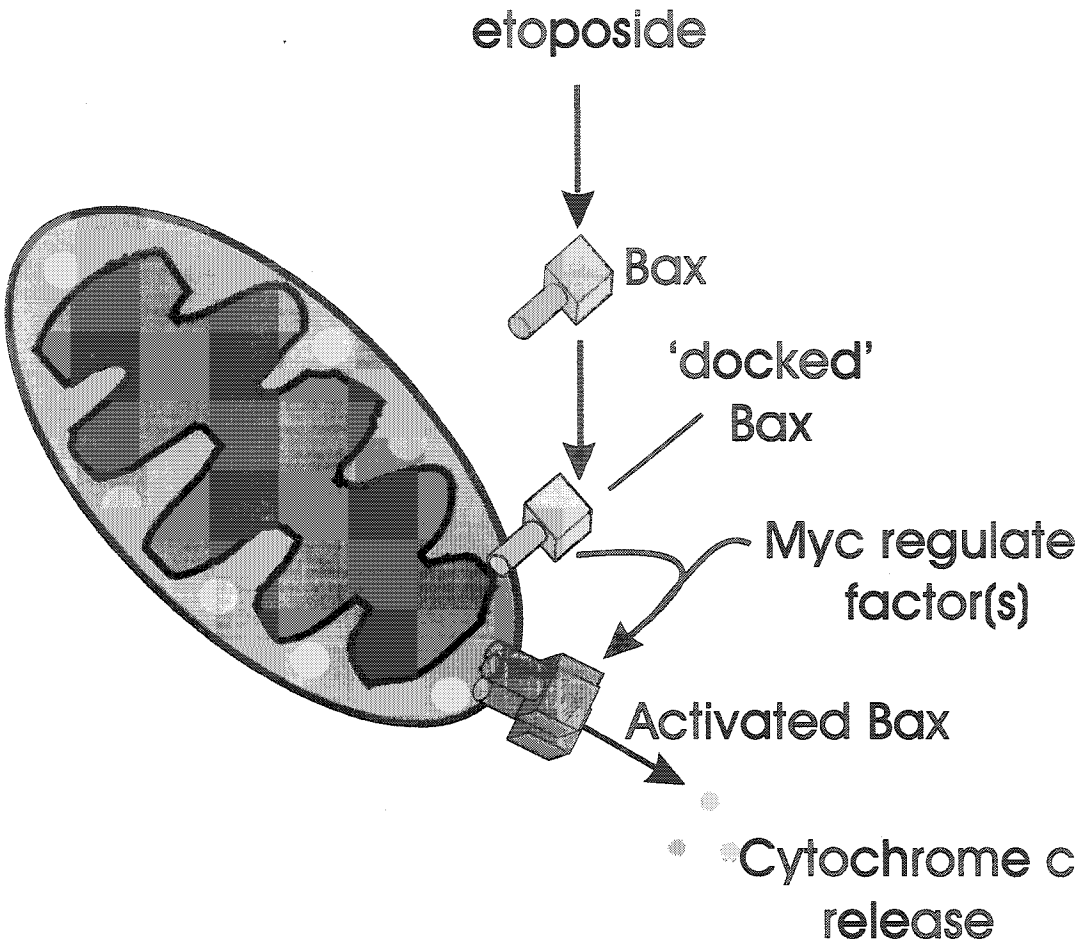


Figure 9. Expression Myc affects Bax activation. Etoposide (or Taxol) induced translocation of Bax occurs irrespective of Myc expression. Once docked onto the mitochondria (docked Bax) Myc regulated factors are required for the activation and oligomerization of Bax and subsequent release of cytochrome c.

How does Myc influence Bax activity? The absence of Bax oligomerization could be due to presence of an inhibitor or the absence of activators and may be directly related to the conformational change detected by 6A7. Although Bax can induce cytochrome *c* release from isolated mitochondria (Antonsson et al., 2000), in cells both a membrane component (Roucou et al., 2002) and a cytosolic component (Kluck et al., 1999) are required for efficient cytochrome *c* release. Thus Myc may influence the activity or expression of these unidentified factor(s).

Previously, I demonstrated Bcl-2 expression inhibits Bax oligomerization in Rat-1 fibroblasts (Chapter Three, Figure 8). However, the endogenous level of Bcl-2 protein is not altered by Myc in these cell lines (Soucie et al., 2001). In addition, Bcl-2 expression halted the oligomerization of Bax at a stages when Bax was partially multimerized (presumably with Bcl-2, see Figure 8 in Chapter Three); however in the HO15.19 cells Bax remains as a monomer on the membrane (Figure 7). This in combination with the observation that exogenous Bcl-2 expression did not inhibit apoptosis in the HO15.19 cells suggests that the block is not mediated through the activity of anti-apoptotic Bcl-2 proteins.

The multi-BH-domain protein Bak co-migrates with Bax during etoposide cell death (Annis MG, unpublished data) and expression of Bax and Bak together enhances etoposide induced cell death(Wei et al., 2001); thus it was possible that a deficiency in Bak may explain why Bax does not oligomerize in the HO15.19 cells. However there was no appreciable difference in the amount of Bak in treated HO15.19 and HOmyc3 cells (data not shown).

The BH3 only protein Bid is a known activator of Bax and co-incubation of t-Bid with Bax can trigger the release of cytochrome *c* from isolated mitochondria (Desagher et al., 1999). However, mouse embryonic fibroblasts null for Bid respond equally well to etoposide induced cell death as their wild-type counterparts (Wei et al., 2001). In addition, Caspase activation, and hence t-Bid production, is not required for cytochrome *c* release in etoposide treated Rat-1 fibroblasts (Chapter Three, Figure 4C). Taken together these observations suggest that a deficiency in Bid not responsible for the block in Bax oligomerization in the HO15.19 cells.

Since Myc accelerated apoptosis can proceed in the absence of de novo protein synthesis (Evan et al., 1992), it seems likely that Myc expression would pre-sensitize a cell to apoptosis by ensuring the necessary components for cell death are abundant and available. Unfortunately, initial micro-array analysis has not provided insight into how Myc influences Bax activity (EL Soucie and LZ Penn, unpublished data).

Interestingly, Myc is not the only proto-oncogene to influence Bax activity, a heterokaryon fusion between E1A expressing cells and primary cells revealed the presence of a factor, IODA (inhibitor of oncogene-dependent apoptosis), that inhibited E1A induced cytochrome *c* release (Duelli and Lazebnik, 2000). In these heterokaryons, etoposide induces the same phenotype as I observed with the Myc deficient cells, mitochondrial translocation of Bax without the release of cytochrome *c*. Interestingly, 96 hours after fusion these heterokaryons become susceptible to apoptotic stimuli, however it is unclear whether this is do to: increased protein synthesis of an activator; increased turnover of an inhibitor of Bax; or repression of the expression of an inhibitor

of Bax. Clearly, these observations suggest that Myc sensitization of cells to apoptosis is not a unique feature restricted to Myc. Intriguingly, both E1A function and etoposide induced cell death require the activity of p53 (Lowe et al., 1993; Villunger et al., 2003).

Although p53 activity has historically been restricted to gene expression (Villunger et al., 2003), direct activation of Bax by p53 has recently been reported (Chipuk et al., 2004). In the absence of nuclear import p53 accumulates in the cytoplasm where it can directly activate Bax to promote the release of cytochrome *c*. This activation does not require any additional factors and can proceed *in vitro*. Although this novel function for p53 is controversial (Baptiste and Prives, 2004), a deficiency in the cytoplasmic activity of p53 is consistent with the apoptotic phenotype seen in the HO15.19 cells. Interestingly, HO15.19 cells respond equally well to DNA damage induced up regulation of p53 expression as their wild-type counterparts (Grassilli et al., 2004), suggesting the deficiency in the HO15.19 is not related to p53 induction. Clearly, the mechanism underlying Myc activity in the acceleration of cell death warrants further examination.

In summary, by examining the requirement for Myc expression in drug induced cell death we have identified novel regulated stages in the activity of Bax. Previously, Bax activity was thought to occur in one concerted step, however it is now clear that Bax function is a highly regulated process. Myc expression is required for the oligomerization and activation of Bax during etoposide induced cell death. In addition, the absence of Bcl-2 activity in the HO15.19 cells suggests that one function of Bcl-2

involves the inhibition of etoposide induced cell death by preventing Bax oligomerization. This is consistent with observations made in Chapter Three of this thesis.

Chapter Five

Mapping the membrane topology of Bax

Introduction:

Apoptosis is a genetically encoded distinct form of cell death characterized by several specific morphological and biochemical changes. An individual cell's fate is regulated by several factors; however one potent regulator of cell survival is the Bcl-2 family of proteins (Adams and Cory, 2001). This family is sub-divided into pro- and anti-apoptotic members based on function. This antagonistic relationship between members is thought to regulate cell fate.

Although cellular functions are well recognized, the precise molecular mechanism by which these proteins regulate cell death (either positively or negatively) is controversial and the subject of many ongoing investigations. However, one insight into the activity of these proteins is the requirement for many of them to be membrane localized (Zhu et al., 1996; Wolter et al., 1997). Indeed, in the previous two chapters I have shown the Bcl-2 family of proteins regulate apoptosis pathways at different subcellular locations (ER and mitochondria) and described a role for the proto-oncogene Myc in regulating activation of Bax at mitochondria. In this chapter I investigate the contribution of Bax membrane topology in the pore forming activity of this pro-apoptotic protein.

The three dimensional structures of Bcl-X_L (Muchmore et al., 1996), Bid (Chou et al., 1999), Bax (Suzuki et al., 2000), Bcl-2 (Petros et al., 2001), and Bcl-w (Denisov et al., 2003; Hinds et al., 2003) are all organized around a core structure that resembles the transmembrane domain of diphtheria toxin (Muchmore et al., 1996). Each protein has two central relatively hydrophobic helices surrounded by amphipathic helices in a structure

that is more similar than predicted from primary sequence alignments. Consistent with the known structures, there is good *in vitro* evidence that some these proteins can form pores or channels in lipid bilayers [reviewed in (Desagher and Martinou, 2000)].

The dynamics of Bax pore formation *in vitro* are controversial. Initial investigations utilized a truncated version of Bax (BaxΔCT) where the hydrophobic carboxyl terminus was removed to facilitate purification. Under certain conditions BaxΔCT will spontaneously target to membranes and liposomes *in vitro* and form channels/pores in the bilayer (Jurgensmeier et al., 1998; Antonsson et al., 1997). However, full length Bax, like its cellular counterpart, does not spontaneously insert into mitochondria (Goping et al., 1998) or endoplasmic reticulum (ER) derived microsomes (Annis MG, unpublished data). Full length Bax with an amino-terminal His tag can be targeted to mitochondria if it is pretreated with the detergent octyl-glucoside (Antonsson et al., 2000). Under these conditions Bax is a homo-oligomer and can induce the release of cytochrome c from mitochondria and fluorescent dextrans from liposomes. Monomeric Bax does not induce cytochrome *c* release from mitochondria or release of dextrans from liposomes (Antonsson et al., 2000). These observations promoted the concept that oligomerization is essential for Bax pore formation and hence cytochrome *c* release. Although the use of artificially activated Bax only demonstrates the *capabilities* of this protein and not necessarily its activity *in vivo*; a requirement for Bax oligomerization in the release of cytochrome has also been demonstrated in cultured cells (Chapters Three and Four of this thesis and (Wang et al., 2003)).

What regulates Bax pore formation in cells? In the absence of apoptosis Bax is located in the cytoplasm. Therefore, it is reasonable to assume that the initial interaction of Bax with membranes is a candidate step for regulation. In Chapter Four I demonstrated a requirement for *Myc* expression for Bax activity. In the absence of *Myc* expression (HO15.19 cells), etoposide treatment caused Bax to translocate to membranes in response to an apoptotic stimulus; however cytochrome *c* was not released. When *Myc* was expressed constitutively in these cells (HOmyc3), etoposide induced Bax translocation, activation, oligomerization and release of cytochrome *c*. These observations reveal that activation and/or oligomerization is a second regulated step in Bax pore formation. Combining these observations we have identified three forms of Bax; cytoplasmic, membrane bound, and 'activated', each of which can be isolated from cells. This allows the unique opportunity to analyze, separately, the membrane topology of membrane bound and activated Bax and thereby determine if changes to the membrane topology correlate with Bax activity.

The mechanism of Bax targeting to mitochondria is controversial. Despite containing a predicted tail-anchor sequence Bax remains in the cytoplasm until after the induction of apoptosis. Targeting to mitochondria during cell death requires not only the tail-anchor sequence (Wolter et al., 1997; Nechushtan et al., 1999), but the central α5 and α6 helices (Nouraini et al., 2000) as well as regions of the amino-terminus(Goping et al., 1998; Cartron et al., 2003). However, targeting of Bcl-2 is mediated exclusively by the tail-anchor sequence and Bcl-2 is in a 'tail-anchor' topology (Figure 1B) when targeted to membranes *in vitro* ((Chen-Levy and Cleary, 1990) and Kim et al., in press). If Bax

targeting is similar to Bcl-2 then a 'tail-anchor' topology would be predicted for membrane bound Bax prior to activation. To promote cytochrome *c* release Bax forms a channel or pore in the membrane, and therefore this activity is thought to require insertion of helices α5 and α6 into the membrane. Thus, a 'pore-like' topology would be adopted when Bax is forming a channel/pore in the membrane (Figure 1B).

Based on this I predicted that when Bax first binds to membranes it is likely in a 'tail-anchor' topology and when it oligomerizes to form a pore its topology changes to a 'pore-like' topology. Based on this hypothesis I would predict that in the absence of Myc expression etoposide treatment would result in Bax adopting a tail-anchor topology in which helices α5 and α6 would not be inserted into the lipid bilayer. However, in the Myc expressing cells all three helices (α5, α6, and α9) would be inserted into the lipid bilayer following etoposide treatment.

The topology of a membrane protein can be assessed by various biochemical techniques including: protease protection, fusion protein tags, fluorescent probes, or chemical labeling. Previously we have utilized chemical labeling to monitor topology changes in the anti-apoptotic protein Bcl-2 during apoptosis (Kim et al, in press). This technique has many advantages; most importantly, the topology adopted in living cells can be assessed. This is critical for a protein like Bax, which is not membrane bound unless stimulated with an apoptotic agonist.

