REGULATION OF CYTOCHROME C RELEASE BY BCL-2, BAX, AND tBID

INVESTIGATING THE MOLECULAR MECHANISM OF BCL-2, BAX, AND tBID IN THE REGULATION OF CYTOCHROME C RELEASE FROM MITOCHONDRIA

By

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ABSTRACT

Proteins in the Bcl-2 family are important regulators of apoptosis, but their exact mechanism of action remains unknown. To study the interactions of the anti-apoptotic protein Bcl-2 and the pro-apoptotic proteins Bax and tBid, a cell free assay was developed using mitochondria isolated from cultured cells. Using this assay we were able to show that tBid activates Bax, resulting in the oligomerization of Bax on the membrane. Bcl-2 inhibits Bax oligomerization by sequestering tBid-activated Bax.

Bcl-2 is a transmembrane protein that adopts a tail-anchored topology with the hydrophobic carboxyl-terminus of helix 9 inserted into the membrane in healthy cells. After exposure to apoptotic stimuli, Bcl-2 changes conformation such that cysteine 158 that is located in helix 5 is inserted into the lipid bilayer. The experiments presented here show that exposure of Bcl-2 in isolated heavy membranes to either recombinant tBid or a peptide corresponding to the BH3 region of Bim triggered a similar conformational change. The data from the cell free assay showed that Bcl-2 proteins that have inserted cysteine 158 into the membrane are competent to prevent cytochrome c release *in vitro*. Taken together, these results suggest a model of Bcl-2 function, in which the conformationally modified form of Bcl-2 binds to Bax to prevent it from oligomerizing to form large molecular weight multimers that are competent release pro-apoptotic factors from the inter-membrane space of mitochondria. Thus these studies have provided valuable insight into both the complexity and molecular mechanisms of the Bcl-2 family of proteins in the regulation of apoptosis.

The ability of Bcl-2 expression to inhibit apoptosis has been linked to the expression of the proto-oncogene Myc. In cells, the absence of myc is associated with a marked decrease in the ability to undergo apoptosis. The effects of Myc were studied using the cell free assay with mitochondria isolated from Myc overexpressing and Myc null cells. The results show that mitochondria from Myc null cells are dramatically more resistant to tBid and Bax induced cytochrome c release than mitochondria from Myc overexpressing cells. Cytosol switching experiments confirmed that the defect in cytochrome c release after exposure to apoptotic stimuli in cells lacking myc is inherent to the mitochondria.

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ABBREVIATIONS USED IN THIS THESIS

AIF	Apoptosis inducing factor
Bcl-2ΔTM	Bcl-2 without the transmembrane domain
BH	Bcl-2 homology
CARD	Caspase recruitment domain
Cb5	Cytochrome b5
DISC	Death inducing signal complex
ER	Endoplasmic reticulum
IAP	Inhibitor of Apoptosis Proteins
IASD	4-acetamido-4'-iodoacetly-amino stilbene-2,2'-disulfonic acid
PTC	Permeability transition complex
SDS-PAGE	Sodium dodecyl sulfate – polyacrylamide gel electrophoresis
tBID	Truncated Bid
TM	Transmembrane
VDAC	Voltage dependent anion channel
$\Delta\Psi_m$	Inner mitochondrial membrane potential

Chapter One

General Introduction

Apoptosis, or programmed cell death, is an essential process that plays a critical role in the development and maintenance of eukaryotic organisms. Proper embryonic development, tissue homeostasis and the maturation of the immune system all depend on programmed cell death and a number of diseases are associated with defects in the regulation of apoptosis (reviewed in Desagher, 2000). Several neurodegenerative diseases such as Parkinson's disease, Huntington's disease and muscular degeneration arise when apoptosis occurs at inappropriate times. In contrast, the inability of a cell to undergo apoptosis when required is a contributing factor in allowing host cells to live thereby propagating viral infections, and cancer (Thompson, 1995). A cell undergoing apoptosis exhibits characteristic morphological and biochemical changes including cellular blebbing, membrane rearrangement, cell shrinkage, chromatin condensation, DNA cleavage, cleavage of specific cellular proteins, and the formation of apoptotic bodies (Wyllie, 1980 and reviewed by Jacobson, 1997).

While the events that occur during apoptosis have been extensively studied there is still much to be discovered about how the process is regulated. One group of proteins that regulate apoptosis is the Bcl-2 family of proteins. Bcl-2 was the first member to be identified. In human follicular B cell lymphoma a chromosomal rearrangement places Bcl-2 near the immunoglobin heavy chain enhancer, thereby increasing its expression (Cleary, 1986 and Tsujimoto, 1986). Later investigations revealed that the consequence of this was inhibition of apoptosis (Vaux *et al.,* 1988).

Since that time many other Bcl-2 related proteins have been discovered. The Bcl-2 family can be divided into two groups, those that inhibit apoptosis (anti-apoptotic) and

those that promote apoptosis (pro-apoptotic) (Figure 1). The antagonistic relationship between the family members determines whether a cell will live or die. The prevailing theory for the mechanism by which the Bcl-2 family anti-apoptotic proteins control apoptosis is through sequestration of pro-apoptotic family members by direct binding. Therefore the ratio between the anti-apoptotic and pro-apoptotic proteins determines the susceptibility of a cell to a death signal. Specific cell death signals activate pro-apoptotic proteins, which in turn initiate the disintegration of a cell by disrupting mitochondrial function.

Mitochondria

The Bcl-2 family of proteins regulates apoptosis by modifying processes at the mitochondria. During apoptosis mitochondria lose the electrochemical gradient across the inner membrane and several pro-apoptotic factors from the intermembrane space including cytochrome c (Liu *et al.,* 1996), AIF (Susin *et al.,* 1996) and Smac/Diablo (Du *et al.,* 2000; Verhagen *et al.,* 2000).

The release of cytochrome c activates caspases (cysteine aspartate-specific proteases), the executioners of apoptosis (Liu *et al.,* 1996; Li *et al.,* 1997). Caspases exist as pro-enzymes; after a pro-apoptotic signal, they are proteolytically activated (reviewed in Thornberry *et al.,* 1998). Cytosolic cytochrome c forms a complex with Apaf-1, a protein containing caspase recruitment domain (CARD), and nucleotide-binding domains. The formation of the cytochrome c/Apaf-1 complex triggers the binding of dATP, which causes oligomerization of cytochrome c/Apaf-1 to form a large multimeric

complex termed the apoptosome (Zhu *et al.,* 1999). As the CARD domains of Apaf-1 become exposed in the apoptosome, they recruit multiple procaspase-9 molecules to the complex and facilitate autoactivation. Only the caspase 9 bound to the apoptosome efficiently cleaves and activates downstream executioner caspases (Rodrigues *et al.,* 1999). These executioner caspases target a diverse group of proteins involved in DNA replication and repair, chromatin condensation, cytoskeleton regulation, and adhesion. The consequences of unopposed caspase activation are the irreversible biochemical and morphological changes in the cell that are the hallmark of apoptosis (reviewed in Hengartner, 2000).

Smac/Diablo is also released from the mitochondria into the cytosol during apoptosis. Smac/Diablo (second mitochondrial-derived activator of caspases/ direct IAP binding protein with low pI) promotes apoptosis by preventing IAPs from binding to capsases (Du *et al.,* 2000; Verhagen *et al.,* 2000). IAPs (Inhibitors of Apoptosis Proteins) are a family of intracellular proteins that bind to and inactivate caspases by altering the conformation or sterically preventing substrate binding (Shiozaki *et al.,* 2003).

AIF (apoptosis inducing factor) released from the mitochondria translocates to the nucleus where it causes chromatin condensation and cleavage of DNA into large molecular weight fragments (Susin *et al.,* 1999; Joza *et al.,* 2001). The mechanism by which AIF initiates DNA fragmentation is unknown, but it is independent of caspase activity (Miramar *et al.,* 2001; Yu *et al.,* 2002).

The mechanism by which these large molecules pass through the outer mitochondrial membrane during apoptosis remains controversial. However, it is clear that the Bcl-2 family of proteins regulates this process.

The Bcl-2 family of proteins

The Bcl-2 family of proteins is divided into members with either pro- or anti-apoptotic function. All members of the Bcl-2 family contain at least one of the four motifs called the Bcl-2 Homology (BH) domains (BH 1-4) (reviewed in Adams, 1998). However as these "domains" are not structurally defined they are more appropriately referred to as BH regions. Most anti-apoptotic proteins contain BH1 and BH2 regions, and some proteins like Bcl-2, Bcl-x_L and Bcl-w contain all four regions. The pro-apoptotic proteins can be further sub-divided into two groups: multiregion and BH3 only protein. The multiregion pro-apoptotic proteins such as Bax and Bak contain BH1, BH2 and BH3 regions while BH3 only proteins such as Bid, Bad, and Bim only share the BH3 regions.

Multiregion pro-apoptotic proteins

Bax and Bak

Bax (Bcl-2-associated protein X) and Bak (Bcl-2 antagonistic killer) contain three BH regions and a carboxyl-terminal tail anchor sequence (Figure 1). The importance of these proteins in apoptosis was demonstrated in Bax and Bak double knockout mice. Inactivation of Bax affected apoptosis only slightly and disruption of Bak had no

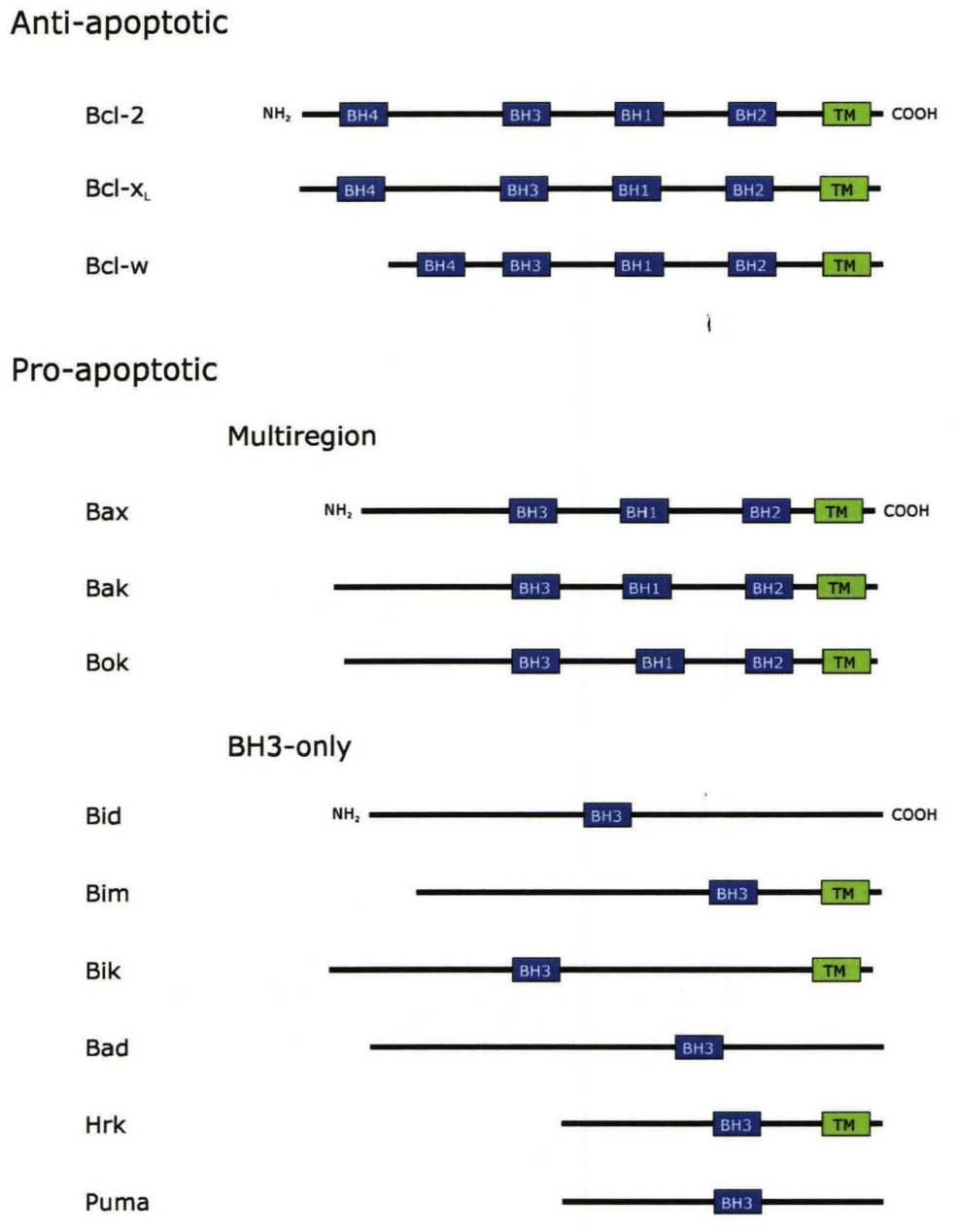

Figure 1 **The Bcl-2 protein family.** A schematic representation of anti-apoptotic and pro-apoptotic members of the Bcl-2 protein family and the location of Bcl-2 homology (BH) regions and predicted transmembrane domains (TM) (Adapted from Gross *et al.*, 1999).

discernible effect. However, loss of both genes dramatically impaired apoptosis in many tissues (Lindsten *et al.,* 2000; Wei *et al.,* 2001).

Bax and Bak function at the mitochondria. However, recent investigations have demonstrated that Bax and Bak also modulate endoplasmic reticulum (ER) calcium levels (reviewed in Annis *et al.,* 2004). Although the functional roles for Bcl-2 family proteins at the ER are emerging, mitochondria are clearly an important target for the activity of Bax, Bak and other Bcl-2 family proteins in controlling cell fate.

Bax and Bak differ in their initial cellular location. Bak is constitutively integrated into the lipid bilayer of membranes including the mitochondria outer membrane (Chittenden, 1995), whereas Bax is cytosolic or loosely bound to membranes (Wolter *et al.,* 1997; Goping *et al.,* 1998). Following an apoptotic stimulus Bax translocates to the mitochondria and becomes an integral membrane protein (Wolter *et al.,* 1997; Goping *et al.,* 1998; Gesagher *et al* 1999) where it oligomerizes and allows the release of pro-apoptotic factors from the intermembrane space (Juergensmeier *et al.,* 1998; Antonsson *et al.,* 2000). The activated form of Bax on the mitochondria can be distinguished from the cytosolic and loosely attached Bax by a conformational change in the amino-terminus that exposes the 6A7 epitope (amino acids 13 to 19) (Nechushtan *et al.,* 1999; Hsu *et al.,* 1998; Desagher *et al.,* 1999). Like Bax, Bak homo-oligomerizes during apoptosis (Wei *et al.,* 2000; Mikhailov *et al.,* 2003) and exposes an amino-terminal epitope (Griffiths *et al.,* 1999).

Although membrane permeabilization by Bax/Bak has been well documented, the molecular mechanism underlying this activity remains controversial. There are two

dominant theories for how Bax/Bak control membrane permeability: by forming pores/channels in the membrane or by interacting with and regulating pre-existing mitochondrial membrane pores.

One proposed hypothesis is that Bax/Bak induce the rupture of the mitochondrial outer-membrane through an interaction with the permeability transition pore complex (PTC). The PTC is a channel that forms in response to certain apoptotic stimuli connecting the inner and outer mitochondrial membranes (Marzo *et al.,* 2000). The formation of the PTC is characterized by massive swelling of the mitochondria, depolarization of the inner mitochondrial membrane and the uncoupling of oxidative phosphorylation (Reviewed in Zamzami *et al.,* 2001). Ultimately this leads to the rupture of the outer mitochondrial membrane and release of pro-apoptotic proteins. Bax may induce the opening of the PTC in cells upon the induction of apoptosis. Consistent with this, inhibitors of the PTC prevent Bax induced release of cytochrome c (Narita *et al.,* 1998).

Evidence also exists suggesting that Bax/Bak can form homo-oligomeric pores. In vitro, detergent exposure induces recombinant Bax to form homo-oligomers which create pores in artificial membranes without any additional proteins (Antonsson *et al.,* 1997). These pores can release encapsulated cytochrome c (Saito *et al.,* 2000; Antonsson *et al.,* 2000). Furthermore, it has been observed that mitochondria can be depleted of cytochrome c yet retain inner mitochondrial membrane potential ($\Delta\Psi_m$) (von Ahsen *et al.,* 2000). This would suggest that the activation of the PTC is a secondary event, at

least in some forms of apoptosis, since the opening of the PTC leads to the rapid loss of $\Delta\Psi_m$ and rupture of the mitochondrial outer-membrane.

BH3 only pro-apoptotic proteins

The BH3 only family of proteins includes proteins such as Bik, Hrk, Bad, Bid and Bim (Figure 1). These proteins share an amino acid similarity only within the short (9-16 amino acids) BH3 region. The amphipathic helix formed by the BH3 doman of pro-apoptotic Bcl-2 family members can bind to a hydrophobic groove formed by the anti-apoptotic Bcl-2 family members (Fesik *et al.,* 2000). Leucine and aspartic acid are conserved in the BH3 regions (Figure 2) and have been identified as the crucial resides in both heterodimer formation and death promotion. However the remaining amino acids vary between proteins and may explain the different interactions with selected multiregionBcl-2 family members that the BH3 region only proteins display (O'Connor *et al.,* 1998; Oda *et al.,* 2000; Wang *et al.,* 1996), and suggests a reason for the apparent redundancy of the BH3 only family. Yeast two hybrid screens, co-immunoprecipitation experiments from cell lysates and in vitro pull down experiments show most BH3 only proteins interact with Bcl-2 and Bcl-x_L (reviewed in Huang and Strasser 2000; Kelekar and Thompson 1998). However only Bid (Wei *et al.,* 2000; Wang *et al.,* 1996) and Bim (Marani *et al.,* 2002; Yamaguchi et al., 2002) interact directly with Bax or Bak. Using short peptides representing the BH3 regions of various BH3 only proteins, it has been suggested that two categories of BH3 only proteins exist (Letai *et al.,* 2002). One

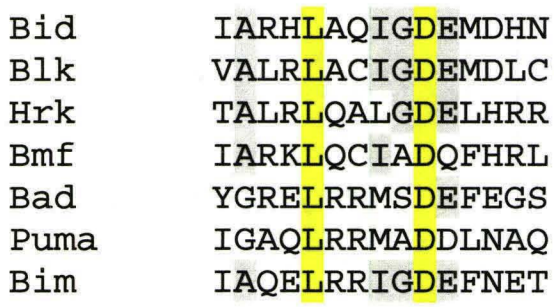

Figure 2 **Alignment of the BH3 regions of BH3 only pro-apoptotic proteins.** An alignment of the amino acid residues in the BH3 regions of mouse BH3 only pro-apoptotic proteins using clustal w algorithm. Identical (yellow) and conserved (gray) amino acids are highlighted.

category (Bim and Bid) directly activate Bax and Bak, monitored by oligomerization. The other category (Bad, Bik and Noxa) do not directly activate Bax and Bak, but rather bind to Bcl-2 and displace Bid or Bim which can then activate Bax or Bak. Consistent with this model, mutations in the second class of BH3 only proteins that prevent binding to anti-apoptotic Bcl-2 family members disrupts the ability to promote cell death (Kelekar *et al.,* 1998).

Bid

Bid (BH3 interacting domain death agonist) is a BH3-only protein that links the Fas receptor with the release of cytochrome c (Figure 3) (Li *et al.,* 1998 and Luo *et al.,* 1998). Fas is a transmembrane protein belonging to the Tumor Necrosis Factor receptor superfamily. Following binding of Fas ligand or agonistic antibody, the Fas receptor trimerizes and activates the apoptotic pathway by recruiting several proteins to form the death-inducing signal complex (DISC). In the DISC, procaspase 8 is processed by proteolysis to its active form (Budihardjo *et al.,* 1999). Activated caspase 8 cleaves the cytosolic form of Bid (p22) between amino acids 59 and 60 (Li *et al.,* 1998) generating a p7 and a p15 fragment. The carboxyl-teminal part of Bid (p15), referred to as truncated Bid (tBid) translocates to the mitochondria and induces cytochrome c release (Wei *et al.,* 2000; Gross *et al.,* 1999; Madesh *et al.,* 2002; Lutter *et al.,* 2001; Grinberg *et al.,* 2002). In vitro, tBid is 100-fold more potent than full length Bid at inducing cytochrome c release (Madesh *et al.,* 2002). However, recent evidence shows that full length Bid is also important in the regulation of apoptosis (Sarig *et al.,* 2003; Valentijn *et al.,* 2004).

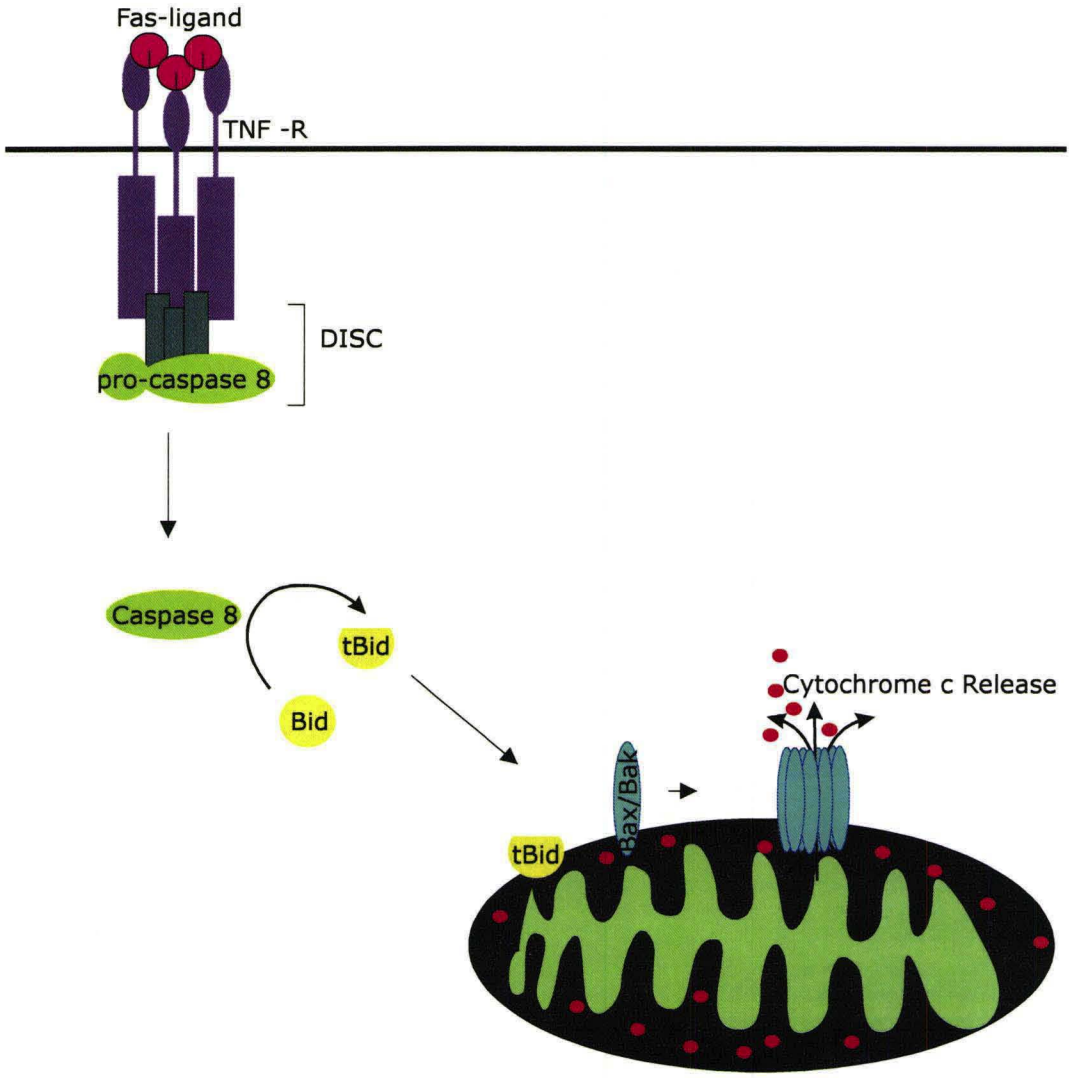

Figure 3 **Model for tBid as an intracellular messenger linking Fas receptor activation to cytochrome c release from the mitochondria.** Ligand binding induces oligomerization of the Fas receptor and the recruitment of several proteins to form the death-inducing signal complex (DISC). In the DISC, procaspase 8 is processed by proteolysis to its active form. Activated caspase 8 cleaves Bid to generate tBid. tBid translocates to the mitochondria and induces Bax/Bak conformational changes and oligomerization leading to cytochrome c release. Cytochrome c released from the mitochondrial intermembrane space into the cytosol activates the downstream caspase cascade.

Many different mechanisms have been postulated to explain tBid mediated cytochrome c release, including ion channel formation (Schendel *et al.,* 1999), destabilization of the lipid bilayer directly by tBid (Kudla *et al.,* 2000), interactions of tBid with multiregion pro-apoptotic proteins Bax and Bak (Wang *et al.,* 1996; Desagher *et al.,* 1999; Eskes *et al.,* 2000; Wei *et al* 2000) or with components of the PTC (Zamzami *et al.,* 2000). Even though the sequence homology between Bid and the Bcl-2 protein family is limited to the BH3 region, the three dimensional structure is similar to that of Bcl-2, Bcl-x_L and Bax, including the two central hydrophobic helices which are similar to the pore-forming domain of diphtheria toxin (Chou *et al.,* 1999; McDonnell *et al.*, 1999). Notably, tBid can form pores (Yang *et al.,* 2003) and ion channels (Schendel *et al.,* 1999) in artificial lipid bilayers. Moreover, tBid becomes an alkali resistant, integral membrane protein following translocation to the mitochondria (Gross *et al.,* 1999; Yi *et al.,* 2003). Thus, in vitro studies suggest that tBid alone may induce cytochrome c release from mitochondria. However, in cells tBid is unable to cause cytochrome c release in the absence of Bax and Bak (Wei *et al.,* 2000; Wei *et al* 2001). Mutants of tBid that do not bind to Bax, Bak and Bcl-2 cannot release cytochrome c (Wang *et al.,* 1996). Moreover, tBid releases cytochrome c before the loss of any $\Delta\Psi_m$ (Mandesh *et al.,* 2002) and tBid mediated cytochrome c release is not blocked by the PTC inhibitor cyclosporin A (Wei *et al.,* 2000), suggesting that tBid requires Bak and Bax to release cytochrome c in a PTC-independent manner.

tBid can activate Bax (Desagher *et al.,* 1999; Eskes *et al.,* 2000; Ruffolo *et al.,* 2000; Roucou *et al.,* 2002; Yi *et al.,* 2003) and Bak (Wei *et al.,* 2000; Brustovetsky *et al.,*

2003, Carton *et al.,* 2003) by inducing Bax translocation, membrane insertion and Bax/Bak oligomerization. These effects likely require direct binding of tBid to Bax/Bak, as the ability of various Bid chimeric and mutant proteins to release cytochrome c depends on the ability to bind to and oligomerize Bax/Bak (Wang *et al.,* 1996; Wei *et al.,* 2000). However tBid does not remain bound to Bak after oligomerization, suggesting a "kiss and run" model in which tBid no longer binds after activation.

As an alternate mechanism, tBid can destabilize lipid membranes *in vitro* by increasing the permeabilization (Kudla *et al.,* 2000) and negative curvature (Epand *et al.,* 2002) of liposomes. Thus tBid binding may destabilize the mitochondrial outer membrane with Bak/Bax activation as an indirect consequence (Lutter *et al.,* 2001). Although the intervening steps are controversial, tBid functions by activating multiregion pro-apoptotic proteins Bax/Bak.

Bim

Bim (Bcl-2 interacting mediator of cell death) is a BH3 only protein that binds to and antagonizes the anti-apoptotic activity of Bcl-2 (O' Connor *et al.,* 1998). All three isoforms (Bim_{EL}, Bim_L and Bim_S) interact with Bcl-2 and promote apoptosis, although they differ in efficacy (O' Connor *et al.,* 1998), with Bim_S being the most potent. In cells Bim is regulated by sequestration to the cytoskeleton. In healthy cells Bim_{EL} and Bim_L are bound to microtubular dynein motor complexes through interactions with the light chain LC8 (Puthalakath *et al.*, 1999). Growth factor deprivation and other apoptotic stimuli release Bim from the dynein motor complex which can then translocate to

mitochondria and induce apoptosis. Bim_S does not bind to the dynein motor complex which may explain why it is more potent than Bim_L and Bim_{EL}.

Bim was originally discovered because of its interaction with Bcl-2 however, it can also activate Bax/Bak directly. Bim_S and the novel isoform Bim_{AD} bind to Bax leading to a conformational change in Bax associated with activation (Marani et al., 2002). Therefore Bim, like Bid, can interact with both Bcl-2 and Bax/Bak.

Anti-apoptotic proteins

The anti-apoptotic Bcl-2 family of proteins are hypothesized to prevent cell death via heterodimerization with pro-apoptotic family members by an interaction mediated by BH regions (Figure 1). The structure of Bcl-x_L shows that BH1, BH2 and BH3 regions create an elongated hydrophobic cleft to which the amphipathic α-helix of a BH3 region of a pro-apoptotic protein can bind (Salter *et al.,* 1997).

Bcl-2

Bcl-2 (B-cell lymphoma protein-2) was identified as a proto-oncogene in human follicular B cell lymphoma (Clearly *et al.,* 1986; Tsujimoto *et al.,* 1986). 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 ER, nuclear envelope and mitochondria (Krajewski *et al.*, 1993). This broad localization allows Bcl-2 to function at spatially

distinct regions of the cell, thus enhancing its anti-apoptotic activity (Zhu *et al.,* 1996; Annis *et al.*, 2001; Besik *et al.,* 2004).

Bcl-2 at the ER has been shown to regulate intercellular calcium homeostasis (Baffy *et al.*, 1992; Lam *et al.*, 1994; Magnelli *et al.,* 1994). However the effects of Bcl-2 on the concentration of ER luminal calcium are controversial and the literature contains conflicting data. In some cell lines Bcl-2 over-expression increases or preserves ER calcium (Zhong et al., 1993; Lam et al., 1994; Distelhorst et al., 1996; He et al., 1997), whereas in other cases Bcl-2 decreases ER calcium stores (Pinton *et al.*, 2000; Foyouzi-Youssefi *et al.*, 2000). In the latter circumstance, it is hypothesized that Bcl-2 protects cells from apoptosis by limiting calcium release into the cytoplasm decreasing the amount of calcium available for uptake by the mitochondria thereby mitigating PTC formation (reviewed in Samaili *et al.,* 2003). Despite the controversy surrounding the exact nature of calcium regulation, it is clear that changes in the cytoplasmic calcium levels can trigger apoptosis and Bcl-2 prevents this.

