A MITOCHONDRIAL SYSTEM FOR THE STUDY OF APOPTOSIS

THE CHARACTERIZATION OF A MITOCHONDRIAL SYSTEM FOR THE STUDY OF APOPTOSIS AND ITS INHIBITORS

By

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Abstract

The study of apoptosis is a rapidly growing field due to its relevance not only to development, but also its relationship to several diseases such as cancer. The Bcl-2 family of proteins function at the mitochondrial membrane, where many apoptotic stimuli converge. There are three main theories on the regulation of the Bcl-2 family proteins, supported by several methods of *in vitro* and cell-based studies.

Mitochondria isolated from wild type and Bak-/- C57BL6 mouse liver are free of Bax, Bcl-XL and Bid as determined by immunoblotting. A comparison of the two membranes and the use of recombinant proteins demonstrated that tBid activated Bak or Bax to permeabilize the membrane in this system, and lower concentrations of tBid were required for membrane permeabilization in the presence of both Bak and Bax. Recombinant Bcl-XL inhibited this process, indicated by a decrease in cytochrome c release. Mutant recombinant proteins demonstrated that Bcl-XL inhibits cytochrome c release through interactions with Bax/Bak and tBid, and by a third protein-independent mechanism. Together, this supports the Embedded Together Model.

The mitochondrial system also functions as an intermediate in the study of inhibitors of the Bcl-2 family of proteins. Potential inhibitors 3e and 3e-D2 previously demonstrated to bind Bcl-XL through fluorescence polarization were shown to have a Bcl-2 protein-independent method of membrane permeabilization that has not yet been determined. 3e also functioned as an activator of Bax and Bak. Similar to fluorescence polarization experiments, the dimeric compound 3e-D2 was more potent than monomeric 3e.

A comparison of membranes in the presence and absence of Bak provides a robust system in which to study multiple facets of apoptosis, including but not limited to regulation of Bcl-2 proteins and the development of protein-specific inhibitors.

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Chapter 1: General Introduction

Apoptosis, or programmed cell death, is a process of cell death that does not illicit an inflammatory response. Proper regulation is important in development and maintenance of eukaryotic organisms, and instances of too much or too little apoptosis have clinical consequences such as cancer, AIDS and other viral diseases, diabetes, mitochondrial diseases, neurodegenerative diseases, ischemic diseases, immunity and autoimmunity (Chen *et al.* 2006; Gotsiridze *et al.* 2007; Grodzicky *et al.* 2002; Jones *et al.* 2007; Reed *et al.* 2005; Yamada *et al.* 2007). Apoptosis is marked biochemically by a loss of sialic acid, fragmentation of nuclear DNA, and translocation of phosphatidylserine to the outer plasma membrane (reviewed in Henry-Mowatt *et al.* 2004). The morphological hallmarks of apoptosis include vacuolization, chromatin condensation, blebbing of the plasma membrane, and the formation of apoptotic bodies (reviewed in Henry-Mowatt *et al.* 2004).

Mitochondria in the Extrinsic Pathway

There are two pathways by which apoptosis is activated, an extrinsic pathway initiated by a ligand binding to a death receptor on the plasma membrane, and an intrinsic pathway sensitive to intracellular homeostasis (reviewed in Henry-Mowatt *et al.* 2004). In the extrinsic pathway, a caspase cascade amplifies an external death signal, whereas caspase-8 is directly activated in the intrinsic pathway (reviewed in Kim 2005). One focus of the extrinsic pathway has been the mitochondria where multiple pathways converge. Mitochondria are involved in apoptosis in response to several factors such as DNA damage, UV irradiation, activation of tumor suppressors including p53, inappropriate expression of c-myc, chemotherapeutic agents, and lipid mediators (reviewed in Henry-Mowatt *et al.* 2004). At the mitochondria, a group of proteins referred to as the Bcl-2 family of proteins plays a significant role in determining if the extrinsic signal will continue to cell death. A simplified outline of the extrinsic pathway of apoptosis begins at the cell membrane where an external signal induces oligomerization of a transmembrane death receptor, which initiates the formation of the DISC complex bound to the intracellular domain(s) of the death receptor (reviewed in Grodzicky *et al.* 2002). This leads to cleavage of caspase-8 possibly by induced proximity (Yan *et al.* 2005). Active caspase-8 cleaves Bid into its active form tBid which then interacts with other Bcl-2 proteins, ultimately leading to the release of proteins from the mitochondrial intermembrane space including Endonuclease G (EndoG), AIF (apoptosis-inducing factor),