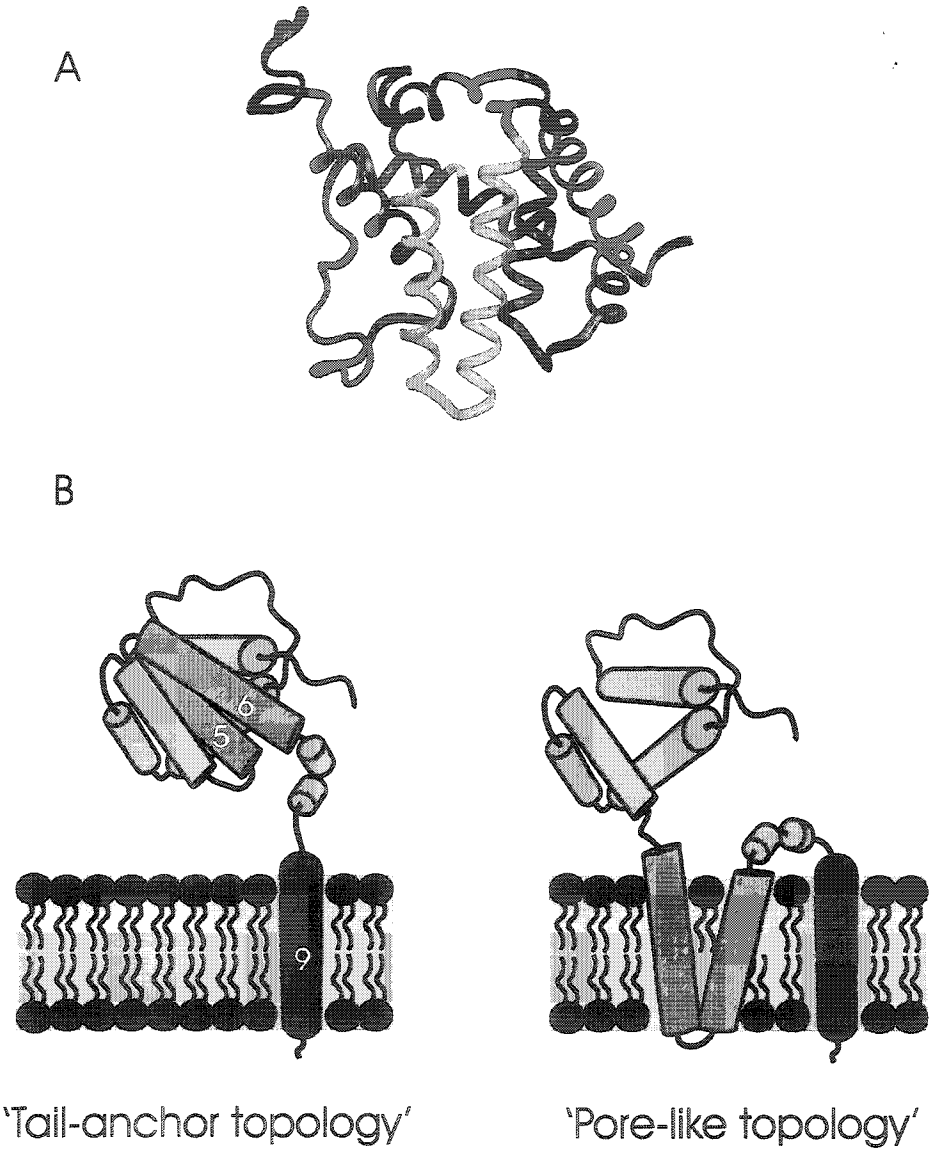


Figure 1. NMR solution structure of Bax and predicted membrane topologies for Bax. (A) NMR structure for full length human Bax. Bax is a bundle of nine alpha helices. The putative pore forming domain, comprised of helices α5 and α6, are highlighted in red. The locations of the two endogenous cysteines of Bax are highlighted in blue. (B) Two predicted membrane topologies for Bax. As Bax contains a hydrophobic carboxyl-terminus (helix α9) it would be predicted to target to membranes by this sequence and thus adopt a 'tail-anchor topology'. When Bax is triggered to form a pore on the membrane it would adopt a 'pore-like topology' with the insertion of helices α5 and α6.

Chemical labeling and site directed mutagenesis were used to determine the membrane topology of human Bax in the HO15.19 and HOmyc3 cells. Surprisingly, following etoposide treatment Bax adopts a 'pore-like topology' both in the presence and absence of Myc expression. Thus insertion of helices α5 and α6 is not a commitment to release cytochrome *c*; and changes to the oligomeric state of Bax are what determine the membrane activity of this protein.

Results:

Methodology

The membrane topology of Bax was assessed using the lipid bilayer impermeant cysteine modifying reagent 4-acetamido-4'-iodoacetyl-amino stilbene-2.2'-disulfonic acid (IASD, Figure 2B). Human Bax contains two endogenous cysteine residues (at amino acid positions 62 and 126) the locations of these two residues are highlighted in blue in Figure 1A. These two endogenous cysteines were sequentially removed and replaced with alanine. From this cysteine-less mutant, several single cysteine mutants were generated throughout the putative pore forming domain (helices α5 and α6). As controls, single cysteine mutants were generated with the cysteine positioned within the putative membrane anchor (helix α9); as well as in several regions predicted to remain exposed to the cytoplasm (Figure 2A, Table I). Utilizing the human Bax protein allows unique identification of the mutant proteins expressed in Rat cells using a monoclonal antibody (2D2, (Hsu and Youle, 1997)) specific for human Bax.

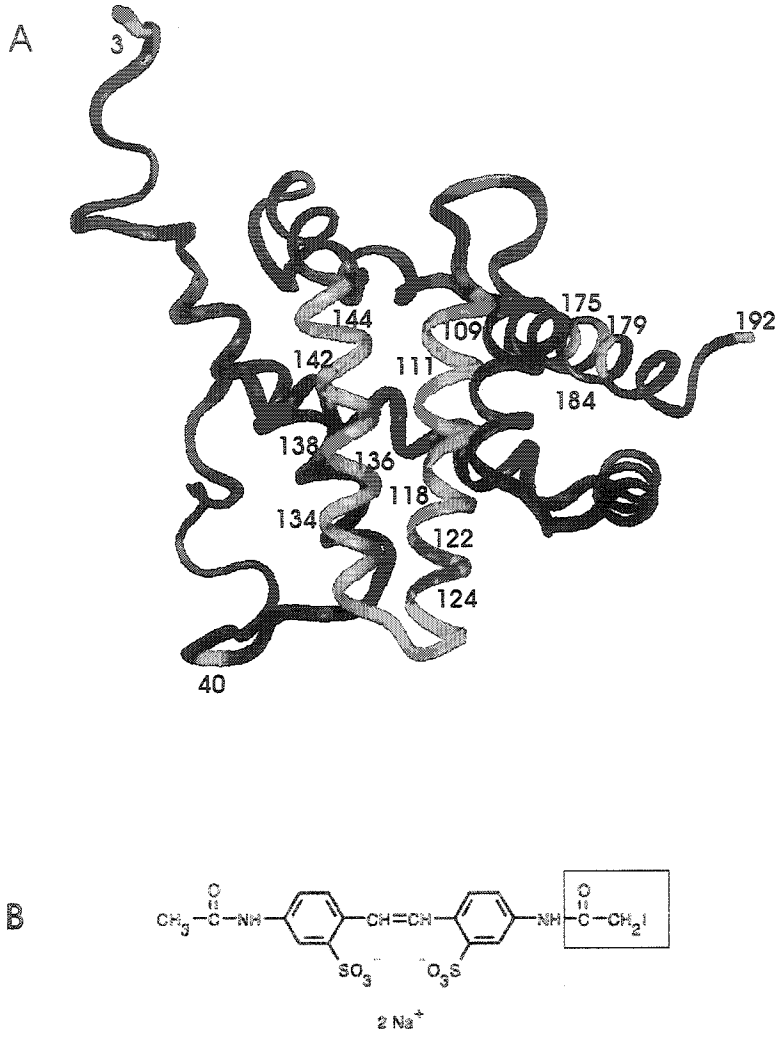


Figure 2. Single cysteine mutants of Bax and the structure of IASD. (A) The positions of all eighteen single single cysteine mutations are indicated. (B) The chemical structure of IASD. The reactive iodo-acetyl group is boxed.

The human protein is predicted to behave identically to rat Bax, as human and rat Bax are 91 % identical in amino-acid sequence and 100% identical throughout helix α5, α6, and α9 and all other positions mutated.

Human Bax protein and the mutants in which the endogenous cysteines were converted to alanines behaves similarly to the endogenous rat Bax in that it is cytoplasmic in healthy cells and translocates to membranes following an apoptotic stimulus (Figure 3). Thus the human protein mimics the activity as the endogenous Rat protein.

IASD was used to determine the environment of cysteine residues. In aqueous environments, cysteine residues are modified by IASD; however if a cysteine residue is buried in the lipid bilayer it is not accessible to IASD (Kim et al., in press). Iodoacetimides can modify cysteine, histidine, methionine, and lysines residues (Gurd FRN, 1967). One can skew this reaction towards cysteine by ensuring the pH of the reaction is close to neutral (this limits the reaction with lysine residues) and as Bax contains no histidines this is not a concern for this protein. Reaction between IASD and methionine is normally slower then with cysteine (Gundlach et al., 1959) and as our reaction time is short (15 minutes) this is not a problem. IASD has been used to determine the membrane topology of several proteins ((Krishnasastry et al., 1994), (Grundling et al., 2000), and Kim et al., in press). Modification with IASD adds two negative charges to the protein via the sulfonic acid groups. Thus, labeled and unlabeled

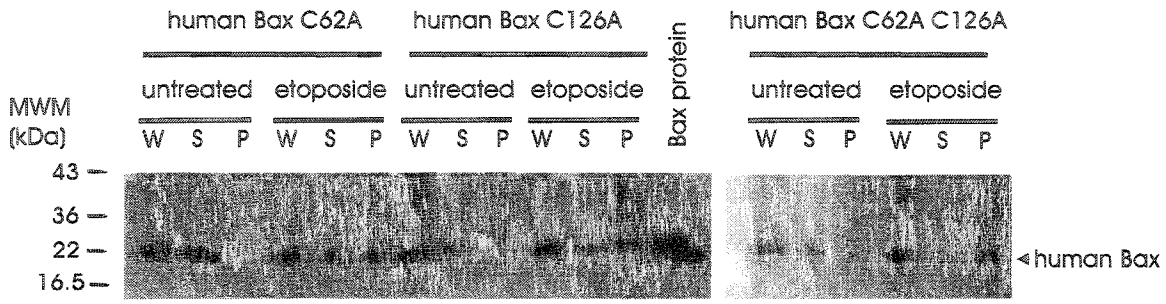


Figure 3. Human Bax translocates to membranes in etoposide treated HOmyc3 cells. Cells were treated with 6 μM etoposide or left untreated then subjected to nitrogen cavitation to yield a whole cell lysate (W). Samples were divided into soluble protein (S) and membrane proteins (P) by centrifugation at 1 000 x g for 30 minutes. Ten micrograms of whole cell lysate and an equivalent volume of supernatant and pellet were separated on a Tris-Tricene gel followed by immunoblotting with the human specific Bax antibody (2D2). The mobility of human Bax is indicated on the right of the blot. Five nanograms of purified recombinant Bax protein was loaded as a mobility control. There was incomplete transfer in the untreated C126A untreated samples (S fraction) as this difference was not seen in other blots with this mutant.

proteins can be separated by a gel shift assay (Krishnasastry et al., 1994). Previously, high percentage gradient SDS-PAGE have been used to resolve the gel shift for Bcl-2 (Kim et al., in press); however perhaps due to its acidic pI (~4.9) gel shifts for Bax were not resolved with this technique (data not shown). Instead, after extensive optimization, isoelectric focusing was used to resolve IASD labeled Bax from unlabeled Bax.

To determine the environment of an individual cysteine residue both cytosolic and membrane Bax were labeled with IASD for each cell line analyzed. Comparing the labeling pattern after 15 minutes incubation with IASD for cytosolic Bax with membrane bound Bax allowed me to determine the environment of individual cysteine residues. As a control both samples were solubilized with detergent (2% CHAPS, 4% IGEPAL) to liberate membrane inserted regions of the protein. Labeling of residues only after detergent solubilization of the membrane confirms that protection from labeling was due to the lipid bilayer rather than the surrounding amino acid sequence, and also demonstrates that the concentration of IASD is not limiting the reaction. Cysteine residues in an aqueous environment were labeled after 15 minutes in both the cytosolic and membrane bound protein (illustrated diagrammatically in Figure 4). Residues that become membrane integrated were accessible to IASD in the cytosolic fractions, but protected in the membrane fraction until the membrane was solubilized with detergent (Figure 4, membrane integrated residue). Reaction conditions were established using position 175 (helix α9) as a control for a membrane integrated residue. Conditions were optimized such that the cytosolic version of this mutant was completely labeled in 15 minutes.

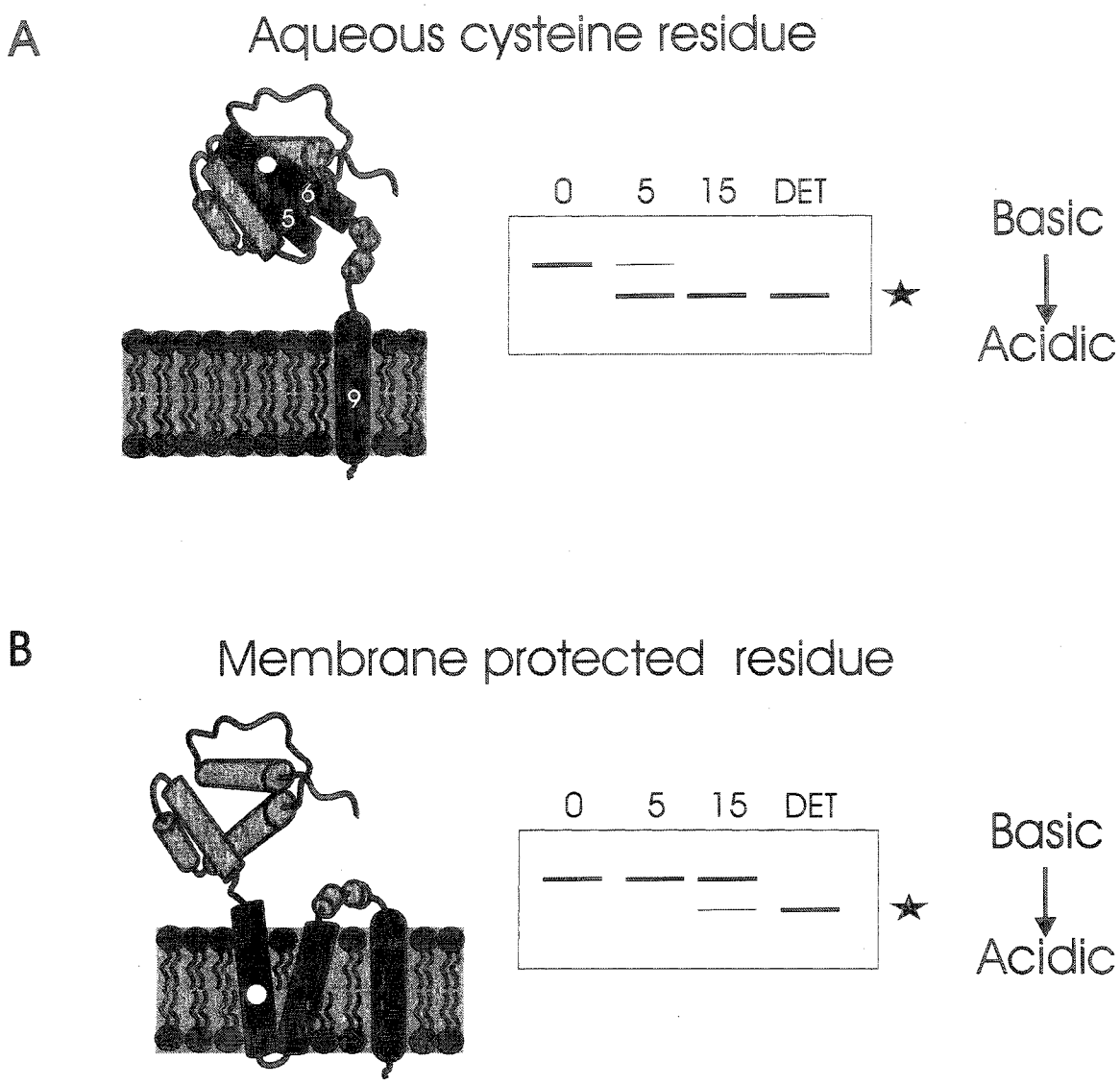


Figure 4. Theoretical labeling reactions for aqueous and membrane inserted cysteine residues. Samples are either untreated (0) or incubated with IASD for 5 and 15 minutes (an additional sample is solubilized in detergent and labeled for 15 minutes (DET)). Samples are then separated on the IEF system and blotted for Bax. (A) **Cysteine in an aqueous environment**. If a cysteine is in an aqueous environment then it will be readily modified by IASD and thus a gel shift towards the acidic end of the IEF gel will occur. (B) **Membrane protected cysteine**. If a cysteine residue is embedded into the lipid bilayer then it will be protected from modification by IASD. Thus a gel shift will only occur when this residue is solubilized in detergent (DET). A gel shift is indicated with a star.