Bcl-2 at the mitochondria prevents the release of pro-apoptotic factors and maintains the $\Delta\Psi_m$. These effects are thought to be mediated by heterodimerization with the Bcl-2 family pro-apoptotic proteins. Similar to Bcl-x_L, the three dimensional structure of Bcl-2 shows that BH1, BH2 and BH3 regions are proximal to each other and create an elongated hydrophobic groove on the surface of the protein (Petros *et al.*, 2001). This groove is the interaction site for pro-apoptotic members of the Bcl-2 family (Sattler *et al.*, 1997; Petros *et al.*, 2000; Liu *et al.*, 2003). The consequence of this binding is still unclear: one postulated mechanism is to bind and sequester BH3 only pro-apoptotic

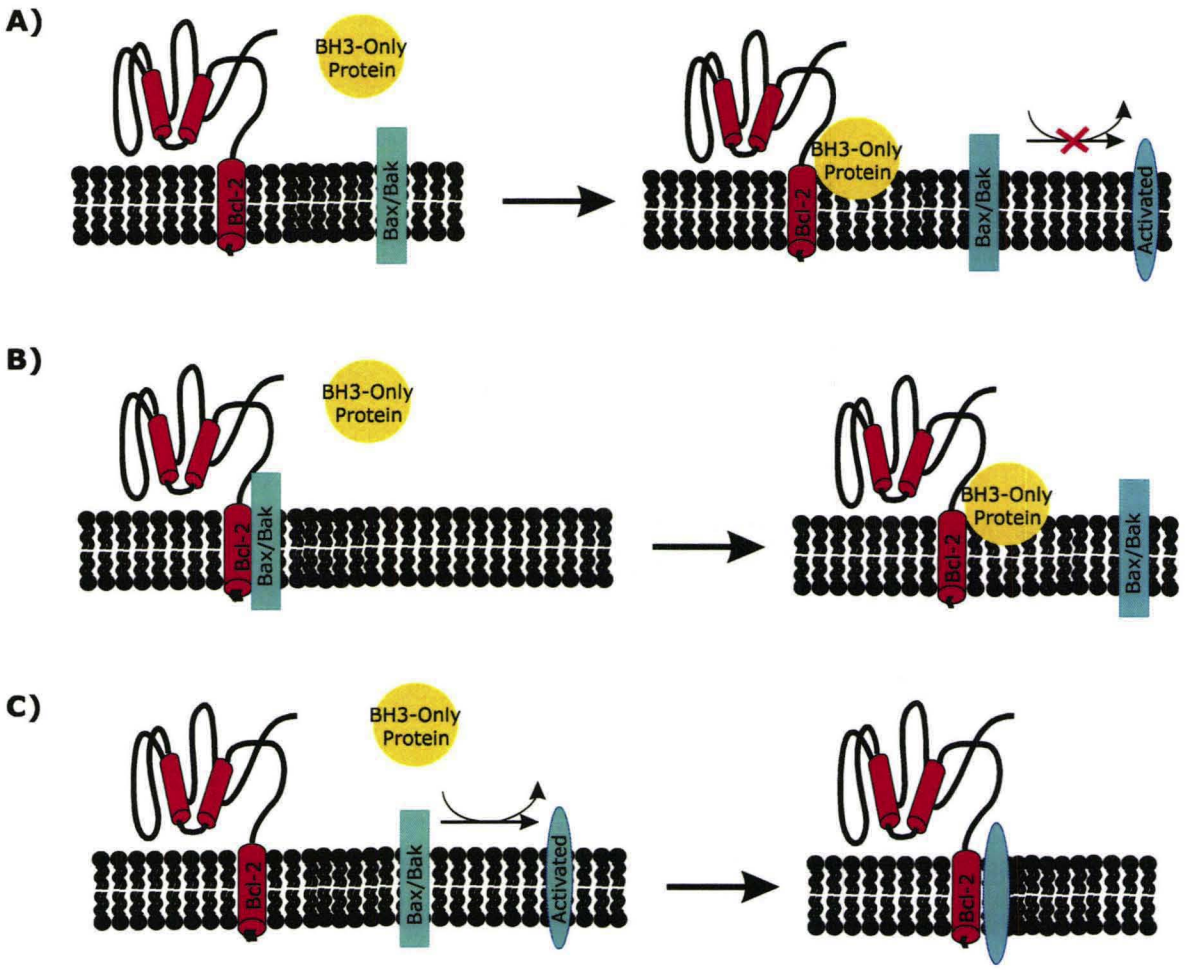

Figure 4 **Proposed mechanisms of action for the anti-apoptotic protein Bcl-2. A)** Bcl-2 binds and sequesters BH3-only pro-apoptotic proteins, preventing them from activating Bax/Bak. **B)** Bcl-2 sequesters Bax/Bak. The binding of BH3-only proteins to Bcl-2 displaces Bax/Bak, liberating them. **C)** Bcl-2 preferentially interacts with activated Bax/Bak preventing pore formation.

proteins, preventing them from activating Bax/Bak (Figure 4A) (Cheng *et al*, 2001; Letai *et al.*, 2002; Yi *et al.*, 2003). Another model suggests that Bcl-2 sequesters Bax/Bak and that the binding of BH3-only proteins to Bcl-2 displaces Bax/Bak, liberating them to initiate apoptosis (Figure 4B) (Zong *et al.*, 2001). A third model proposed is that Bcl-2 preferentially interacts with activated Bak preventing pore formation (Figure 4C) (Perez *et al.,* 2000; Ruffolo *et al.,* 2003).

Investigations presented in this thesis

The Bcl-2 family of proteins plays an important role in the control of apoptosis (reviewed in Antonsson 2004). However due to the complexity and redundancy of the mammalian cell it is extremely difficult to tease out the function of individual proteins within a live cell. Therefore, to study the interactions between Bcl-2, Bax and tBid, a cell free assay is desirable. In chapter three of this thesis, I discuss the development of a cell free assay using isolated heavy membranes from Rat1-MycER^{TAM}. By comparing the release of cytochrome c from mitochondria isolated from Rat1-MycER^{TAM} cells expressing Bcl-2 with mitochondria isolated from cells transfected with an empty vector, we confirmed that Bcl-2 inhibited tBid and Bax induced cytochrome c release in this system. Examining the proteins separately showed that the addition of tBid resulted in the oligomerization of Bax on the membrane and that Bcl-2 inhibited Bax oligomerization. In addition, I demonstrated that Bcl-2 prevents apoptosis by inhibiting the formation of Bax oligomers through the sequesteration of tBid-activated Bax.

Bcl-2 is a transmembrane protein found at both the ER and Mitochondria (Krajewski *et al.*, 1993). Bcl-2 has been shown to undergo a conformational change on the membrane upon the induction of apoptosis (Kim *et al.*, 2004). In healthy cells Bcl-2 adopts a tail-anchored topology with the hydrophobic carboxyl-terminus inserted into the membrane. Upon induction of apoptosis, Bcl-2 changes to a conformation where cysteine 158 (helix 5) is inserted into the lipid bilayer. This change was monitored using a cysteine specific lipid impermeant reagent. In chapter four the conformational change in Bcl-2 is further investigated. In this analysis the addition of purified, recombinant tBid or a peptide corresponding to the BH3 region of Bim to isolated heavy membranes triggered a conformational change. I showed that Bcl-2 proteins that have inserted cysteine 158 into the membrane are competent to prevent cytochrome c release, suggesting that the conformational change is important for Bcl-2 function.

The ability of Bcl-2 expression to inhibit apoptosis has been linked to the expression of the proto-oncogene Myc (Vaux et al., 1988; Fanidi et al., 1992; Soucie et al., 2001). Myc is a regulator of gene transcription that, by itself, does not induce apoptosis; rather Myc sensitizes cells to apoptotic stimuli. Soucie *et al* found that Myc potentiates apoptosis by enhancing signals for Bax activation at the mitochondria. In chapter five of this thesis I examine the difference between mitochondria isolated from Myc over-expressing and Myc null cells using the cell free assay developed in chapter three. The results show that mitochondria from Myc null cells are dramatically more resistant to tBid and Bax induced cytochrome c release than mitochondria from Myc over-expressing cells. Cytosol switching experiments demonstrated that the inhibition of

cytochrome c release is inherent to the mitochondria and not conferred by a factor in the cytosol of either cell line.

Chapter Two

Materials and Methods

2.1 General Materials

The chemical reagents used were obtained from Sigma Chemicals or Gibco-Life Science unless otherwise noted. The BL21-SI strain of *Escherichia coli* used was from Statagene.

2.2 Peptides

Peptides corresponding to the BH3 domain of Bim, a BH3 only pro-apoptotic protein, used to induce a conformational change in Bcl-2 and promote cytochrome c release from mitochondria were purchased from Dalton Chemical Laboratories Inc. The Bim peptide (Ac-MRPEIWIAQELRRIGDEFNA-amide) and the inactive point mutant peptide Bim Mut (Ac-MRPEIWIAQEARRIGDEFNA-amide) were blocked at both ends because they correspond to a Bim internal sequence.

2.3 Antibodies

The monoclonal antibodies used in these experiments were: 5C8 (Ex-alpha Biologicals), a mouse antibody against tBid (1:4,000) and 2D2, a human Bax specific mouse antibody (1:10,000) (Hsu et al, 1997). Donkey anti-mouse conjugated to HRP (1:10,000) was used as a secondary antibody for the above mentioned primary antibodies. To monitor cytochrome c release a purified sheep anti-cytochrome c primary antibody (1:3,000) (Ex Alpha Biologicals) and donkey anti-sheep HRP secondary antibody (1:10,000) were used. Stan, a rabbit polyclonal antibody specific for Bcl-2 (1:10,000) made in Dr. David Andrews Lab (McMaster University) was used with goat anti-rabbit

HRP (1:10,000) secondary antibody to immunoblot for Bcl-2. All secondary antibodies were purchased from Jackson Immuno Research Laboratories Inc. All antibodies were diluted in TBS-T with 1%BSA.

2.4 General Methods

2.4.1 Protein Electrophoresis and Immunoblotting

Protein samples were separated by SDS-PAGE using 10% Tricene gels (Schagger and von Jagow, 1987). The proteins were visualized either by staining with Coomassie brilliant blue dye or immunoblotting. For immunoblotting proteins were electrophoretically transferred to PVDF membrane (Gilman) for the detection of proteins other than cytochrome c where nitrocellulose membrane (Gilman) was used. The proteins were transferred onto the membranes for 1 hour at 50 mA per membrane using a Hoefer semi-dry transfer apparatus (Pharmacia Biotech). The membranes were blocked in Blocking Buffer (10 mM K_2PO_4 pH 7.4, 140 mM NaCl, 0.02% NaN_3 and 5g/L powdered milk) at room temperature for 30 minutes. The blots were incubated overnight at 4°C in primary antibody diluted in TBS-T with 1%BSA (10 mM Tris-HCl pH 7.4, 500 mM NaCl, 0.2%Tween 20 and 1% BSA). The next day the blots were washed three times for a minimum of 5 minutes in TBS-T (10 mM Tris-HCl pH 7.4, 500 mM NaCl and 0.2%Tween 20). The blots were then incubated in horseradish peroxidase (HRP) conjugated secondary antibodies diluted in TBS-T with 1%BSA for 2 hours at room temperature. The blots were then washed three times for a minimum of 5 minutes in

TBS-T and then developed using the Enhanced Chemiluminescence (ECL) method (Perkin Elmer).

2.4.2 Transcription and Translation

Transcription reactions were performed as described in (Gurevich *et al.*, 1991) with SP6 (Fermentas) or T7 (Fermentas) polymerase. Cell-free translation reactions were performed in a rabbit reticulocyte lysate system (Jackson and Hunt, 1983) with S^{35} methionine (NEN Perkin and Elmer) following the method outlined in (Andrews, 1989).

2.4.3 Tissue Cell Culture

Rat1-MycER^{TAM} cells were grown in α-minimal essential medium (αMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, HyClone). HOMyc3 and HO15.19 cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% calf serum (Invitrogen).

2.4.4 Protein Sample Quantification

Protein samples were quantified using the BCA assay (Pierce) or the Bradford assay (Bio-Rad) according to the manufactures protocol. Mitochondrial protein concentration was determined similarly using the Bradford assay. Protein concentrations were assigned by reference to a standard curve constructed using BSA.

2.5 Bid Overexpression and Purification

For overexpression of murine BID, pRSETB-Bid (pMAC1446) (Desagher et al., 1999), was transformed into *E. coli* BL21 SI cells by electroporation using Electroporator 2510 (Eppendorf) according to the manufactures instructions. The cells were grown in LBON medium (10g/L tryptone, 5g/L yeast extract pH 7.0) supplemented with 50 μg/ml ampicillin. Initial 10 ml cultures were grown to saturation from a single colony and used to inoculate 1L of culture. The cells were then grown at 30°C to an OD_{600} of 0.5, at which point the culture was induced by adding NaCl to a final concentration of 0.3 M and the cells were grown for 4 more hours at 30°C. The cells were harvested by centrifugation at 4,400 x g for 10 min and the cell pellet was washed with phosphate buffered saline (PBS). Cells were resuspended in 15 ml of Bid Lysis Buffer (0.01 M $NaPO_4$ pH 8.0, 0.3 M NaCl, 0.03 M imidazole, 1 mM PMSF, 1 mM DTT, 0.1 mg/ml DNase and Complete Protease Inhibitors (Roche). The cell suspension was then lysed by three passes though a French Pressure cell at 20,000psi. Cells that were incompletely lysed and other cellular debris were separated from the lysate by centrifugation at 23,000 x g for 15 minutes. The lysate was then incubated with 1 ml of nickel affinity resin (Qiagen) at 4°C with rotation for 1.5 hours. The resin was then packed in a disposable 10 ml column (BioRad) and washed with Bid Lysis Buffer without PMSF, DTT, DNase, and protease inhibitors. Bid was then eluted with 10 ml of Elution Buffer (10 mM $NaPO_4$ pH 8.0, 300 mM NaCl, 200 mM imidazole, 0.2% CHAPS, 10% glycerol) in 1 ml fractions and stored at -80°C. A typical yield of approximately 2mg of Bid is obtained from a 1L culture.

2.6 Cleavage of Bid with Caspase 8

100 μg of purified recombinant Bid, 500 units of caspase 8 (BIOMOL) and 400 μl of Caspase 8 Assay Buffer (0.05 M Hepes pH 7.4, 0.1 M NaCl, 0.1% CHAPS, 10% glycerol and 0.01 M DTT and 1 mM EDTA) were incubated for 8 to 10 hours at 24°C.

2.7 Purification of tBid

The cleavage reaction described above was incubated with 0.5 ml of nickel affinity resin in 10ml of tBid Wash Buffer (10 mM $NaPO_4$ pH 8.0, 300 mM NaCl) at 4°C with rotation for 2 hours. The resin was then packed in a disposable 10 ml column (BioRad) and washed with tBid Wash Buffer. tBid was then eluted with 3 ml of tBid Elution Buffer (10 mM $NaPO_4$ pH 8.0, 300 mM NaCl, 10 mM imidazole, 1.2% octyl glucoside) collecting 0.5 ml fractions (Zha *et al.,* 2000). Bid elution buffer (5 ml) was used to wash Bid and the p7 fragment off the Ni column.

Before using tBid, the octyl glucoside was removed from the buffer. Fractions containing tBid were pooled together and brought up to 15 ml with (10 mM $NaPO_4$ pH 8.0, 300 mM NaCl) Buffer. The 15 ml were concentrated down to 1 ml using a 10,000 kDa cut off spin concentrator (Millipore). This was repeated once more and tBid was store in (10 mM $NaPO_4$ pH 8.0, 300 mM NaCl) Buffer at -80°C.

2.8 Bax Overexpression and Purification

Bax was purified using an intein fusion system. This system involves the fusion of a target protein to a protein element called intein (protein intron). This precursor fusion protein can be induced to undergo an autocatalytic splicing reaction that cleaves the intein segment from the target protein. A small chitin binding domain (CBD) from *Bacillus circulans* (NEB Impact manual) was added to the intein to allow purification of the fusion protein using a chitin column. The intein initiates the self-cleavage reaction in the presence of thiols, such as DTT, β-mercaptoethanol, and cysteine. The intein undergoes specific self-cleavage, which releases the target protein from the chitin bound intein tag.

The vector that expresses full-length human Bax as a carboxyl- terminal intein fusion, pTXB3-Bax (pMAC1572) (Yethon *et al.,* 2003), was transformed into *E. coli* BL21 SI cells by electroporation, as above. The cells were grown in LBON medium (10g/L tryptone, 5g/L yeast extract pH 7.0) supplemented with 50 μg/ml ampicillin. 10ml cultures were grown from a single colony and used to inoculate 1L of culture. The cells were then grown at 30°C to an OD_{600} of 0.6, at which point the culture was induced using 0.3 M NaCl and grown for 4 more hours at 30°C. The cells were then harvested by centrifugation at 4,400 x g for 10 min. The cell pellet was washed with PBS and centrifuged again at 4,400 x g for 10min. Cells were resuspended in 30 ml of Bax Lysis Buffer (20 mM Tris-HCl pH 8, 500 mM NaCl, 0.5 mM EDTA, 0.05% CHAPS, 1mM PMSF, Complete Protease Inhibitors (Roche) and 0.1mg/ml DNase). The cell suspension was then lysed by three passes though a French Pressure cell at 20,000psi. Incompletely

lysed cells and other cellular debris were separated from the lysate by centrifugation at 23,000 x g for 15 min. The lysate was then incubated with 2.5 ml of chitin resin (New England Biolabs) at 4°C with rotation for 1.5 hours. The resin was then packed in a 20 ml disposable column (BioRad) and washed with 35 ml of Bax Lysis Buffer without DTT and DNAse. The column buffer was exchanged to Bax Column Buffer (10 mM Hepes pH 7.4, 100 mM NaCl, 0.2 mM EDTA, 20% glycerol). To induce intein self cleavage, 5 ml of Bax Column Buffer containing 100 mM β-mercaptoethanol was applied over the column and, with approximately 1 ml of buffer remaining above the resin, the column was capped and kept at 4°C for 36 hours. Bax was eluted with 3 ml of Bax Column Buffer and then applied to a DEAE-Sepharose column (Amersham Biosciences). Bax does not bind this column, and was collected in the flowthrough with residual impurities remaining bound to the column. The eluted Bax was then dialyzed 2 times for 8 hours against 4L of Bax Column Buffer to remove β-mercaptoethanol and stored at -80°C.

2.9 Co-precipitation Assay

200μg of Bid or tBid were coupled to Affigel 10 beads by incubation for 4 hours at 4°C with 100μl of a 50% slurry of Affigel 10 beads (Biorad). Prior to coupling the beads were washed three times in 0.5M Sodium Acetate pH 4.5. After coupling the remaining unbound reactive esters on the beads, were blocked by incubation for another hour at 4°C with 0.1 M ethanolamine. As a negative control beads were incubated with a 5M excess of ethanolamine instead of protein (typically 500μl of 1 M ethanolamine for 100 μl of a 50% slurry of beads). The beads were then washed three times with Co-

Precipitation (CP) Buffer (10 mM Hepes pH 7.5, 150 mM NaCl, 0.2% Triton X-100 or 0.5% CHAPS). 5µl of a 50% slurry of beads was diluted in 400µl of CP buffer and 10µl of *in vitro* synthesized protein (Bax, Bak, Bcl-x_L, or Bcl-2) (see transcription and translation section above) and incubated overnight at 4°C with rotation. The resin was then washed by resuspension and sedimentation three times in CP buffer and twice in CP buffer with no detergent. The final pellet was resuspended in 30 µL of Tricine Loading Buffer (TLB) (4% SDS, 0.1 M Tris-HCl pH8.9, 2 mM EDTA, 0.1% bromophenol blue, 20% glycerol and 0.25 M DTT) and boiled for 10 min at 90°C for SDS-PAGE analysis. Following electrophoresis the gel was dried and exposed to Kodak BioMax autoradiography film.

2.10 Isolation of Mitochondria

Heavy membranes enriched in mitochondria were isolated by a modification of a previously published protocol (Eskes et al., 2000). Cells were harvested using a rubber policeman and pelleted by centrifugation in a clinical centrifuge for 3 min at 4°C. The cell pellets were washed twice in mitochondrial buffer (MB) (210 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM HEPES pH 7.5). The final pellet was resuspended in MB supplemented with Complete Protease Inhibitor Cocktail (Roche) and lysed by nitrogen cavitation at 150 psi for 15 min on ice in a 45 ml Nitrogen Bomb (Parr Instrument) (Annis *et al.*, 2001). The debris was separated from the lysate by centrifugation at 2,000 x g for 4 minutes. Mitochondria and other heavy membranes were pelleted at 13,000 x g (17,000 rpm using a TLA120.2 rotor) for 10 minutes at 4°C in a 1ml pollyallomer

centrifuge tube. The pellet was resuspended in MB-EGTA (MB with 0.5 mM EGTA instead of EDTA, 0 mM sucrose and 150 mM KCl) and the protein concentration was determined using a Bradford assay (Bio-Rad) according to the manufactures protocol. The isolated heavy membranes were used within four hours.

2.11 *In vitro* Assay for Cytochrome c Release

Isolated heavy membranes were diluted to a concentration of 1mg/ml of mitochondrial proteins in using a mixture of MB-EGTA and MCB buffer (210 mM mannitol, 150 mM KCl, 0.5 mM EGTA, 10 mM HEPES pH 7.5, 4 mM $MgCl_2$, 5 mM Na_2PO_4, 5 mM succinate and 5 μM rotenone) to give a final concentration of (210 mM mannitol, 150 mM KCl, 0.5 mM EGTA, 10 mM HEPES pH 7.5, 2 mM $MgCl_2$, 2.5 mM Na_2PO_4, 2.5 mM succinate and 2.5 μM rotenone) and incubated with Bim peptide and/or purified proteins for 1 hour at 30°C. The reactions were then centrifuged at 13,000 x g (17,000 rpm using TLA120.2 rotor) for 10 minutes in 1 ml pollyallomer centrifuge tubes. The supernatants and pellets were analyzed by immunoblotting with sheep anti-cytochrome c antibodies for cytochrome c release. In parallel samples the pellets were resuspended in 35 μl of cell buffer (250 mM sucrose, 20 mM Hepes pH 7.5, 2 mM $MgCl_2$, 1 mM EDTA, 1mM PMSF, 1mM DTT and complete protease inhibitor cocktail (Roche)) and then labeled with IASD as described below.

2.12 Chemical Labeling of Cysteine Residues in Bcl-2

The chemical labeling gel shift assay for cysteine residues in Bcl-2 was adapted by Dr. Peter Kim (Kim *et al.*, 2004) from that described in (Krishnasastry *et al.*, 1994). To monitor the change in topology of Bcl-2 in the membrane, cell lysates and isolated mitochondria (see above) were incubated with IASD (4-acetamido-4'((iodoacetyl)amino)stilbene-2,2'-disulfonic acid)(Molecular probes). To prepare cell lysates, Rat –1 MycER^{TAM} cells were harvested and washed twice in cell buffer. The final pellet was resuspended in cell buffer supplemented with complete protease inhibitor cocktail (Roche) and lysed by nitrogen cavitation (as above). The suspension was centrifuged at 500 x g to remove cell debris and nuclei. Cell lysates (150 μg of proteins) were incubated with peptide and/or purified pro-apoptotic proteins for 30 min at 24°C. The membranes were isolated from the reactions by centrifugation at 131,000 x g (55,000 rpm using a TLA100 rotor) for 45 min. The pellet was resuspended in 35 μl of cell buffer and equal volume of 8M urea buffer (cell buffer pH 9.5 supplemented with 8M urea) was added. 20 μl was removed and kept as an unlabelled negative control, 20 μl was incubated with 16mM IASD for 15 min and 20 μl was incubated with 16 mM IASD and 1% Triton X-100 for 15 min.

After labeling, samples were analyzed by SDS-PAGE on 12 cm 16% acrylamide Laemmli gels. Resolution of the small shift due to labeling required separation for 15 hours at 10 mA constant current. Bcl-2 was visualized by immunoblotting with rabbit anti-Bcl-2 antibodies.

2.13 Alkaline Extractions

Heavy membrane pellets were resuspended in 40µl of MB Buffer (the original volume of the reaction) and 710µl of freshly made extraction buffer (200 mM Na_2CO_3, 10 mM DTT, 2% glycerol) was added. The reaction was incubated on ice for 30 minutes. The alkaline extraction reaction was overlaid onto a 250 µl cushion (200 mM Na_2CO_3, 10 mM DTT, 2% glycerol and 500 mM sucrose) in a 1ml pollyallomer tube and spun at 225,000 x g (80,000 rpm using TLA120.2 rotor) for 30 min at 4°C. The supernatant (750µl) was removed and 450 µl of 50% trichloroacetic acid (TCA) and 13µl of glacial acetic acid were added. Two hundred µl of 50% TCA and 5 µl of glacial acetic acid were added to the pellet and cushion. The reactions were left on ice for 15 minutes. To pellet the precipitated protein, the reactions were centrifuged at full speed in a microfuge for 15 minutes. The supernatant was discarded and the pellet was washed with 500 µl of cold 50:50 ethanol:ether. To repellet the precipitated proteins the reaction were spun for 15 minutes at full speed in a microfuge. The supernatant was then removed and the pellet was dried and resuspended in TLB (20-40µl). The samples were then separated by SDS-PAGE and immunoblotted using the appropriate antibodies.

2.14 Quantification of *In Vitro* Translation Product of Bcl2-cb5

As described in (Legate *et al.*, 2000) 1µl of the products of a coupled transcription translation reaction of Bcl2-cb5 fusion protein in pSPUTK (pMAC652) (Zhu *et al.*, 1996) was added to 50µl of 200 mM NaOH and 2.5 µl of H_2O_2. The mixture was then spotted onto glass microfiber filters (Whatman) and dried. The filters were soaked in 10%

trichloroacetic acid for 10 minutes. Subsequently the filters were washed three times in 5% trichloroacetic acid, once in 95% ethanol and dried. Once dry, the filters were added to scintillation fluid, and the radioactivity was measured in a Beckman Coulter LS 6500 liquid scintillation counter. Radioactive counts were converted to femtomoles of protein by Equations 1 and 2.

Equation 1

$$Ci = \frac{\left(\frac{CPMx\%P}{M}\right)xEF}{2.2x10^{12}}$$

In equation 1 *Ci* is the number of curies per sample, *%P* is the percentage of total labeled protein in the translated sample which corresponds to the protein of interest as measured in Molecular Dynamics Storm 820 phosophoimager, *M* is the number of methionines in the protein, and *EF* is the efficiency factor of the scintillation counter.

Equation 2

$$fmoles\_protein = \left(\frac{Ci}{\frac{f}{1/SA_o - (1-f)/1494}} \right) x10^{12}$$

In equation 2 f is the decay factor and SA_o is the specific activity of the isotope.

2.15 Quantitative Immunoblot Analysis

Known quantities of purified Bax and Bcl-2ΔTM (provided by Dr. Jialing Lin) were used as standards that were used to determine the amount of Bax targeted to the mitochondria in a cytochrome c release assay, and how much Bcl-2 was present of mitochondria of Rat-1 MycER^{TAM} cells over expressing human Bcl-2. Serial dilutions of know amounts of Bax or Bcl-2 were loaded on the same gel as the unknown samples, and then analyzed by immunoblotting. The immunoblots were visualized and captured using the Kodak Image Station system (440CF). Analysis of the band intensity was performed using the associated Image Analysis software. The camera was set for optimal chemiluminescence capture conditions (F-stop: 1.2 zoom 35, no filter, focus: 1.8) and 10 captures of the images were taken for 8 sec. Lanes, bands and background were selected using the Kodak Image Station software. The background readings were subtracted from the band intensity and net band intensities were recorded. A linear regression analysis was performed on the net intensity reading of the standards and only lines of best fit with R^2 values of ≥ 0.95 were accepted to ensure that the reading were within a linear range of the equipment. The protein concentration of the unknown samples was determined using the equation for the line of best fit of the standards.

2.16 Gel Filtration Chromatography to Monitor Oligomerization

The oligomeric state of Bcl-2 and Bax on the mitochondrial membrane was analyzed by gel filtration. Two hundred and fifty μg of mitochondrial protein at a concentration of 1mg/ml was incubated with purified recombinant tBid and/or Bax for 1 hour at 30 °C as described in the cytochrome c release assay. Heavy membranes were pelleted by centrifugation of 13,000 x g for 10 minutes and solubilized in SB buffer (250 mM Sucrose, 20 mM Hepes pH 7.5, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 300 mM NaCl, 2% CHAPS and protease inhibitor cocktail (Roche)) and incubated for 30 min on ice. Samples were centrifuged at 90,000 x g for 20 min to remove any unsolubilized membranes and applied to a Superdex 200 HR 10/30 column (Amersham Biosciences). The column was equilibrated and run in (20 mM Hepes pH 7.5, 300 mM NaCl, 0.2 mM DTT and 2% CHAPS) and 400μl fractions were collected. Proteins in every other fraction were precipitated with trichloroacetic acid and analyzed by immunoblotting.

2.17 Immunoprecipitation

To determine if Bcl-2 and Bax interact, 100μg of mitochondrial protein at a concentration of 1mg/ml was incubated with purified recombinant tBid and/or Bax for 1 hour at 30 °C as described in the cytochrome c release assay. Heavy membranes were pelleted by centrifugation at 13,000 x g for 10 minutes and solubilized in SB buffer for 30 min on ice. Samples were centrifuged at 90,000 x g for 20 min to eliminate any unsolubilized membranes and pre-treated with 5 μl of washed protein G agarose beads

(Amersham Biosciences) for 1 hour to reduce unspecific binding. After pelleting the protein G beads, the remaining supernatant was incubated with 5µl of Sheep anti-Bcl-2 antibodies overnight at 4°C with rotation. The following morning 5µl of new protein G agarose beads were added, followed by a two hour incubation at 4°C with rotation. The beads were then washed three times with SB buffer followed by two washes with SB buffer without CHAPS. Samples were tested for the presence of Bax by immunoblotting with a monoclonal mouse anti-Bax antibody (2D2).

2.18 Liposome Preparation

Lipids were mixed in the appropriate ratio from stocks dissolved in chloroform. Liposomes used were made up of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and cholesterol (7:8:1:4 molar ratio). The chloroform was removed by evaporation under a stream of nitrogen gas, followed by a two hour incubation in a vacuum to ensure complete solvent removal. The lipid films were overlayed with argon gas and stored at -20°C for a maximum of seven days.

Lipid films were rehydrated in retic buffer (50 mM KCl, 2 mM $MgCl_2$, 10 mM Tris pH7.5 and 1 mM DTT) and subjected to 10 freeze-thaw cycles. Large unilamellar vesicles were formed by extrustion through a 100 nm Nucleopore polycarbonate membrane (Whatman). The phospholipid vesicles were used within 24 hours.

2.19 Gel Filtration Assay for Membrane Targeting

Bcl2-cb5 synthesized in a rabbit reticulocyte lysate (50 µl) was incubated with phospholipid vesicles (40 µl) for 1 hour at 30°C. To separate the Bcl2-cb5 protein that targeted to liposomes from the non-targeted protein, the reaction was loaded onto a 2.5ml CL2B-Sepharose (Amersham Biosciences) gel filtration column equilibrated with Column Buffer (50 mM TEA-Ac, 150 mM NaCl and 1 mM EDTA). The CL2B-Sepharose resin was packed in a 3ml syringe (B-D) with nytex cloth attached at the tip and inserted into a Bio-Rad low pressure column fixture to form a seal. Fourteen 0.2ml fractions were collected, with the Bcl2-cb5 protein that targeted to liposomes eluting in fractions 3 and 4.

Chapter Three

Development of a cell free assay to study the functions of Bcl-2, Bax and tBid *in vitro*

INTRODUCTION:

The first insight into the genetic basis of apoptosis was gained from studies using *Caenorhaditis elegans*. Investigations revealed that four genes are central to the regulation of cell fate: *ced-3, ced-4, ced-9 and egl-1*. Ced-9 encodes a cell survival protein (Hengarter *et al.*, 1994) that inhibits cell death by sequestering the adaptor protein Ced-4 (Chinnaiyan *et al.*, 1997) and the cellular protease Ced-3 (Xue *et al.*, 1995). To induce death the pro-apoptosis protein Egl-1 binds to Ced-9 displacing Ced-4. Released Ced-4 undergoes oligomerization and bound Ced-3 is autocatalytically activated. This leads to down stream apoptotic events.

The relevance of this pathway was established in higher organisms as homologues of *C. elegans* apoptotic proteins exist in mammals: Bcl-2 (Ced-9), BH3-only proteins (Egl-1), Caspases (Ced-3) and Apaf-1 (Ced-4). However, the regulation of cell death in mammals is far more complex than that of the nematode. For example, unlike in *C. elegans,* interactions between anti-apoptotic proteins and pro-apoptotic proteins do not directly trigger the activation of caspases. Rather pro-apoptotic proteins promote the disruption of the mitochondrial membrane, releasing cytochrome c, which in turn triggers the activation of caspases through the apoptosome complex. There are also at least 20 Bcl-2 related proteins in mammalian cells. There are 6 anti-apoptotic proteins, 3 multiregion pro-apoptotic proteins and no less then 11 BH3 only pro-apoptotic proteins (review in Kuwana, 2003) and more proteins continue to be discovered.

This chapter focuses on three Bcl-2 family proteins: Bcl-2, tBid and Bax (reviewed in Adams, 1998). Bcl-2, an anti-apoptotic protein, inhibits apoptosis induced

by pro-apoptotic proteins like Bax and tBid. tBid is a BH3 only pro-apoptotic protein that is hypothesized to activate Bax. Bax is a multiregion pro-apoptotic protein that upon activation changes conformation and forms homo-oligomers (reviewed in Scorrano et al, 2003).

Due to the complexity and redundancy of the mammalian cell it is extremely difficult to tease out the function of individual proteins within a live cell. Therefore, to determine the mechanism of action of Bcl-2 in preventing tBid and Bax induced apoptosis, I established a cell free assay using cell fractions from the cells used extensively in the laboratory to study Bcl-2 proteins. Ideally we would have used a system based on purified recombinant proteins. As reported in this chapter I was able to purify Bax and tBid. However, the purification of recombinant full-length Bcl-2 has proven problematic. As an alternate source of Bcl-2 I used mitochondria isolated from cells overexpressing Bcl-2, which has the additional advantage of allowing convenient monitoring of Bcl-2 function by measuring cytochrome c release from the mitochondria. However, if release of cytochrome c is used to measure apoptosis, then the mitochondria must be energized to prevent non-specific release due to loss of ATP generation. In this chapter my adaptation of published assays to isolate energized mitochondria from cultured cells that overexpress Bcl-2 is discussed as well as the results that were obtained when purified recombinant tBid and Bax were added to the mitochondria.

RESULTS AND DISCUSSION

Overexpression and purification of recombinant Bid and tBid

Lysates from *E.coli* BL21 SI cells transformed with pRSETB-Bid showed a prominent band on an SDS-polyacrylamide gel consistent with the predicted molecular mass (21 kDa) of the hexahistadine-tagged Bid protein (Figure 1). Amino-terminal (N-terminal) hexahistadine-tagged Bid was purified using nickel affinity chromatography as described in chapter 2 section 2.5 (Figure 1).

Purification of recombinant tBid

Obtaining purified recombinant tBid proved harder than anticipated. The first two hexahistadine-tagged tBid constructs made (pRSETB tBid (pMAC1634) and pET28a tBid (pMAC1635)) did not express in *E. coli*. It was then decided to try the IMPACT^{TM}-CN System (New England BioLabs). However regardless of the intein vector or cleavage conditions used, the self-cleavage of the intein-tBid fusion was very inefficient (refer to the appendix). Therefore, an alternate technique was used to obtain tBid.

In the cell Bid is cleaved by caspase 8 between amino acids 59 and 60 to generate tBid (Figure 2A) (Li *et al.*, 1998). The availability of a commercial source for hexahistadine-tagged enzymatically active caspase 8 allowed me to generate tBid from full length Bid by enzymatic digestion *in vitro* (Figure 2B) as described in chapter 2 section 2.6. After 0.5 hours the appearance of tBid (15 kDa) and the p7 fragment (the N-terminus of Bid, 7 kDa) was observed. At later time points a decrease in Bid with concomitant increase in tBid and the p7 fragment was evident. After 16 hours the

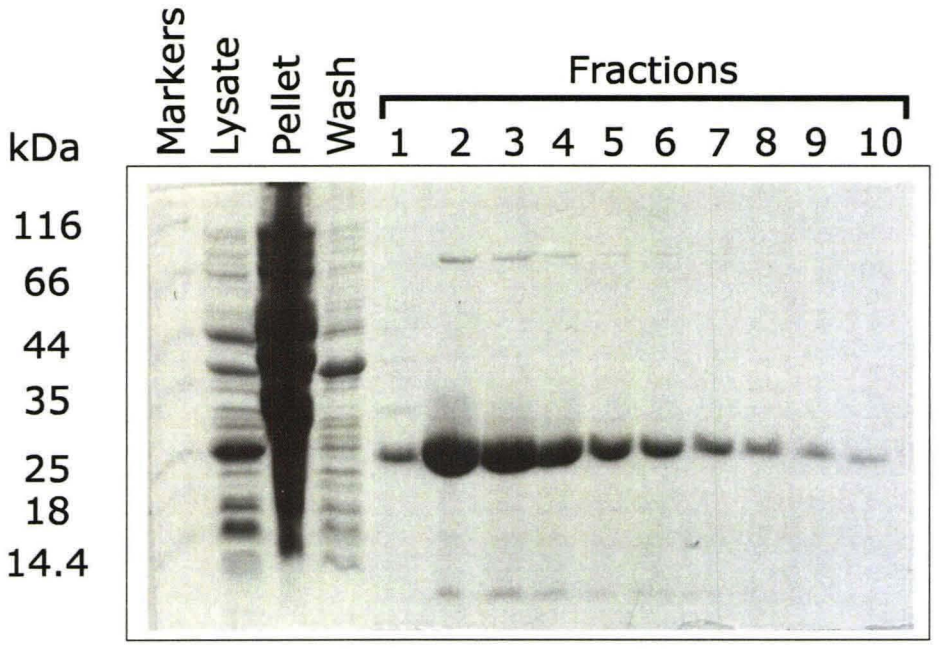

Figure 1 **Purification of Bid.** Protein samples from various stages of the purification of Bid separated by SDS-PAGE and stained by Coomassie Blue. Hexahistidine-tagged Bid expressed in *E.coli* BL21 SI cells (Lysate) was applied to a 1ml nickel affinity resin. Nonspecifically bound proteins were eluted with Bid Lysis Buffer (wash). Bid was eluted with Bid Elution Buffer and collected in ten 1ml fractions (Fractions 1-10).

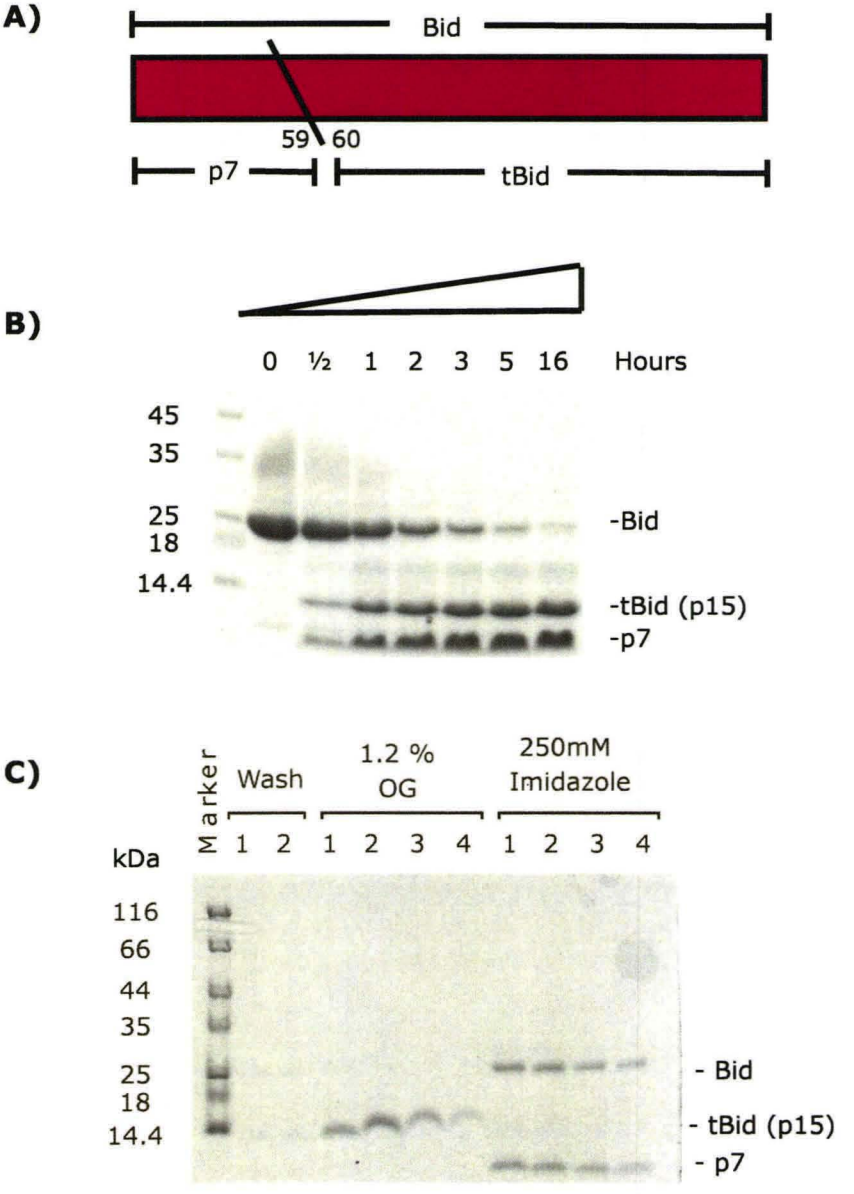

Figure 2 **The cleavage of Bid by caspase 8 and purification of tBid. A)** A schematic diagram of the cleavage of Bid by caspase 8. Two cleavage products are generated: tBid (15kDa) and a 7kDa fragment referred to as p7. **B) C**leavage of Bid monitored by Coomassie Blue staining. 250µg of Bid were incubated with 1,000 units of caspase 8 for the times indicated. **C)** The cleavage reaction was incubated with 0.5mL of nickel affinity resin. The resin was then packed and washed with tBid Wash Buffer (Wash fractions 1, 2). To elute tBid 1.2 % octyl glucoside buffer was used (1.2% OG fractions 1, 2, 3, 4). Bid and p7 were removed from the nickel affinity resin with 250mM imidazole (250mM imidazole fractions 1, 2, 3, 4).

majority of Bid had been converted to tBid and p7 (Figure 2B 16 hour lane). However, it is known that tBid and p7 remain bound together after cleavage *in vitro* and that p7 inhibits the pro-apoptotic activity of tBid. Therefore, the final step in tBid purification was to separate tBid from the components of the cleavage reaction. Bid contained an N-terminal hexahistadine-tag and the cleaved p7 fragment retains this tag. The purchased caspase 8 also contained a carboxyl-terminal hexahistadine-tag, while tBid did not. Thus all the unwanted components bound to a nickel affinity column and tBid flowed through (Figure 2C). However, since tBid and p7 remain in a noncovelent complex after cleavage, 1.2% octyl glucoside was added to the elution buffer to disrupt the complex and elute tBid. The shift in migration of tBid in 1.2% OG lanes 1-4 is most likely due to the increase in detergent concentration. Nearly all the cleaved Bid could be isolated using this protocol (Figure 2C lanes 250mM imidazole). Before using tBid in the mitochondrial release assay, the octyl glucoside was removed by buffer exchange as described in chapter 2 section 2.7.

Overexpression and purification of recombinant Bax

Bax was purified using the IMPACT^{TM}-CN system (chapter 2, section 2.8). Intein self cleavage was induced with 100mM ß-mercaptoethanol and recombinant full-length Bax with no additional amino acid residues was eluted off the chitin column (Figure 3 chitin elution). The eluted protein was applied to a DEAE column and collected from the flow-though, with residual impurities remaining bound to the column (Figure 3 DEAE flow-through). Eluted Bax was dialyzed to remove the ß-

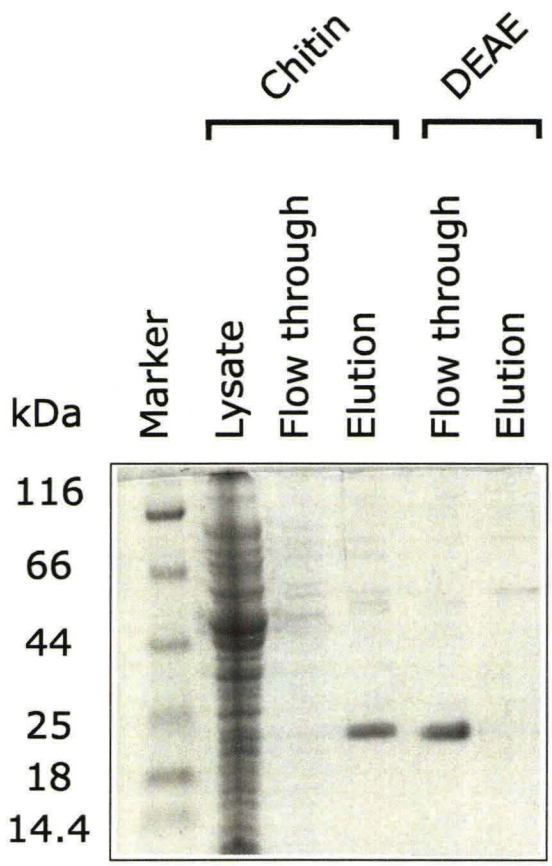

Figure 3 **Purification of Bax.** Proteins samples were analyzed by SDS-PAGE and visualized with Coomassie Blue Stain. Lanes contain a clarified cell lysate of *E. coli* BL21-SI cells expressing Bax as a carboxyl-terminal intein fusion (Lysate), flow through containing proteins that did not bind to the chitin resin (Chitin Flow through), eluted Bax after intein self cleavage was induced with 100mM ß-mercaptoethanol (Chitin Elution) and Bax collected in the flow through of a DEAE column.

mercaptoethanol. Using this protocol 0.5 mg of Bax protein was purified to near homogeneity.

Bid and tBid antibody testing

To determine the interactions between Bcl-2, Bax and tBid a good antibody that could detect small amounts of each protein was required. Bcl-2 and Bax have been studied previously in the lab and therefore high affinity antibodies had already been obtained for these two proteins. However, we have been unable to make a good antibody to tBid. Therefore, purified recombinant Bid protein was sent to ExAlpha Biologicals where monoclonal antibodies were generated.

The determine if any of the monoclonal antibodies generated could be used to immuno-precipitate Bid specifically (Figure 4A), Bid and Bcl-x_L were synthesized in a rabbit reticulocyte lysate in the presence of S^{35} methionine and incubated with the Bid antibodies. Bcl-x_L was added as a negative control to test the specificity of the antibodies for the Bid protein. Bcl-x_L was chosen for this purpose as the protein is a member of the Bcl-2 family that is structurally similar although unrelated in sequence to Bid. All the antibodies tested were specific for Bid including the polyclonal rabbit antibody (rb1) generated previously. The intensity of the Bid band was the highest when immuno-precipitated with the 1H5 antibody (figure 4A lane 1H5).

Bid (9 ng), as well as 9 ng of caspase 8 cleaved Bid and 200 μg of Rat-1MycER^{TAM} whole cell lysate were separated by SDS-PAGE and immunoblotted with the monoclonal antibodies (Figure 4B). One antibody (7D9) detected only full length Bid

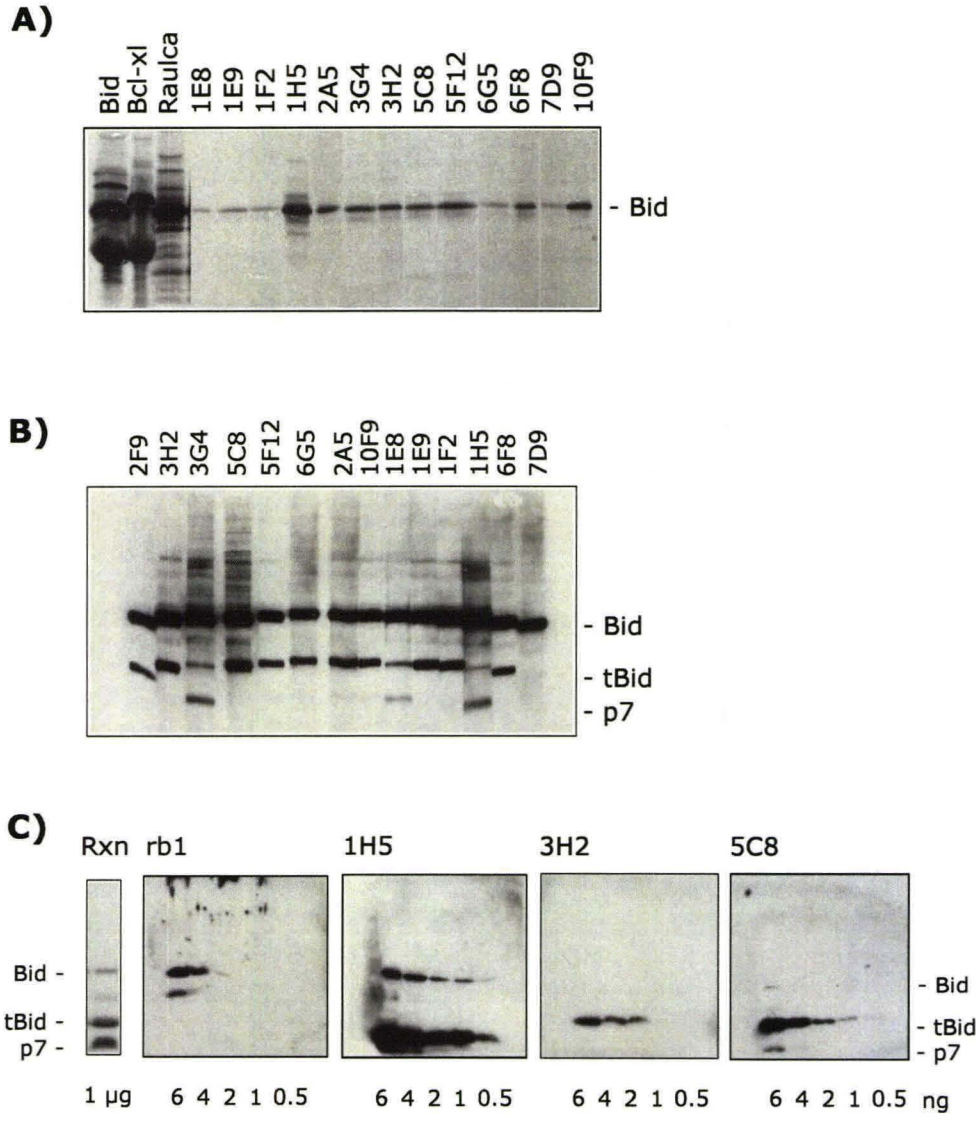

Figure 4 **Bid and tBid antibody testing. A)** An audioradiograph of the results of an immuno-precipitation using the anti-Bid antibodies listed above the gel. Bid and Bcl-x_L were synthesized in a rabbit reticulocyte lysate in the presence of S^{35} methionine and incubated with the antibody listed above the lane to test the specificity of the antibodies for Bid. **B)** 9ng of Bid as well as 9ng of caspase 8 cleaved Bid and 200µg of Rat1-MycER^{TAM} whole cell lysate were separed by SDS-PAGE and immunoblotted with the monoclonal antibody specified above the lane. **C)** Left panel (Rxn): an SDS-PAGE gel visualized by Coomassie Blue staining showing the cleavage of Bid by caspase 8 (5µg of total protein). Following panels: samples of the cleave reaction containing 6, 4, 2, 1 and 0.5 ng of total protein was separated by SDS-PAGE and immunoblotted with rb1, 1H5, 3H2 and 5C8 antibodies.

suggesting that the epitope may be located near the cleavage site. Several antibodies (2F9, 3H2, 5C8, 5F12, 6G5, 2A5, 10F9, 1E9, 1F2, 6F8) detected both Bid and tBid. Finally, 3G4, 1E8 and 1H5 detected Bid and p7 demonstrating that these antibodies recognize one or more epitopes located near the N-terminus of Bid.

Antibodies 1H5, 3H2 and 5C8 were chosen for further testing (Figure 4C). Out of all the antibodies that recognized Bid and tBid the band intensity was the greatest when immunoblotted with 3H2 and 5C8 (Figure 4B lanes 3H2 and 5C8). Antibody 1H5 recognized Bid and p7 (Figure 4B lane 1H5) and was the preferred antibody for Bid immuno-precipitations (Figure 4A). Bid (50ug) was cleaved by caspase 8 (Figure 4C Rxn) and aliquots of the reaction mixture (containing Bid, tBid and p7) corresponding to 6, 4, 2, 1 and 0.5 ng of total protein were separated by SDS-PAGE, electroblotted onto PVDF membranes and immunoblotted with rb1, 1H5, 3H2 and 5C8 antibodies. In this way the specificity and detection sensitivity of each antibody could be compared directly. The polyclonal antibody rb1 detected only Bid. This may be because the rb1 antibody was raised against the full length protein, and after cleavage of Bid the antibody epitopes may no longer be present. The 1H5 antibody detected Bid and the p7 fragment from all the samples including the one corresponding to 0.5 ng of Bid. Thus the lower limit for detection with this antibody is less than 0.5 ng of protein. The antibodies 3H2 and 5C8 both detected tBid and the lower limit of detection for each antibody was less than 2 and 0.5 ng tBid, respectively. Because 5C8 could be used to detect smaller quantities of tBid it was selected for further experiments.

Conformation specific protein interactions

The conformation of some Bcl-2 family members can be altered by specific types of detergents (Hsu, *et al.,* 1998). For example exposure of Bax to Triton X-100 alters the conformation of Bax such that the protein is bound by the conformation sensitive monoclonal antibody 6A7. In this conformation (referred to here as the 6A7 positive conformation) Bax can be immuno-precipitated with the 6A7 antibody. The epitope recognized by 6A7 is located near the N-terminus of Bax, residues 13 to 19 (Hsu, *et al.,* 1998). Non-ionic detergents also induce conformational changes in some members of the Bcl-2 family that lead to the formation of homo- and hetero-dimers (Hsu, *et al.,* 1999). To test if pro-apoptotic proteins Bax and Bak and anti-apoptotic proteins Bcl-x_L and Bcl-2 interact with Bid or tBid and to determine if these interactions are conformation specific, co-precipitation experiments were performed. In these experiments Bid or tBid was covalently attached to Affigel 10 beads and incubated with *in vitro* synthesized in a rabbit reticulocyte lysate system in the presence of S^{35} methionine Bax, Bak, Bcl-x_L or Bcl-2 in CP buffer (chapter 2, section 2.9) with either 0.2% Triton X-100 or 0.5% CHAPS. Proteins that bound to the Bid or tBid beads were eluted by incubation in SDS-PAGE loading buffer and identified by SDS-PAGE and autoradiography.

Both Bak and Bcl-2 are found constitutively on membranes in cells. Both proteins also co-precipitated with Bid and tBid only in 0.5% CHAPS (Figure 5, panels labeled Bak and Bcl-2). Bax and Bcl-x_L are proteins predominantly located in the cytoplasm of unstressed cells and certain apoptotic stimuli trigger migration of these

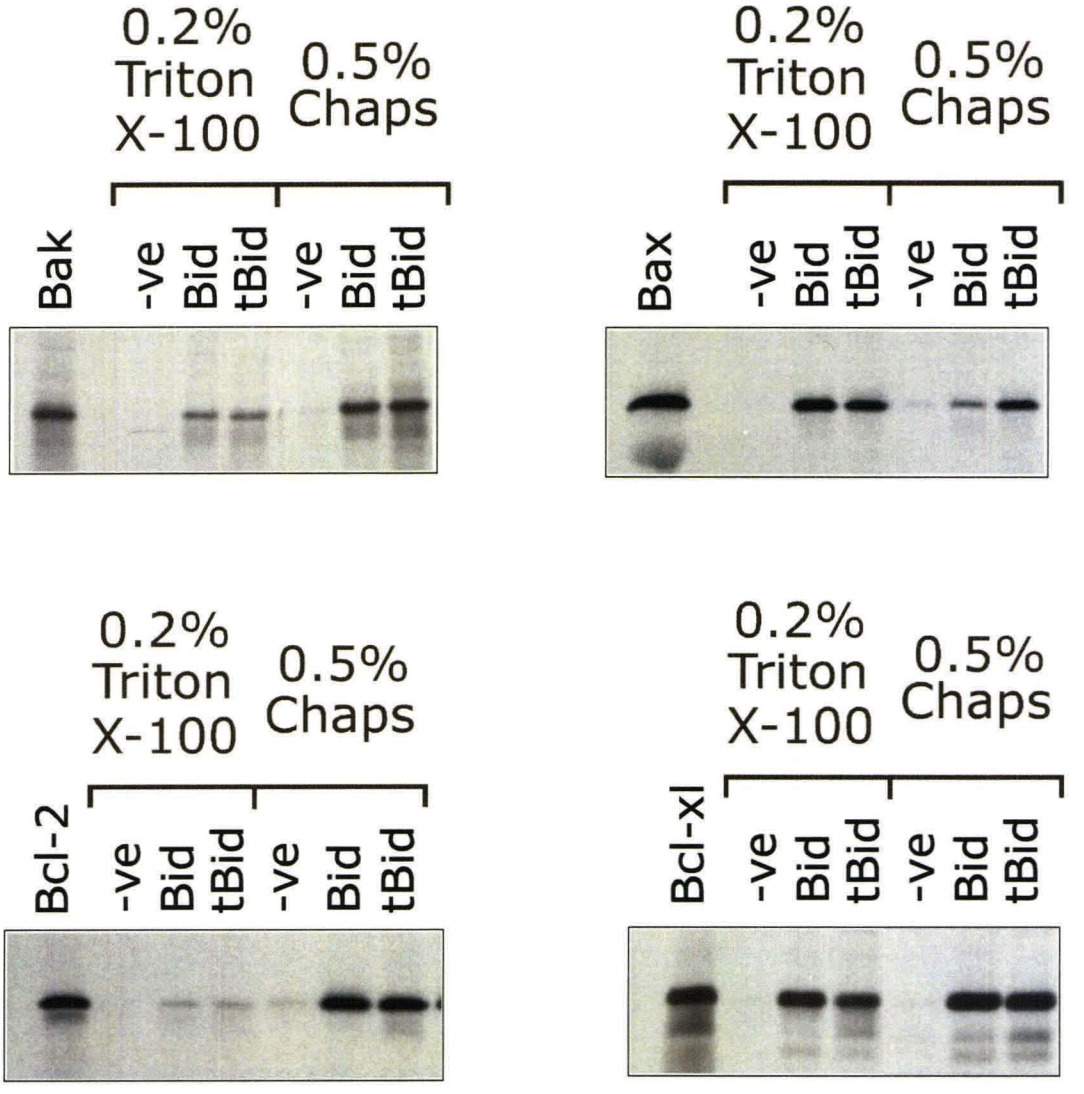

Figure 5 **Identifying the binding partners of Bid through co-precipitation.** Bid or tBid bound to Affigel 10 beads were incubated with ^{35}S labeled Bak, Bax, Bcl-2 and Bcl-x_L synthesized in rabbit reticulocyte lysate in KP buffer containing either 0.2% Triton X-100 or 0.5% CHAPS. Bak, Bax, Bcl-2 and Bcl-x_L synthesized in rabbit reticulocyte lysate were also added to beads that did not have Bid or tBid bound to them (-ve control). Tricine Loading Buffer was added to washed Affigel 10 resin and 10µl of the sample was loaded on the SDS-PAGE gel. Following electrophoresis the gel was dried and exposed to autoradiography film.

proteins to the mitochondria and/or ER. Bax and Bcl-x_L interacted with Bid and tBid in both detergents (Figure 5 Bax and Bcl-x_L).

These results show that all of the proteins tested (Bax, Bak, Bcl-x_L and Bcl-2) can bind to Bid and tBid. However, the binding of Bak and Bcl-2 with Bid/tBid was detergent specific. This indicates that after exposure to Triton X-100 either Bak and Bcl-2, or Bid and tBid, or all four proteins underwent a conformational change that inhibited the heterodimerization of the proteins. Unfortunately, without the aid of conformation specific antibodies it is hard to interpret these results further. Therefore a better system was needed to monitor these interactions and their effect on apoptosis.

Using isolated mitochondria to monitor the interactions between Bcl-2, Bax and tBid

Assay Development

Despite numerous attempts by many different laboratories across the world, no one has been able to purify functional full length Bcl-2. Therefore I developed a cell free assay in which the mitochondria added were isolated from cells transfected with an empty control vector or from cells overexpressing Bcl-2. In this way Bcl-2 function can be examined in a relatively native context. A further benefit of using isolated mitochondria to monitor the interactions between Bcl-2, Bax and tBid is that the extent of apoptosis can be conveniently measured by assaying cytochrome c release from the mitochondria.

Using mitochondria isolated from live animals to study the Bcl-2 family of proteins is a well established assay (Li et al., 1998; Wei et al., 2000; Lutter et al., 2001).

However, these mitochondria must be used within 4 hours of preparation and therefore an animal must be killed for each experiment. Using mitochondria isolated from cultured cells is less common, but Eskes *et al* have successfully used this technique. Unfortunately, the mitochondria used in (Eskes *et al.*, 1999) were isolated from HeLa cells and these mitochondria were shown to have peripherally associated Bax (Desagher *et al.*, 1999). We needed mitochondria that would incorporate expressed exogenous Bcl-2 but were free of Bax so that purified Bax could be added. Rat1-MycER^{TAM} cells were chosen as the source of mitochondria for routine assays. Rat1-MycER^{TAM} cells are Rat-1 fibroblasts with a version of c-myc proto-oncogene which becomes activated after addition of tamoxifen (Littlewood *et al.,* 1995). In these cells the endogenous Bax is located in the cytoplasm and translocates to mitochondria only after an apoptotic stimulus (Annis *et al.*, 2001). Therefore, it is possible to isolate mitochondria from these cells that do not contain detectable amounts of Bax.

In the protocol described by Eskes *et al* cells were broken by shear forces generated by passing the cells six times through a 25 gauge needle using a syringe (Eskes *et al.*, 1999). We wanted to break the cells using nitrogen cavitation since the amount of force is much more reproducible using nitrogen cavitation, as the pressure can be set the same each time. Nitrogen cavitation therefore results in decreased rupture of outer mitochondria membranes as compared to mechanical homogenization (Adachi *et al.*, 1998) and this meant amending the previous protocol. To ensure this modification did not change the efficacy of the protocol, some of the previously published data (Eskes *et al.*, 1999) was repeated. As previously observed (Eskes *et al.*, 1999) the addition of tBid

to isolated heavy membranes enriched with mitochondria from HeLa cells induced cytochrome c release (Figure 6A). Thus the modified protocol was validated in HeLa cells.

Unfortunately, the addition of tBid and Bax to the heavy membranes isolated from Rat1-MycER^{TAM} cells did not induce complete cytochrome c release (Figure 6B). Even when membranes were solubilized by 1% Triton X-100, some of the cytochrome c remained in the pellet fraction (Figure 6B).

Similar results were obtained using isolated rat liver mitochondria (Ott *et al.*, 2002). These authors showed that cytochrome c released from mitochondria involves a two-step process. Their data suggested that there are two distinct pools of cytochrome c in the mitochondria. The first pool is loosely bound to cardiolipin by electrostatic interactions, and consequently is sensitive to alteration in ionic strength. The second pool is partially embedded in the membrane via hydrophobic interactions. Therefore the cytochrome c that was released from the mitochondria of the Rat1-MycER^{TAM} cells was from the second pool that was detached because of disturbances in membrane structure, but the first pool remained bound to the cardiolipin. To perturb the electrostatic interaction between cytochrome c and cardiolipin, heavy membranes were incubated in a buffer containing 150 mM KCl, an ionic composition previously used to detach loosely bound cytochrome c from the inner membrane (Figure 6C) (Cortese *et al.*, 1998). Thus our cell free assay differs from that used by Eskes *et al* in that nitrogen cavitation was used to rupture the outer cell membrane, and 150 mM KCl was added to disrupt the

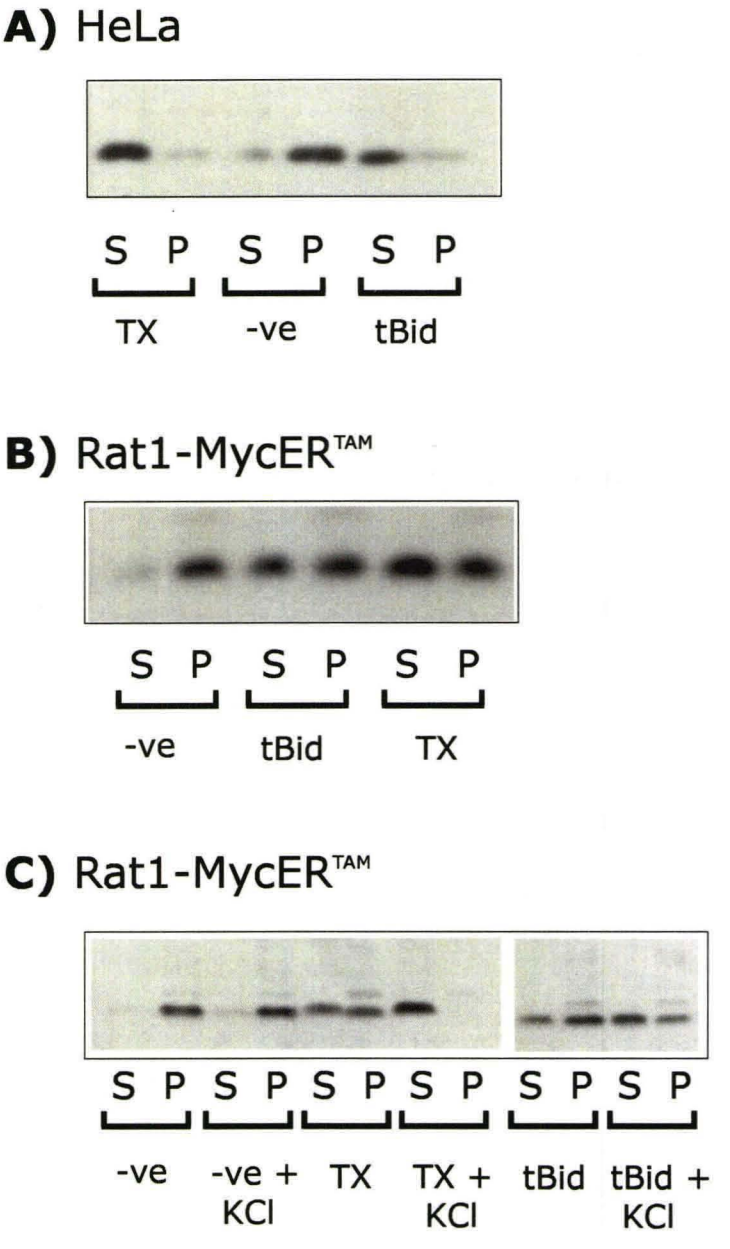

Figure 6 **Development of the assay to isolate heavy membranes.** Heavy membranes isolated from **A)** HeLa and **B)** Rat-1MycER^{TAM} cells (as described in Eskes *et al.*, 1999) except lysed by nitrogen cavitation) were incubated with 1% Triton X-100 (TX), buffer alone (-ve) or 6nM tBid (tBid). The reaction was fractionated into supernatant (S) and pelleted mitochondria (P) by centrifugation and cytochrome c release was monitored by immunoblotting with a sheep anti-cytochrome c antibody. **C)** Heavy membranes isolated from Rat-1MycER^{TAM} cells were incubated in MCB buffer without (-ve) and with 150mM KCl (-ve KCl) with 1% Triton X-100 (TX) and 6nM tBid (tBid).

electrostatic interactions between cytochrome c and cardiolipin in the mitochondrial membrane.