As controls lysates from cell lines expressing wild-type human Bax and cysteine-less Bax were labeled with IASD (Figure 5). Cytosolic and membrane fractions from etoposide treated HO15.19 cells expressing these Bax proteins were labeled with IASD, membranes were solubilized, proteins were separated on IEF gels transferred to PVDF and immunoblotted for Bax (Figure 5). Wild-type human Bax contains two cysteine residues, and thus functions as a control for the resolution range of the gel system. Two shifts are apparent with cytosolic and, after solubilizing the membrane, for membrane fraction membrane bound wild-type Bax. Thus with this gel system unlabeled, single and double labeled proteins can be resolved. Lysates from cells expressing cysteine-less Bax were labeled to determine if under my labeling conditions IASD was modifying other residues in the Bax protein. No shift in the mobility of Bax was observed, confirming that IASD specifically modifies cysteine residues in my reaction conditions (Figure 5).

Membrane topology of Bax in etoposide treated HO15.19 cells

Labeling reactions were performed using the HO15.19 cells first as I have shown that Bax remains monomeric when these cells were etoposide treated (Chapter Four) and this eliminates the possibility of oligomerization of Bax reducing the accessibility of the cysteine to IASD and complicating the interpretation of results. Eighteen single cysteine mutants were expressed in HO15.19 and HOmyc3 cells by infection with the appropriate recombinant retroviruses. Unfortunately four cysteine mutants (positions 111, 136, 138 and 179) did not express to detectable levels. Seven other mutants expressed well, but

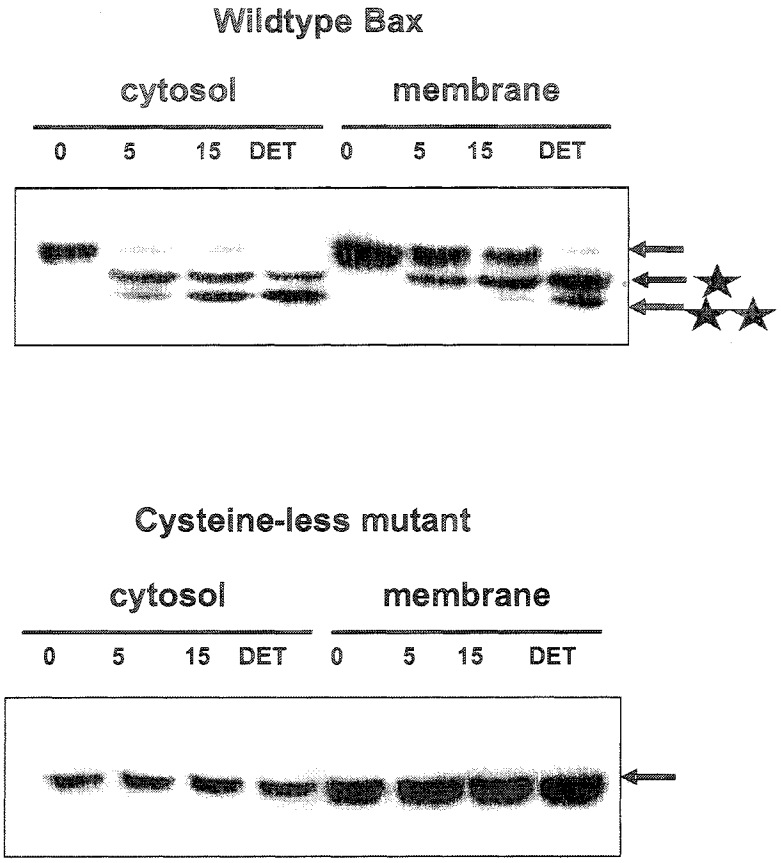


Figure 5. Labeling reactions of wild-type human Bax and cysteine-less Bax in etoposide treated HO15.19 cells. Reactions were performed as described in the materials and methods. Samples were left unlabeled (0) or incubated in the presence of IASD for 5 or 15 minutes (indicated 5 or 15). As a control, samples were solubilized in detergent (DET) to indicate that complete modification of an individual mutant is possible. Unlabeled Bax is indicated with an arrow. IASD modified Bax is indicated with an arrow and star. The pH range for these gels is from 4 - 6. Top of the gel is basic and the bottom of the gel is acidic. For clarity this label has been omitted from these figures. Wild-type human Bax contains two endogenous cysteine residues each of which can be modified by IASD as indicated by a double shift on the IEF gel system (two stars). Cysteine-less human Bax is not modified by IASD and thus appears as a single band on this gel system.

the cysteines at these positions were not labeled by IASD even for cytosolic Bax and, in some cases, detergent solubilized membrane bound Bax. Therefore, the labeling reactions for these mutants were not interpretable. Representative IEF gel analyses of these mutants are shown in Appendix A.

Labeling reactions were performed on the remaining seven single cysteine mutants (Figures 6-9). Initially I had predicted that Bax would adopt a 'tail-anchor topology' in the etoposide treated HO15.19 cells. However, the labeling reactions for the various single cysteine mutants of Bax suggest that membrane bound Bax is in a more 'pore-like' membrane topology. For each mutant a schematic of the membrane topology of Bax is presented and the location of that mutant indicated. The environment for each mutant was determined by comparing the extent of labeling at 15 minutes between the cytosolic and membrane bound Bax proteins (see below).

Position 40 which is part of the unstructured loop between helix α1 and α2 was selected as an aqueous control (Figure 6A). This mutant is readily modified by IASD in both the cytosolic and membrane fractions and behaves as expected for a residue in an aqueous environment.

Position 122, located in 'bottom' portion of helix α5, was readily labeled in Bax molecules in the cytosol; however it was essentially inaccessible to IASD when Bax was on the membrane. However, this residue was modified when detergent was added to solubilize the membrane confirming that protection was due to insertion of the cysteine into the bilayer (Figure 6B). Thus position 122 is predicted to be in the lipid bilayer when Bax is on the membrane.

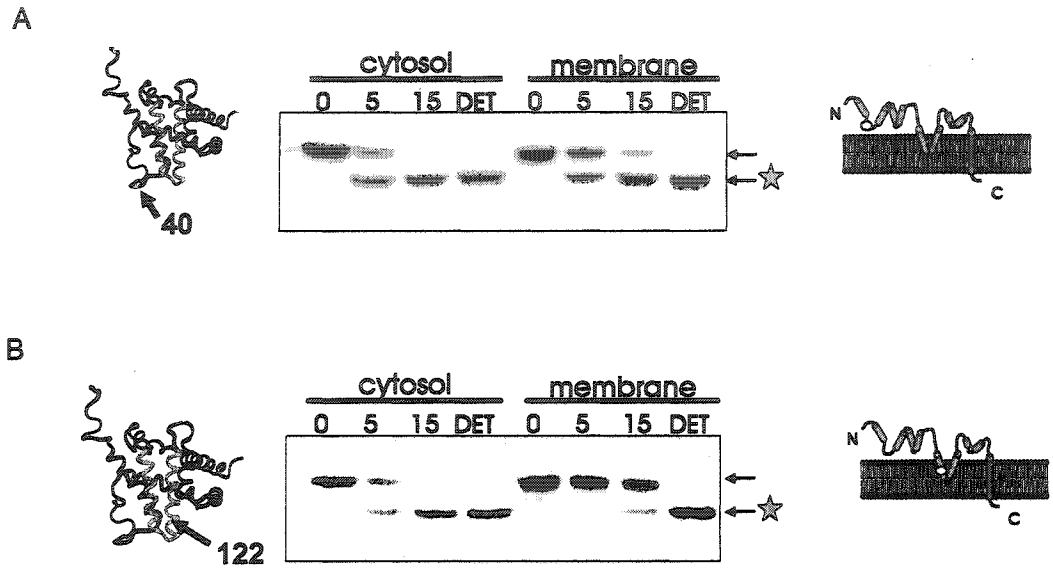


Figure 6. Labeling reactions for single cysteine mutants at positions 40 and 122. The location of an individual residue is indicated in the structure of Bax (right side). The location of this residue in the membrane topology of Bax appears to the left of the labeling reaction. Unlabeled Bax is indicated with an arrow. IASD modified Bax is indicated with an arrow and star. These are representative image of at least three separate experiments. (A) Position 40 is located in the loop between helices α1 and α2. This residue is readily modified by IASD in both the cytosolic and membrane fractions and thus appears to be in an aqueous environment. (B) Position 122 is located in the middle of helix α5 of Bax. This mutant is modified in the cytosol, but protected on the membrane.

Position 126, located at the 'bottom-end' of helix α5, was modified by IASD in the cytosolic form, however the cysteine at position 126 was partially protected from the reagent when membrane bound Bax was assayed (Figure 7A). Thus this position of Bax protein is in the lipid bilayer.

Position 134, located in the 'bottom' portion of helix α6, was almost completely modified in its cytosolic form and partially protected in its membrane form. Thus this position of the membrane bound Bax is likely inserted into the lipid bilayer (Figure 7B).

Positions 142 and 144, located in the top third of helix α6, were modified by IASD in both the cytosolic and membrane fractions. It is unclear why the mobility of cytosolic Bax D142C appears to shift towards the acidic end of the gel; elimination of an aspartic acid residue would alter the calculated pI from ~4.9 to ~5.0. Despite this both bands of the cytosolic protein are labelled with IASD (both bands shift) and the membrane bound Bax is also labelled. Taken together, these residues are in an aqueous environment (Figure 8A and B).

Lastly, position 175 is located in the middle of helix α9, the putative tail-anchor of Bax. In cytosolic Bax position 175 was labelled by IASD, however on the membrane this position was partially protected from IASD. Thus this position of Bax protein is predicted to be inserted into the lipid bilayer (Figure 9A).

Each of these immunoblots is a representative from at least three separate labelling reactions. To quantify the accessibility of IASD to specific residues, immunoblots were developed directly on the Typhoon9200 imaging station and the band intensities quantified using ImageQuant software. From these intensities the percent

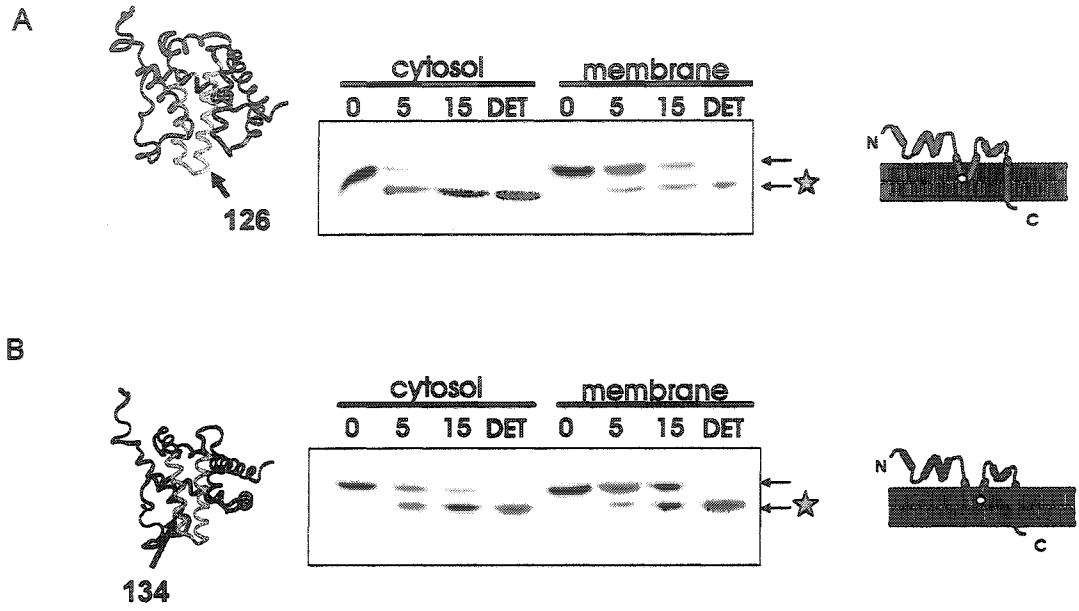


Figure 7. Labeling reactions for single cysteine mutants at positions 126 and 134. The location of an individual residue is indicated in the structure of Bax (right side). The location of this residue in the membrane topology of Bax appears to the left of the labeling reaction. Unlabeled Bax is indicated with an arrow. IASD modified Bax is indicated with an arrow and star. These are representative image of at least three separate experiments. (A) Position 126 is located in the 'bottom' of helix α5. This residue is readily modified by IASD in the cytosol, but partially protected in the membrane fractions. (B) Position 134 is located in the middle of helix α6 of Bax. This mutant is modified in the cytosol, but protected on the membrane.

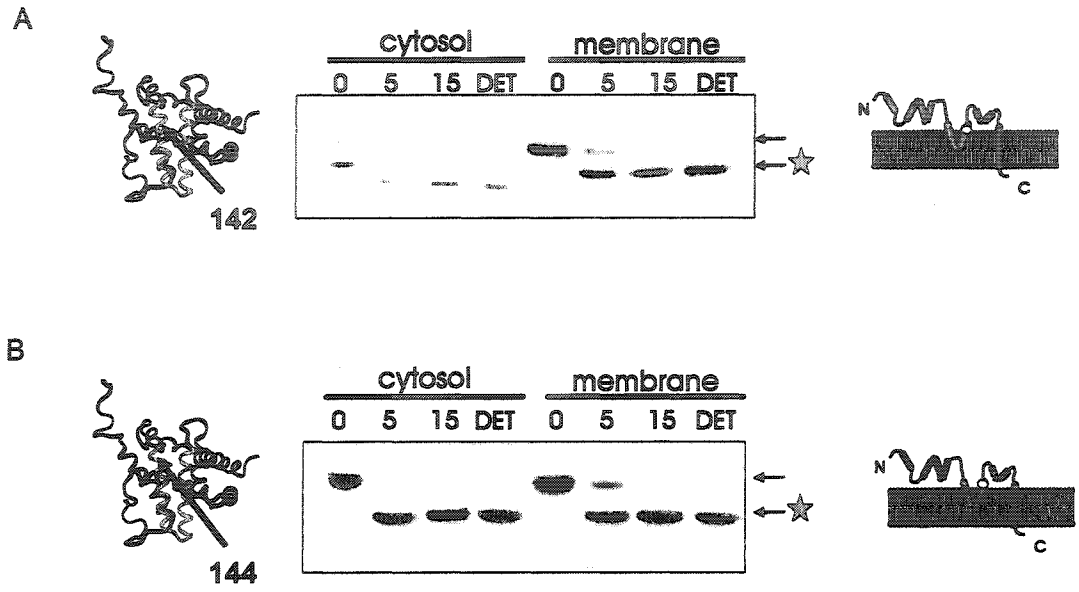
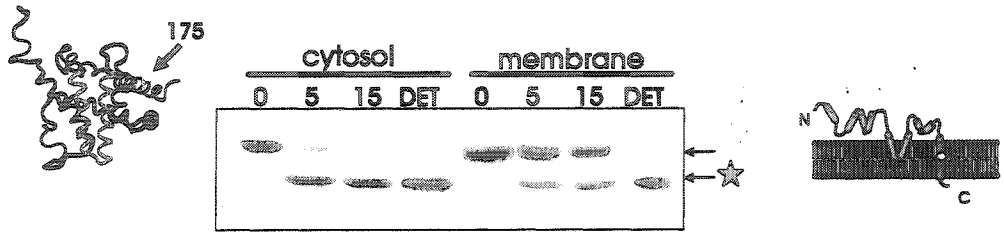


Figure 8. Labeling reactions for single cysteine mutants at positions 142 and 144. The location of an individual residue is indicated in the structure of Bax (right side). The location of this residue in the membrane topology of Bax appears to the left of the labeling reaction. Unlabeled Bax is indicated with an arrow. IASD modified Bax is indicated with an arrow and star. These are representative image of at least three separate experiments. (A) Position 142 is located in the top third of helix α6. This residue is readily modified by IASD in both the cytosolic and membrane fractions and thus appears to be in an aqueous environment. (B) Position 144 is located in the top porition of helix α6 of Bax. This mutant is modified in both the cytosol and membrane fractions.