Bcl-2 can inhibit tBid induced cytochrome c release

To determine if Bcl-2 can inhibit tBid induced cytochrome c release, heavy membranes were isolated from Rat1-MycER^{TAM} cells overexpressing Bcl-2 or, as a control, from cells transfected with an empty vector. These heavy membranes were incubated with increasing amounts of purified tBid and Bax and monitored for the release of cytochrome c. Because the regulation of cytochrome c release by Bcl-2 family proteins is concentration dependent the effect of varying the amounts of tBid and Bax was examined quantitatively (Figure 7).

Polster *et al* calculated that in neuronal cells 0.7-1.1 μg of cellular Bax was present per milligram of mitochondrial proteins. However, Bax is up regulated in many acute and chronic neurodegenerative diseases therefore Poster *et al* concluded that the addition of 8 μg of Bax /mg of mitochondrial protein is within the range of concentrations that exist in the cell under pathological conditions (Polster *et al.*, 2003). In my assay, the heavy membranes were used at a concentration of 1mg of mitochondrial protein/ml, the addition of 8 μg of Bax per mg of mitochondrial protein would result in a concentration of 333 nM Bax. Hence, the upper limit of 521 nM Bax used in my experiments with isolated heavy membranes agrees reasonably with the published data.

After the addition of 0.83 nM tBid and 260 nM Bax, over 50% of the cytochrome c was released from the vector control mitochondria (Figure 7). A significant amount of

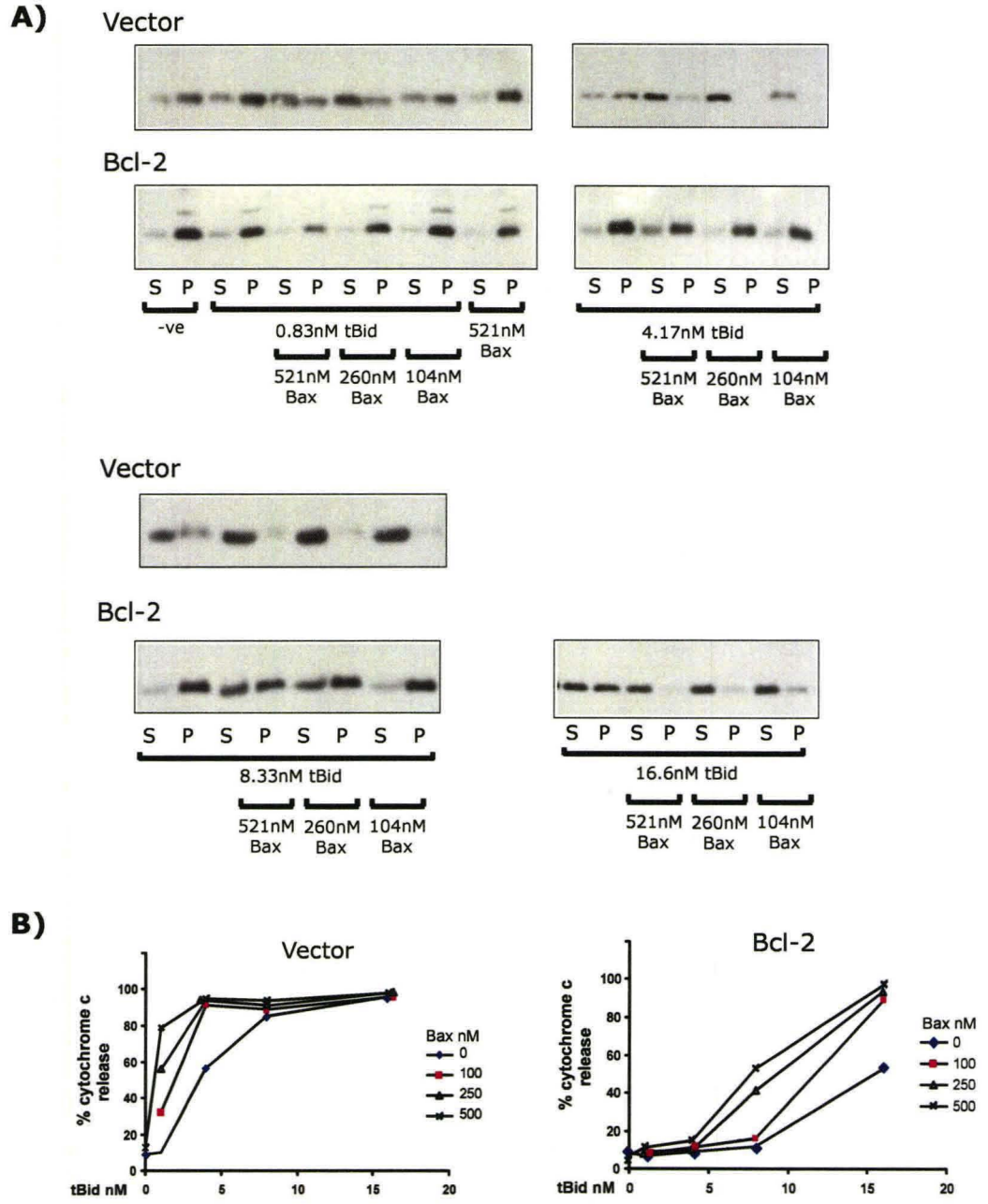

Figure 7 **Bcl-2 inhibits tBid and Bax induced cytochrome c release.** Heavy membranes were isolated from Rat-1MycER^{TAM} cells transfected with an empty expression vector (Vector) or expressing Bcl-2 (Bcl-2) and incubated with different amounts of purified tBid and Bax. The reaction was fractionated into supernatant (S) and pelleted mitochondria (P) by centrifugation. **A)** Cytochrome c release was monitored by immunoblotting with a sheep anti-cytochrome c antibody **B)** and quantified by densitometry (n > 3).

cytochrome c was not released from the mitochondria from Bcl-2 expressing cells until 8.33 nM tBid and 521 nM Bax were added (Figure 7). Therefore, Bcl-2 can inhibit tBid and Bax induced cytochrome c release from the mitochondria.

The mitochondria isolated from Rat1-MycER^{TAM} cells contain endogenous Bak on the membrane. Oligomerization and activation of Bak induced by tBid also releases cytochrome c from mitochondria. Despite early reports suggesting that tBid alone can trigger cytochrome c release from mitochondria (Zhai *et al.,* 2000), experiments with mitochondria from Bax and Bak double knock out cells clearly indicate that mitochondria are resistant to tBid unless Bax or Bak are present (Wei *et al.,* 2001). Consistent with this result, tBid will induce pore formation in membranes only at concentrations of tBid much higher than observed in cells (Zhai *et al.*, 2000). Since Rat1-MycER^{TAM} cells express Bak it should be possible to trigger it to release cytochrome c from mitochondria using only tBid. In my experiments 4.17 nM tBid alone released 57% of the cytochrome c from the vector control mitochondria and after addition of 8.33 nM tBid cytochrome c was detected mainly in the supernatant fraction (Figure 7). In contrast, cytochrome c was not released from mitochondria isolated from cells expressing Bcl-2 until 16.6 nM tBid was added, when approximately 50% of the cytochrome c was located in the supernatant. This shows that Bcl-2 can also inhibit tBid and Bak induced cytochrome c release from mitochondria.

The isolated heavy membranes from Rat1-MycER^{TAM} cells are enriched in mitochondria; however they may also contain ER. Recent investigations have shown that Bax and Bak also affect ER Calcium (Ca^{2+}) levels (reviewed in Annis *et al.*, 2004).

Consequently tBid-activated Bax or Bak could cause the release of the Ca^{2+} from the ER microsomes causing a corresponding increase in uptake of Ca^{2+} into the mitochondria, thereby leading to loss of mitochondrial membrane potential and release of pro-apoptotic factors including cytochrome c. Alternatively, tBid may release Ca^{2+} from the ER directly. However, the buffer used in the cytochrome c release assays contains 0.5 mM EDTA or 1mM EGTA - amounts that should be sufficient to chelate any Ca^{2+} released from the ER, preventing its entry into the mitochondria and inhibiting down stream events. Cytochrome c is only located within the mitochondria and having eliminated the possibility of Ca^{2+} influx, the molecular interactions leading to the release of cytochrome c are most likely to be between Bcl-2 family proteins on the mitochondria membrane.

These studies are consistent with the observation that Bcl-2 can prevent tBid and Bax induced cytochrome c release (Figure 7) but are not sufficient to resolve the mechanism of action of these proteins. For example, my results do not clearly distinguish whether Bcl-2 inhibited tBid induced cytochrome c release by binding tBid to prevent its interaction with Bax, or if Bcl-2 sequestered tBid-activated Bax inhibiting pore formation by Bax or if Bcl-2 functioned through an indirect mechanism.

Quantification of Bcl-2 on the membrane

To further characterize the events leading to cytochrome c release in the cell free assay and to begin to resolve the mechanism of action whereby Bcl-2 inhibits cytochrome c release, it was necessary to determine the amount of Bcl-2 present on the membranes used in these reactions. To date none of the cell free systems for apoptosis have used

known amounts of Bcl-2, Bax and tBid; therefore, the stoichiometry of these interactions has not been determined. The recombinant proteins tBid and Bax purified from *E. coli* lysates can be quantified using a variety of assays. However full length recombinant Bcl-2 cannot be purified as a functional protein. Therefore, to study Bcl-2 we took advantage of the fact that Bcl-2 binds tightly to membranes and isolated mitochondria from cells over expressing Bcl-2.

Quantitative immunoblotting was used to determine the amount of Bcl-2 present on the isolated heavy membranes. Serial dilutions of known quantities of Bcl-2ΔTM were used as standards to measure the amount of Bcl-2 on isolated heavy membranes (Figure 8A). Using the Kodak Image station, net intensity readings for the standards as well as the samples were obtained. The net intensity readings of the standards were used to construct a standard curve (Figure 8B), from which the amounts of Bcl-2 in the unknown samples could be calculated (Table 1). The amount of Bcl-2 on the heavy membranes, determined for 15 individual samples was 20 +/- 6 nmoles/L of reaction when the Bcl-2 enriched heavy membranes were used at a concentration of 1 mg of total membrane protein/ml.

Quantification of Bax on the membrane

Known concentrations of Bax and heavy membranes were added to the cell free reactions. As Bax is active only when it is bound to membranes, we measured this fraction by quantitative immunoblotting. Serial dilutions of known quantities of Bax were used as standards to determine the amount of Bax that had bound to the heavy

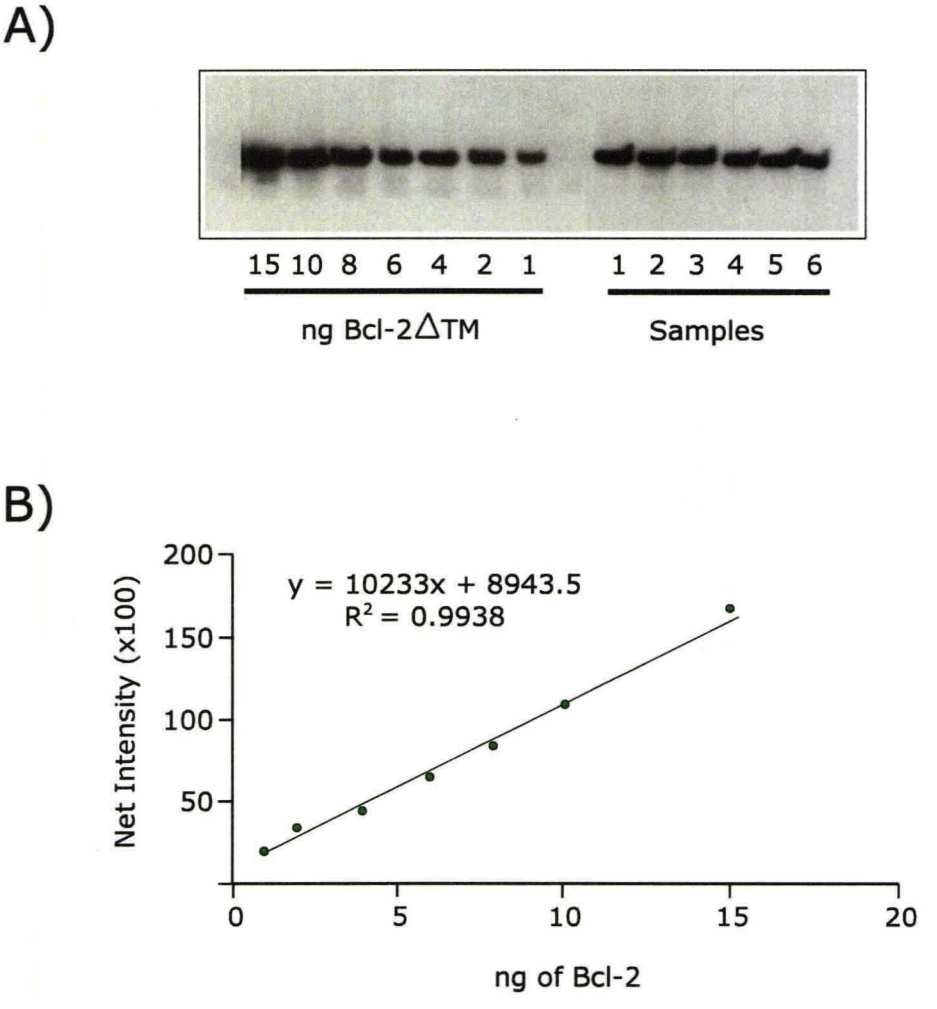

Figure 8 **Quantification of Bcl-2 in heavy membrane fractions**. **A)** Serial dilutions of purified Bcl-2ΔTM (ng Bcl-2ΔTM) were resolved and immunoblotted on the same SDS-PAGE gel as samples containing isolated heavy membranes from Rat-1Myc ER^{TAM} cells expressing Bcl-2 (10μg of total heavy membrane protein). **B)** The immunoblots were visualized and captured using the Kodak Image Station system (440CF). A linear regression analysis was performed on the net intensity reading of the standards and the protein concentration of the unknown samples listed in Table 1 was determined using the equation for the line of best fit of the standards.

Table 1 **Quantification of Bcl-2 on the heavy membranes isolated from Bcl-2 expressing Rat1-MycER^{TAM} cells**

	nmoles/L of the reaction
	28
	26
	22
	18
	14
	20
	23
	27
	9
	9
	22
	30
	19
	23
	17
Avg	**20**
Stdev	6

membranes in a pelleting reaction (Figure 9A and 9B). The amount of Bax bound to the membrane was compared for heavy membranes isolated from vector control and Bcl-2 expressing cells in the absence and presence of 0.83 nM or 8.33 nM tBid (Table 2 and 3, pH 7.5). 0.83 nM tBid was the minimum concentration required to induce cytochrome c release from mitochondria isolated from vector control cells in the presence of added Bax. Incubation of 0.83 nM tBid plus 521 nM Bax with vector control mitochondria caused the release of approximately 80% of cytochrome c, and 0.83 nM tBid plus 260 nM Bax caused 55% of cytochrome c to be released (Figure 7). To induce the release of 50% cytochrome c from mitochondria isolated from Bcl-2 expressing cells, 8.33 nM tBid plus 521 nM Bax was required (Figure 7). Table 4 showed there was virtually no difference between the amount of Bax that bound to heavy membranes from vector control cells compared those from cell expressing Bcl-2. This result demonstrates that Bcl-2 does not inhibit Bax binding to the membrane. Surprisingly, the same amount of Bax was found at the membrane in the presence or absence of tBid (10% of the added Bax).

Although Bax is cytoplasmic in most healthy cells and cell lines, in some cell lines a fraction of the Bax is constitutively bound to mitochondria (Goping *et al.*, 1997; Desagher *et al.*, 1999). In these cell lines Bax is peripherally bound to the membranes as it can be released by alkaline extraction. To determine if tBid caused Bax integration into membranes in our system, we performed alkaline extraction of the membrane pellets, and measured the amount of Bax by quantitative immunoblotting (Table 2 and 3, pH 11.5). To ensure that our conditions extracted peripheral but not integral membrane proteins, we

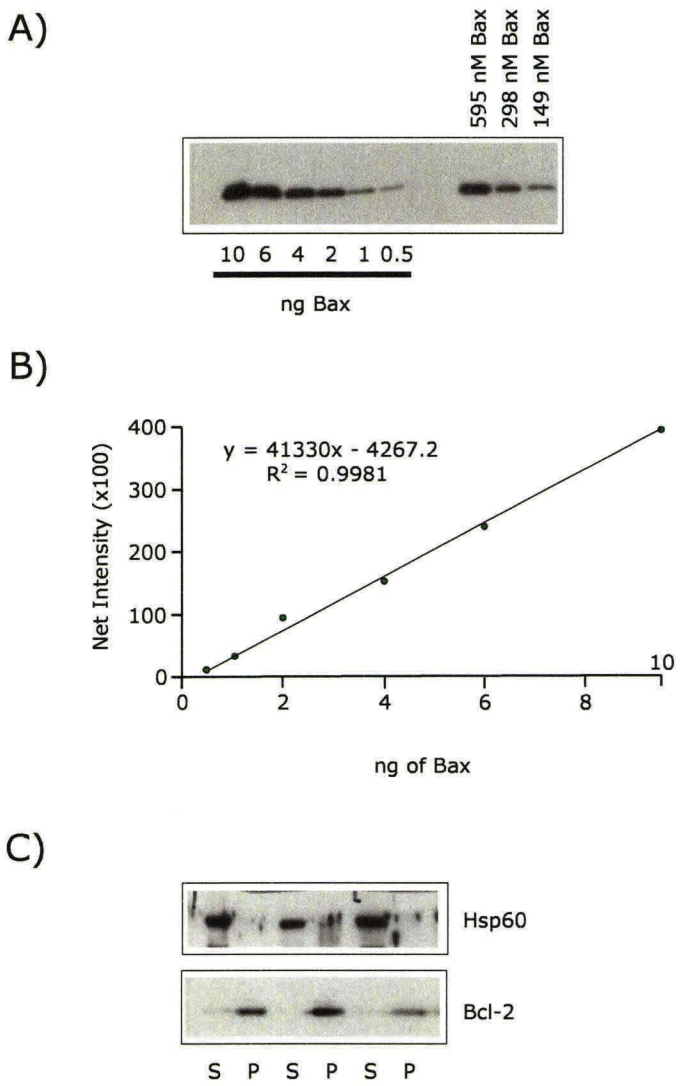

Figure 9 **Quantification of Bax targeted to heavy membranes. A)** Serial dilutions of purified Bax (ng Bax) were resolved and immunoblotted on the same SDS-PAGE gel as samples containing isolated heavy membranes from Rat1-Myc ER^{TAM} cells after incubation with 595nM, 298nM or 149nM Bax (loading 10μg of total heavy membrane protein). **B)** The immunoblots were visualized and captured using the Kodak Image Station system (440CF). A linear regression analysis was performed on the net intensity reading of the standards and the protein concentration of the unknown samples listed in Tables 2 and 3 was determined using the equation for the line of best fit of the standards. **C)** To confirm the efficiency of alkaline extractions used in Table 2 and 3, the supernatant (S) and pellet (P) were separated on an SPS-PAGE gel and immunoblotted with anti-Bcl-2 or anti-Hsp-60 antibodies as indicated to the left of the panels.

Table 2 **Quantification of Bax targeted to the heavy membranes isolated from Bcl-2 overexpressing Rat1-MycER^{TAM} cells**

pH 7.5	nmoles of Bax on membrane/L of reaction					
					Avg	Stdev
521 nM Bax	63	41	75	49	**57**	15
260 nM Bax	27	22		20	**23**	3
104 nM Bax	15	17	16	11	**15**	2
0.83 nM tBid + 521 nM Bax	64	58	48		**56**	8
0.83 nM tBid + 260 nM Bax	33	27	19		**27**	7
0.83 nM tBid + 104 nM Bax	21	21	19		**20**	1
8.33 nM tBid + 521 nM Bax	51	67	57	34	**52**	14
8.33 nM tBid + 260 nM Bax	26	42		31	**33**	8
8.33 nM tBid + 104 nM Bax	18	19	16	15	**17**	2

pH 11.5						
					Avg	Stdev
521 nM Bax	3.8	5.1	4.7		**4.5**	0.7
260 nM Bax	2.5	2.2	2.5		**2.4**	0.2
104 nM Bax	1.0	1.1	1.3		**1.1**	0.1
0.83 nM tBid + 521 nM Bax	7.7	5.9	4.8		**6.1**	1.5
0.83 nM tBid + 260 nM Bax	2.6	3.6	2.5		**2.9**	0.6
0.83 nM tBid + 104 nM Bax	3.3	2.2	1.4		**2.3**	1.0
8.33 nM tBid + 521 nM Bax	19.8	19.2	17.3		**18.7**	1.3
8.33 nM tBid + 260 nM Bax	9.0	6.4	7.7		**7.7**	1.3
8.33 nM tBid + 104 nM Bax	2.0	2.7	2.0		**2.3**	0.4

Table 3 **Quantification of Bax targeted to the heavy membranes isolated from Rat1-MycER^{TAM} cells transfected with an empty vector**

pH 7.5	nmoles of Bax on membrane/L of reaction					
					Avg	Stdev
521 nM Bax	24	24	32	40	**30**	7
260 nM Bax	18	12	9	24	**16**	6
104 nM Bax	9	7	7	14	**9**	3
0.83 nM tBid + 521 nM Bax	50	36	48	48	**45**	7
0.83 nM tBid + 260 nM Bax	33	13	20	14	**20**	9
0.83 nM tBid + 104 nM Bax	18	6	18	11	**13**	6
8.33 nM tBid + 521 nM Bax	59	41	71	76	**62**	15
8.33 nM tBid + 260 nM Bax	33	21	30	38	**31**	7
8.33 nM tBid + 104 nM Bax	16	9	13	25	**15**	7

pH 11.5						
					Avg	Stdev
521 nM Bax	5.2	8.6	4.3	6.4	**6.1**	1.8
260 nM Bax	2.2	2.0	2.1	2.5	**2.2**	0.2
104 nM Bax	1.0	0.8	1.5	1.2	**1.1**	0.3
0.83 nM tBid + 521 nM Bax	4.9	5.2	6.5	4.2	**5.2**	1.0
0.83 nM tBid + 260 nM Bax	3.7	1.9	2.6	2.1	**2.6**	0.8
0.83 nM tBid + 104 nM Bax	1.5	1.5	2.0	2.0	**1.7**	0.3
8.33 nM tBid + 521 nM Bax	6.0	12.8	7.5	6.5	**8.2**	3.1
8.33 nM tBid + 260 nM Bax	4.7	4.4	4.0	5.3	**4.6**	0.6
8.33 nM tBid + 104 nM Bax	2.3	3.7	2.1	4.1	**3.1**	1.0

Table 4 **Comparison and summary of the quantification of Bax targeted to the heavy membranes isolated from Rat1-MycER^{TAM} cells transfected with an empty vector or expressing Bcl-2**

	Bcl-2		Vector	
	nmoles of Bax on membrane/L of reaction			
pH 7.5				
	Avg	*Stdev*	***Avg***	*Stdev*
521 nM Bax	**57**	*15*	**30**	*7*
260 nM Bax	**23**	*3*	**16**	*6*
104 nM Bax	**15**	*2*	**9**	*3*
0.83 nM tBid + 521 nM Bax	**56**	*8*	**45**	*7*
0.83 nM tBid + 260 nM Bax	**27**	*7*	**20**	*9*
0.83 nM tBid + 104 nM Bax	**20**	*1*	**13**	*6*
8.33 nM tBid + 521 nM Bax	**52**	*14*	**62**	*15*
8.33 nM tBid + 260 nM Bax	**33**	*8*	**31**	*7*
8.33 nM tBid + 104 nM Bax	**17**	*2*	**15**	*7*

pH 11.5				
	Avg	*Stdev*	**Avg**	*Stdev*
521 nM Bax	**4.5**	*0.7*	**6.1**	*1.9*
260 nM Bax	**2.4**	*0.2*	**2.2**	*0.2*
104 nM Bax	**1.1**	*0.1*	**1.1**	*0.3*
0.83 nM tBid + 521 nM Bax	**6.1**	*1.5*	**5.2**	*1.0*
0.83 nM tBid + 260 nM Bax	**2.9**	*0.6*	**2.6**	*0.8*
0.83 nM tBid + 104 nM Bax	**2.3**	*1.0*	**1.7**	*0.3*
8.33 nM tBid + 521 nM Bax	**18.7**	*1.3*	**8.2**	*3.2*
8.33 nM tBid + 260 nM Bax	**7.7**	*1.3*	**4.6**	*0.6*
8.33 nM tBid + 104 nM Bax	**2.3**	*0.4*	**3.1**	*1.0*

monitored the mitochondrial matrix protein Hsp 60 and the integral membrane protein Bcl-2 as positive and negative controls, respectively (Figure 9C). Interestingly 1% of the added Bax (which represents 10% of the membrane bound Bax) was alkaline resistant, regardless of the presence of tBid or Bcl-2 (Table 4). There was one exception to this observation: after the addition of 8.33 nM tBid to Bcl-2 expressing membranes, 3% of the Bax added (which represents 30% of the membrane bound Bax) was alkaline resistant. A possible explanation for this apparently discordant result is that at these concentrations of tBid and Bax the structural integrity of the vector control membranes is compromised, causing Bax to 'leak out' during the alkaline extraction. Because the membranes are protected from pore formation when Bcl-2 is present, the same loss of membrane integrity is not evident. This is consistent with the fact that 100% of cytochrome c is released from vector control mitochondria in the presence of 8.33 nM tBid and 521 nM Bax, while under the same conditions only 50% of cytochrome c is released from Bcl-2 enriched mitochondria. To determine if the heavy membranes isolated from vector control cells are compromised in the presence of 8.33nM tBid and 521nM Bax, the integrity of the membranes should be tested by alkaline extraction of an unrelated mitochondrial integral membrane protein. If the membrane protein is released into solution, this would suggest that the membranes are compromised and alkaline extraction experiments should be carried out using lower tBid concentrations in which the vector control heavy membranes are not disrupted. However, if this membrane protein is found exclusively in the membrane pellet, then the membranes would be intact as far as can be determined by this assay, and other explanations would have to be tested.

The data presented in Table 4 shows that Bax bound and inserted into the mitochondrial membranes without inducing cytochrome c release. The addition of 521 nM Bax to vector control or Bcl-2 enriched mitochondria did not cause cytochrome c release (Figure 7). By the results described above, we can infer that in this case 5 nM Bax (or 1%) was integrated into the heavy membranes (Table 4). Despite the presence of membrane integrated Bax, cytochrome c release was not observed until either 0.83 nM or 8.33 nM tBid was added mitochondria from control or Bcl-2 expressing cells, respectively. These results are consistent with a model in which tBid is required to activate Bax integrated in outer mitochondrial membrane, and if the amount of activated Bax exceeds a threshold, then cytochrome c was released. Presumably Bcl-2 at the mitochondria inhibited the activation of Bax by tBid and thereby prevented the release of cytochrome c (Figure 7).

Bcl-2 inhibits the oligomerization of tBid-activated Bax

In some forms of apoptosis Bid is cleaved to tBid by caspase 8, and the tBid translocates to mitochondria. In this system Bcl-2 does not influence the amount of tBid binding to mitochondria (Gross *et al.*, 1999). However, tBid binds directly to Bcl-2 (Figure 5) (Wang *et al.,* 1996), and one hypothesis for Bcl-2 function is that it sequesters tBid thereby preventing it from activating Bax/Bak. To test this hypothesis in our system, the pellet fraction of mitochondria from which cytochrome c has been released was probed for the presence of tBid. No tBid was detected, suggesting that tBid is not sequestered by Bcl-2 on the membrane (Figure 10).

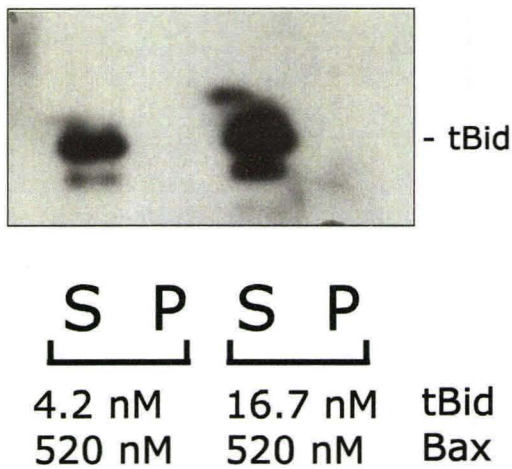

Figure 10 **tBid is not found on the mitochondria.** Heavy membranes isolated from Rat-1MycER^{TAM} cells overexpressing Bcl-2 were incubated with 4.2nM tBid plus 520nM Bax or 16.7 nM tBid plus 520nM Bax. The reactions were fractionated into supernatant (S) and pelleted mitochondria (P) by centrifugation and tBid assayed by immunoblotting with mouse 5C8 Bid antibody.

Detecting small amounts of tBid is a problem. To overcome this, researchers have used radioactively labeled tBid (Lutter *et al.*, 2001; Wei *et al.*, 2000; Zha *et al.*, 2000; Grinberg *et al.*, 2002), GST-tagged tBid (Hu *et al.*, 2003), or large amounts of protein (Eskes *et al.*, 2000). In order to use physiologically relevant amounts of untagged tBid a new anti-tBid antibody (5C8) was generated that is sensitive enough to detect small amounts of tBid (Figure 4). The lower limit of detection of tBid by the 5C8 Bid antibody is 0.5 ng tBid (Figure 4), which is equivalent to the amount of tBid at 0.83 nM in our system. However, even at 10ng of tBid (equivalent to 16.7 nM) which is well above the detection limit of the 5C8 antibody, no tBid was detected in the pellet fraction (Figure 10). This data suggests that tBid sequestration is not the mechanism by which Bcl-2 inhibited cytochrome c release.

Another way that Bcl-2 might inhibit cytochrome c release is by inhibiting the activation of Bax. Activated Bax oligomerizes to form a channel/pore allowing the release of cytochrome c from the mitochondria (Antonsson *et al.*, 2000). Bcl-2 binds to Bax directly under some circumstances (Oltvai *et al.*, 1993), and may interfere with the oligomerization of Bax. To test the hypothesis that Bcl-2 sequesters activated Bax the heavy membranes were solubilized in 2% CHAPS and the oligomeric status of the proteins was determined by gel filtration chromatography (Superdex 200). This detergent was chosen because higher molecular weight complexes of Bax are stable under these conditions (Antonsson *et al.,* 2000). The eluted fractions were immunoblotted using antibodies specific for Bax and Bcl-2. Molecular weight complexes larger than 600 kDa are excluded from the matrix of the column and hence eluted in fractions 1 and 2,

whereas complexes between 10-600 kDa are efficiently sieved by the resin and elute in later fractions.

When heavy membranes containing Bcl-2 were isolated and incubated with 520 nM Bax, Bcl-2 eluted in fractions 16-22 corresponding to Bcl-2 monomers and dimmers (Figure 11, 520nM Bax). The membrane bound Bax eluted predominantly in fractions 22-24 in the presence or absence of Bcl-2 (Figure 11, 520 nM Bax) suggesting that Bax remains monomeric even when bound to membranes containing Bcl-2. When heavy membranes from vector transfeced control cells were incubated with 4.2 nM tBid and 520 nM Bax cytochrome c was released indicating that Bax had formed pore-forming oligomers. Consistent with this interpretation higher molecular weight complexes were detected (fractions 2-12) as well as Bax monomers (fractions 22-24) (Figure 11, 4.2 nM tBid + 520 nM Bax). However, most of the Bax is still monomeric, suggesting that only a small proportion of membrane integrated Bax is required to form functional oligomers or that Bax oligomers are not stable in CHAPS.