A



B

Position	%Unlabelled in cytosol	% Protected on membrane	Predicted Location	n
40	7% +/- 1%	17%+/- 6%	cytosolic	3
122	29% +/- 5%	73%+/- 1%	membrane	3
126	26% +/- 6%	64%+/- 6%	membrane	2
134	27% +/- 6%	51%+/- 7%	membrane	3
142	7% +/- 2%	11%+/- 1%	cytosolic	2
144	7% +/- 4%	9%+/- 4%	cytosolic	3
175	23%+/- 7%	51% +/- 7%	membrane	3

Figure 9. Labeling reaction for single cysteine mutant at positions 175 and Percent protection for the assayed mutants. The location of an individual residue is indicated in the structure of Bax (right side). The location of this residue in the membrane topology of Bax appears to the left of the labeling reaction. Unlabeled Bax is indicated with an arrow. IASD modified Bax is indicated with an arrow and star. This is a representative image of at least three separate experiments. (A) Position 175 is located in the middle of helix α9, the putative tail anchor for Bax. This residue is readily modified by IASD in the cytosol, but protected on the membrane. (B) The percentage protection of individual residues in HO15.19 cells are indicated +/- the standard deviation, the predicted location stated, and number of images quantified (n). For residues 126 and 142 only two images were quantified and thus the range between these two numbers, and not the standard deviation, is presented. Highlighted are those residues that are protected from IASD modification and thus are considered membrane integrated.

protection from IASD after 15 minutes incubation for each of these mutants was calculated (Figure 9B). Residues that were interpreted, by qualitative judgement, as inserted into the lipid bilayer were 51-73 % protected from IASD modification; whereas residues that were interpreted as aqueous were only 9-17 % protected from IASD. In the cytosolic fractions positions interpreted to be aqueous were efficiently labelled with IASD (only 7% remained unlabelled after 15 minutes) whereas those that were judged to be protected by the lipid bilayer were all partially protected from modification (23-29% unlabelled by IASD after 15 minutes). Decreased labeling in the cytosolic protein may indicate that these residues are in a hydrophobic environment and thus not solvent exposed; however all of these positions are on the surface of the solution structure (data not shown). Although it is unclear why specific residues are not readily modified by IASD the difference in the accessibility of these residues from the cytosolic to membrane bound form is higher for membrane integrated vs. aqueous residues (24-44% vs. 2-10%, respectively). Considering the limited interpretable data in this analysis it is premature to state a numerical threshold or 'cut-off' value to indicate whether a residue is either in the lipid bilayer or an aqueous environment. Gründling et al. have used IASD to determine the membrane topology of the poly-topic membrane protein lambda S holin (Gründling et al., 2000). For their analysis the extent of labeling for single cysteine mutants is presented and the results for interpreted membrane integrated residues confirmed by detergent solubilization. Combining this approach, with a qualitative interpretation a labeling data schematic of the membrane topology is presented (Figure 10). The data

collected thus far suggest that in etoposide treated HO15.19 cells Bax adopts a 'pore-like' topology.

Membrane topology of Bax in etoposide treated HOmyc3 cells

In the presence of Myc expression Bax forms an oligomer in etoposide treated cells and cytochrome c is released from mitochondria. Similar to the HO15.19 cells several of the mutant proteins either did not express or were poorly labelled by IASD in the HOmyc3 cells. The labelling reactions for eight single cysteine mutants are presented in Figure 11.

In the HOmyc3 cells the cytosolic Bax for some single cysteine mutants run as multiple distinct species (bands) on the isoelectric focusing gels prior to the addition of IASD. These multiple bands likely originate from the post-translational modification of Bax. Although these multiple forms make the interpretation of these mutants difficult; positions 40, 122, 142, and 175 have a similar labeling pattern to the HO15.19 cells. Interestingly, positions 3 and 192 are efficiently modified in the HOmyc3 cell lysates (compared to the HO15.19 cells where they are only partially modified, see Appendix A) and these cysteines both appear to be in aqueous environments. Position 109 could not be labeled in the standard reactions (data not shown); even performing the labeling reactions in presence 4M urea (Figure 11, position 109) only slightly increased the accessibility of this position to IASD. Despite the apparent difference in activity (Chapter Four), the membrane topology of Bax appears similar in both a monomeric and oligomeric form.

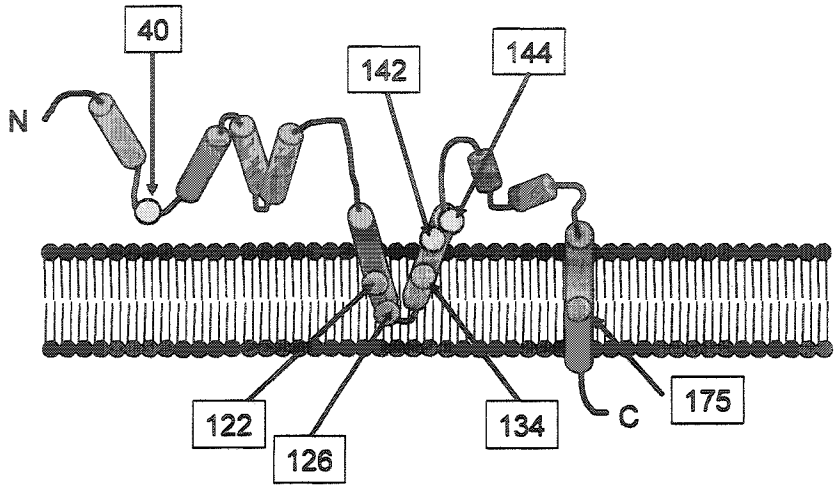


Figure 10. Schematic membrane topology of Bax in etoposide treated HO15.19 cells. This topology is based on the labeling pattern for the several mutants (indicated) presented in Figures 6-9 and the NMR structure of Bax.

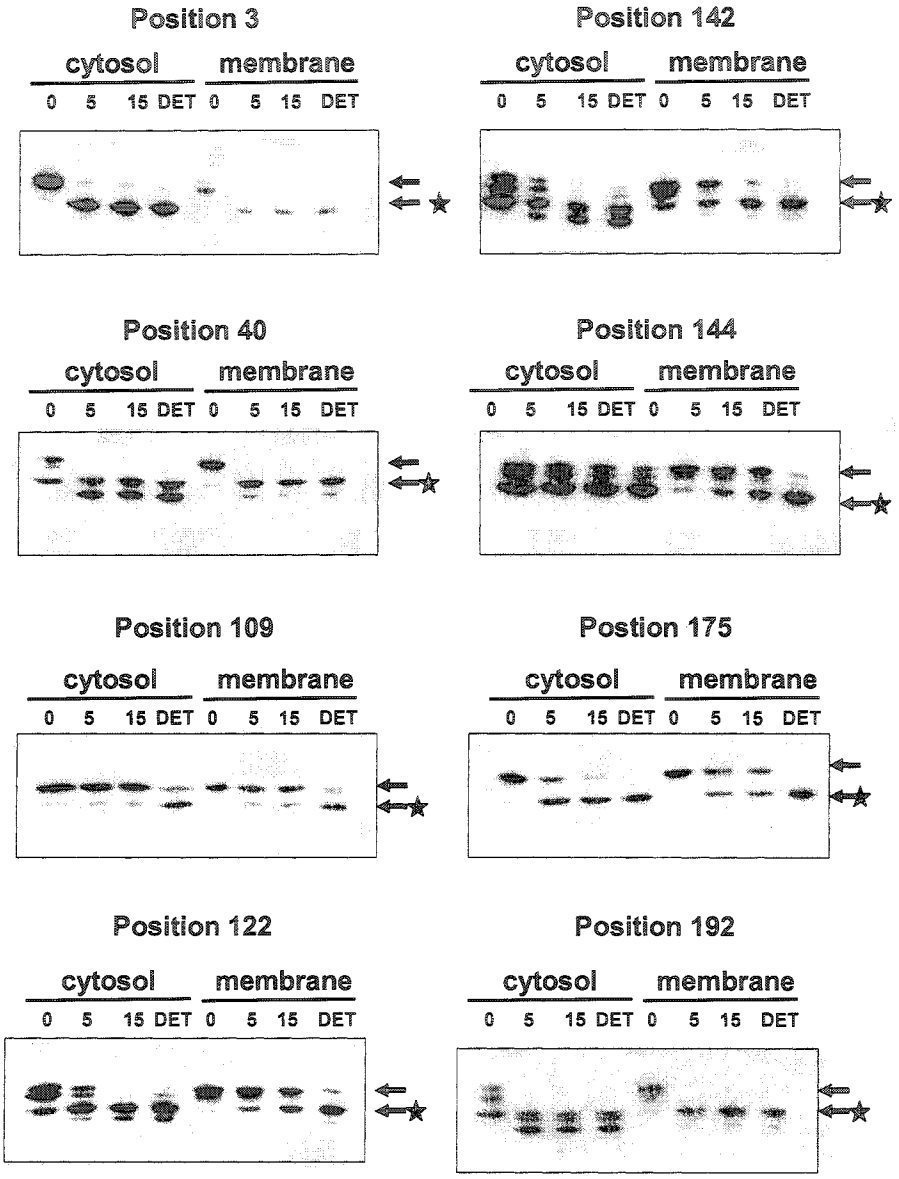


Figure 11. Labeling reactions of single cysteine Bax mutants in the HOmyc3 cells. The location of individual residues is indicated in Figure 2. Reactions were performed as described in the materials and methods. Samples were left unlabeled (0) or incubated in the presence of IASD for 5 or 15 minutes (indicated 5 or 15). As a control, samples were solublized in detergent (DET) to indicate that complete modification of an individual mutant is possible. Unlabeled Bax is indicated with an arrow. IASD modified Bax is indicated with an arrow and star. The pH range for these gels is from 4 - 6. Top of the gel is basic and the bottom of the gel is acidic. For clarity this label has been omitted from these figures. Position 109 was labeled in 4M UREA.

Discussion:

Bax induction of cell death is manifested through its oligomerization and pore formation in mitochondrial membranes. The molecular mechanism behind the activity of Bax is unknown, however its oligomerization is critical for cytochrome c release (Soucie et al., 2001; Antonsson et al., 2000). Bax pore formation was thought to be facilitated through the membrane insertion of two relatively hydrophobic helices (α5 and α6) into the lipid bilayer (Nouraini et al., 2000). Previously, I have demonstrated that in the absence of Myc, Bax is membrane integrated, but monomeric and does not induce cytochrome c release. With constitutive Myc expression Bax oligomerizes and cytochrome is released from mitochondria. Thus in this cell system I can obtain Bax in two different membrane bound forms - monomeric and oligomeric. By isolating these two forms of Bax I addressed whether membrane topology changes in Bax are required for pore formation.

Using chemical labeling and site directed mutagenesis we have established the approximate membrane topology of Bax in its monomeric and oligomeric state. Surprisingly these two topologies appear to be identical with three alpha helices (α5, α6, and α9) at least partially inserted into the membrane (Figure 10). Unfortunately, several of the mutants prepared for this analysis were poorly expressed or difficult to label and thus a more precise map of the topology of Bax was not obtained. Nevertheless, the membrane topology does not change significantly with Myc expression (at least in the regions of Bax that are thought to be membrane bound). Despite the apparent similarity, Bax does undergo a structural change to expose an amino-terminal epitope when it is an

oligomer (Chapter Four). Intriguingly, the amino-terminus does appear protected from modification in the HO15.19 cells (Position 3, Appendix A), however this residue is accessible in the HOmyc3 cells. This difference may be due to the presence of an additional protein binding and thus blocking the amino-terminus of Bax. A possible candidate is Ku70, which interacts with the amino-terminus of Bax. However, interaction with Ku70 was shown in to prevent Bax translocation, rather than activation (Sawada et al., 2003)

In determining the topology of Bax, certain residues were predicted to be membrane integrated, e.g. position 175 of helix α9. Even though this residue was predicted to be protected from modification, it was still partially modified (~50 %) by IASD. This may indicate a mixed population of membrane integrated and loosely bound Bax molecules. Carbonate extractions, which remove peripherally bound proteins, did remove a small amount of Bax protein; however the carbonate resistant molecules were still modified to the same extent with IASD (data not shown). Partial labeling with IASD would also be consistent with a residue being part of an aqueous channel with limited accessibility. Recently, Dr. Richard Epand and colleagues demonstrated that Bax alone is capable of disrupting the integrity of liposomes; however in this state the pore formed by Bax is not uniform or large enough for the passage of cytochrome *c* (Epand et al., 2002). However these small disruptions in the lipid bilayer may allow the passage of a small molecule like IASD and thus explain the partial labeling seen with certain mutants. Additional mutations flanking those used in this analysis would verify if the partial

labeling seen is due to the location of the residue in a helix lining a pore as there would be a periodicity of IASD accessibility around the alpha helix.

My results suggest a multi-spanning membrane topology for a tail-anchor protein. Bax targeting is thought to regulated by the amino terminus (Goping et al., 1998), helices α5 and α6 (Nouraini et al., 2000), and the carboxyl-terminal hydrophobic sequence (Nechushtan et al., 1999). If Bax were to behave like other tail-anchor proteins then targeting to membranes would first involve the integration of the hydrophobic carboxyl-terminal sequence. Alternatively, Bax may insert all three helices at the same time. With the current analysis we cannot differentiate these two Bax targeting mechanisms; however the anti-apoptotic protein Bcl-2 also undergoes a conformational change during apoptosis that is consistent with insertion of helices α5 and α6 (Kim et al., in press). Bcl-2 is targeted to the membrane via its carboxyl-terminal insertion sequence (Janiak et al., 1994) and exists in healthy cells in a 'tail-anchor' topology. Initiation of apoptosis by pro-apoptotic Bcl-2 family members induces a change in Bcl-2 membrane topology to a 'pore-like' topology. Interestingly, this change in Bcl-2 topology occurs when Bax is partially oligomerized on mitochondria (Chapter Three, Figure 8,), suggesting Bcl-2 may interact with Bax when it is in this conformation.