These amounts of tBid and Bax were not sufficient to cause cytochrome c release from Bcl-2 enriched mitochondria (Figure 7), and Bcl-2 prevented the formation of large Bax oligomers (Figure 11, 4.2 nM tBid + 520 nM Bax). However, when Bcl-2 was unable to prevent cytochrome c release (16.7 nM tBid and 520 nM Bax), high molecular weight oligomers of Bax were detected in fractions 2-6 and 10-12. Furthermore, Bax and Bcl-2 eluted together in fractions 14-18 (Figure 11, 16.7 nM tBid + 520 nM Bax). Fractions 14-18 correspond to an approximate molecular weight range of 66 to 31 kDa, and may be due to Bcl-2/Bax heterodimers or low molecular weight Bax oligomers

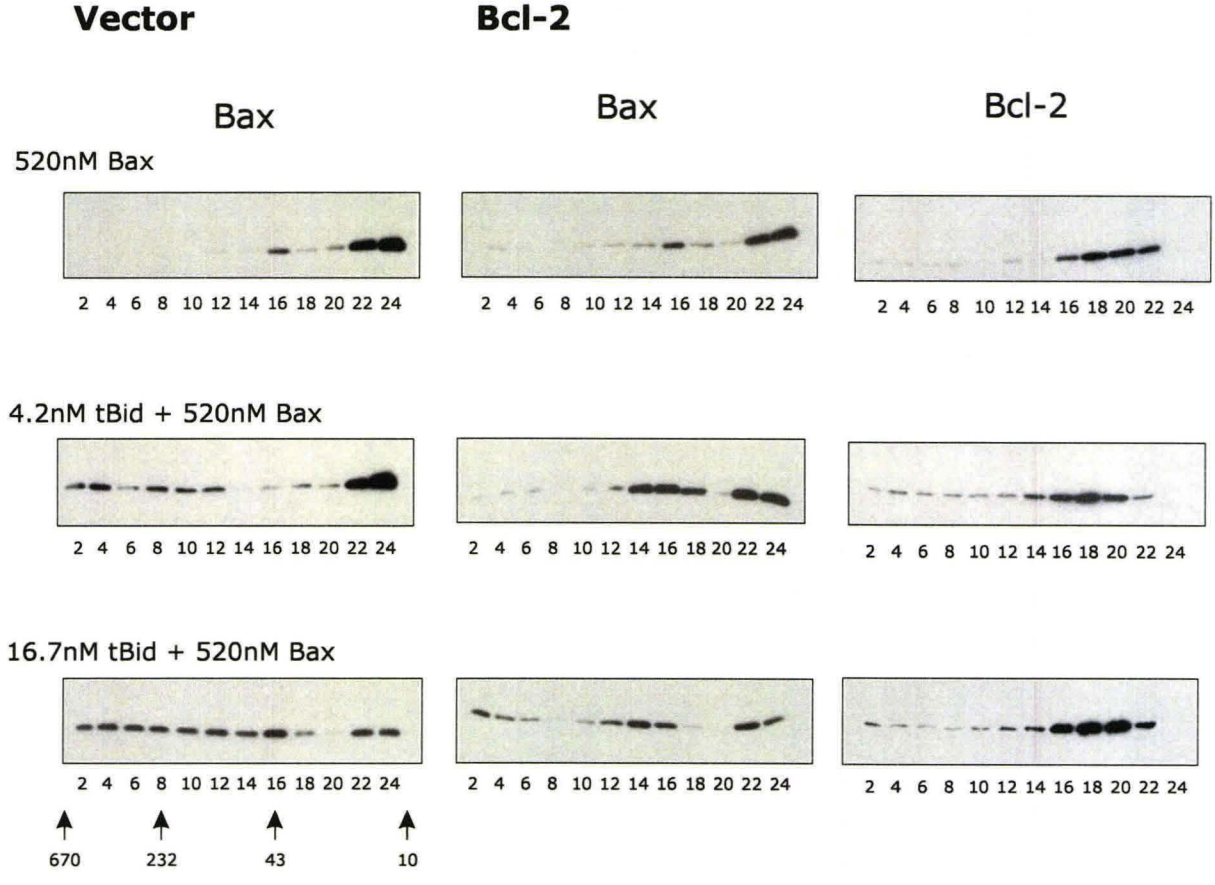

Figure 11 **Bcl-2 inhibits the oligomerization of tBid-activated Bax.** Isolated heavy membranes from Rat1-MycER^{TAM} cells transfected with an empty expression vector (Vector) or overexpressing Bcl-2 (Bcl-2) were incubated with increasing amounts of purified tBid and 520nM Bax. After an incubation of 1 hour at 30°C, the membrane fractions were solubilized in 2% CHAPS and subjected to gel filtration chromatography. Twenty-four fractions were collected (0.4ml) and proteins in every other fraction were precipitated with trichloroacetic acid and immunoblotted for Bcl-2 and/or Bax. Fraction numbers are indicated below the immunoblots and the elution positions of calibrated strandards are shown blow the fraction numbers of the immunoblot at the lower left.

(homodimers or homotrimers) that are incapable of forming pores. A third possibility is that the complex is formed by Bcl-2 (26kDa), tBid (15kDa) and Bax (21kDa); however as mentioned above, tBid could not be detected. Direct binding of Bcl-2 to Bax was assayed by co-immuno-precipitation. Mitochondria from vector transfected (control) and Bcl-2 expressing cells were incubated with tBid alone or tBid and Bax and then solubilized in 2% CHAPS. Immunoprecipitation with a Bcl-2 antibody followed by Western blotting with Bax antibody showed an interaction between Bcl-2 and Bax only after incubation with tBid (Figure 12). Taken together these results suggest that Bcl-2 inhibits cytochrome c release by binding directly to activated Bax thereby suppressing the formation of large molecular weight Bax oligomers, and that this inhibition can be overcome by forming more activated Bax when increasing amounts of tBid are added.

tBid is a BH3 only protein for which a direct interaction with Bax and Bak has been published (Wang *et al*, 1996). The interaction of tBid with Bax and Bak induces conformational changes in Bax and Bak associated with exposure of a previously cryptic amino terminal epitopes in both cases (in Bax this epitope is recognized by the antibody 6A7 and encompasses amino acids 13-19). tBid does not remain bound to Bak after it has been activated, and a "kiss and run" model for activation of Bax/Bak by tBid has been postulated (Wang *et al*, 1996). Consistent with this model, tBid has not been detected in Bax and Bak oligomers. A variation of this basic model is suggested by the observation that a modified Bak protein can induce the exposure of the amino-terminus of endogenous Bak in the absence of an apoptotic stimulus, thereby propagating this conformational change (Ruffolo *et al.*, 2003). Since Bax and Bak proteins are very

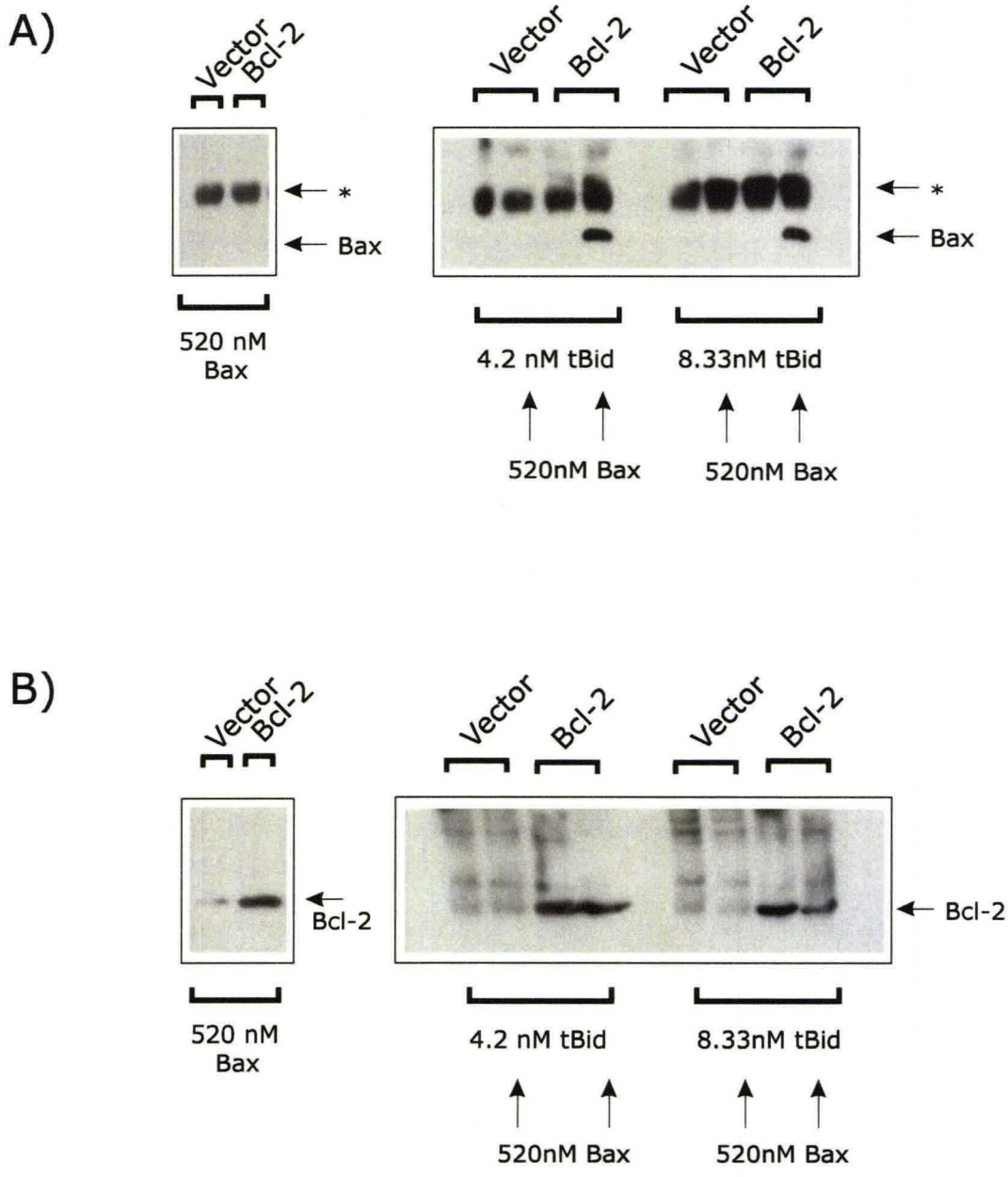

Figure 12 **Bcl-2 immunoprecipitates with tBid-activated Bax**. To determine if Bcl-2 and Bax interact heavy membranes isolated from Bcl-2 overexpressing cells, 100μg of total mitochondrial protein were incubated with purified recombinant tBid and/or Bax for 1 hour at 30 °C. Heavy membranes were pelleted by centrifugation and solubilized in 2% CHAPS. **A)** The samples were immunoprecipitated with a sheep anti-Bcl-2 antibody, analyzed by SDS-PAGE and immunoblotting with a mouse anti-Bax antibody and **B)** a rabbit anti-Bcl-2 antibody. * denotes a cross-reacting band.

similar it is possible that tBid-activated Bax is the physiologic trigger for a Bax conformational change that leads to auto-oligomerization. In this case, a small amount of tBid might remain bound to the Bak or Bax oligomer but not be detectable by current techniques. Our results (Figure 11) cannot distinguish between these models, but do strongly suggest that Bcl-2 preferentially interact with tBid-activated Bax blocking formation of large pore-forming multimers, thereby preventing the release of cytochrome c.

Summary

A cell free assay for apoptosis was developed to investigate the interactions between tBid, Bax and Bcl-2. Recombinant, in vitro cleaved Bid (tBid) activated recombinant Bax to form oligomers that release cytochrome c from the intermembrane space of isolated mitochondria. Because full length recombinant Bcl-2 is not soluble, mitochondria from cell expressing Bcl-2 were used. By using well defined amounts of the recombinant proteins and quantitative immunoblotting of membrane fractions using internal standards, the exact amount of each of these three proteins was measured in different experimental conditions. This system allowed me to test models of Bcl-2 function that would not be easily distinguished using experiments with whole cells where it is difficult to express controlled amounts of different proteins. The data I obtained using this cell free assay, combined with findings in the published literature suggested the model for Bax activation shown in Figure 13, in which tBid binding to Bax on the

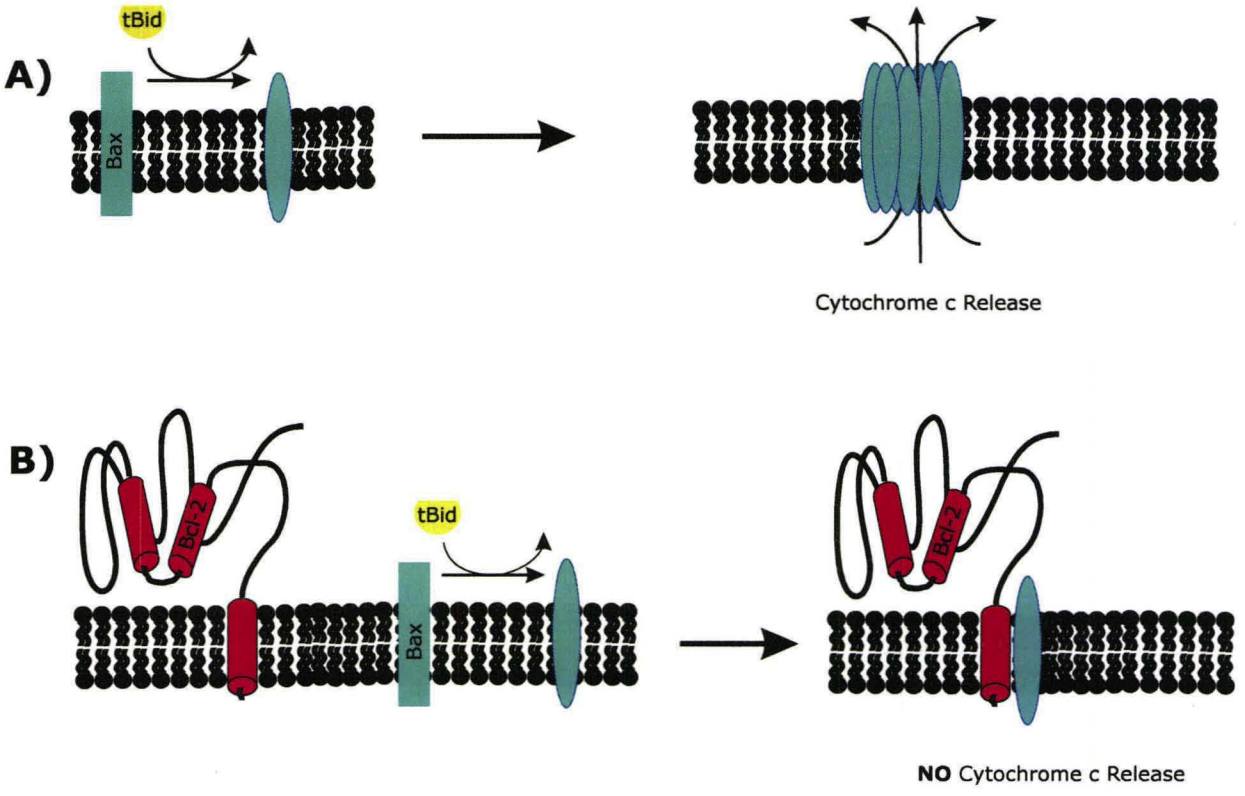

Figure 13 **Model for Bcl-2 inhibition of tBid and Bax induced cytochrome c release.** **A)** tBid binds to Bax on the membrane and activates the protein, leading to Bax oligomerization and cytochrome c. **B)** Bcl-2 inhibits the formation of Bax oligomers by sequestering tBid-activated Bax and antagonizing the release of cytochrome c.

membrane causing it to oligomerize and form a pore through which cytochrome c is released (Figure 11).

Bcl-2 inhibits the formation of Bax oligomers by sequestering tBid-activated Bax (Figure 11, 12) which prevents oligomerization of Bax, and possibly also the activation of other Bax monomers. The result of this interaction(s) is to prevent the release of cytochrome c and other factors from the intermembrane space of mitochondria, that otherwise would activate downstream effectors of apoptosis.

Chapter Four

Monitoring the membrane topology of Bcl-2

INTRODUCTION:

Although the cellular functions of the Bcl-2 family of proteins are well recognized, the precise molecular mechanisms by which these proteins determine cell fate remains unclear. Bcl-2 is an anti-apoptotic transmembrane protein found at both the ER and mitochondria (Krajewski *et al.*, 1993); however, the exact topology of Bcl-2 at the membrane is controversial. Bcl-2 contains a carboxyl-terminal trasmembrane (TM) domain that may confer a tail-anchored topology (Figure 1A). The three dimensional structure of Bcl-2 has two central, relatively hydrophobic helices (helix 5 and 6) surrounded by amphipathic helices (Figure 13). Helix 5 and helix 6 of Bcl-2 have been proposed to be membrane spanning domains based on the structural similarity of Bcl-2 to diptheria toxin, a pore forming protein. Thus, Bcl-2 may have a multispanning transmembrane topology in which helix 5 and 6 are transmembrane sequences (Figure 1A). We have shown that Bcl-2 is in a tail-anchored topology in the cell and upon the induction apoptotis undergoes a conformational change that is consistent with a multispanning transmembrane topology (Kim *et al.*, 2004). This conformational change was monitored by 4-acetamido-4'-iodoacetly-amino stilbene-2,2'-disulfonic acid (IASD) labeling.

IASD is a cysteine specific, lipid impermeable reagent (Figure 2A). IASD has been used to determine the topology of several proteins (Krishnansastry *et al.*, 1994; Grundling *et al.*, 2000 and Kim *et al*, 2004). Protein labeling by this reagent can be detected by a shift in the migration of the protein on a 16% SDS-PAGE gel (Figure 2B).

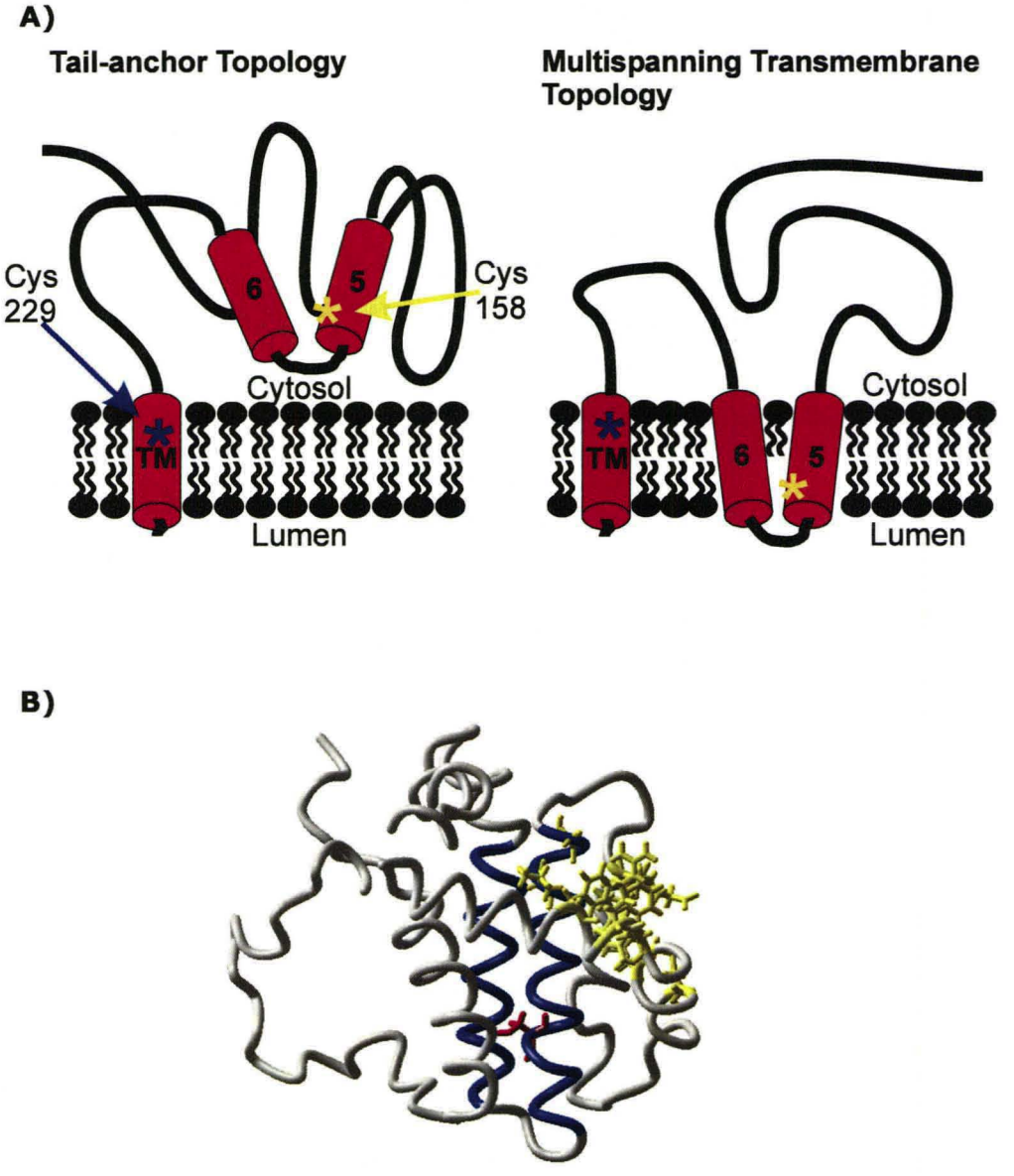

Figure 1 **Models for the topology of membrane bound Bcl-2 and NMR solution structure of Bcl-2. A)** Two predicted membrane topologies for Bcl-2. Tail-anchored topology is typical of proteins with carboxyl-terminal transmembrane (TM) regions. The multispanning transmembrane topology is based on the similarity of Bcl-2 to pore forming Diptheria toxin. In this topology the TM and the putative pore forming domains (ά-helix 5 and ά-helix 6) are inserted into the lipid bilayer of the membrane. **B)** Model of the NMR structure of Bcl-2 with helix 5 and 6 shown in blue and cysteine 158 in helix 5 highlighted in red. Residues equivalent to those in Bcl-x_L that have been shown to be important for heterodimerization with other Bcl-2 family members are shown in yellow.

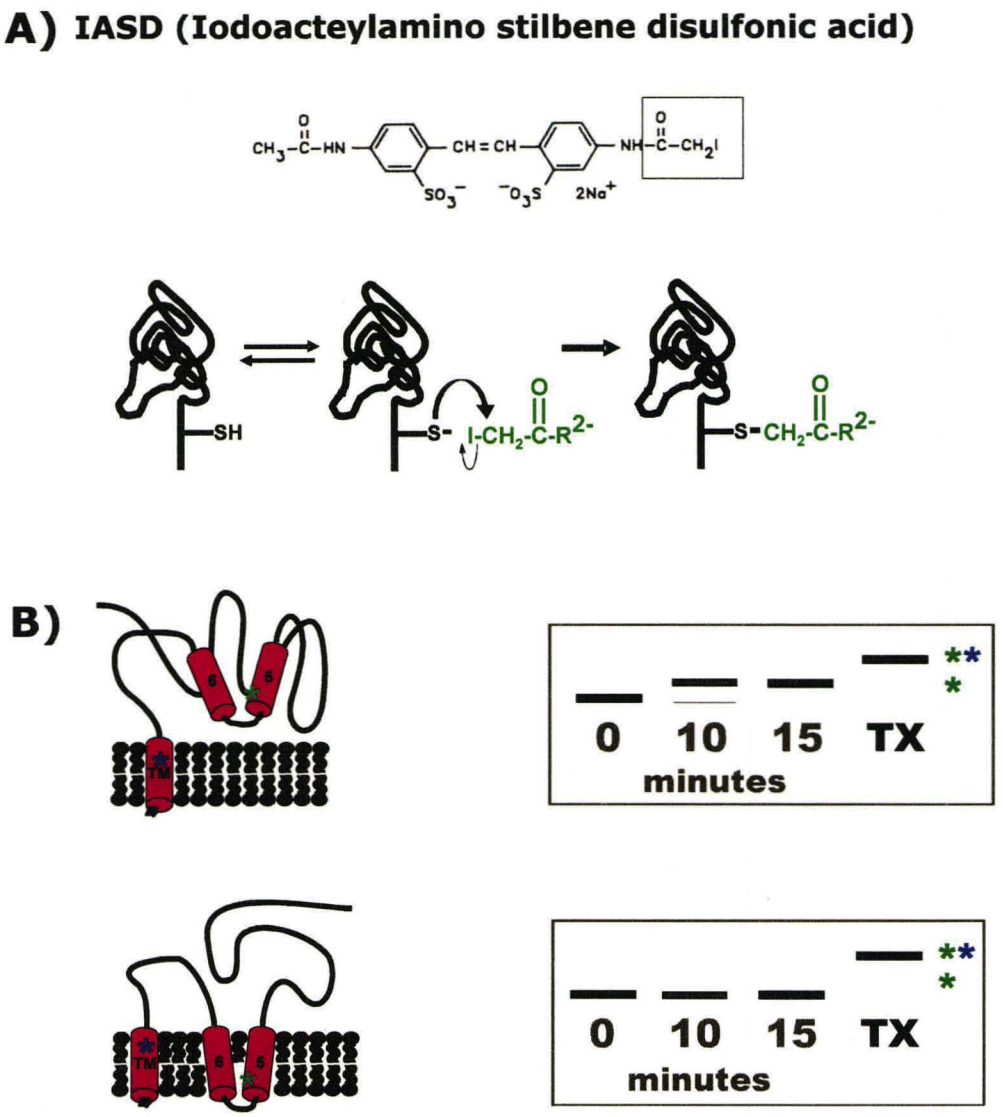

Figure 2 **The structure of IASD and schema for interpreting labeling reactions for aqueous and membrane inserted cysteine residues.** **A)** The chemical structure of IASD. The reactive iodo-acetyl group is boxed and reacts with the thiol group of cysteins. **B)** Protein labeling by IASD can be detected by a shift in the migration of the protein on a SDS-PAGE gel. Samples are either untreated (0) or incubated with IASD for 10 or 15 minutes. An additional sample is solubilized in 1% Triton X-100 and labeled for 15 minutes (TX). The samples are then separated on a 16% SDS-PAGE gel and immunoblotted for Bcl-2. If Bcl-2 is in a tail-anchor topology, cysteine 158 is in an aqueous environment it will be readily modified by IASD and a gel shift in migration will occur (*). If Bcl-2 adopts a multispanning transmembrane topology cysteine 158 will be embedded into the lipid bilayer and protected from modification by IASD. Therefore a shift in the migration of the protein will only occur when the protein is solubilized in detergent (TX). Solibilization in 1% Triton X-100 causes a double shift in the migration as both cysteine 158 and 229 accessible to modification by IASD (**).

Bcl-2 contains two endogenous cysteines (Figure 1A). Cysteine at position 229 is located within the tail-anchor of Bcl-2 and the other cysteine residue (158) is located near the base of helix 5. If Bcl-2 adopts a tail-anchored topology, only cysteine 229 would be protected from IASD labeling (Figure 1A). In a multispanning transmembrane topology, the TM and the putative pore forming domains (helix 5 and 6), would be inserted into the bilayer of the membrane (Figure 1A). Therefore both cysteine residues would be protected from IASD.

Recently we showed that Bcl-2 undergoes a conformational change on the membrane upon induction of apoptosis (Kim *et al.,* 2004). Dr. Peter Kim and Dr. Matthew Annis showed that in untreated cells Bcl-2 cysteine 158 is accessible to labeling by IASD indicating a tail-anchored topology. However, drug treatment of the cells triggered a change in conformation of Bcl-2 such that cysteine 158 was protected from modification by IASD. This suggests that Bcl-2 has undergone a conformational change from a tail-anchored topology to a multispanning transmembrane topology with cysteine 158 inserted into the lipid bilayer, although we cannot rule out a conformational change that protects only cysteine 158 from IASD modification and does not involve the complete insertion of helix 5 and 6 into the bilayer. The cell free assay described in chapter 3 combined with the IASD labeling results presented in this chapter show that tBid and Bim peptide can provoke a change in conformation of Bcl-2 on the membranes and that this conformational change is a necessary part of Bcl-2 function. These results support the data acquired by Dr. Peter Kim and Dr. Matthew Annis, and were included in the published manuscript (Kim *et al.*, 2004).

RESULTS AND DISCUSSION:

In the absence of membranes Bcl-2 cysteine 158 is accessible to IASD labeling, even after incubation with tBid.

The structure of Bcl-2 shows that cysteine 158 is buried within the core of the protein and predicts that this residue would be inaccessible to IASD in the absence of denaturants (Figure 1B) (Petros *et al.*, 2001). Therefore labeling reactions were carried out in 4M urea which makes Bcl-2 cysteine 158 IASD accessible by unfolding the protein. The BH1, BH2 and BH3 regions of Bcl-2 create an elongated hydrophobic cleft to which the BH3 region amphipathic α-helix of a pro-apoptotic protein can bind (Petros *et al.*, 2001). Disruptions of the BH1 and BH2 regions of Bcl-2 have shown that these regions are essential for its interaction with pro-apoptotic proteins (Yin *et al*, 1994). Cysteine 158 of Bcl-2 is not located in these regions and therefore it is unlikely that the direct binding of a pro-apoptotic protein like tBid to Bcl-2 would inhibit the labeling of cysteine 158 (Figure 1B). Furthermore, since 4M urea was sufficient to unfold Bcl-2, it is less likely that other proteins would remain bound to Bcl-2 in this altered conformation. To show unequivocally that binding of tBid to Bcl-2 in 4M urea would not mask cysteine 158, a control experiment was carried out in which the two proteins were mixed together in the absence of membranes. To test this, we used Bcl2-cb5, which is a functional mutant of Bcl-2 in which the carboxyl terminus tail-anchor (containing cysteine 229) has been exchanged with the carboxyl terminus tail-anchor of the ER specific isoform of rat cytochrome b5. The carboxyl terminus tail-anchor of cytochrome b5 does not contain any cysteines (Figure 3A) and therefore Bcl2-cb5 only has the helix 5

cysteine (cysteine 158) of Bcl-2. In cells the Bcl2-cb5 fusion protein is targeted exclusively to the ER and inhibits cell death induced by apoptotic stimuli that perturb ER function (Zhu *et al.*, 1996; Hacki *et al.*, 2000; Rudner *et al.*, 2001; Thomenius *et al*; 2003). Because Bcl-cb5 is as effective as wild type Bcl-2 at preventing apoptosis in these cases (Fiebig *et al.*, submitted), it is likely that they adopt similar functional conformations. Therefore if the binding of tBid to Bcl2-cb5 does not inhibit IASD labeling of cysteine 158 in this mutant, it is unlikely that it would mask cysteine 158 in wild type Bcl-2.

To test if the binding of tBid to Bcl2-cb5 rather than membrane insertion prevents the modification of cysteine 158 with IASD, S^{35} labeled Bcl2-cb5 protein was synthesized in reticulocyte lysate and incubated with purified recombinant tBid at various concentrations in the absence of membranes. To find the ratio of tBid to Bcl2-cb5 that could be incubated together without the inhibition of IASD labeling of cysteine 158 the amount of Bcl2-cb5 sythesized in a rabbit reticulocyte lysate was quantified. It was determined that 2 ± 0.2 fmole of Bcl2-cb5 was synthesized/µl of a translation reaction. Bcl2-cb5 was used at a concentration of 10 nM. After 1 hour of incubation of Bcl2-cb5 with tBid the samples were treated with IASD for 15min in 4M urea. The audioradiograph in figure 3 demonstrates that if tBid remains bound to Bcl-2 in 4M urea, this binding does not inhibit the IASD labeling of Bcl-2 cysteine 158. Even when tBid was present in 150-fold excess of Bcl2-cb5, which is a higher concentration then used in the experiments described below, almost all of cysteine 158 was modified by IASD (Figure 3, *).

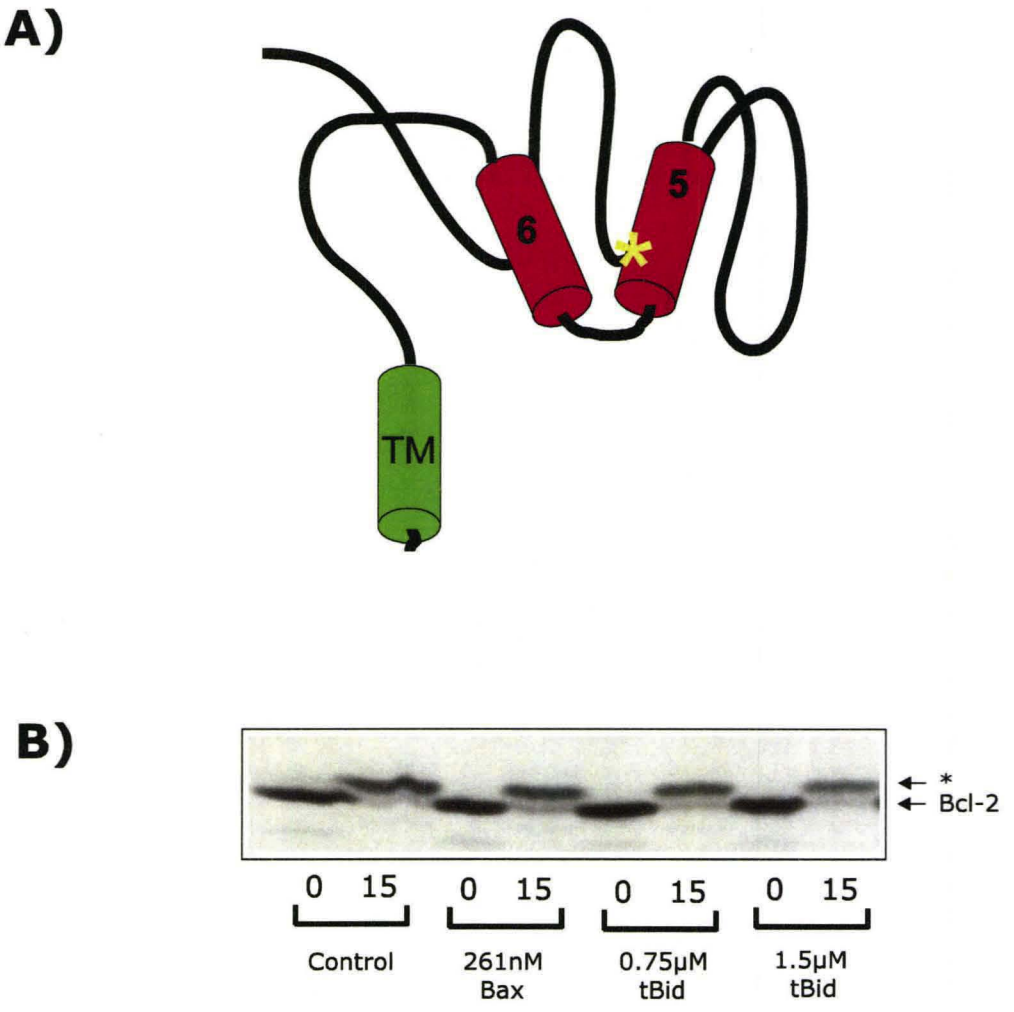

Figure 3 **Direct binding of tBid to Bcl-2 does not result in the protection of cysteine 158 from IASD labeling. A)** A schematic representation of the fusion protein Bcl2-cb5. Bcl2-cb5 is a version of Bcl-2 in which the TM and carboxyl terminus (containing cysteine 229) have been exchanged with the TM and carboxyl terminus of an ER isoform of cytochrome b5 that is cysteineless, shown in green. **B)** An autoradiograph of S^{35} labeled Bcl2-cb5 protein synthesized in rabbit reticulocyte lysate, which was incubated with various concentrations of purified tBid in the absence of membranes. After 1 hour at 30°C of incubation the samples were treated with IASD for 15 minutes and separated by SDS-PAGE. The single asterisk indicates that cysteine 158 has been modified, as per schema shown in Figure 2.

Bcl-2 changes conformation on the membrane in response to tBid

To determine if tBid induces a conformational change in Bcl-2 topology, tBid was added to lysates of Rat1-MycER^{TAM} cells. After the addition of 1 μM tBid approximately 70% of cysteine 158 in Bcl-2 was inaccessible to IASD (Figure 4A), as shown by the increase in intensity of the unlabelled band with a corresponding decrease in the shifted band (*). As a control, when membranes were solubilized with detergent (1% Triton X-100) both cysteines were modified by the IASD reagent, seen by the double shift on the SDS-PAGE gel (Figure 4A, TX,**). This shows that the addition of tBid to whole cell lysates triggered a change in Bcl-2 conformation that led to the protection of cysteine 158 from IASD. This protection is likely due to the insertion of cysteine 158 into the bilayer of the membrane and not due to the binding of tBid to Bcl-2 (Figure 3). When the membranes were solublized by 1% Triton X-100, protection of the cysteine was abolished (Figure 4A, **). This corroborates the idea that protection of cysteine 158 was due to membrane bilayer and not protein binding, as this concentration of detergent does not interfere with the binding of pro-apoptotic family members to Bcl-2 (Hsu et al., 1997).

These experiments were performed using whole cell lysates. Therefore, to determine whether cytosolic proteins were required for the conformational change in Bcl-2, tBid was added to the isolated membrane fraction. When the accessibility of cysteine 158 of Bcl-2 was examined using the membrane fractions isolated from Bcl-2 expressing cells, cysteine 158 was labeled by IASD (Figure 4B control). In the presence of 521 nM

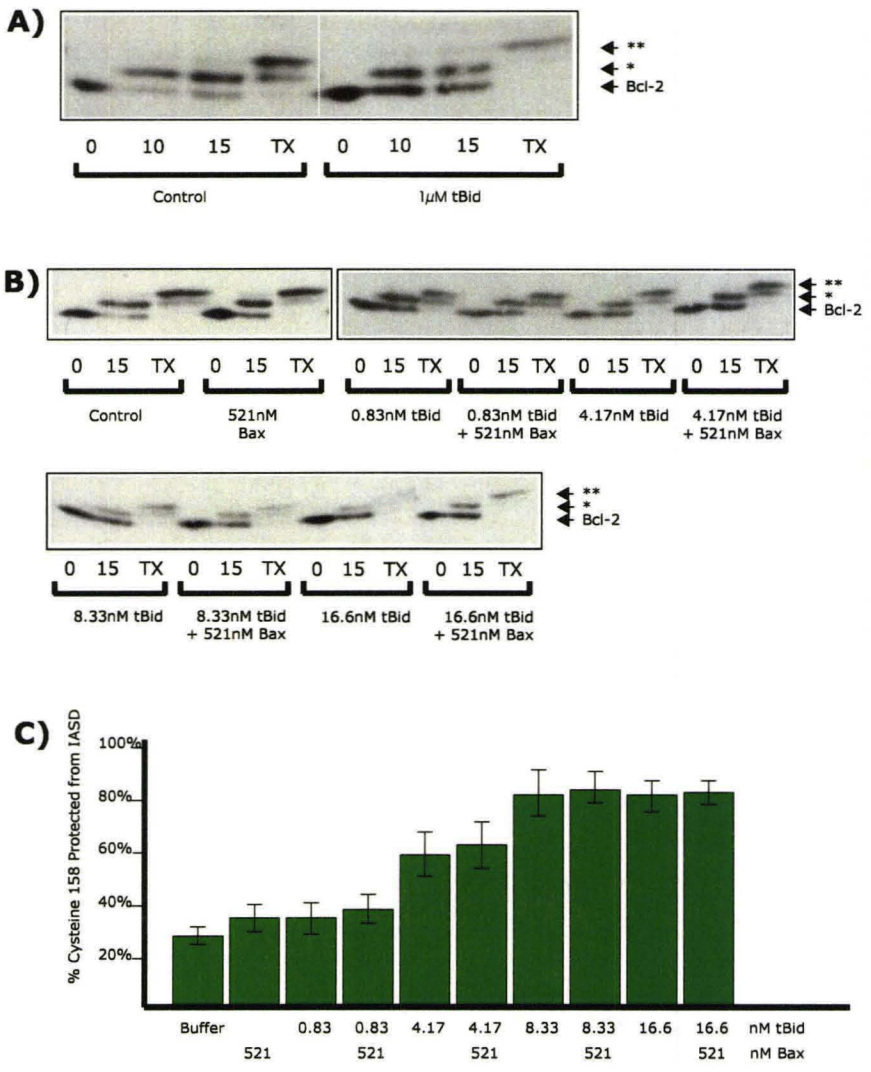

Figure 4 **Exposure to tBid triggers a conformational change in Bcl-2 monitored by IASD labeling. A)** Lysates of Rat-1MycER^{TAM} cells overexpressing Bcl-2 (175μg of total protein) were incubated with tBid buffer (Control) or 5μM tBid for 30min at 24°C. **B)** Isolated heavy membranes from Rat-1MycER^{TAM} cells overexpressing Bcl-2 (50μg of total membrane protein) were incubated with increasing amounts of tBid and/or 521nM Bax for 1 hour at 30°C. The membranes from A and B were pelleted, resuspended in cell buffer and incubated with IASD for the time indicated. The samples were separated by SDS-PAGE and immunoblotted for Bcl-2. Bcl-2 molecules with cysteine 158 modified by IASD are indicated by (*), and (**) indicates labeling of both cysteine 158 and 229 in control reactions containing 1% Triton X-100 (TX). **C)** The percentage of cysteine 158 protected from IASD modification after 15 minutes of labeling was determined by densitometry of data obtained from three individual experiments. Error bars indicate standard deviation, except for samples 8.33nM tBid plus 521nM Bax and 16.6nM tBid plus 521nM Bax. For these samples only two images were quantified and thus the range between the two numbers is presented.

Bax, Bcl-2 cysteine 158 was still accessible to modification by IASD (Figure 4B*). However, the addition of tBid to isolated heavy membranes triggered the insertion of Bcl-2 cysteine 158 into the membrane, confirming that cytosolic proteins including Bax are not required for the conformational change (Figure 4B, 4C).

After the addition of 0.83 nM tBid and 521 nM Bax a slight change in the amount of cysteine 158 that was protected from IASD modification was observed. However, 59% of cysteine 158 was protected upon the addition of 4.2 nM tBid, and when the concentrations was raised to 8.33 nM tBid or above more than 80% (16nM) of the Bcl-2 had undergone a conformational change (Figure 4C). The addition of Bax to tBid in the reaction did not potentiate the Bcl-2 conformational change (Figure 4C). My data also demonstrated that Bcl-2 inhibited tBid induced cytochrome c release up to 16.6 nM tBid (Chapter 3 Figure 7). Only 50% of cytochrome c was released after adding 8.33 nM tBid and 521 nM Bax to the Bcl-2 enriched mitochondria compared to complete cytochrome c release observed from vector control mitochondria at the same or lower concentrations of tBid and Bax (Chapter 3 Figure 7). Hence even after Bcl-2 has changed conformation as detected by IASD labeling of cysteine 158 (that we suggest corresponds to Bcl-2 adopting a multispanning transmembrane topology), it still inhibits apoptosis. This suggests that either this change is required for function or that the conformational change is a result of it having carried out its function.

Bim BH3 peptide provokes a conformational change in Bcl-2

The results of an experiment performed by Dr. Mathew Annis indicated that in Rat-1MycER^{TAM} cells tBid is not the mediator of etoposide induced apoptosis or the conformational change in Bcl-2. Inhibition of caspases in the cells did not prevent the insertion of cysteine 158 into membranes. This suggests that insertion of cysteine 158 into the bilayer is independent of Bid activation by caspase 8. To extend these findings, another well characterized inducer of apopotosis was used, a peptide corresponding the BH3 region of the pro-apoptotic BH3 only protein Bim (Marani *et al.,* 2002; Yamaguchi et al., 2002; Letai *et al.,* 2002). As a control, a peptide containing an inactivating Leu to Ala mutation equivalent to position 94 in Bim (Bim Mut) was used.

Since Bim peptide binds to and antagonize the anti-apoptotic activity of Bcl-2, control reactions were carried out (Letai et al., 2002). To determine whether binding of Bim peptide to Bcl-2 would prevent the IASD labeling of cysteine 158 in 4M urea, 1 nM S^{35} labeled Bcl2-cb5 protein synthesized in reticulocyte lysate was incubated with Bim peptide at various concentrations in the absence of membranes. The audioradiograph in figure 5A demonstrates that the presence of Bim peptide does not protect cysteine 158 of Bcl2-cb5 from IASD modification. The same ratios of Bim peptide to Bcl-2 were used in subsequent reactions discussed below.

Similar to the results obtained with tBid, the addition of 40 μM Bim peptide to cell lysates triggered a conformational change in Bcl-2 and led to the protection of cysteine 158 from IASD (Figure 5B). Protection from labeling was not observed when 40 μM Bim Mut was added to the lysates (Figure 5B). To determine if cytosolic proteins

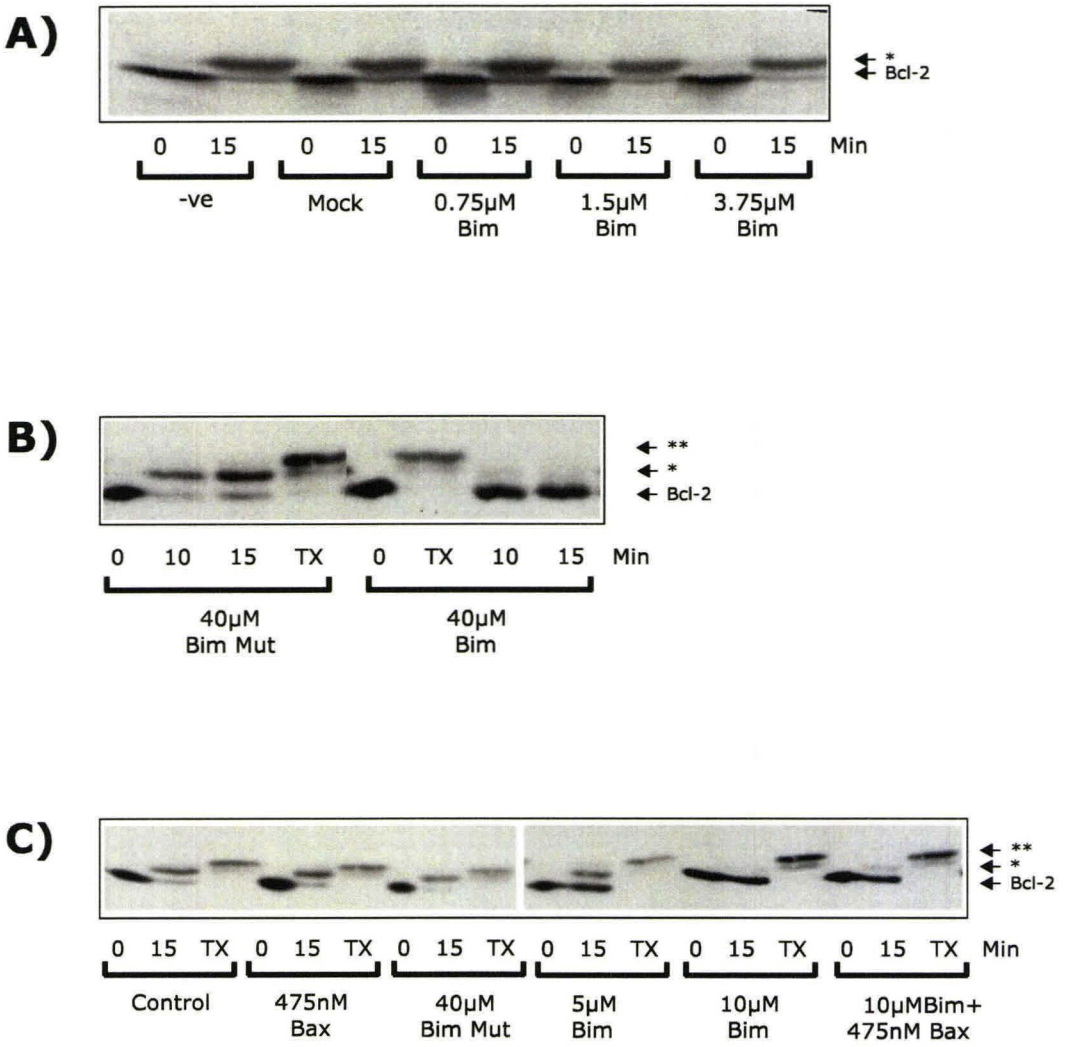

Figure 5 **Bim peptide triggers a confromational change in Bcl-2 on the membrane. A)** An autoradiograph of S^{35} labeled, Bcl2-cb5 protein synthesized in rabbit reticulocyte lysate which was incubated with Bim peptide at various concentrations in the absence of membranes. After 1 hour of incubation samples were treated with IASD for 15 min. **B)** Lysates of Rat1-MycER^{TAM} cells overexpressing Bcl-2 (175μg of total protein) were incubated with 40μM of Bim or Bim Mutant (Bim Mut) peptide for 30min at 24°C. **C)** Isolated heavy membranes from Rat1-MycER^{TAM} cells overexpressing Bcl-2 (50μg of total membrane protein) were incubated with 5μl of Buffer as a negative control, purified Bax, Bim Mut peptide, or Bim peptide as indicated for 1 hour at 30°C. The membranes were pelleted, resuspended in cell buffer and incubated with IASD for the time indicated. The samples were separated by SDS-PAGE and immunoblotted for Bcl-2. Bcl-2 molecules with cysteine 158 modified by IASD are indicated by (*), and (**) indicates labelling of both cysteine 158 and 229 in control reactions containing 1% Triton X-100 (TX).

are required for the Bim peptide triggered Bcl-2 conformational change, Bim peptide was added to isolated heavy membranes. Bcl-2 cysteine 158 was accessible to IASD independent of the presence of either Bax or Bim Mut peptide. The addition of Bim peptide to isolated heavy membranes triggered the protection of Bcl-2 cysteine 158 from IASD modification (Figure 5C), confirming that cytosolic proteins were not required for Bcl-2 to undergo the conformational change. After the addition of 5 μM Bim peptide, protection of Bcl-2 cysteine 158 from IASD was observed (Figure 5C), and at a concentration of 10 μM Bim peptide or higher (18nM) ~90% of Bcl-2 cysteine 158 was inserted into the membrane (Figure 5C and 6A).

To determine if Bcl-2 inhibited Bim induced cytochrome c release, heavy membranes were isolated from Rat1-MycER^{TAM} cells transfected with either control vector or expressing Bcl-2 (as described in chapter 3) and incubated with increasing amounts of Bim peptide. Cytochrome c release (~60%) was observed from vector control mitochondria after the addition of 10 μM Bim peptide, while Bcl-2 expressing mitochondria did not release cytochrome c after exposure to10 μM Bim peptide, with significant inhibition still evident at even higher concentrations of Bim peptide (25 μM) (Figure 6B). Even under conditions where almost all of the Bcl-2 (~90 % of total) exhibited the altered conformation, Bim induced apoptosis was still repressed. This result supports the hypothesis that Bcl-2 in a multispanning transmembrane topology inhibits apoptosis.

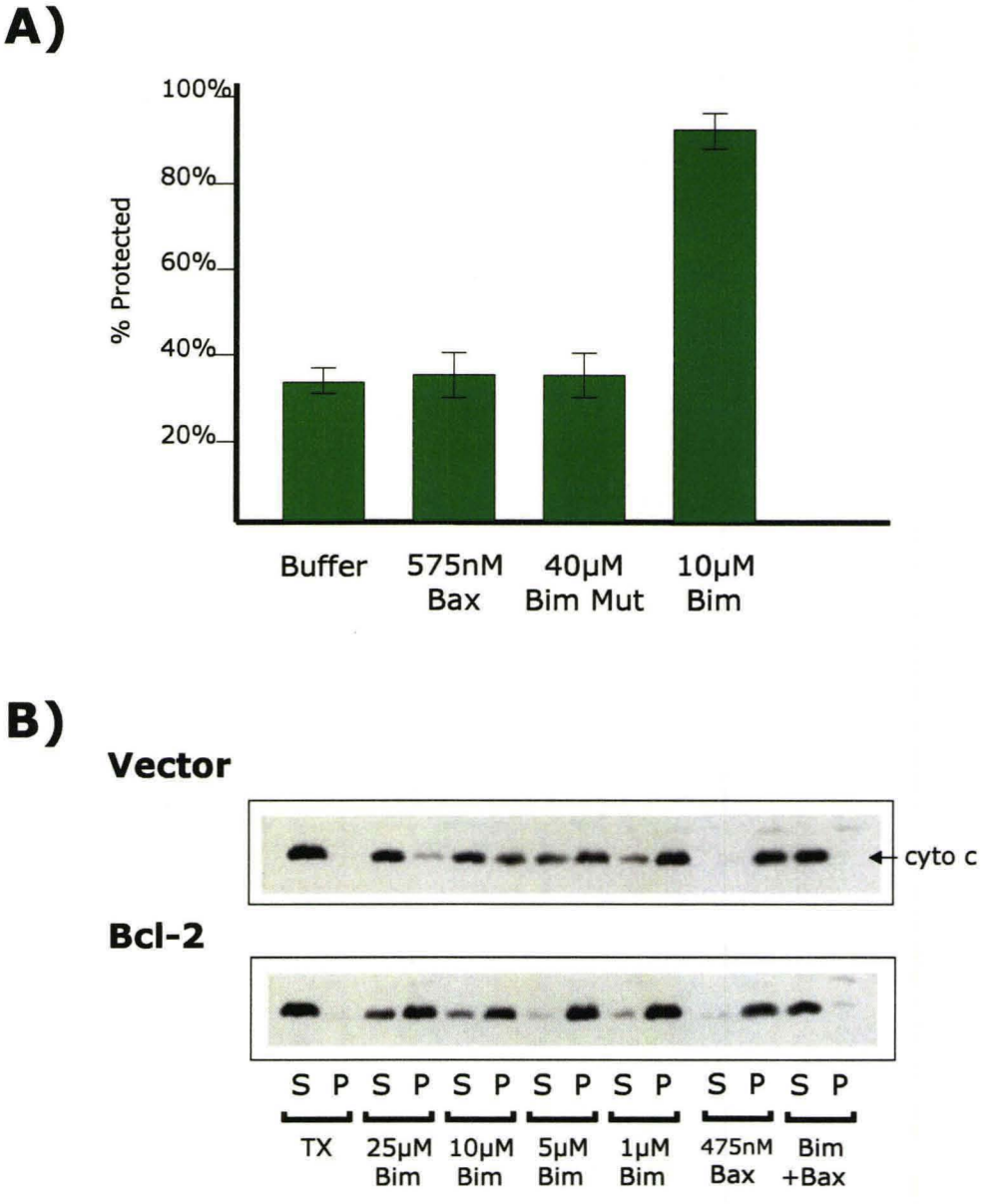

Figure 6 **Bcl-2 topology change triggered by Bim peptide is evident in conditions in which Bcl-2 is functional. A)** The percentage cysteine 158 protected from IASD modification after 15 minutes of labeling was determined by densitometry of data obtained from three individual experiments, error bars indicate standard deviation. **B)** Bim peptide alone, or with 475nM Bax (Bim +Bax) were added to heavy membranes isolated from Rat1-MycER^{TAM} cells overexpressing Bcl-2 (Bcl-2) or transfected with an empty expression vector (Vector). Cytochrome c release was monitored by immunoblotting the reaction supernatants (S) and pelleted mitochondria (P).

Membrane proteins are needed for Bcl-2 to undergo a conformational change on the membrane

Bcl-2 expressed in *E. coli* is not soluble and the isolated refolded protein does not prevent apoptosis in the cell free assay, as described in Chapter 3. Therefore, we used two approaches to obtain Bcl-2 that could be assayed in vitro. First we expressed Bcl-2 in cells and then isolated the mitochondria from these cells. By incubating tBid or Bim peptide with the isolated mitochondria we were able to show cytosolic proteins are not required for Bcl-2 function and the conformational change observed. However, this approach cannot be used to determine the importance of other identified (Bak) or unidentified membrane proteins as partners in this process. Full length Bcl-2 can be made in rabbit reticulocyte lysate. However, in this form Bcl-2 does not bind to liposomes. Therefore, to determine if membrane proteins are required for the conformational change Bcl2-cb5 was used. The cytochrome b5 (cb5) tail-anchor inserts spontaneously into liposomes. Hence, S^{35} labelled Bcl2-cb5 synthesized in reticulocyte lysate was targeted to liposomes (Figure 7B). After binding to liposomes, Bcl2-cb5 elutes in fractions 3 and 4 of a gel filtration column (Figure 7). Therefore IASD labeling reactions were carried out with the contents of fraction 3 (Figure 7E). Bcl2-cb5 was also targeted to liposomes in the presence of Bim peptide (Figure 7C) or Bim peptide plus purified recombinant Bax protein (Figure 7D).

In the absence of liposomes Bim does not prevent the IASD labeling of cysteine 158 of Bcl2-cb5 (Figure 7E and Figure 5A). After targeting Bcl2-cb5 to liposomes, a fraction of the protein contained cysteine 158 which was protected from IASD labeling

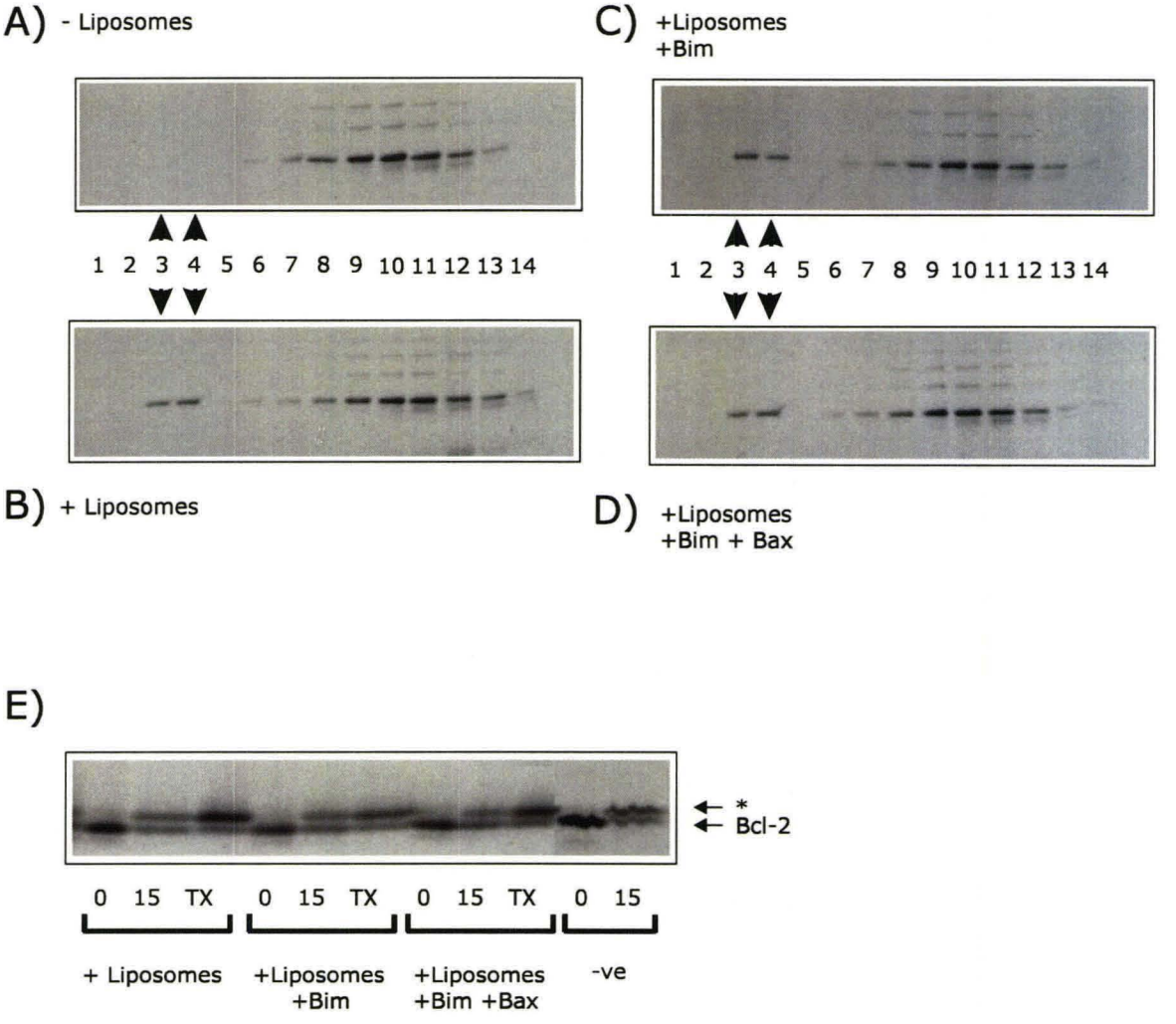

Figure 7 **Membrane proteins are need for the conformational change of Bcl-2. A)** Gel filtration of Bcl2-cb5 was synthesized in rabbit reticulocyte lysate or **B)** incubated with liposomes, or **C)** lipsomes plus 20μM Bim peptide or **D)** liposomes plus 20μM Bim peptide and 521nM Bax. The reactions were incubated for 1 hour at 30°C and applied to a 2.5ml CL2B gel filtration column, and fourteen 200μl fractions were collected (listed between the panels). 10μl of each fraction was separated by SDS-PAGE. Following electrophoresis the gels were dried and exposed to autoradiography film. **E)** Autoradiograph of IASD labelling of the contents of fraction 3 of samples A-D. Bcl2-cb5 molecules IASD labelled at cysteine 158 are indicated by *.

(Figure 7E, labeled Bcl-2). However, this fraction did not increase with the addition of Bim peptide or Bim peptide and Bax protein (Figure 7E). This suggests that additional membrane proteins are required for the Bcl-2 conformational change. It is interesting to note that even after the liposomes were solubilized by 1% Triton X-100, not all of cysteine 158 was modified by IASD (Figure 7E TX lanes). However the detergent control shows a clear increase in IASD labeling.

Model for the function of Bcl-2 in a multispanning transmembrane topology

Previously published data does not distinguish whether the conformational change in Bcl-2 is triggered directly by an interaction with an activated BH3 only protein (Bim peptide or tBid) or indirectly via an interaction with activated Bax/Bak. Our data suggests that Bcl-2 conformational change is due to the direct interaction of Bcl-2 with an activated BH3 only protein. After the addition of tBid to the isolated heavy membranes Bcl-2 changed conformation, and the addition of Bax did not potentiate this response (Figure 4B). This strongly suggests that more Bax does not increase the amount of Bcl-2 in a multispanning transmembrane topology, but increasing tBid causes more Bcl-2 to change conformation.

In the previous chapter it was determined that Bcl-2 interacts with Bid and tBid in the presence of CHAPS, but when the detergent was switched to Triton X-100 the interaction between the proteins was abolished (Chapter 3 Figure 5). One interpretation of this data is that after Bcl-2 changes conformation, tBid is released from Bcl-2. Therefore tBid interaction with Bcl-2 provoked a conformation change in Bcl-2, after

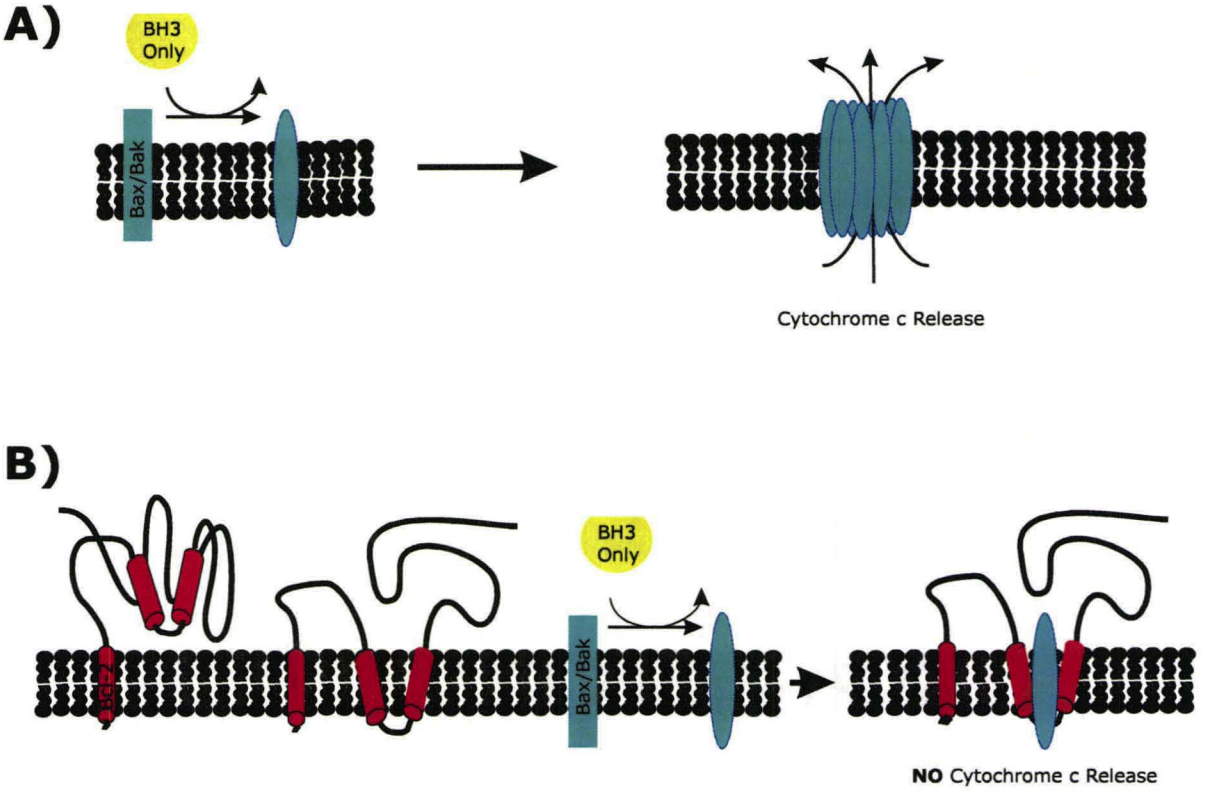

Figure 8 **Model for the function of Bcl-2 in a multispanning transmembrane topology. A)** tBid binds to Bax on the membrane and activates the protein, leading to Bax oligomerization and cytochrome c. **B)** tBid interacts with Bcl-2, provokes a conformation change in Bcl-2 from the tail-anchor topology to the multispanning transmembrane topology after which the tBid is released. Bcl-2 in the multispanning transmembrane topology inhibits the formation of Bax oligomers by sequestering tBid-activated Bax and antagonizing the release of cytochrome c.

which tBid was released. This "transient interaction" model would account for the stoichiometry we observed: exposure to 8.33nM tBid changes the conformation of 16nM Bcl-2. The fraction of Bcl-2 that has adopted the multispanning transmembrane topology could then bind tBid-activated Bax (Chapter 3 Figure 12). Consistent with this observation, Bcl-2 does bind to Bax in Triton X-100 and other non-ionic detergents (Hsu *et al.*, 1997; Mikhailov *et al.*, 2001). Taken together my data from this and the previous chapter suggest that the molecular mechanism for Bcl-2 inhibition of apoptosis is by Bcl-2 adopting a multispanning transmembrane topology. The most likely explanation for my data is that in the altered conformation Bcl-2 inhibits pore formation by binding pro-apoptotic molecules Bax and Bak that have themselves been activated by a BH3 only protein (Figure 8).