I have observed multiple migrating species of Bax in HOmyc3 cells. Human Bax appears as a single band on standard SDS-PAGE gels (Figure 3); however multiple bands are apparent when this protein is separated by iso-electric focusing (Figure 11). These multiple forms of Bax are variable, but abundant in the cytosolic fraction. The majority of these additional forms for Bax appear to have a lower pI then Bax indicating the

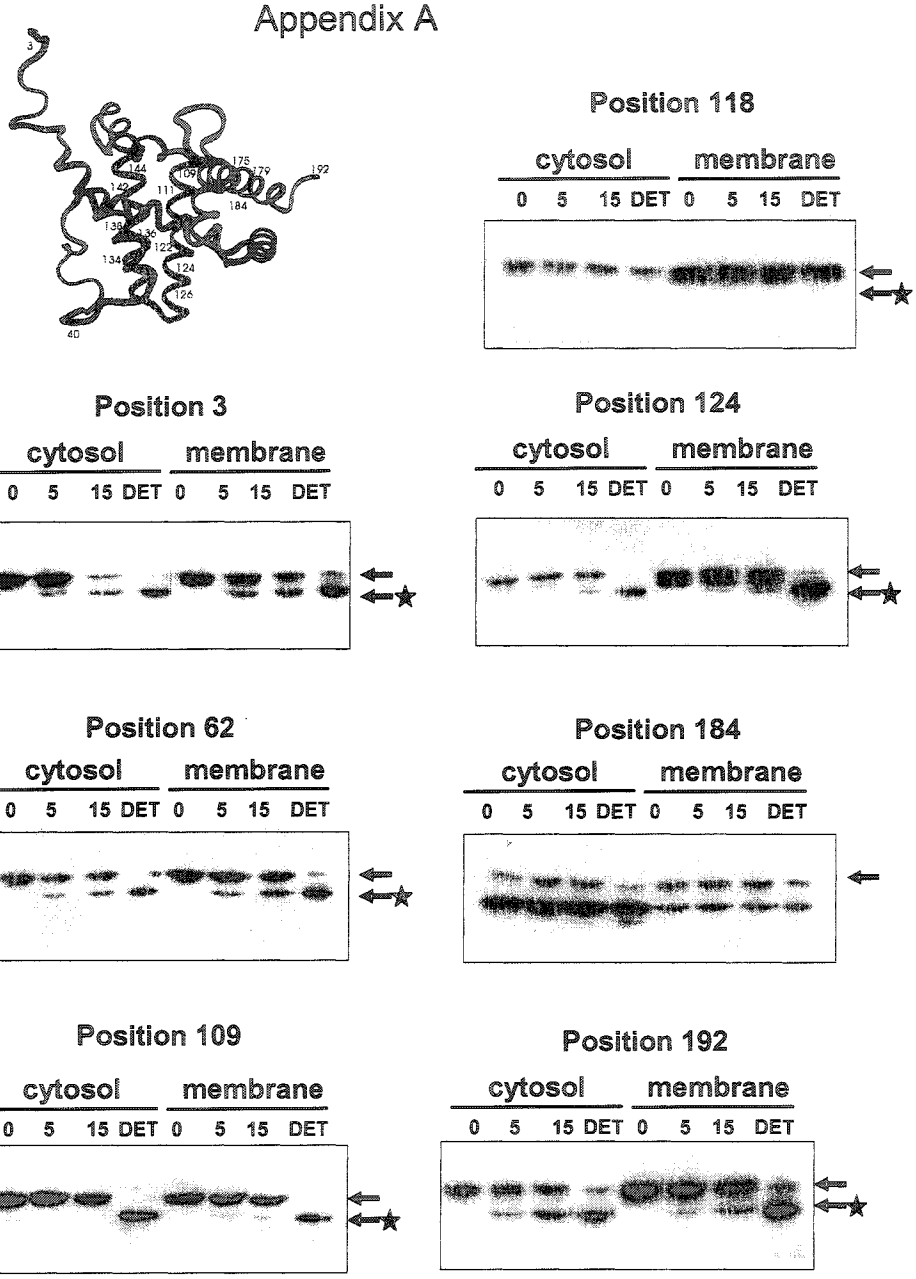
addition of negative charges, consistent with phosphorylation. Bax phosphorylation has been reported in hypoxic newborn piglets (Ashraf et al., 2001), however this was determined indirectly by immunoprecipiting phosphorylated proteins followed by immunoblotting for Bax. Interestingly, these modified Bax proteins are not abundant in the membrane fraction, suggesting the removal of post-translational modification of Bax is required for membrane translocation. Consistent with this is the recent observations that the phosphorylation of Bax by Akt (on Ser184) inhibits Bax activity at the mitochondria (Gardai et al., 2004).

The general membrane topology of Bax has three alpha helices partially inserted into the lipid bilayer; however the extent to which each of these helices is inserted remains to be determined. In addition, between helices α5 and α6 is a short three amino-acid loop containing one lysine residue. It would be interesting to determine the environment of this loop region and determine if helices α5 and α6 traverse the lipid-bilayer. In addition, the extent to which helix α9 is inserted into lipid bilayer also requires further analysis. Unfortunately, attempts to generate cell lines expressing these additional mutants have not been successful.

The simple model that Bax translocation, activation, and cytochrome c release all occur as one concerted step is invalid. Clearly we have demonstrated that there are several individual steps to Bax activity and perhaps multiple activators and inhibitors along the pathway leading to cytochrome c release. Dramatic changes to the membrane topology of Bax are not likely critical to its oligomerization. The precise mechanism of

Bax oligomerization remains to be determined; however this limited membrane topology analysis has provided valuable insight into the mechanism of Bax activity.

Appendix A



Appendix A. Labeling reactions of single cysteine Bax mutants in the HO15.19 cells. The location of individual residues is indicated in Figure 2. Samples were left unlabeled (0) or incubated in the presence of IASD for 5 or 15 minutes (indicated 5 or 15). As a control, samples were solublized in detergent (DET) to indicate that complete modification of an individual mutant is possible. Unlabeled Bax is indicated with an arrow. IASD modified Bax is indicated with an arrow and star. The pH range for these gels is from 4 - 6. Top of the gel is basic and the bottom of the gel is acidic. For clarity this label has been omitted from these figures.

MUTANT	LOCATION	EXPRESSION HOmyc3	EXPRESSION HO15.19	Environment
G3C	loop	☑	☑	?
G40C	loop	☑	☑	Aqueous
62C (C126A)	BH3	low	☑	Can't label
R109C	5	☑	☑	Can't label
V111C	5	X	X	No Exp.
S118C	5	low	☑	Can't label
L122C	5	☑	☑	membrane
A124C	5	X	☑	Can't label
126C (C62A)	5	☑	☑	membrane
R134C	6	low	☑	membrane
I136C	6	X	X	No Exp.
G138C	6	X	X	No Exp.
D142C	6	☑	☑	Aqueous
L144C	6	☑	☑	Aqueous
I175C	9	☑	☑	membrane
G179C	9	X	X	No Exp.
S184C	9	X	☑	Can't label
G192C	tail	☑	☑	?

Table 1. Single cysteine mutants of human Bax. The first column indicates the mutant (the endogenous cysteine positions are indicated in bold). The location of the single cysteine mutation is indicated in the second column. In the third and fourth column the level of expression in the HOmyc3 and HO15.19 cells are indicated [a check mark indicates good expression, low indicates low expression and an 'X' means no detectable expression]. The environment of the cysteine is indicated in column five.

Chapter Six

Discussion

Apoptosis is required for the normal development and tissue homeostasis of most multi-cellular organisms. The importance of apoptosis is revealed when the normal regulation of this process is disrupted. Increased apoptosis results in degenerative phenotypes as seen in the human neurological disorders Alzheimer or Huntington. In contrast, decreased cell death contributes to the development of human cancers. Thus, understanding the regulation of cell death both positively and negatively has implications in several human diseases.

One potent regulator of apoptosis is the Bcl-2 family of proteins. Indeed, expression of Bcl-2 inhibits cell death elicited by a variety of apoptotic agonists (Chapter Three and (Annis et al., 2001; Vaux et al., 1988; Soucie et al., 2001)). How does the expression of a single protein provide resistance to such disparate apoptotic agonists? Clearly, these agonists engage the endogenous cell death machinery through the activation of pro-apoptotic members of the Bcl-2 family. As demonstrated in this thesis, the antagonistic relationship between Bcl-2 family members is critical for many aspects of their function. Mitochondria are a central target for the activity of the pro-apoptotic members and, as demonstrated in Chapters Three and Four, mitochondria undergo several dysfunction events during the initiation of cell death. However, mitochondria are not the only target of pro-apoptotic proteins; the endoplasmic reticulum (ER) also serves as a battlefield for the Bcl-2 family.

Bcl-2 is located at the ER, nuclear envelope and mitochondria in mammalian cells. This broad localization, not common for tail anchor proteins, is thought to contribute the potent anti-apoptotic activity of this protein. To address this theory mutants of Bcl-2 that

target exclusively to either the ER or mitochondria were generated. Expression of Bcl-2-Acta, a mitochondria targeted mutant, confers resistance to many apoptotic agonists; however, in contrast, ER localized Bcl-2-cb5 is only functional with specific apoptotic agonists (Chapter Three and (Zhu et al., 1996; Lee et al., 1999; Annis et al., 2001)). These observations are consistent with mitochondria serving as a central target in apoptosis. In addition, these results also demonstrate that events at the ER are equally important in regulation of apoptosis depending on the apoptotic stimulus. In Chapter Three I presented two intrinsic cell death pathways: in one pathway, initiated by serum starvation (with deregulated Myc expression) or ceramide, loss of inner mitochondrial membrane potential ($\Delta\Psi_m$) precedes the release of cytochrome *c*; in the other pathway, elicited by etoposide, the early translocation and activation of Bax at mitochondria occurs coincident with cytochrome *c* release and prior to the loss of $\Delta\Psi_m$. These two pathways are considered spatially distinct as the former is inhibited by ER localized Bcl-2 (Bcl-2-cb5) and the latter is not. Thus two spatially distinct apoptosis pathways termed, for the purpose of discussion, the ER pathway and Mitochondria pathway have been identified.

The ER pathway

The signal to induce the early loss of $\Delta\Psi_m$, characteristic of the ER pathway, remains unresolved; however Ca^{2+} is a probable candidate. Indeed, increases in cytoplasmic Ca^{2+} levels can lead to the loss of $\Delta\Psi_m$, a process that is inhibited by blocking Ca^{2+} uptake into mitochondria. And Ca^{2+} has been implicated as an important signaling molecule in ceramide induced cell death. However, the contribution of Bcl-2 in

regulating ER Ca^{2+} stores at steady state is very controversial. As well the contribution of the pro-apoptotic members, Bak and Bax, in the regulation of ER Ca^{2+} is equally unclear (see Introduction). Thus it is hard to identify the mechanism governing the release of Ca^{2+} in the ER pathway.

One possible target for Bcl-2 activity is the ER Ca^{2+} pump, SERCA. The direct interaction between SERCA and Bcl-2 suggests that Bcl-2 may augment the activity of SERCA directly. This provides an intriguing model where Bcl-2 enhances SERCA activity and thus inhibits increases in cytoplasmic Ca^{2+} levels; however disruption of Bcl-2 activity, through interaction with pro-apoptotic members, inhibits the SERCA pump leading to increased cytoplasmic Ca^{2+} levels (through leakage from the ER). The pro-apoptotic protein, Bad is a good candidate for the regulation of the ER pathway. Bad is normally sequestered in the cytoplasm due to a phosphorylation dependent interaction with 14-3-3(Datta et al., 1997; Zha et al., 1996). However, in the absence of growth factors Bad is dephosphorylated and is now free to bind to Bcl-2. This interaction inhibits Bcl-2, in part, by preventing Bcl-2 from binding additional pro-apoptotic proteins or, potentially, non-family members (SERCA?) as well. Although not empirically tested in these cell lines, Bad activity has been implicated in serum starvation and ceramide induced cell death and Bcl-2-cb5 expression can inhibit Bad induced cell death, thus Bad may be the critical pro-apoptotic Bcl-2 member mediating the ER cell death pathway.

It is interesting to note that Bak/Bax null mouse embryonic fibroblasts were resistant to serum starvation induced cell death (Wei et al., 2001). Since changes to localization or activation of Bax were not observed in serum starved/myc (or ceramide

treated) Rat-1 fibroblasts (Chapter Three), this implicates Bak as the mediator of cell death in the ER pathway. Similar to Bax, specific cell death agonists will trigger a conformational change in Bak (Griffiths et al., 1999). Unfortunately, Bak conformation specific antibodies do not cross-react with Rat Bak, thus it is difficult to determine if a change in Bak conformation is required in the cell death pathways examined in this thesis.

The mitochondria pathway and Myc

The mitochondria pathway, initiated by etoposide, is characterized by the membrane translocation of the normally cytoplasmic protein, Bax. After translocation to the mitochondrial membrane, Bax oligomerizes and is activated to induce the release of cytochrome *c*. Inhibition of this pathway requires mitochondrial localization of Bcl-2, suggesting a direct interaction between Bcl-2 and Bax is required for Bcl-2 function. Consistent with this, expression of a Bcl-2 mutant (Bcl-2-G145A), that does not hetero-dimerize with Bax, did not inhibit etoposide induced Bax oligomerization or Caspase activation in Rat-1 fibroblasts (Chapter Three).

Recently, Scorrano *et al.* reported that etoposide induced cell death in mouse embryonic fibroblasts requires both an ER (Ca^{2+}) and mitochondrial component (Bax) (Scorrano et al., 2003). However, in Rat-1 fibroblasts Bcl-2-cb5 expression was ineffective at inhibiting cell death induced by etoposide (Chapter Three). Thus either Bcl-2-cb5 is incapable of inhibiting the release of ER Ca^{2+} or that inhibition of Ca^{2+} release is not necessary to prevent etoposide induced cell death. Consistent with the latter, expression of Bcl-2 does not appear to inhibit the release of Ca^{2+} from the ER in either

doxorubicin (Marin et al., 1996) or thapsigargin (Srivastava et al., 1999) induced cell death. Thus the critical location for Bcl-2 activity in etoposide induced cell death is mitochondria.

On mitochondria Bcl-2 and Bax co-migrate into multimeric structures following etoposide treatment. The formation of these larger Bcl-2/Bax complexes is coincident with activation of Caspases; suggesting that at some point these Bax/Bcl-2 complexes allow the release of cytochrome *c* and hence the activation of Caspases (Model proposed in Introduction, Figure 4). Although it is not possible to prove that these larger complexes are permeable to cytochrome *c* with this experimental system, these observations are consistent with a rheostat model of cell death regulation, where Bcl-2 becomes overwhelmed by multi Bax proteins.

It is interesting to note that expression of Bcl-2 did not inhibit etoposide induced cell death in the absence of Myc expression (Soucie et al., 2001), and that expression of Myc is required for the oligomerization of Bax in etoposide treated cells (Chapter Four). Together with the observations seen with Bcl-2-G145A in Rat-1 fibroblasts (Chapter Three), this suggests that the critical site of Bcl-2 function is to inhibit the oligomerization of Bax in etoposide induced cell death.

In the cytoplasm, two proteins, Ku70 and Humanin prevent agonist induced mitochondrial Bax translocation (Sawada et al., 2003; Guo et al., 2003). The signal for Bax translocation in etoposide induced cell death remains unresolved; however pro-apoptotic Bcl-2 proteins can mediate this change. For example, incubation of purified recombinant t-Bid with recombinant Bax will promote the release of cytochrome *c* from

isolated mitochondria (Desagher et al., 1999). In etoposide treated cells t-Bid is not the mediator of Bax translocation, as Caspase activation is not required for cytochrome c release. Thus an alternative pro-apoptotic Bcl-2 member is likely initiating Bax translocation. However, mitochondrial translocation of Bax, although necessary, is not sufficient for cytochrome c release.

Once on mitochondria, etoposide induced cytochrome c release requires a Myc dependent component (Chapter Four). This Myc dependent factor regulates Bax oligomerization and activation. Interestingly, mitochondria isolated from *Myc* null cells have impaired cytochrome *c* release induced by purified recombinant Bax and t-Bid (Paulina Dlugosz, unpublished data) suggesting the defect in the *Myc* null cells is a mitochondrion associated factor. Identifying this factor will provide valuable insight into the regulation of Bax (and perhaps Bcl-2) activity.

Membrane topology of Bax

Monomeric membrane bound Bax has a multi-spanning membrane topology with three helices, α5, α6 and α9 partially inserted into lipid bilayer (Chapter Five). This topology does not appear to change significantly with the oligomerization of Bax. Thus the critical switch for Bax activation is through its oligomerization. The importance of this step is highlighted by the fact that Bcl-2 functions to inhibit the oligomerization of Bax in etoposide induced apoptosis.

In collaboration with Dr. Peter Kim, we investigated the membrane topology of Bcl-2 in Rat-1 fibroblasts during cell death (Kim et al., in press). By applying similar

techniques to those used to map the membrane topology of Bax we determined that in healthy cells Bcl-2 adopts a typical tail-anchor membrane topology (see Chapter Five, Figure 1 for schematic). However, similar to Bax, during apoptosis Bcl-2 enters a multi-spanning membrane topology. These changes in Bcl-2 membrane topology are coincident with Bcl-2:Bax complex formation (as seen in Chapter Three, Figure 8). This raises the intriguing possibility that interactions between Bcl-2 and Bax only occur after Bcl-2 changes membrane topology. Consistent with this is the observation that non-ionic detergents artificially induce conformational changes to Bax structure (making it 6A7 positive) and allow it to interact with Bcl-2 (Hsu and Youle, 1997) suggesting that conformational changes in these proteins are required for hetero-dimerization.

Interaction between Bcl-2 family members is principally mediated by the hydrophobic BH regions of these proteins. Intriguingly, the hydrophobic face of the Bax BH3 region is buried in the solution structure and thus inaccessible to Bcl-2. However, once on the membrane the BH3 is likely in a different orientation as helices α5, α6 and α9, which interact with the BH3 in solution, are now inserted into the membrane. However, an additional conformational change may be required for Bax to interact with Bcl-2, as these two proteins do not appear to co-migrate until Bax forms larger molecular weight oligomers. Thus, at a molecular level, changes to Bcl-2 membrane topology may be required to inhibit Bax oligomerization. Consistent with this is that Bcl-2-G145A does not undergo a change in membrane topology and does not inhibit Bax oligomerization.

Apoptosis in the absence of Myc Expression

Although delayed, etoposide induced cell death in the absence of Myc is still apoptosis (determined by morphological changes, PARP cleavage and DNA fragmentation (Soucie et al., 2001)). Thus Bax activity is not the only target in the etoposide induced cell death pathway. Etoposide is reported to stabilize the topoisomerase II- DNA complex and thus initiated apoptosis through DNA damage; this DNA damage is recognized by the tumor suppressor, p53. P53 initiates apoptosis through increased expression of Puma and Noxa (Villunger et al., 2003; Nakano et al., 2001); additionally p53 can activate Bax directly. Thus in the absence of Bax activation, as seen in the Myc knockouts, p53 may initiate cell death through the transcription of *puma* and *noxa*. Because the targets of Puma and Noxa activity are Bax and Bak (Liu et al., 2003), changes to Puma and Noxa expression alone do not explain the mechanism of etoposide induced cell death in the Myc knockouts. Another possible target for etoposide is engagement of the extrinsic cell death pathway, as this pathway is not inhibited by Bcl-2 activity and does not require Bak/Bax expression (in Type I cells, see Introduction). Clearly, an alternative, uncharacterized, Bcl-2 independent mechanism, mediates etoposide induced cell death in the absence of Myc expression.

The investigations presented in this thesis have addressed several controversial aspects of cell death regulation namely: the contribution of the ER to the regulation of cell death; the target of Myc in sensitizing cells to apoptosis; and the contribution of Bax membrane topology and oligomerization to pro-apoptotic activity. I have demonstrated that the ER functions as a critical location in the regulation specific apoptosis pathways

and that Bcl-2 located at this organelle can prevent early changes to mitochondrial function. In addition, I have shown that Bax activity at mitochondria, and Bcl-2 function, requires a Myc regulated factor. Finally, I have identified a critical event in Bax activation is mediated through its oligomerization rather than a change in its membrane topology. Combined these studies have provided valuable insight into both the complexity and molecular mechanisms of Bcl-2 proteins in the regulation of apoptosis.

References

Achenbach,T.V., Muller,R., and Slater,E.P. (2000). Bcl-2 independence of flavopiridol-induced apoptosis. Mitochondrial depolarization in the absence of cytochrome c release. J. Biol. Chem. *275*, 32089-32097.

Adachi,S., Gottlieb,R.A., and Babior,B.M. (1998). Lack of release of cytochrome C from mitochondria into cytosol early in the course of Fas-mediated apoptosis of Jurkat cells. J. Biol. Chem. *273*, 19892-19894.

Adams,J.M. and Cory,S. (2001). Life-or-death decisions by the Bcl-2 protein family. Trends Biochem. Sci. *26*, 61-66.

Annis,M.G., Yethon,J.A., Leber,B., and Andrews,D.W. (2004). There is more to life and death than mitochondria: Bcl-2 proteins at the endoplasmic reticulum. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research *1644*, 115-123.

Annis,M.G., Zamzami,N., Zhu,W., Penn,L.Z., Kroemer,G., Leber,B., and Andrews,D.W. (2001). Endoplasmic reticulum localized Bcl-2 prevents apoptosis when redistribution of cytochrome c is a late event. Oncogene *20*, 1939-1952.

Antonsson,B., Conti,F., Ciavatta,A., Montessuit,S., Lewis,S., Martinou,I., Bernasconi,L., Bernard,A., Mermod,J.J., Mazzei,G., Maundrell,K., Gambale,F., Sadoul,R., and Martinou,J.C. (1997). Inhibition of Bax channel-forming activity by Bcl-2. Science *277*, 370-372.

Antonsson,B., Montessuit,S., Lauper,S., Eskes,R., and Martinou,J.C. (2000). Bax oligomerization is required for channel-forming activity in liposomes and to trigger cytochrome c release from mitochondria. Biochem. J. *345 Pt 2*, 271-278.

Arnoult,D., Gaume,B., Karbowski,M., Sharpe,J.C., Cecconi,F., and Youle,R.J. (2003). Mitochondrial release of AIF and EndoG requires caspase activation downstream of Bax/Bak-mediated permeabilization. EMBO J. *22*, 4385-4399.

Ashraf,Q.M., Zanelli,S.A., Mishra,O.P., and Delivoria-Papadopoulos,M. (2001). Phosphorylation of Bcl-2 and Bax proteins during hypoxia in newborn piglets. Neurochem. Res. *26*, 1-9.

Baffy,G., Miyashita,T., Williamson,J.R., and Reed,J.C. (1993). Apoptosis induced by withdrawal of interleukin-3 (IL-3) from an IL-3-dependent hematopoietic cell line is associated with repartitioning of intracellular calcium and is blocked by enforced Bcl-2 oncoprotein production. J. Biol. Chem. *268*, 6511-6519.

Baptiste,N. and Prives,C. (2004). p53 in the Cytoplasm; A Question of Overkill? Cell *20;116*, 487-489.

Basu,S., Bayoumy,S., Zhang,Y., Lozano,J., and Kolesnick,R. (1998). BAD enables ceramide to signal apoptosis via Ras and Raf-1. J. Biol. Chem. *273*, 30419-30426.

Bissonnette,R.P., Echeverri,F., Mahboubi,A., and Green,D.R. (1992). Apoptotic cell death induced by c-myc is inhibited by bcl-2. Nature *359*, 552-554.

Boatright,K.M. and Salvesen,G.S. (2003). Mechanisms of caspase activation. Curr. Opin. Cell Biol. *15*, 725-731.

Bossy-Wetzel,E., Newmeyer,D.D., and Green,D.R. (1998). Mitochondrial cytochrome c release in apoptosis occurs upstream of DEVD-specific caspase activation and independently of mitochondrial transmembrane depolarization. EMBO J. *17*, 37-49.

Bouchard,V.J., Rouleau,M., and Poirier,G.G. (2003). PARP-1, a determinant of cell survival in response to DNA damage. Exp. Hematol. *31*, 446-454.

Breckenridge,D.G., Stojanovic,M., Marcellus,R.C., and Shore,G.C. (2003). Caspase cleavage product of BAP31 induces mitochondrial fission through endoplasmic reticulum calcium signals, enhancing cytochrome c release to the cytosol. J. Cell Biol. *160*, 1115-1127.

Bush,A., Mateyak,M., Dugan,K., Obaya,A., Adachi,S., Sedivy,J., and Cole,M. (1998). c-myc null cells misregulate cad and gadd45 but not other proposed c-Myc targets. Genes Dev. *12*, 3797-3802.

Cande,C., Cohen,I., Daugas,E., Ravagnan,L., Larochette,N., Zamzami,N., and Kroemer,G. (2002). Apoptosis-inducing factor (AIF): a novel caspase-independent death effector released from mitochondria. Biochimie *84*, 215-222.

Cardone,M.H., Roy,N., Stennicke,H.R., Salvesen,G.S., Franke,T.F., Stanbridge,E., Frisch,S., and Reed,J.C. (1998). Regulation of cell death protease caspase-9 by phosphorylation. Science *282*, 1318-1321.

Cartron,P.F., Priault,M., Oliver,L., Meflah,K., Manon,S., and Vallette,F.M. (2003). The N-terminal End of Bax Contains a Mitochondrial-targeting Signal. J. Biol. Chem. *278*, 11633-11641.

Cavalieri,F. and Goldfarb,M. (1987). Growth factor-deprived BALB/c 3T3 murine fibroblasts can enter the S phase after induction of c-myc gene expression. Mol. Cell Biol. *7*, 3554-3560.

Chen-Levy,Z. and Cleary,M.L. (1990). Membrane topology of the Bcl-2 proto-oncogenic protein demonstrated in vitro. J. Biol. Chem. *265*, 4929-4933.

Cheng,E.H., Levine,B., Boise,L.H., Thompson,C.B., and Hardwick,J.M. (1996). Bax-independent inhibition of apoptosis by Bcl-XL. Nature *379*, 554-556.

Chinnaiyan,A.M., Chaudhary,D., O'Rourke,K., Koonin,E.V., and Dixit,V.M. (1997). Role of CED-4 in the activation of CED-3. Nature *388*, 728-729.

Chipuk,J.E., Kuwana,T., Bouchier-Hayes,L., Droin,N.M., Newmeyer,D.D., Schuler,M., and Green,D.R. (2004). Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. Science *303*, 1010-1014.

Chittenden,T., Harrington,E.A., O'Connor,R., Flemington,C., Lutz,R.J., Evan,G.I., and Guild,B.C. (1995). Induction of apoptosis by the Bcl-2 homologue Bak. Nature *374*, 733-736.

Chmura,S.J., Nodzenski,E., Beckett,M.A., Kufe,D.W., Quintans,J., and Weichselbaum,R.R. (1997). Loss of ceramide production confers resistance to radiation-induced apoptosis. Cancer Res. *57*, 1270-1275.

Chou,J.J., Li,H., Salvesen,G.S., Yuan,J., and Wagner,G. (1999). Solution structure of BID, an intracellular amplifier of apoptotic signaling. Cell *96*, 615-624.

Cohen,J.J. (1991). Programmed cell death in the immune system. Adv. Immunol. *50:55-85.*, 55-85.

Cory,S., Huang,D.C., and Adams,J.M. (2003). The Bcl-2 family: roles in cell survival and oncogenesis. Oncogene *22*, 8590-8607.

Datta,S.R., Dudek,H., Tao,X., Masters,S., Fu,H., Gotoh,Y., and Greenberg,M.E. (1997). Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell *91*, 231-241.

Davis,A.C., Wims,M., Spotts,G.D., Hann,S.R., and Bradley,A. (1993). A null c-myc mutation causes lethality before 10.5 days of gestation in homozygotes and reduced fertility in heterozygous female mice. Genes Dev. *7*, 671-682.

Degterev,A., Boyce,M., and Yuan,J. (2003). A decade of caspases. Oncogene *22*, 8543-8567.

Denisov,A.Y., Madiraju,M.S., Chen,G., Khadir,A., Beauparlant,P., Attardo,G., Shore,G.C., and Gehring,K. (2003). Solution structure of human BCL-w: modulation of ligand binding by the C-terminal helix. J. Biol. Chem. ..

Desagher,S. and Martinou,J.C. (2000). Mitochondria as the central control point of apoptosis. Trends Cell Biol. *10*, 369-377.

Desagher,S., Osen-Sand,A., Nichols,A., Eskes,R., Montessuit,S., Lauper,S., Maundrell,K., Antonsson,B., and Martinou,J.C. (1999). Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome c release during apoptosis. J. Cell Biol. *144*, 891-901.

Dowd,D.R., MacDonald,P.N., Komm,B.S., Haussler,M.R., and Miesfeld,R.L. (1992). Stable expression of the calbindin-D28K complementary DNA interferes with the apoptotic pathway in lymphocytes. Mol. Endocrinol. *6*, 1843-1848.

Du,C., Fang,M., Li,Y., Li,L., and Wang,X. (2000). Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell *102*, 33-42.

Duelli,D.M. and Lazebnik,Y.A. (2000). Primary cells suppress oncogene-dependent apoptosis. Nat. Cell Biol. *2*, 859-862.

Ellis,H.M. and Horvitz,H.R. (1986). Genetic control of programmed cell death in the nematode C. elegans. Cell *44*, 817-829.

Enari,M., Sakahira,H., Yokoyama,H., Okawa,K., Iwamatsu,A., and Nagata,S. (1998). A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature *391*, 43-50.

Epand,R.F., Martinou,J.C., Montessuit,S., Epand,R.M., and Yip,C.M. (2002). Direct evidence for membrane pore formation by the apoptotic protein Bax. Biochem. Biophys. Res. Commun. *298*, 744-749.

Eskes,R., Desagher,S., Antonsson,B., and Martinou,J.C. (2000). Bid induces the oligomerization and insertion of Bax into the outer mitochondrial membrane. Mol. Cell Biol. *20*, 929-935.

Evan,G.I. and Vousden,K.H. (2001). Proliferation, cell cycle and apoptosis in cancer. Nature *411*, 342-348.

Evan,G.I., Wyllie,A.H., Gilbert,C.S., Littlewood,T.D., Land,H., Brooks,M., Waters,C.M., Penn,L.Z., and Hancock,D.C. (1992). Induction of apoptosis in fibroblasts by c-myc protein. Cell *69*, 119-128.