Chapter Five

Myc expression affects tBid and Bax induced cytochrome c release

INTRODUCTION

The cellular myc gene (*c-myc*) is a oncogene first linked to human cancer in the context of a chromosomal translocation in Burkitt's Lymphoma (Dalla-Favera *et al.*, 1982). The *c-myc* gene encodes a basic helix-loop-helix leucine zipper transcription factor, which can function as an activator or repressor of transcription (Reviewed in Patel *et al.*, 2004). Myc-responsive targets include representatives of virtually every major biochemical and regulatory process in the cell.

Cells lacking Myc are impared for growth and proliferation, and Myc knockout mice die during early embryogenensis (Davis *et al.*, 1992). Deletion of Myc in Rat-1 fibroblasts results in the extension of both the G_1 and G_2 phases of the cell cycle (Mateyak *et al.,* 1997). Independent of its effect on cell proliferation, myc expression also sensitizes cells to apoptotic stimuli (Wagner *et al.*, 1994; Soucie *et al.*, 2001; Askew *et al.*, 1991; Evan *et al.*, 1992, James *et al.*, 2002).

To study the influence of Myc expression on apoptosis our lab has compared an immortalized Rat-1 fibroblast cell line (HO15.19) in which both Myc alleles have been deleted through homologous recombination with an isogenic line in which an exogenous *c-myc* allele was reintroduced though retroviral infection (HOMyc3) (Mateyak *et al.*, 1997). The results indicate that Myc potentiates apoptosis by allowing Bax activation after translocation to mitochondria. Thus, in both cell lines Bax migrated to mitochondria after exposure to etoposide or taxol (apoptosis inducing drugs). However, Bax activation, as monitored by the detection of a normally inaccessible amino-terminal epitope using a monoclonal anitobody (6A7, Nechushtan *et al.*, 1999), was only evident in HOMyc3 cells

(Soucie *et al.*, 2001). Furthermore, release of cytochrome c only occurred after Bax activation (Soucie *et al.*, 2001). In mitochondria from Myc expressing cells, Bax formed higher molecular weight complexes following etoposide treatment, whereas in the absence of Myc only Bax monomers were detected in cells (Annis *et al.*, submitted). In this chapter I extend these observations by showing that mitochondria isolated from HO15.19 cells are resistant to tBid and Bax induced cytochrome c release in our purified cell free system. The data in this chapter and together with these previous observations demonstrate there may be an inhibitor of Bax oligomerization present on the HO15.19 mitochondria, and have been submitted for publication (Annis *et al.*, submitted).

RESULTS AND DISCUSSION

Myc expression sensitizes mitochondria to tBid and Bax induced cytochrome c release

Heavy membranes were isolated from HOMyc3 cells and HO15.19 cells and tested for sensitivity to tBid and Bax induced cytochrome c release to determine the role of Myc in this process. There was no cytochrome c released by 0.42 nM tBid (Figure 1A,C). However, a small amount of cytochrome c was released from the HOMyc3 mitochondria after exposure to 0.83 nM tBid, and was increased when 1.67 nM tBid was added (Figure 1A). Since these mitochondria do not contain detectable Bax but do contain Bak, cytochrome c release was likely due to tBid activation of Bak. When 0.42 nM tBid was added with 104 nM Bax most of the cytochrome c was released (Figure 1A,

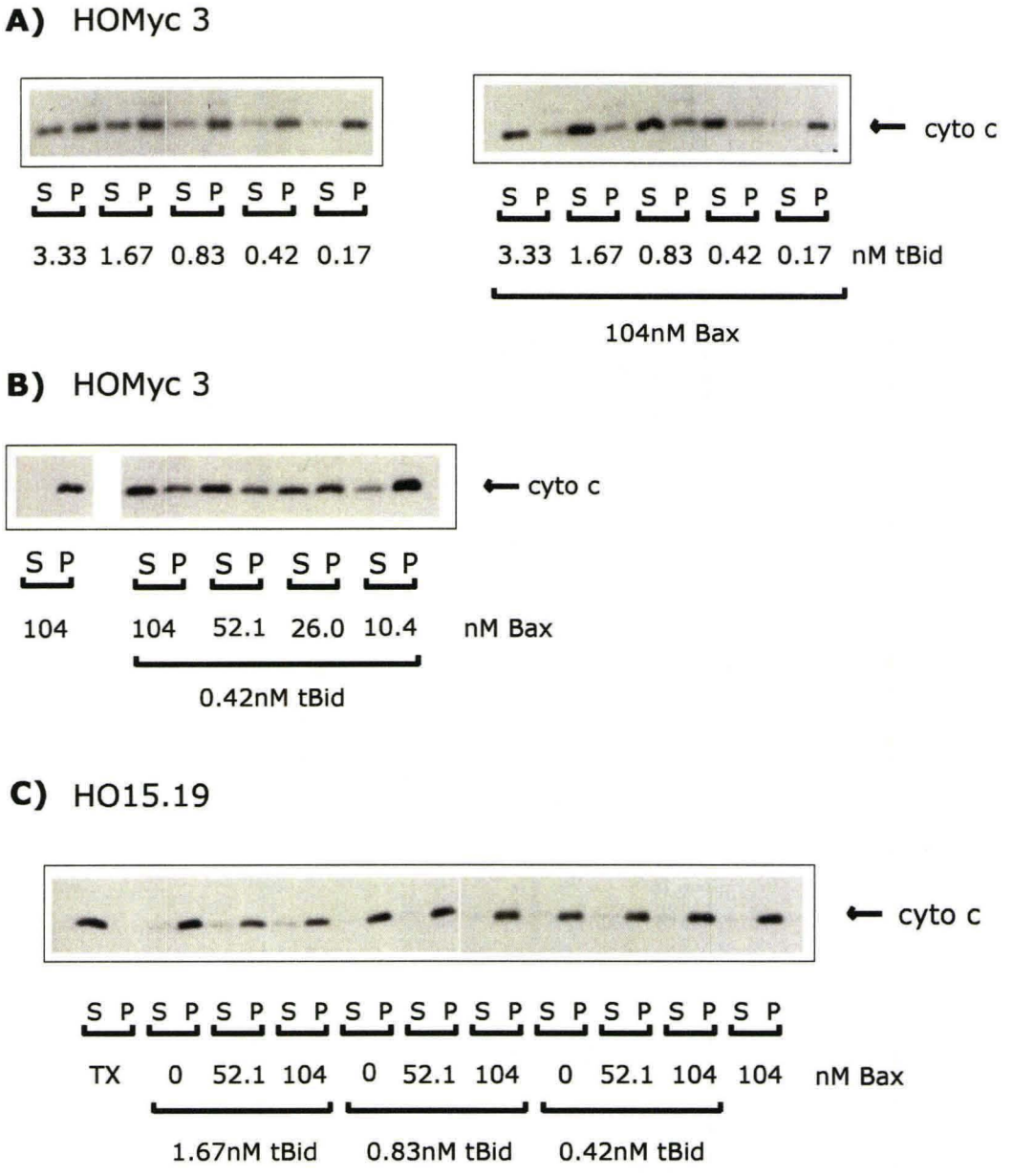

Figure 1 **HOMyc3 mitochondria are more sensitive than HO15.19 mitochondria to tBid induced cytochrome c release.** HOMyc3 heavy membranes (40µg of total membrane proteins) were incubated with **A)** increasing amounts of tBid with or without 104nM Bax and **B)** decreasing amounts of Bax in the presence and absence of 0.42nM tBid. **C)** HO15.19 heavy membranes (40µg of total membrane proteins) were incubated with tBid with or without Bax, as indicated. 1% Triton X-100 (TX) was used as positive control to cause the release of cytochrome c by detergent solubilization of the outer mitochondrial membrane. Cytochrome c (cyto c) release was monitored by immunoblotting the reaction supernatants (S) and repelleted mitochondria (P).

B). Because 0.42 nM tBid released cytochrome c only when Bax was added we conclude that cytochrome c release was primarily due to activation of Bax (rather than Bak) by tBid. In contrast, mitochondria isolated from HO15.19 cells were less sensitive, as 1.67 nM tBid with 104 nM Bax did not cause cytochrome c release (Figure 1C), and cytochrome c was only partially released after incubation with 8 nM tBid and 520nM Bax. Complete release of cytochrome c required 80 nM tBid with 520 nM Bax (Figure 2B). The increased sensitivity of the HOMyc3 mitochondria to tBid and Bax induced cytochrome c release is not due to increased Bak on the membrane as the amount of Bak does not differ in the two cell lines (Matthew Annis, PhD thesis, Dept. of Biochemistry, McMaster University). Surprisingly, HO15.19 cells were also more resistant to tBid without added Bax. Even after 833 nM tBid was added to the isolated heavy membranes no cytochrome c release was detected. Therefore Myc expression presumably sensitizes mitochondria to endogenous activated Bak as well as activated Bax.

The Rat-1MycER^{TAM} cells used in chapter 3 contain a chimeric fusion of Myc to the estrogen receptor (MycER^{TAM}) (Littlewood et al, 1995). This fusion protein has minimal transcriptional activity until the addition of tamoxifen, which binds to estrogen receptor moiety, changing the conformation of the fusion protein such that Myc becomes functional. However, this is a 'leaky' system with continued expression of endogenous Myc and partial activity of the MycER^{TAM} fusion protein even in the absence of tamoxifen (Mateyak et al, 1997). The MycER^{TAM} cells are therefore intermediate between the HO15.19 and HOMyc3 cells in expression of Myc. Exposure to 0.83 nM tBid and 260 nM Bax induces the release of cytochrome c from the Rat-1MycER^{TAM}

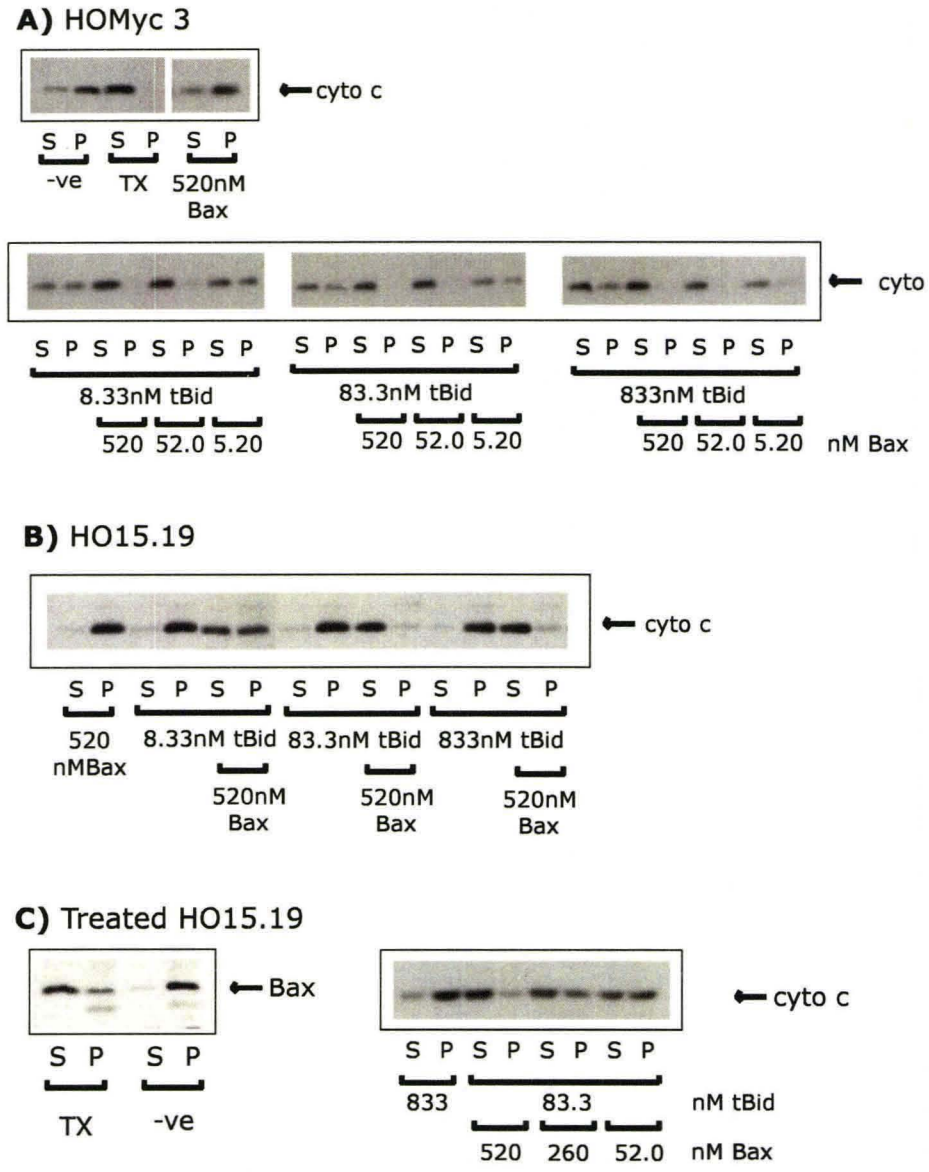

Figure 2 **HO15.19 mitochondria are resistant to tBid induced cytochrome c release even after 48 hour treatment with etoposide.** Cytochrome c (cyto c) release was monitored by immunoblotting the reaction supernatants (S) and pelleted mitochondria (P) from **A)** HOMyc3 and **B)** HO15.19 heavy membranes that were incubated with tBid with and without Bax, as indicated. The highest concentration of Bax was added to the isolated heavy membranes to determine its effect on the mitochondria. 1% Triton X-100 (TX) was used as positive control. **C)** Mitochondria isolated from HO15.19 cells that had been treated with etoposide for 48 hours were immunoblotted for the presence of endogenous Bax. The isolated heavy membranes were incubated with tBid and Bax as indicated and monitored for cytochrome c release (cyto c) by immunoblotting.

mitochondria (Figure 7 in chapter 3), twice the amount of tBid and Bax required to release cytochrome c from the HOMyc3 mitochondria, but significantly less than for HO15.19 mitochondria. Therefore, even in this cell line where there is relatively little deregulated Myc expression, mitochondria are sensitized to tBid and Bax induced cytochrome c release.

After HO15.19 cells have been exposed to etoposide for 48 hours, 40% of the total amount of Bax in the cell has migrated to the mitochondria (Soucie et al, 2001). Therefore mitochondria from these cells contain endogenous Bax (Figure 2C). Despite the presence of Bak and Bax, these mitochondria were insensitive to tBid (and Bax) induced cytochrome c release (Figure 2C) suggesting that Myc-controlled factor(s) determine sensitivity to tBid and Bax induced cytochrome c release.

An inhibitor of Bax oligomerization is present on the HO15.19 mitochondria

To determine whether the factor(s) inhibiting cytochrome c release was present in mitochondria or if either sensitization or inhibition was conferred by something in the cytosol of either cell line I took advantage of the cell free system described in Chapter 3 to perform cytosol switching experiments. Exposure of HO15.19 mitochondria with HOMyc3 cytosol to the combination of 1.67 nM tBid and 104 nM Bax did not release cytochrome c (Figure 3A sample 3). Moreover, cytosol from HO15.19 does not inhibit tBid and Bax induced cytochrome c release from HOMcy3 mitochondria (Figure 3B sample 6). Therefore, either mitochondria from HOMyc3 cells contain an activator or

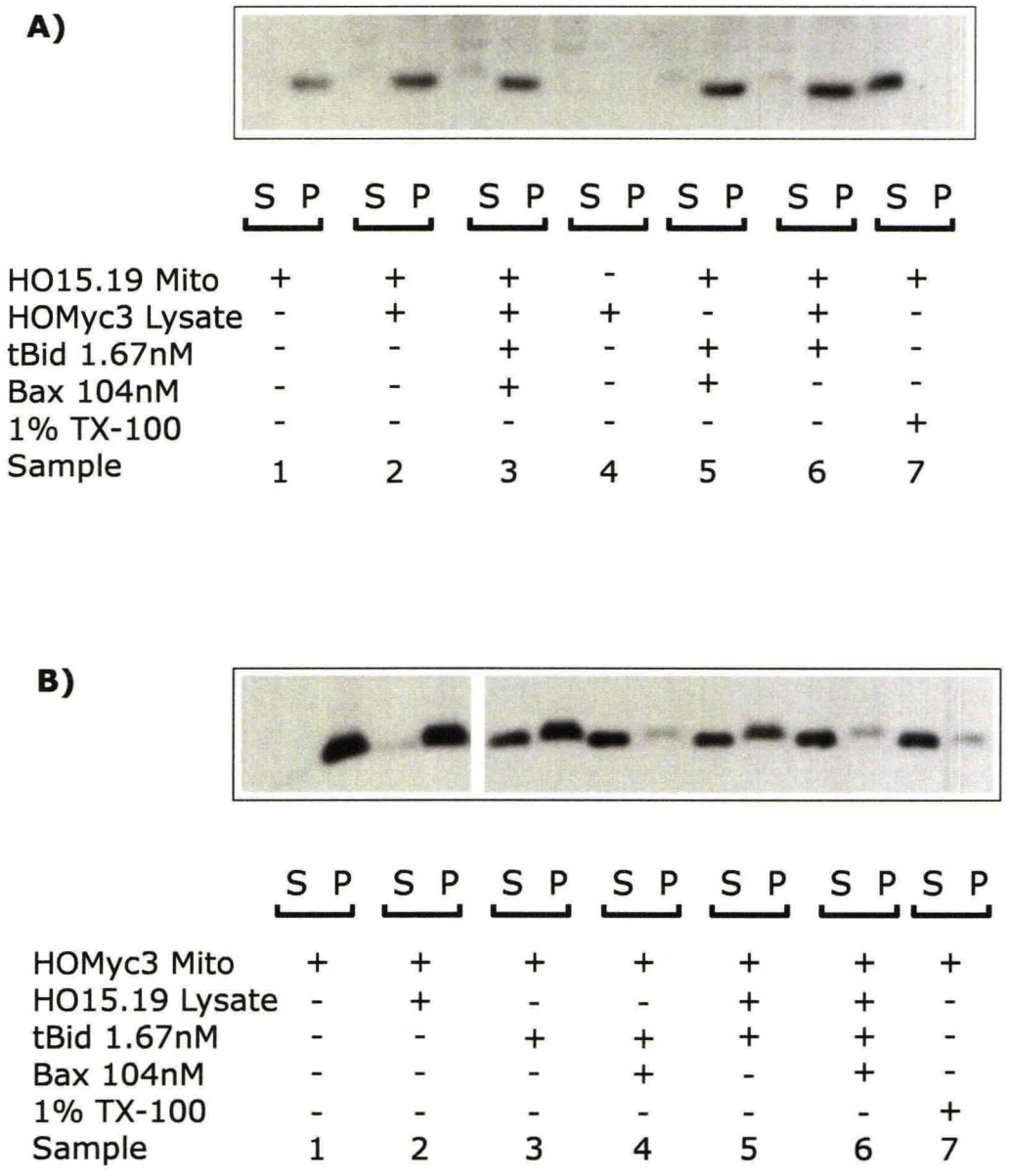

Figure 3 **HOMyc3 cytosol is unable to sensitize HO15.19 heavy membranes to tBid and Bax induced cytochrome c release.** Heavy membranes isolated from **A)** HO15.19 or **B)** HOMyc3 cells were incubated with tBid, Bax and cytosol as indicated below the immunoblots. Cytochrome c release was monitored by pelleting the mitochondria and visualizing the cytochrome c in the supernatant (S) and pellet (P) fractions after SPS-PAGE and immunoblotting. 1% Triton X-100 (TX-100) was used as a positive control to solubilize the mitochondria.

HO15.19 mitochondria contain one or more components that inhibit tBid and Bax mediated cytochrome c release.

These results are consistent with *in vitro* observations made using the cell lines. In Myc expressing cells Bax formed higher molecular weight complexes following etoposide treatment, consistent with other reports in which oligomerization is required for the cytochrome c releasing activity of Bax. In HO15.19 cells Bax oligomerization was blocked and only Bax monomers were found on the mitochondrial membrane after etoposide treatment (Annis *et al.*, submitted). The simplest interpretation of the results obtained using cell lines and the cell free system is that an inhibitor was present on the mitochondria of HO15.19 cells that was repressed in HOMyc3 cells, or that Myc positively regulates the expression of a component necessary for Bax oligomerization.

In vitro, tBid-activated Bax has been shown to form large molecular weight oligomers in liposomes (Kuwana *et al.*, 2002; Yethon *et al.*, 2003) that permeabilized liposomes to allow release of dextrans up to 2000 kDa (Kuwana *et al.*, 2002). Thus in this system other proteins are not required for tBid-activated Bax oligomerization or pore formation. Therefore, the most likely explanation for the resistance of HO15.19 mitochondria to tBid and Bax induced cytochrome c release is the presence of an inhibitor. As a transcription factor, Myc has been shown to both activate and repress the transcription of many target genes both directly and indirectly. Thus, our current interpretation of the results in Figures 2 and 3 is that Myc represses the transcription of an inhibitor of Bax oligomerization.

Chapter Six

Discussion

Apoptosis is an essential process that plays a critical role in the normal development and tissue homeostasis of multi-cellular organisms. The importance of apoptosis is revealed when the normal regulation of the process is disrupted. Increased apoptosis results in several neurodegenerative diseases such as Parkinson's disease, Huntington's disease and muscular degeneration. In contrast, decreased cell death is a contributing factor in propagating viral infections and cancers (Thompson, 1995). Thus understanding the regulation of apoptosis has implications in several human diseases.

One potent regulator of apoptosis is the Bcl-2 family of proteins (reviewed in Antonsson 2004). As demonstrated by the results presented in this thesis, the antagonistic relationship between Bcl-2 family members is critical for the regulation of cell death. Mitochondria are a central target for the activity of these proteins and therefore a cell free assay was developed in which isolated heavy membranes from Rat1-MycER^{TAM} cells were used to study the interactions between Bcl-2, Bax and tBid.

Assay Development

Due to the complexity and redundancy of a mammalian cell it is extremely difficult to tease out the function of individual proteins within a live cell. Therefore, to examine the function of Bcl-2 in preventing tBid and Bax induced apoptosis, a cell free assay was needed. An assay based entirely on purified recombinant proteins would have been ideal; however purification of functional recombinant full-length Bcl-2 protein is problematic. As an alternate source of Bcl-2, mitochondria were isolated from cells overexpressing Bcl-2. The use of isolated mitochondria also allowed a convenient

measure of Bcl-2 function, as apoptosis can be monitored by assaying cytochrome c release from mitochondria. Therefore a cell free assay was developed using isolated energized mitochondria that are susceptible to physiologically relevant release of cytochrome c during apoptosis (Chapter 3) to monitor the interaction and function of Bcl-2, Bax and tBid.

Bcl-2 inhibits tBid and Bax induced cytochrome c

Bax alone is unable to induce cytochrome c release from isolated mitochondria. However, the addition of tBid and Bax together leads to complete cytochrome c release. Bcl-2 can inhibit tBid and Bax induced cytochrome c release in this system (Chapter 3). Examining the proteins separately showed that the addition of tBid resulted in the oligomerization of Bax on the membrane and that Bcl-2 inhibited Bax oligomerization and prevented Bax pore formation. Bcl-2 and Bax formed a complex after the addition of tBid, suggesting that Bcl-2 prevents apoptosis by inhibiting the formation of Bax oligomers through the sequesteration of tBid-activated Bax.

Activated Bak protein can induce oligomerization and activation of other Bak molecules (Ruffolo *et al.*, 2003). Since Bax and Bak proteins are very similar, it is possible that tBid activated Bax can also trigger Bax auto oligomerization. Therefore Bcl-2 may preferentially interact with tBid-activated Bax by blocking oligomerization and further activation of other Bax proteins, thereby preventing the release of cytochrome c.

Bcl-2 inhibition of Bax oligomerization and cytochrome c release can be overcome by increasing the amount of tBid added. After the addition of tBid and Bax under conditions where Bcl-2 was unable to prevent cytochrome c release, high molecular weight oligomers of Bax were detected. These observations are consistent with a threshold model of cell death regulation, in which Bcl-2 inhibits apoptosis until there is an excess of tBid-activated Bax proteins.

Membrane topology of Bcl-2

Bcl-2 undergoes a conformational change on the membrane after exposure to apoptotic stimuli. In healthy cells Bcl-2 adopts a tail-anchored topology with the hydrophobic carboxyl-terminus in helix 9 inserted into the membrane. After induction of apoptosis, Bcl-2 changes to a conformation in which cysteine 158 in helix 5 is inserted into the lipid bilayer (Kim *et al.*, 2004). Exposure of Bcl-2 in isolated heavy membranes to either recombinant tBid or a peptide corresponding to the BH3 domain of Bim triggered a similar conformational change. (Chapter 4). Bcl-2 proteins that have inserted cysteine 158 into the membrane are competent to prevent cytochrome c release. This suggests that either this change is required for the function of Bcl-2 or that the conformational change is a result of Bcl-2 having carried out its function.

The changes in Bcl-2 membrane topology appear to be coincident with a Bcl-2-Bax complex formation. (Chapter 3, Figure 11). This raises the intriguing possibility that the interaction between Bcl-2 and Bax only occurs after Bcl-2 changes topology. Consistent with this is the observation that non-ionic detergents artificially induce

changes to Bax (making it 6A7 positive) and allow it to bind with Bcl-2 (Hsu *et al.*, 1997) suggesting that conformational changes in both these proteins are required for heterodimerization. Binding between the Bcl-2 family members is mediated by the BH domains of these proteins. In the solution structure of Bax, the BH3 domain is buried and therefore inaccessible to Bcl-2. However, after activation by tBid, the Bax BH3 domain may be in a different orientation in which it is available to bind to Bcl-2. Bcl-2 may also need to change membrane topology to bind activated Bax, thereby inhibiting Bax oligomerization and preventing cytochrome c release.

Cytochrome c release in the absence of Myc

When Myc is not expressed in cells, the mitochondria contain one or more components that inhibit tBid and Bax mediated cytochrome c release (Chapter 5) compared to the response of mitochondria from Myc over-expressing cells. Cytosol switching experiments confirmed that the defect in cytochrome c release after exposure to apoptotic stimuli in cells lacking myc is inherent to the mitochondria and not conferred by a factor in the cytosol of either cell line. Therefore it is possible that Myc expression represses the transcription of a membrane bound inhibitor of Bax oligomerization.

In this thesis the development of a cell free assay is presented. This assay can be used to study apoptosis and analyze the role of specific proteins in a controlled fashion. The results obtained with this cell free assay have further elucidated the mechanism of action of Bcl-2, Bax and tBid. I have shown that Bcl-2 inhibits the oligomerization of tBid-activated Bax. In addition I have demonstrated that Bcl-2 changes topology on the

membrane as part of its anti-apoptotic function. Finally, I have shown that the tBid/ Bax induced cytochrome c release from mitochondria requires an organelle-intrinsic Myc-dependent factor.

REFERENCES

Adachi, S., R. A. Gottlieb, and B. M. Babior. 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.

Andrews, D. 1989. Examining protein translocation in cell-free systems and microinjected Xenopus oocytes. Biotechniques 7:960-962, 964-967.

Annis, M. G., E. L. Soucie, P. J. Dlugosz, L. Z. Penn, B. Leber, and D. W. Andrews. Bax intergrates into membranes as a multi-spanning membrane protein that subsequently oligomerizes to form pores during apoptosis. *Submitted.*

Annis, M. G., J. A. Yethon, B. Leber, and D. W. Andrews. 2004. There is more to life and death than mitochondria: Bcl-2 proteins at the endoplasmic reticulum. Biochim Biophys Acta 1644:115-123.

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

Antonsson, B. 2004. Mitochondria and the Bcl-2 family proteins in apoptosis signaling pathways. Mol Cell Biochem 256-257:141-155.

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

Antonsson, B., S. Montessuit, S. Lauper, R. Eskes, and J. C. Martinou. 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., B. Gaume, M. Karbowski, J. C. Sharpe, F. Cecconi, and R. J. Youle. 2003. Mitochondrial release of AIF and EndoG requires caspase activation downstream of Bax/Bak-mediated permeabilization. EMBO J 22:4385-4399.

Askew, D. S., R. A. Ashmun, B. C. Simmons, and J. L. Cleveland. 1991. Constitutive c-myc expression in an IL-3-dependent myeloid cell line suppresses cell cycle arrest and accelerates apoptosis. Oncogene 6:1915-1922.

Baffy, G., T. Miyashita, J. R. Williamson, and J. C. Reed. 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.

Bassik, M. C., L. Scorrano, S. A. Oakes, T. Pozzan, and S. J. Korsmeyer. 2004. Phosphorylation of BCL-2 regulates ER Ca(2+) homeostasis and apoptosis. EMBO J 23:1207-1216.

Brustovetsky, N., J. M. Dubinsky, B. Antonsson, and R. Jemmerson. 2003. Two pathways for tBID-induced cytochrome c release from rat brain mitochondria: BAK-versus BAX-dependence. J Neurochem 84:196-207.

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

Cartron, P. F., P. Juin, L. Oliver, S. Martin, K. Meflah, and F. M. Vallette. 2003. Nonredundant role of Bax and Bak in Bid-mediated apoptosis. Mol Cell Biol 23:4701-4712.

Cheng, E. H., M. C. Wei, S. Weiler, R. A. Flavell, T. W. Mak, T. Lindsten, and S. J. Korsmeyer. 2001. BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. Mol Cell 8:705-511.

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

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

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

Cleary, M. L., S. D. Smith, and J. Sklar. 1986. Cloning and structural analysis of cDNAs for bcl-2 and a hybrid bcl-2/immunoglobulin transcript resulting from the t(14;18) translocation. Cell 47:19-28.

Cortese, J. D., A. L. Voglino, and C. R. Hackenbrock. 1998. Multiple conformations of physiological membrane-bound cytochrome c. Biochemistry 37:6402-6409.

Dalla-Favera, R., E. P. Gelmann, S. Martinotti, G. Franchini, T. S. Papas, R. C. Gallo, and F. Wong-Staal. 1982. Cloning and characterization of different human sequences related to the onc gene (v-myc) of avian myelocytomatosis virus (MC29). Proc Natl Acad Sci U S A 79:6497-6501.

Davis, A. C., M. Wims, G. D. Spotts, S. R. Hann, and A. Bradley. 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.

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

Distelhorst, C. W., M. Lam, and T. S. McCormick. 1996. Bcl-2 inhibits hydrogen peroxide-induced ER Ca2+ pool depletion. Oncogene 12:2051-2055.

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

Epand, R. F., J. C. Martinou, M. Fornallaz-Mulhauser, D. W. Hughes, and R. M. Epand. 2002. The apoptotic protein tBid promotes leakage by altering membrane curvature. J Biol Chem 277:32632-32639.

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

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

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

Fesik, S. W. 2000. Insights into programmed cell death through structural biology. Cell 103:273-282.

Fiebig, A., W. Zhu, C. Hollerbach, B. Leber, and D. W. Andrews. Bcl-XL is more potent an inhibitor of apoptosis and differs qualitatively from Bcl-2 in breast cancer cells. *Submitted*

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

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

Griffiths, G. J., L. Dubrez, C. P. Morgan, N. A. Jones, J. Whitehouse, B. M. Corfe, C. Dive, and J. A. Hickman. 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.