Fanidi,A., Harrington,E.A., and Evan,G.I. (1992). Cooperative interaction between c-myc and bcl-2 proto-oncogenes. Nature *359*, 554-556.

Ferrari,D., Pinton,P., Szabadkai,G., Chami,M., Campanella,M., Pozzan,T., and Rizzuto,R. (2002). Endoplasmic reticulum, Bcl-2 and Ca(2+) handling in apoptosis. Cell Calcium *32*, 413-420.

Ferri,K.F. and Kroemer,G. (2001). Organelle-specific initiation of cell death pathways. Nat. Cell Biol. *3*, E255-E263.

Finucane,D.M., Waterhouse,N.J., Amarante-Mendes,G.P., Cotter,T.G., and Green,D.R. (1999). Collapse of the inner mitochondrial transmembrane potential is not required for apoptosis of HL60 cells. Exp. Cell Res. *251*, 166-174.

Fischer,U., Janicke,R.U., and Schulze-Osthoff,K. (2003). Many cuts to ruin: a comprehensive update of caspase substrates. Cell Death. Differ. *10*, 76-100.

Foyouzi-Youssefi,R., Arnaudeau,S., Borner,C., Kelley,W.L., Tschopp,J., Lew,D.P., Demaurex,N., and Krause,K.H. (2000). Bcl-2 decreases the free Ca2+ concentration within the endoplasmic reticulum. Proc. Natl. Acad. Sci. U. S. A *97*, 5723-5728.

Fridman,J.S. and Lowe,S.W. (2003). Control of apoptosis by p53. Oncogene *22*, 9030-9040.

Gardai,S.J., Hildeman,D.A., Frankel,S.K., Whitlock,B.B., Frasch,S.C., Borregaard,N., Marrack,P., Bratton,D.L., and Henson,P.M. (2004). Phosphorylation of Bax serine 184 by Akt regulates its activity and apoptosis in neutrophils. J. Biol. Chem. 279: 21085 – 21095.

Goping,I.S., Gross,A., Lavoie,J.N., Nguyen,M., Jemmerson,R., Roth,K., Korsmeyer,S.J., and Shore,G.C. (1998). Regulated targeting of BAX to mitochondria. J. Cell Biol. *143*, 207-215.

Grassilli,E., Ballabeni,A., Maellaro,E., Del Bello,B., and Helin,K. (2004). Loss of Myc confers resistance to doxorubicin-induced apoptosis by preventing the activation of multiple serine protease-and caspase-mediated pathways. J. Biol. Chem. ..

Griffiths,G.J., Dubrez,L., Morgan,C.P., Jones,N.A., Whitehouse,J., Corfe,B.M., Dive,C., and Hickman,J.A. (1999). Cell damage-induced conformational changes of the pro-apoptotic protein Bak in vivo precede the onset of apoptosis. J. Cell Biol. *144*, 903-914.

Grundling,A., Blasi,U., and Young,R. (2000). Biochemical and genetic evidence for three transmembrane domains in the class I holin, lambda S. J. Biol. Chem. *275*, 769-776.

Gundlach,H.G., Moore,S., and Stein,W.H. (1959). The reaction of iodoacetate with methionine. J. Biol. Chem. *234*, 1761-1764.

Guo,B., Zhai,D., Cabezas,E., Welsh,K., Nouraini,S., Satterthwait,A.C., and Reed,J.C. (2003). Humanin peptide suppresses apoptosis by interfering with Bax activation. Nature *423*, 456-461.

Gurd FRN (1967). Carboxymethylation. Methods in Enzymology *11*, 532-541.

Hacki,J., Egger,L., Monney,L., Conus,S., Rosse,T., Fellay,I., and Borner,C. (2000). Apoptotic crosstalk between the endoplasmic reticulum and mitochondria controlled by Bcl-2. Oncogene *19*, 2286-2295.

Hajnoczky,G., Davies,E., and Madesh,M. (2003). Calcium signaling and apoptosis. Biochem. Biophys. Res. Commun. *304*, 445-454.

He,H., Lam,M., McCormick,T.S., and Distelhorst,C.W. (1997). Maintenance of calcium homeostasis in the endoplasmic reticulum by Bcl-2. J. Cell Biol. *138*, 1219-1228.

Hengartner,M.O. and Horvitz,H.R. (1994). C. elegans cell survival gene ced-9 encodes a functional homolog of the mammalian proto-oncogene bcl-2. Cell *76*, 665-676.

Hinds,M.G., Lackmann,M., Skea,G.L., Harrison,P.J., Huang,D.C., and Day,C.L. (2003). The structure of Bcl-w reveals a role for the C-terminal residues in modulating biological activity. EMBO J. *22*, 1497-1507.

Horvitz,H.R. (2003). Worms, Life, and Death (Nobel Lecture). Chembiochem. *4*, 697-711.

Hsu,Y.T. and Youle,R.J. (1997). Nonionic detergents induce dimerization among members of the Bcl-2 family. J. Biol. Chem. *272*, 13829-13834.

Irmler,M., Thome,M., Hahne,M., Schneider,P., Hofmann,K., Steiner,V., Bodmer,J.L., Schroter,M., Burns,K., Mattmann,C., Rimoldi,D., French,L.E., and Tschopp,J. (1997). Inhibition of death receptor signals by cellular FLIP. Nature *388*, 190-195.

Jacobson,M.D., Weil,M., and Raff,M.C. (1997). Programmed cell death in animal development. Cell *88*, 347-354.

Janiak,F., Leber,B., and Andrews,D.W. (1994). Assembly of Bcl-2 into microsomal and outer mitochondrial membranes. J. Biol. Chem. *269*, 9842-9849.

Juin,P., Hunt,A., Littlewood,T., Griffiths,B., Swigart,L.B., Korsmeyer,S., and Evan,G. (2002). c-Myc functionally cooperates with Bax to induce apoptosis. Mol. Cell Biol. *22*, 6158-6169.

Jurgensmeier,J.M., Xie,Z., Deveraux,Q., Ellerby,L., Bredesen,D., and Reed,J.C. (1998). Bax directly induces release of cytochrome c from isolated mitochondria. Proc. Natl. Acad. Sci. U. S. A *95*, 4997-5002.

Kane,L.P., Shapiro,V.S., Stokoe,D., and Weiss,A. (1999). Induction of NF-kappaB by the Akt/PKB kinase. Curr. Biol. *9*, 601-604.

Kim,P.K., Janiak-Spens,F., Trimble,W.S., Leber,B., and Andrews,D.W. (1997). Evidence for multiple mechanisms for membrane binding and integration via carboxyl-terminal insertion sequences. Biochemistry *36* , 8873-8882.

Kim, PK, Annis MG, Dlugosz PJ, Leber B, Andrews DW (2004) During Apoptosis Bcl-2 Changes Membrane Topology at Both the Endoplasmic Reticulum and Mitochondria. Mol. Cell *In Press*

Kluck,R.M., Esposti,M.D., Perkins,G., Renken,C., Kuwana,T., Bossy-Wetzel,E., Goldberg,M., Allen,T., Barber,M.J., Green,D.R., and Newmeyer,D.D. (1999). The pro-apoptotic proteins, Bid and Bax, cause a limited permeabilization of the mitochondrial outer membrane that is enhanced by cytosol. J. Cell Biol. *147*, 809-822.

Kolesnick,R. and Fuks,Z. (2003). Radiation and ceramide-induced apoptosis. Oncogene *22*, 5897-5906.

Korsmeyer,S.J., Shutter,J.R., Veis,D.J., Merry,D.E., and Oltvai,Z.N. (1993). Bcl-2/Bax: a rheostat that regulates an anti-oxidant pathway and cell death. Semin. Cancer Biol. *4*, 327-332.

Krajewski,S., Tanaka,S., Takayama,S., Schibler,M.J., Fenton,W., and Reed,J.C. (1993). Investigation of the subcellular distribution of the bcl-2 oncoprotein: residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes. Cancer Res. *53*, 4701-4714.

Krishnasastry,M., Walker,B., Braha,O., and Bayley,H. (1994). Surface labeling of key residues during assembly of the transmembrane pore formed by staphylococcal alpha-hemolysin. FEBS Lett. *356*, 66-71.

Kroemer,G. (1999). Mitochondrial control of apoptosis: an overview. Biochem. Soc. Symp. *66*, 1-15.

Kuo,T.H., Kim,H.R., Zhu,L., Yu,Y., Lin,H.M., and Tsang,W. (1998). Modulation of endoplasmic reticulum calcium pump by Bcl-2. Oncogene *17*, 1903-1910.

Kutay,U., Hartmann,E., and Rapoport,T.A. (1993). A class of membrane proteins with a C-terminal anchor. Trends in Cell Biology *3*, 72-75.

Kuwana,T., Mackey,M.R., Perkins,G., Ellisman,M.H., Latterich,M., Schneiter,R., Green,D.R., and Newmeyer,D.D. (2002). Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. Cell *111*, 331-342.

Kuwana,T. and Newmeyer,D.D. (2003). Bcl-2-family proteins and the role of mitochondria in apoptosis. Curr. Opin. Cell Biol. *15*, 691-699.

Laurie,S.A., Logan,D., Markman,B.R., Mackay,J.A., and Evans,W.K. (2004). Practice guideline for the role of combination chemotherapy in the initial management of limited-stage small-cell lung cancer. Lung Cancer *43*, 223-240.

Lee,S.T., Hoeflich,K.P., Wasfy,G.W., Woodgett,J.R., Leber,B., Andrews,D.W., Hedley,D.W., and Penn,L.Z. (1999). Bcl-2 targeted to the endoplasmic reticulum can inhibit apoptosis induced by Myc but not etoposide in Rat-1 fibroblasts. Oncogene *18*, 3520-3528.

Li,H., Zhu,H., Xu,C.J., and Yuan,J. (1998). Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell *94*, 491-501.

Li,K., Li,Y., Shelton,J.M., Richardson,J.A., Spencer,E., Chen,Z.J., Wang,X., and Williams,R.S. (2000). Cytochrome c deficiency causes embryonic lethality and attenuates stress-induced apoptosis. Cell *101*, 389-399.

Li,P., Nijhawan,D., Budihardjo,I., Srinivasula,S.M., Ahmad,M., Alnemri,E.S., and Wang,X. (1997). Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell *91*, 479-489.

Liston,P., Fong,W.G., and Korneluk,R.G. (2003). The inhibitors of apoptosis: there is more to life than Bcl2. Oncogene *22*, 8568-8580.

Littlewood,T.D., Hancock,D.C., Danielian,P.S., Parker,M.G., and Evan,G.I. (1995). A modified oestrogen receptor ligand-binding domain as an improved switch for the regulation of heterologous proteins. Nucleic Acids Res. *23*, 1686-1690.

Liu,F.T., Newland,A.C., and Jia,L. (2003). Bax conformational change is a crucial step for PUMA-mediated apoptosis in human leukemia. Biochem. Biophys. Res. Commun. *310*, 956-962.

Liu,X., Kim,C.N., Yang,J., Jemmerson,R., and Wang,X. (1996). Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell *86*, 147-157.

Liu,X., Zou,H., Slaughter,C., and Wang,X. (1997). DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. Cell *89*, 175-184.

Lowe,S.W., Ruley,H.E., Jacks,T., and Housman,D.E. (1993). p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell *74*, 957-967.

Marin,M.C., Fernandez,A., Bick,R.J., Brisbay,S., Buja,L.M., Snuggs,M., McConkey,D.J., von Eschenbach,A.C., Keating,M.J., and McDonnell,T.J. (1996). Apoptosis suppression by bcl-2 is correlated with the regulation of nuclear and cytosolic Ca2+. Oncogene *12*, 2259-2266.

Martinou,J.C. and Green,D.R. (2001). Breaking the mitochondrial barrier. Nat. Rev. Mol. Cell Biol. *2*, 63-67.

Marzo,I., Brenner,C., Zamzami,N., Susin,S.A., Beutner,G., Brdiczka,D., Remy,R., Xie,Z.H., Reed,J.C., and Kroemer,G. (1998). The permeability transition pore complex: a target for apoptosis regulation by caspases and bcl-2-related proteins. J. Exp. Med. *187*, 1261-1271.

Mateyak,M.K., Obaya,A.J., Adachi,S., and Sedivy,J.M. (1997). Phenotypes of c-Myc-deficient rat fibroblasts isolated by targeted homologous recombination. Cell Growth Differ. *8*, 1039-1048.

Messam,C.A. and Pittman,R.N. (1998). Asynchrony and commitment to die during apoptosis. Exp. Cell Res. *238*, 389-398.

Mihara,M., Erster,S., Zaika,A., Petrenko,O., Chittenden,T., Pancoska,P., and Moll,U.M. (2003). p53 has a direct apoptogenic role at the mitochondria. Mol. Cell *11*, 577-590.

Mikhailov,V., Mikhailova,M., Pulkrabek,D.J., Dong,Z., Venkatachalam,M.A., and Saikumar,P. (2001). Bcl-2 prevents Bax oligomerization in the mitochondrial outer membrane. J. Biol. Chem. *276*, 18361-18374.

Minn,A.J., Kettlun,C.S., Liang,H., Kelekar,A., Vander Heiden,M.G., Chang,B.S., Fesik,S.W., Fill,M., and Thompson,C.B. (1999). Bcl-xL regulates apoptosis by heterodimerization-dependent and -independent mechanisms. EMBO J. *18*, 632-643.

Minn,A.J., Velez,P., Schendel,S.L., Liang,H., Muchmore,S.W., Fesik,S.W., Fill,M., and Thompson,C.B. (1997). Bcl-x(L) forms an ion channel in synthetic lipid membranes. Nature *385*, 353-357.

Miyashita,T., Krajewski,S., Krajewska,M., Wang,H.G., Lin,H.K., Liebermann,D.A., Hoffman,B., and Reed,J.C. (1994). Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. Oncogene *9*, 1799-1805.

Muchmore,S.W., Sattler,M., Liang,H., Meadows,R.P., Harlan,J.E., Yoon,H.S., Nettesheim,D., Chang,B.S., Thompson,C.B., Wong,S.L., Ng,S.L., and Fesik,S.W. (1996). X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death. Nature *381*, 335-341.

Murphy,K.M., Ranganathan,V., Farnsworth,M.L., Kavallaris,M., and Lock,R.B. (2000). Bcl-2 inhibits Bax translocation from cytosol to mitochondria during drug-induced apoptosis of human tumor cells. Cell Death. Differ. *7*, 102-111.

Nakagawa,T. and Yuan,J. (2000). Cross-talk between two cysteine protease families. Activation of caspase-12 by calpain in apoptosis. J. Cell Biol. *150*, 887-894.

Nakano,K. and Vousden,K.H. (2001). PUMA, a novel proapoptotic gene, is induced by p53. Mol. Cell *7*, 683-694.

Nechushtan,A., Smith,C.L., Hsu,Y.T., and Youle,R.J. (1999). Conformation of the Bax C-terminus regulates subcellular location and cell death. EMBO J. *18*, 2330-2341.

Nechushtan,A., Smith,C.L., Lamensdorf,I., Yoon,S.H., and Youle,R.J. (2001) Bax and Bak coalesce into novel mitochondria-associated clusters during apoptosis. JCB 153, 1265-76

Newmeyer,D.D. and Ferguson-Miller,S. (2003). Mitochondria. Releasing power for life and unleashing the machineries of death. Cell *112*, 481-490.

Nouraini,S., Six,E., Matsuyama,S., Krajewski,S., and Reed,J.C. (2000). The putative pore-forming domain of Bax regulates mitochondrial localization and interaction with Bcl-X(L). Mol. Cell Biol. *20*, 1604-1615.

Nutt,L.K., Chandra,J., Pataer,A., Fang,B., Roth,J.A., Swisher,S.G., O'Neil,R.G., and McConkey,D.J. (2002a). Bax-mediated Ca2+ mobilization promotes cytochrome c release during apoptosis. J. Biol. Chem. *277*, 20301-20308.

Nutt,L.K., Pataer,A., Pahler,J., Fang,B., Roth,J., McConkey,D.J., and Swisher,S.G. (2002b). Bax and Bak promote apoptosis by modulating endoplasmic reticular and mitochondrial Ca2+ stores. J. Biol. Chem. *277*, 9219-9225.

O'Brien,K.A., Muscarella,D.E., and Bloom,S.E. Differential induction of apoptosis and MAP kinase signaling by mitochondrial toxicants in drug-sensitive compared to drug-resistant B- lineage lymphoid cell lines.

Oda,E., Ohki,R., Murasawa,H., Nemoto,J., Shibue,T., Yamashita,T., Tokino,T., Taniguchi,T., and Tanaka,N. (2000). Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. Science *288*, 1053-1058.

Oltvai,Z.N., Milliman,C.L., and Korsmeyer,S.J. (1993). Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell *74*, 609-619.

Pan,G., Ni,J., Wei,Y.F., Yu,G., Gentz,R., and Dixit,V.M. (1997). An antagonist decoy receptor and a death domain-containing receptor for TRAIL. Science *277*, 815-818.

Petit,P.X., Goubern,M., Diolez,P., Susin,S.A., Zamzami,N., and Kroemer,G. (1998). Disruption of the outer mitochondrial membrane as a result of large amplitude swelling: the impact of irreversible permeability transition. FEBS Lett. *426*, 111-116.

Petros,A.M., Medek,A., Nettesheim,D.G., Kim,D.H., Yoon,H.S., Swift,K., Matayoshi,E.D., Oltersdorf,T., and Fesik,S.W. (2001). Solution structure of the antiapoptotic protein bcl-2. Proc. Natl. Acad. Sci. U. S. A *98*, 3012-3017.

Pinton,P., Ferrari,D., Magalhaes,P., Schulze-Osthoff,K., Di Virgilio,F., Pozzan,T., and Rizzuto,R. (2000). Reduced loading of intracellular Ca(2+) stores and downregulation of capacitative Ca(2+) influx in Bcl-2-overexpressing cells. J. Cell Biol. *148*, 857-862.

Pinton,P., Ferrari,D., Rapizzi,E., Di Virgilio,F.D., Pozzan,T., and Rizzuto,R. (2001). The Ca2+ concentration of the endoplasmic reticulum is a key determinant of ceramide-induced apoptosis: significance for the molecular mechanism of Bcl-2 action. EMBO J. *20*, 2690-2701.

Roucou,X., Montessuit,S., Antonsson,B., and Martinou,J.C. (2002). Bax oligomerization in mitochondrial membranes requires tBid (caspase-8-cleaved Bid) and a mitochondrial protein. Biochem. J. *368*, 915-921.

Roy,N., Deveraux,Q.L., Takahashi,R., Salvesen,G.S., and Reed,J.C. (1997). The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. EMBO J. *16*, 6914-6925.

Rudner,J., Lepple-Wienhues,A., Budach,W., Berschauer,J., Friedrich,B., Wesselborg,S., Schulze-Osthoff,K., and Belka,C. (2001). Wild-type, mitochondrial and ER-restricted Bcl-2 inhibit DNA damage-induced apoptosis but do not affect death receptor-induced apoptosis. J. Cell Sci. *114*, 4161-4172.

Sakahira,H., Enari,M., and Nagata,S. (1998). Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. Nature *391*, 96-99.

Sawada,M., Sun,W., Hayes,P., Leskov,K., Boothman,D.A., and Matsuyama,S. (2003). Ku70 suppresses the apoptotic translocation of Bax to mitochondria. Nat. Cell Biol. *5*, 320-329.

Scaffidi,C., Fulda,S., Srinivasan,A., Friesen,C., Li,F., Tomaselli,K.J., Debatin,K.M., Krammer,P.H., and Peter,M.E. (1998). Two CD95 (APO-1/Fas) signaling pathways. EMBO J. *17*, 1675-1687.

Schendel,S.L., Xie,Z., Montal,M.O., Matsuyama,S., Montal,M., and Reed,J.C. (1997). Channel formation by antiapoptotic protein Bcl-2. Proc. Natl. Acad. Sci. U. S. A *94*, 5113-5118.

Scorrano,L., Oakes,S.A., Opferman,J.T., Cheng,E.H., Sorcinelli,M.D., Pozzan,T., and Korsmeyer,S.J. (2003). BAX and BAK Regulation of Endoplasmic Reticulum Ca2+: A Control Point for Apoptosis. Science *300*,135-9.

Scorrano,L., Petronilli,V., Colonna,R., Di Lisa,F., and Bernardi,P. (1999). Chloromethyltetramethylrosamine (Mitotracker Orange) induces the mitochondrial permeability transition and inhibits respiratory complex I. Implications for the mechanism of cytochrome c release. J. Biol. Chem. *274*, 24657-24663.

Shimizu,S., Narita,M., and Tsujimoto,Y. (1999). Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. Nature *399*, 483-487.

Shiozaki,E.N., Chai,J., Rigotti,D.J., Riedl,S.J., Li,P., Srinivasula,S.M., Alnemri,E.S., Fairman,R., and Shi,Y. (2003). Mechanism of XIAP-mediated inhibition of caspase-9. Mol. Cell *11*, 519-527.

Smaili,S.S., Hsu,Y.T., Carvalho,A.C., Rosenstock,T.R., Sharpe,J.C., and Youle,R.J. (2003). Mitochondria, calcium and pro-apoptotic proteins as mediators in cell death signaling. Braz. J. Med. Biol. Res. *36*, 183-190.

Smaili,S.S., Hsu,Y.T., Youle,R.J., and Russell,J.T. (2000). Mitochondria in Ca2+ signaling and apoptosis. J. Bioenerg. Biomembr. *32*, 35-46.

Soltys,B.J. and Gupta,R.S. (1996). Immunoelectron microscopic localization of the 60-kDa heat shock chaperonin protein (Hsp60) in mammalian cells. Exp. Cell Res. *222*, 16-27.

Soucie,E.L., Annis,M.G., Sedivy,J., Filmus,J., Leber,B., Andrews,D.W., and Penn,L.Z. (2001). Myc potentiates apoptosis by stimulating Bax activity at the mitochondria. Mol. Cell Biol. *21*, 4725-4736.

Srivastava,R.K., Sollott,S.J., Khan,L., Hansford,R., Lakatta,E.G., and Longo,D.L. (1999). Bcl-2 and Bcl-X(L) block thapsigargin-induced nitric oxide generation, c-Jun NH(2)-terminal kinase activity, and apoptosis. Mol. Cell Biol. *19*, 5659-5674.

St Clair,E.G., Anderson,S.J., and Oltvai,Z.N. (1997). Bcl-2 counters apoptosis by Bax heterodimerization-dependent and -independent mechanisms in the T-cell lineage. J. Biol. Chem. *272*, 29347-29355.

Stehlik,C., de Martin,R., Kumabashiri,I., Schmid,J.A., Binder,B.R., and Lipp,J. (1998). Nuclear factor (NF)-kappaB-regulated X-chromosome-linked iap gene expression protects endothelial cells from tumor necrosis factor alpha-induced apoptosis. J. Exp. Med. *188*, 211-216.

Susin,S.A., Daugas,E., Ravagnan,L., Samejima,K., Zamzami,N., Loeffler,M., Costantini,P., Ferri,K.F., Irinopoulou,T., Prevost,M.C., Brothers,G., Mak,T.W., Penninger,J., Earnshaw,W.C., and Kroemer,G. (2000). Two distinct pathways leading to nuclear apoptosis. J. Exp. Med. *192*, 571-580.

Susin,S.A., Lorenzo,H.K., Zamzami,N., Marzo,I., Snow,B.E., Brothers,G.M., Mangion,J., Jacotot,E., Costantini,P., Loeffler,M., Larochette,N., Goodlett,D.R., Aebersold,R., Siderovski,D.P., Penninger,J.M., and Kroemer,G. (1999). Molecular characterization of mitochondrial apoptosis-inducing factor. Nature *397*, 441-446.

Susin,S.A., Zamzami,N., Castedo,M., Hirsch,T., Marchetti,P., Macho,A., Daugas,E., Geuskens,M., and Kroemer,G. (1996). Bcl-2 inhibits the mitochondrial release of an apoptogenic protease. J. Exp. Med. *184*, 1331-1341.

Suzuki,M., Youle,R.J., and Tjandra,N. (2000). Structure of Bax: coregulation of dimer formation and intracellular localization. Cell *103*, 645-654.

Tewari,M., Quan,L.T., O'Rourke,K., Desnoyers,S., Zeng,Z., Beidler,D.R., Poirier,G.G., Salvesen,G.S., and Dixit,V.M. (1995). Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. Cell *81*, 801-809.

Thomenius,M.J., Wang,N.S., Reineks,E.Z., Wang,Z., and Distelhorst,C.W. (2003). Bcl-2 on the Endoplasmic Reticulum Regulates Bax Activity by Binding to BH3-only Proteins. J. Biol. Chem. *278*, 6243-6250.

Tsujimoto,Y., Finger,L.R., Yunis,J., Nowell,P.C., and Croce,C.M. (1984). Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation. Science *226*, 1097-1099.

Vanden Abeele,F., Skryma,R., Shuba,Y., Van Coppenolle,F., Slomianny,C., Roudbaraki,M., Mauroy,B., Wuytack,F., and Prevarskaya,N. (2002). Bcl-2-dependent modulation of Ca(2+) homeostasis and store-operated channels in prostate cancer cells. Cancer Cell *1*, 169-179.

Vaux,D.L., Cory,S., and Adams,J.M. (1988). Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. Nature *335*, 440-442.

Verhagen,A.M., Ekert,P.G., Pakusch,M., Silke,J., Connolly,L.M., Reid,G.E., Moritz,R.L., Simpson,R.J., and Vaux,D.L. (2000). Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. Cell *102*, 43-53.

Villunger,A., Michalak,E.M., Coultas,L., Mullauer,F., Bock,G., Ausserlechner,M.J., Adams,J.M., and Strasser,A. (2003). p53- and Drug-Induced Apoptotic Responses Mediated by BH3-Only Proteins Puma and Noxa. Science *302*, 1036-1038.

von Ahsen,O., Renken,C., Perkins,G., Kluck,R.M., Bossy-Wetzel,E., and Newmeyer,D.D. (2000). Preservation of mitochondrial structure and function after Bid- or Bax- mediated cytochrome c release.

Wagner,A.J., Kokontis,J.M., and Hay,N. (1994). Myc-mediated apoptosis requires wild-type p53 in a manner independent of cell cycle arrest and the ability of p53 to induce p21waf1/cip1. Genes Dev. *8*, 2817-2830.

Wajant,H. (2002). The Fas signaling pathway: more than a paradigm. Science *296*, 1635-1636.

Wang,B., Nguyen,M., Breckenridge,D.G., Stojanovic,M., Clemons,P.A., Kuppig,S., and Shore,G.C. (2003). Uncleaved BAP31 in association with A4 protein at the endoplasmic reticulum is an inhibitor of Fas-initiated release of cytochrome c from mitochondria. J. Biol. Chem. ..

Wang,K., Gross,A., Waksman,G., and Korsmeyer,S.J. (1998). Mutagenesis of the BH3 domain of BAX identifies residues critical for dimerization and killing. Mol. Cell Biol. *18*, 6083-6089.

Wang,K., Yin,X.M., Chao,D.T., Milliman,C.L., and Korsmeyer,S.J. (1996). BID: a novel BH3 domain-only death agonist. Genes Dev. *10*, 2859-2869.

Wei,M.C., Lindsten,T., Mootha,V.K., Weiler,S., Gross,A., Ashiya,M., Thompson,C.B., and Korsmeyer,S.J. (2000). tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c. Genes Dev. *14*, 2060-2071.

Wei,M.C., Zong,W.X., Cheng,E.H., Lindsten,T., Panoutsakopoulou,V., Ross,A.J., Roth,K.A., MacGregor,G.R., Thompson,C.B., and Korsmeyer,S.J. (2001). Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science *292*, 727-730.

Wolter,K.G., Hsu,Y.T., Smith,C.L., Nechushtan,A., Xi,X.G., and Youle,R.J. (1997). Movement of Bax from the cytosol to mitochondria during apoptosis. J. Cell Biol. *139*, 1281-1292.

Wu,J.C. and Fritz,L.C. (1999). Irreversible caspase inhibitors: tools for studying apoptosis. Methods *17*, 320-328.

Wyllie,A.H., Kerr,J.F., and Currie,A.R. (1980). Cell death: the significance of apoptosis. Int. Rev. Cytol. *68*, 251-306.

Xue,D. and Horvitz,H.R. (1995). Inhibition of the Caenorhabditis elegans cell-death protease CED-3 by a CED-3 cleavage site in baculovirus p35 protein. Nature *377*, 248-251.

Yethon,J.A., Epand,R.F., Leber,B., Epand,R.M., and Andrews,D.W. (2003). Interaction with a membrane surface triggers a reversible conformational change in Bax normally associated with induction of apoptosis. J. Biol. Chem. *278*, 48935-48941.

Yin,X.M., Oltvai,Z.N., and Korsmeyer,S.J. (1994). BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax. Nature *369*, 321-323.

Zamzami,N., Marchetti,P., Castedo,M., Decaudin,D., Macho,A., Hirsch,T., Susin,S.A., Petit,P.X., Mignotte,B., and Kroemer,G. (1995a). Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. J. Exp. Med. *182*, 367-377.

Zamzami,N., Marchetti,P., Castedo,M., Zanin,C., Vayssiere,J.L., Petit,P.X., and Kroemer,G. (1995b). Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death in vivo. J. Exp. Med. *181*, 1661-1672.

Zha,J., Harada,H., Yang,E., Jockel,J., and Korsmeyer,S.J. (1996). Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). Cell *87*, 619-628.

Zha,J., Weiler,S., Oh,K.J., Wei,M.C., and Korsmeyer,S.J. (2000). Posttranslational N-myristoylation of BID as a molecular switch for targeting mitochondria and apoptosis. Science *290*, 1761-1765.

Zhu,L., Ling,S., Yu,X.D., Venkatesh,L.K., Subramanian,T., Chinnadurai,G., and Kuo,T.H. (1999). Modulation of mitochondrial Ca(2+) homeostasis by Bcl-2. J. Biol. Chem. *274*, 33267-33273.

Zhu,W., Cowie,A., Wasfy,G.W., Penn,L.Z., Leber,B., and Andrews,D.W. (1996). Bcl-2 mutants with restricted subcellular location reveal spatially distinct pathways for apoptosis in different cell types. EMBO J. *15*, 4130-4141.

Zong,W.X., Li,C., Hatzivassiliou,G., Lindsten,T., Yu,Q.C., Yuan,J., and Thompson,C.B. (2003). Bax and Bak can localize to the endoplasmic reticulum to initiate apoptosis. J. Cell Biol. *162*, 59-69.