Gross, A., X. M. Yin, K. Wang, M. C. Wei, J. Jockel, C. Milliman, H. Erdjument-Bromage, P. Tempst, and S. J. Korsmeyer. 1999. Caspase cleaved BID targets mitochondria and is required for cytochrome c release, while BCL-XL prevents this release but not tumor necrosis factor-R1/Fas death. J Biol Chem 274:1156-1163.

Gurevich, V. V., I. D. Pokrovskaya, T. A. Obukhova, and S. A. Zozulya. 1991. Preparative in vitro mRNA synthesis using SP6 and T7 RNA polymerases. Anal Biochem 195:207-213.

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

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

Hengartner, M. O. 2000. The biochemistry of apoptosis. Nature 407:770-776.

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

Hsu, Y. T., and R. J. Youle. 1998. Bax in murine thymus is a soluble monomeric protein that displays differential detergent-induced conformations. J Biol Chem 273:10777-10783.

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

Hu, X., Z. Han, J. H. Wyche, and E. A. Hendrickson. 2003. Helix 6 of tBid is necessary but not sufficient for mitochondrial binding activity. Apoptosis 8:277-289.

Jackson, R. J., and T. Hunt. 1983. Preparation and use of nuclease-treated rabbit reticulocyte lysates for the translation of eukaryotic messenger RNA. Methods Enzymol 96:50-74.

James, L., and R. N. Eisenman. 2002. Myc and Mad bHLHZ domains possess identical DNA-binding specificities but only partially overlapping functions in vivo. Proc Natl Acad Sci U S A 99:10429-10434.

Joza, N., S. A. Susin, E. Daugas, W. L. Stanford, S. K. Cho, C. Y. Li, T. Sasaki, A. J. Elia, H. Y. Cheng, L. Ravagnan, K. F. Ferri, N. Zamzami, A. Wakeham, R. Hakem, H. Yoshida, Y. Y. Kong, T. W. Mak, J. C. Zuniga-Pflucker, G. Kroemer, and J. M. Penninger. 2001. Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. Nature 410:549-554.

Kim, P. K., M. G. Annis, P. J. Dlugosz, B. Leber, and D. W. Andrews. 2004. During apoptosis bcl-2 changes membrane topology at both the endoplasmic reticulum and mitochondria. Mol Cell 14:523-239.

Krajewski, S., S. Tanaka, S. Takayama, M. J. Schibler, W. Fenton, and J. C. Reed. 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., B. Walker, O. Braha, and H. Bayley. 1994. Surface labeling of key residues during assembly of the transmembrane pore formed by staphylococcal alpha-hemolysin. FEBS Lett 356:66-71.

Kudla, G., S. Montessuit, R. Eskes, C. Berrier, J. C. Martinou, A. Ghazi, and B. Antonsson. 2000. The destabilization of lipid membranes induced by the C-terminal fragment of caspase 8-cleaved bid is inhibited by the N-terminal fragment. J Biol Chem 275:22713-22718.

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

Lam, M., G. Dubyak, L. Chen, G. Nunez, R. L. Miesfeld, and C. W. Distelhorst. 1994. Evidence that BCL-2 represses apoptosis by regulating endoplasmic reticulum-associated Ca2+ fluxes. Proc Natl Acad Sci U S A 91:6569-6573.

Legate, K. R., D. Falcone, and D. W. Andrews. 2000. Nucleotide-dependent binding of the GTPase domain of the signal recognition particle receptor beta-subunit to the alpha-subunit. J Biol Chem 275:27439-27446.

Letai, A., M. C. Bassik, L. D. Walensky, M. D. Sorcinelli, S. Weiler, and S. J. Korsmeyer. 2002. Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. Cancer Cell 2:183-192.

Lindsten, T., A. J. Ross, A. King, W. X. Zong, J. C. Rathmell, H. A. Shiels, E. Ulrich, K. G. Waymire, P. Mahar, K. Frauwirth, Y. Chen, M. Wei, V. M. Eng, D. M. Adelman, M. C. Simon, A. Ma, J. A. Golden, G. Evan, S. J. Korsmeyer, G. R. MacGregor, and C. B. Thompson. 2000. The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. Mol Cell 6:1389-1399.

Liu, X., S. Dai, Y. Zhu, P. Marrack, and J. W. Kappler. 2003. The structure of a Bcl-xL/Bim fragment complex: implications for Bim function. Immunity 19:341-52.

Lutter, M., G. A. Perkins, and X. Wang. 2001. The pro-apoptotic Bcl-2 family member tBid localizes to mitochondrial contact sites. BMC Cell Biol 2:22.

Madesh, M., B. Antonsson, S. M. Srinivasula, E. S. Alnemri, and G. Hajnoczky. 2002. Rapid kinetics of tBid-induced cytochrome c and Smac/DIABLO release and mitochondrial depolarization. J Biol Chem 277:5651-5659.

Magnelli, L., M. Cinelli, A. Turchetti, and V. P. Chiarugi. 1994. Bcl-2 overexpression abolishes early calcium waving preceding apoptosis in NIH-3T3 murine fibroblasts. Biochem Biophys Res Commun 204:84-90.

Marani, M., T. Tenev, D. Hancock, J. Downward, and N. R. Lemoine. 2002. Identification of novel isoforms of the BH3 domain protein Bim which directly activate Bax to trigger apoptosis. Mol Cell Biol 22:3577-3589.

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

McDonnell, J. M., D. Fushman, C. L. Milliman, S. J. Korsmeyer, and D. Cowburn. 1999. Solution structure of the proapoptotic molecule BID: a structural basis for apoptotic agonists and antagonists. Cell 96:625-634.

Mikhailov, V., M. Mikhailova, K. Degenhardt, M. A. Venkatachalam, E. White, and P. Saikumar. 2003. Association of Bax and Bak homo-oligomers in mitochondria. Bax requirement for Bak reorganization and cytochrome c release. J Biol Chem 278:5367-5376.

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

Narita, M., S. Shimizu, T. Ito, T. Chittenden, R. J. Lutz, H. Matsuda, and Y. Tsujimoto. 1998. Bax interacts with the permeability transition pore to induce permeability transition

and cytochrome c release in isolated mitochondria. Proc Natl Acad Sci U S A 95:14681-14686.

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

O'Connor, L., A. Strasser, L. A. O'Reilly, G. Hausmann, J. M. Adams, S. Cory, and D. C. Huang. 1998. Bim: a novel member of the Bcl-2 family that promotes apoptosis. EMBO J 17:384-395.

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

Ott, M., J. D. Robertson, V. Gogvadze, B. Zhivotovsky, and S. Orrenius. 2002. Cytochrome c release from mitochondria proceeds by a two-step process. Proc Natl Acad Sci 99:1259-1263.

Patel, J. H., A. P. Loboda, M. K. Showe, L. C. Showe, and S. B. McMahon. 2004. Analysis of genomic targets reveals complex functions of MYC. Nat Rev Cancer 4:562-568.

Perez, D., and E. White. 2000. TNF-alpha signals apoptosis through a bid-dependent conformational change in Bax that is inhibited by E1B 19K. Mol Cell 6:53-63.

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

Petros, A. M., D. G. Nettesheim, Y. Wang, E. T. Olejniczak, R. P. Meadows, J. Mack, K. Swift, E. D. Matayoshi, H. Zhang, C. B. Thompson, and S. W. Fesik. 2000. Rationale for Bcl-xL/Bad peptide complex formation from structure, mutagenesis, and biophysical studies. Protein Sci 9:2528-2534.

Petros, A. M., E. T. Olejniczak, and S. W. Fesik. 2004. Structural biology of the Bcl-2 family of proteins. Biochim Biophys Acta 1644:83-94.

Pinton, P., D. Ferrari, P. Magalhaes, K. Schulze-Osthoff, F. Di Virgilio, T. Pozzan, and R. Rizzuto. 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.

Polster, B. M., G. Basanez, M. Young, M. Suzuki, and G. Fiskum. 2003. Inhibition of Bax-induced cytochrome c release from neural cell and brain mitochondria by dibucaine and propranolol. J Neurosci 23:2735-2743.

Puthalakath, H., D. C. Huang, L. A. O'Reilly, S. M. King, and A. Strasser. 1999. The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. Mol Cell 3:287-296.

Rodriguez, J., and Y. Lazebnik. 1999. Caspase-9 and APAF-1 form an active holoenzyme. Genes Dev 13:3179-3184.

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

Rudner, J., A. Lepple-Wienhues, W. Budach, J. Berschauer, B. Friedrich, S. Wesselborg, K. Schulze-Osthoff, and C. Belka. 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.

Ruffolo, S. C., D. G. Breckenridge, M. Nguyen, I. S. Goping, A. Gross, S. J. Korsmeyer, H. Li, J. Yuan, and G. C. Shore. 2000. BID-dependent and BID-independent pathways for BAX insertion into mitochondria. Cell Death Differ 7:1101-1108.

Ruffolo, S. C., and G. C. Shore. 2003. BCL-2 selectively interacts with the BID-induced open conformer of BAK, inhibiting BAK auto-oligomerization. J Biol Chem 278:25039-52045.

Saito, M., S. J. Korsmeyer, and P. H. Schlesinger. 2000. BAX-dependent transport of cytochrome c reconstituted in pure liposomes. Nat Cell Biol 2:553-555.

Sattler, M., H. Liang, D. Nettesheim, R. P. Meadows, J. E. Harlan, M. Eberstadt, H. S. Yoon, S. B. Shuker, B. S. Chang, A. J. Minn, C. B. Thompson, and S. W. Fesik. 1997. Structure of Bcl-xL-Bak peptide complex: recognition between regulators of apoptosis. Science 275:983-986.

Schagger, H., and G. von Jagow. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem 166:368-379.

Schendel, S. L., R. Azimov, K. Pawlowski, A. Godzik, B. L. Kagan, and J. C. Reed. 1999. Ion channel activity of the BH3 only Bcl-2 family member, BID. J Biol Chem 274:21932-21936.

Scorrano, L., and S. J. Korsmeyer. 2003. Mechanisms of cytochrome c release by proapoptotic BCL-2 family members. Biochem Biophys Res Commun 304:437-444.

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

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

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

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

Thomenius, M. J., N. S. Wang, E. Z. Reineks, Z. Wang, and C. W. Distelhorst. 2003. Bcl-2 on the endoplasmic reticulum regulates Bax activity by binding to BH3-only proteins. J Biol Chem 278:6243-6250.

Tsujimoto, Y., and C. M. Croce. 1986. Analysis of the structure, transcripts, and protein products of bcl-2, the gene involved in human follicular lymphoma. Proc Natl Acad Sci 83:5214-5218.

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

von Ahsen, O., C. Renken, G. Perkins, R. M. Kluck, E. Bossy-Wetzel, and D. D. Newmeyer. 2000. Preservation of mitochondrial structure and function after Bid- or Bax-mediated cytochrome c release. J Cell Biol 150:1027-1036.

Wagner, A. J., J. M. Kokontis, and N. Hay. 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.

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

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

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

Xue, D., and H. R. Horvitz. 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.

Yan, L., Q. Miao, Y. Sun, and F. Yang. 2003. tBid forms a pore in the liposome membrane. FEBS Lett 555:545-550.

Yethon, J. A., R. F. Epand, B. Leber, R. M. Epand, and D. W. Andrews. 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.

Yi, X., X. M. Yin, and Z. Dong. 2003. Inhibition of Bid-induced apoptosis by Bcl-2. tBid insertion, Bax translocation, and Bax/Bak oligomerization suppressed. J Biol Chem 278:16992-16999.

Yu, S. W., H. Wang, M. F. Poitras, C. Coombs, W. J. Bowers, H. J. Federoff, G. G. Poirier, T. M. Dawson, and V. L. Dawson. 2002. Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. Science 297:259-263.

Zamzami, N., and G. Kroemer. 2001. The mitochondrion in apoptosis: how Pandora's box opens. Nat Rev Mol Cell Biol 2:67-71.

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

Zhai, D., X. Huang, X. Han, and F. Yang. 2000. Characterization of tBid-induced cytochrome c release from mitochondria and liposomes. FEBS Lett 472:293-296.

Zhong, L. T., T. Sarafian, D. J. Kane, A. C. Charles, S. P. Mah, R. H. Edwards, and D. E. Bredesen. 1993. bcl-2 inhibits death of central neural cells induced by multiple agents. Proc Natl Acad Sci 90:4533-4537.

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

Zhu, W., A. Cowie, G. W. Wasfy, L. Z. Penn, B. Leber, and D. W. Andrews. 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., T. Lindsten, A. J. Ross, G. R. MacGregor, and C. B. Thompson. 2001. BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak. Genes Dev 15:1481-1486.

Zou, H., Y. Li, X. Liu, and X. Wang. 1999. An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. J Biol Chem 274:11549-11556.

Appendix

Intein-tBid Overexpression and Attempted tBid Purification

INTRODUCTION

One of the methods attempted to purify tBid was to use an intein fusion system. This system involves the fusion of a target protein to a protein element called intein (protein intron). This precursor fusion protein can be induced to undergo an autocatalytic splicing reaction that cleaves the intein segment from the target protein. A small chitin binding domain (CBD) from *Bacillus circulans* (NEB Impact manual) was added to the intein to allow purification of the fusion protein using a chitin column. The intein initiates the self-cleavage reaction in the presence of thiols, such as DTT and β-mercaptoethanol. The intein undergoes specific self-cleavage, which releases the target protein form the chitin bound intein tag. The two amino-terminal intein vectors tBid was cloned into, pIMC103 and pTYB12, did not undergo significant amounts of self-cleavage. This method was abandoned as a means of obtaining untagged purified tBid however the following section describes the conditions used in the attempt to induce intein self-cleavage to liberate tBid and the results obtained.

MATERIALS AND METHODS

Intein-tBid overexpression vectors, pIMC103-tBid (pMAC 1633) and pTYB12-tBid (pMAC 1874) were transformed into *E.coli* DH5α cells and *E.coli* BL21 (DE3) cells respectively. The cells were grown in LB medium supplemented with 50μg/ml amplicillin. For the DH5α cells, 10 ml cultures were grown to saturation from a single colony and used to inoculate 1L of culture. The cells were then grown at 30°C for 7.5

hours without induction. The expression of the LacIq gene from the plamsid does not provide stringent repression of the tac promoter, therefore the same amount of the target protein was produced in the presence or absence of the inducer in this case. When using BL21 DE3 cells 10 ml cultures were grown to saturation from a single colony and used to inoculate 1L of culture. The cells were then grown at 30°C to an OD_{600} of 0.6, at which point the culture was induced using 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) and grown for 4 hours. The cells were subsequently pelleted by centrifugation at 4,400 x g for 15 minutes. Cells were resuspended in 25 ml of Intein Lysis Buffer (0.02 M Tris-HCl pH 8.5, 0.5 M NaCl, 0.5 mM EDTA, 0.05% CHAPS, 1 mM PMSF, Complete Protease Inhibitors (Roche) and 0.1mg/ml DNase). The cell suspension was then lysed by three passes though a French Pressure cell at 20,000psi. Unlysed cells were separated from the lysate by centrifugation at 23,000 x g for 30 min. The lysate was incubated with 2.5 ml of Chitin Beads Slurry (New England Biolabs) that had been pre-equilibrated with Intein Lysis Buffer for 1.5 hours at 4°C. The resin was then packed in a 20 ml disposable column (BioRad) and washed with 35 ml of Intein Column Buffer (10 mM Hepes pH 7.4, 100 mM NaCl, 0.2 mM EDTA, 20% glycerol). To induce intein self cleavage 5 ml of Intein Column Buffer containing 100 mM β-mercaptoethanol (for pIMC103-tBid) or 100mM DTT (for pTYB12-tBid) was applied over the column and with approximately 1 ml of buffer remaining above the resin, the column was capped kept at 4°C for 40 hours. tBid was then eluted from the column with Intein Column Buffer.

TLB added to the fractions before running on an SDS-PAGE gel did not contain DTT.

(DTT can stimulate intein self-cleavage which could confound the results obtained under assay conditions.)

Optimization

After the cell lysate was incubated with 2.5 ml of Chitin Beads Slurry that had been pre-equilibrated with Intein Column Buffer for 1.5 hours at 4°C and washed with 35 ml of Intein Lysis Buffer 50 µl of the resin was added to 75 µl of the appropriate cleavage buffer and the reactions were incubated at the appropriate temperature for 40 hours. Reactions were set up according to Table 1. The resin was then spun down in a microfuge at 4,000 rpm for 30 seconds. The supernatant was removed and analyzed by SDS-PAGE and immunoblotted with anti-Bid antibody. TLB added to the fractions before running on an SDS-PAGE gel did not contain DTT.

Table 1 **A summary of the reagent and conditions used to induce intein self-cleavage**

Intein Column Buffer with	**pH**	**Temp-erature**	**Intein Column Buffer with**	**pH**	**Temp-erature**
100mM DTT	8	4°C	100mM Cysteine	8	4°C
		16°C			16°C
		24°C			24°C
		37°C			37°C
	8.5	4°C		8.5	4°C
		16°C			16°C
		24°C			24°C
		37°C			37°C
	9	4°C		9	4°C
		16°C			16°C
		24°C			24°C
		37°C			37°C

Intein Column Buffer with	**pH**	**Temp-erature**	**Intein Column Buffer with**	**pH**	**Temp-erature**
100mM Hydroxy-amine	8	4°C	100mM β-mercapto ethanol	8	4°C
		16°C			16°C
		24°C			24°C
		37°C			37°C
	8.5	4°C		8.5	4°C
		16°C			16°C
		24°C			24°C
		37°C			37°C
	9	4°C		9	4°C
		16°C			16°C
		24°C			24°C
		37°C			37°C

RESULTS AND DISCUSSION

pIMC103-tBid

An amino terminal intein vector, pIMC103 (pMAC1411) had been previously obtained from NEB and tBid was cloned into the vector. *E.coli* DH5α cells were transformed with pIMC103-tBid and the expression of intein-tBid after 7.5 hours of growth was monitored by immunoblotting (Figure 1B Lysate). However, it was found that the intein did not undergo significant amounts of self-cleavage upon activation with 100mM DTT. The fusion protein remained bound to the resin (figure 1A and 1B lane labeled resin) and any free tBid which was eluted could not be visualized by Coomassie blue staining. The small amount of eluted tBid could only be detected by immunoblotting using anti-Bid antibody (figure 1B lanes 1-8).

PTYB12-tBid

Since pIMC103 was only a trial vector, and Dr Jeremy Yethon in our lab found that specific inteins significantly change the expression levels of the fusion protein and self-cleavage of the intein, tBid was subcloned into an alternative intein vector, pTYB12. The pTYB12 intein tBid fusion protein expressed well in *E.coli* BL21 (DE3) cells (Figure 2A, and 2B lysate lane), however self-cleavage of the intein was once again a problem (Figure 2B). To optimize the intein self-cleavage the pTYB12 intein-tBid fusion protein was bound to chitin beads and incubated in the presence of three different thiols (100mM DTT, 100mM cysteine and 100mM β-mercaptoethanol) or 100mM hydroxylamine at three different pHs (8.0, 8.5, 9.0) and at four different temperatures (4°C, 16°C, 24°C and

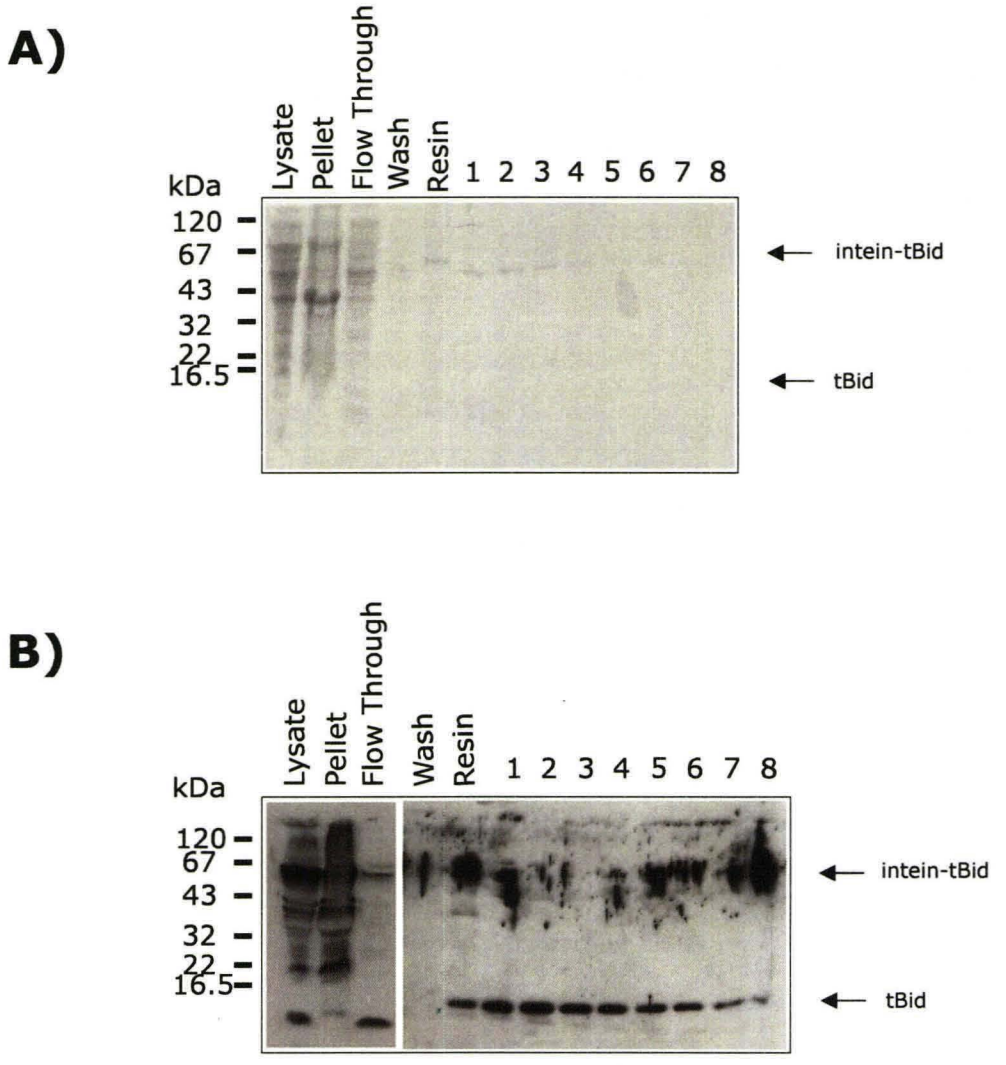

Figure 1 **Purification of tBid using an intein fusion, (pIMC103-tBid)**. An SDS-PAGE gel visualized by **A)** Coomassie Blue staining and **B)** immunoblotting with rabbit anti-Bid (rb1) antibody of the protein samples from various stages of the purification of tBid. *E. coli* DH5 cells expressing the intein-tBid fusion protein were lysed (Lysate) and centrifuged at 4,400rpm for 10 min to remove any unlysed cells (Pellet). The cleared lysate was applied to 2.5 ml of chitin beads and the flowed through was collected. The resin was washed with Intein Lysis Buffer (Wash) and after intein self cleavage was induced with100mM ß -mercaptoethanol, eight 0.5 ml fractions were collected in Intein Elution Buffer (Lanes 1-8). A sample of the remaining resin was also run on the gel (Resin).

37°C). After 40 hours the supernatant of each reaction was tested for the presence of tBid (Figure 2A, B, C and D). Treatment of the beads with 100 mM cysteine (Figure 2B) or 100 mM hydroxylamine (Figure 2D) to induce self-cleavage of the fusion protein was ineffective. Under these conditions the fusion protein remained bound to the chitin resin and was not present in the supernatant of the reaction. β-mercaptoethanol (100 mM) (Figure 2C) was not only ineffective at inducing intein self-cleavage but the fusion protein was also found in the supernatant of the reaction. When the pTYB12 intein-tBid fusion protein was bound to chitin beads and incubated with 100 mM DTT (Figure 2A) the fusion protein was again released into the supernatant however this thiol yielded the most tBid. Therefore DTT was chosen as the thiol to try to induce intein self-cleavage of the pTYB12 intein tBid fusion protein at pH 8.5 in a large scale purification. The reaction temperature did not seem to affect cleavage therefore 4°C was chosen.

The intein-tBid fusion plasmid pTYB12-tBid was transformed into *E. coli* BL21 DE3 cells and a 1.5 L culture was inoculated, induced, harvested and lysed as described above. The lysate was then incubated with the chitin beads for 1.5 hours at 4°C. After packing and washing the column the self-cleavage of the intein was induced with Intein Column buffer pH 8.5 with 100mM DTT. After 40 hours of incubation at 4°C mainly fusion protein was eluted (Figure 3A lanes 1 and 2) and tBid was still only visible by immunoblotting with anti-Bid antibody (Figure 3B lanes 1-7). The chitin resin (5µl) was added to TLB without DTT and separated by SDS-PAGE. It was found that a large amount of the fusion protein remained bound to the chitin resin (figure 3A and 3B resin).

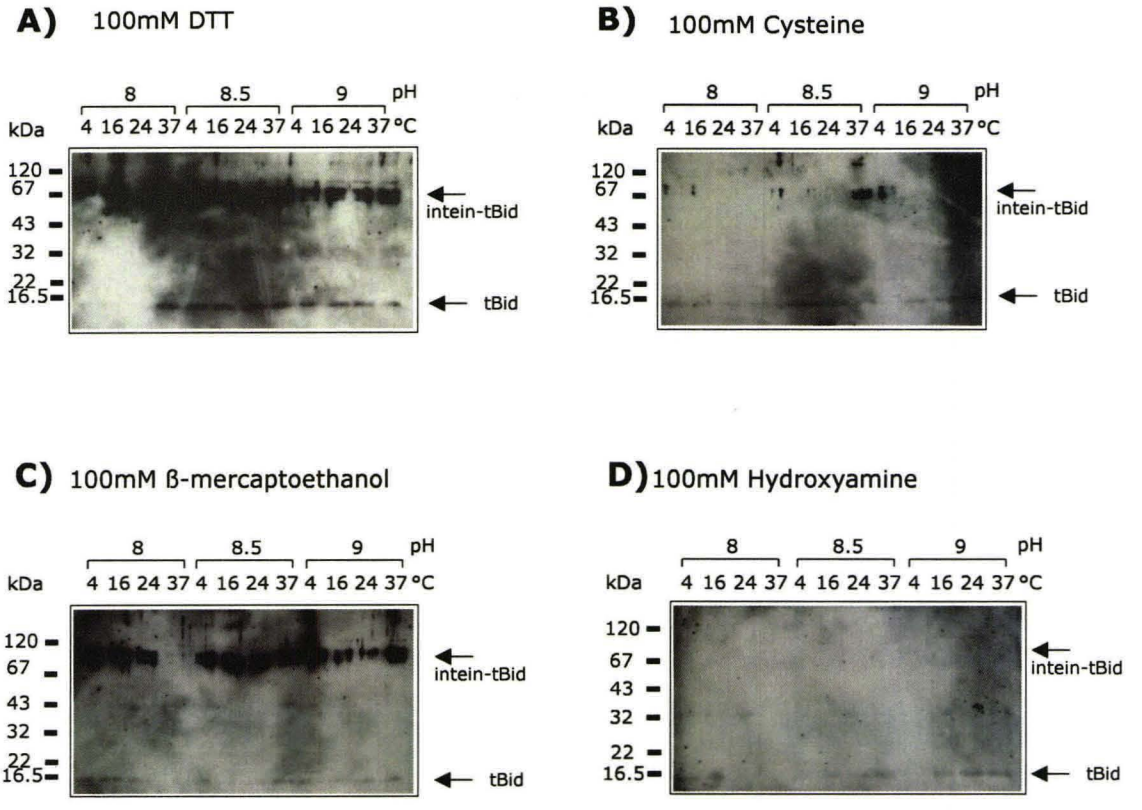

Figure 2 **Optimizing the intein cleavage conditions for fusion protein, (pTYB12-tBid).** Intein-tBid fusion protein was coupled to a chitin column and intein self cleavage was induced at different temperature, pH and by **A)** 100mM DTT, **B)** 100mM cysteine, **C)** 100 mM ß -mercaptoethanol, and **D)** 100mM hydroxylamine for 40 hours. The supernatant of the reaction was then examined for the presence of tBid by immunoblotting using a rabbit anti-Bid (rb1) antibody.

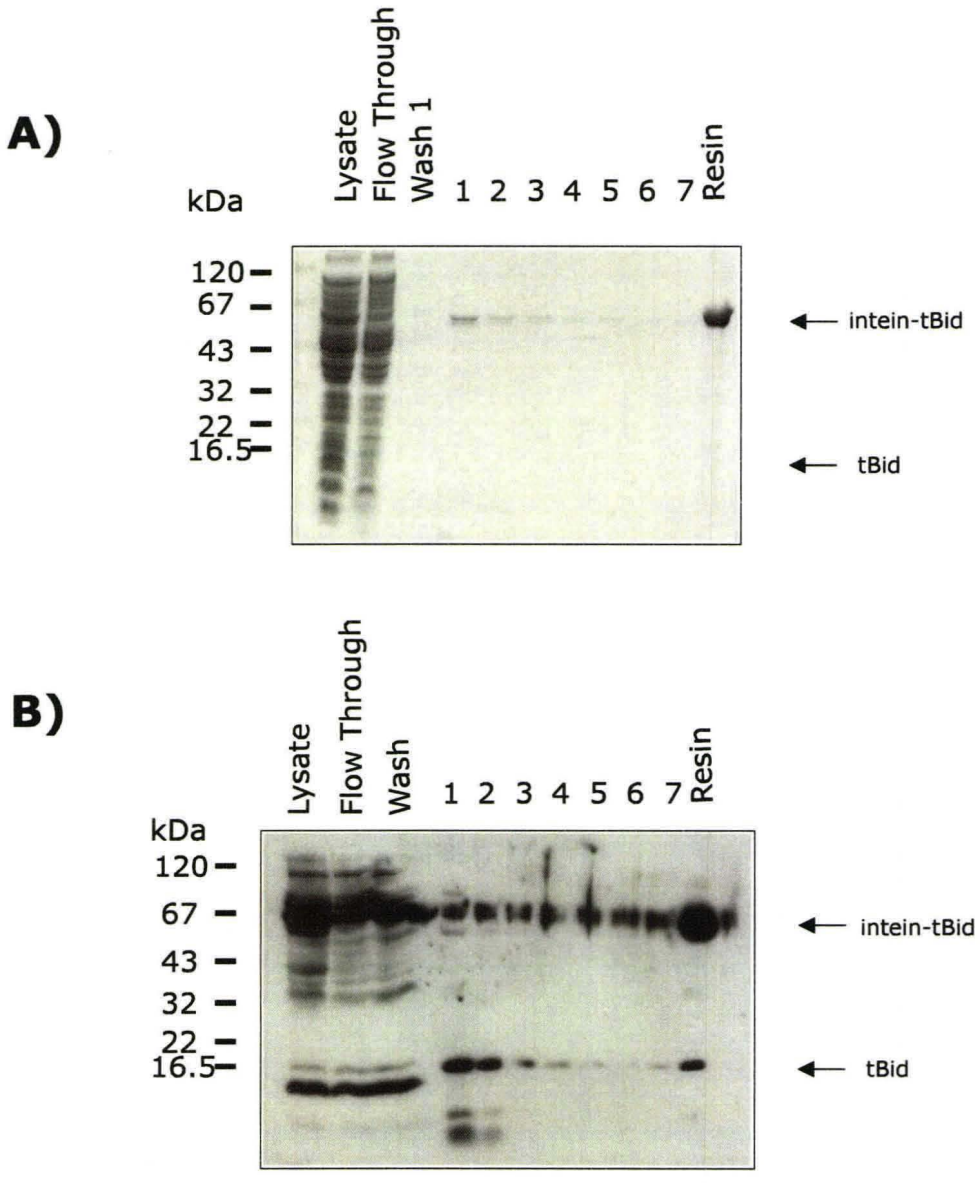

Figure 3 **Purification of tBid using an intein fusion, (pTYB12-tBid)**. An SDS-PAGE gel visualized by **A)** Coomassie Blue staining and **B)** immunoblotting with rabbit anti-Bid (rb1) antibody of the protein samples from various stages of the purification of tBid. *E. coli* BL21 DE3 cells expressing the intein-tBid fusion protein were lysed (Lysate) and unlysed cells and cellular debris was pelleted (Pellet). The cleared lysate was applied to 2.5 ml of chitin beads and the flowed through was collected. The resin was washed with Intein Lysis Buffer (Wash) and after intein self cleavage was induced with 100mM DTT, seven 0.5 ml fraction were collected in Intein Elution Buffer (Lanes 1-7). A sample of the remaining resin was also run on the gel (Resin).

This method was abandoned as a means of obtaining tBid and an alternate method described in chapter 3 section 'purification of recombinant tBid' was used.