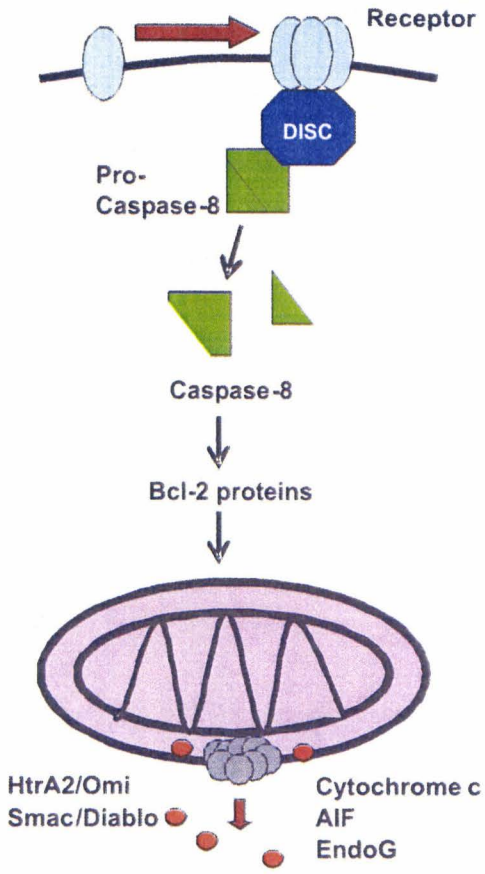

Figure 1.1 The extrinsic pathway of apoptosis. The extrinsic pathway beginning at the cell membrane is shown as a schematic. The external signal is recognized by a receptor at the membrane resulting in an active receptor and the formation of the DISC complex. Pro-caspase-8 is auto-activated and cleaved to form active caspase-8, which can activate Bcl-2 family proteins. A pore at the mitochondrial membrane formed after Bcl-2 family protein activation allows for the release of intermembrane space proteins and eventually cell death.

Smac/Diablo, HtrA2/Omi and cytochrome c (Figure 1.1) (Sharpe *et al.* 2004; Wang 2001). Release of intermembrane space proteins occurs through a pore formed by Bcl-2 proteins Bax and/or Bak (Sharpe *et al.* 2004). The released proteins are responsible for the downstream effects resulting in apoptosis. Endonuclease G and AIF migrate to the nucleus where they mediate chromatin condensation and cleavage of DNA (Li *et al.* 2001; Lorenzo *et al.* 1999; Susin *et al.* 1996). The role of Smac/Diablo and HtrA2/Omi is to inhibit IAP proteins which are themselves caspase inhibitors, finally leading to the activation of caspase-3

(Chai *et al.* 2000; Du *et al.* 2000; Suzuki *et al.* 2001; Verhagen *et al.* 2000). Released cytochrome c forms a multi-protein complex termed the apoptosome with Apaf-1, which functions to activate caspase-9 and eventually caspase-3, an effector caspase (Adrain *et al.* 1999; Cain *et al.* 2002; Hu *et al.* 1998). The activation of caspase-3 is often considered the point of no return (reviewed in Henry-Mowatt *et al.* 2004).

Bcl-2 Proteins

The Bcl-2 family of proteins has been extensively studied for their role in apoptosis and disease. These proteins are also implicated in regulating cellular bioenergetics and cell cycle (Jones *et al.* 2007; Vander Heiden *et al.* 1999; Zinkel *et al.* 2006). The family of proteins is named for their similarity to Bcl-2 (Boise *et al.* 1993; Oltvai *et al.* 1993; Tsujimoto *et al.* 1985). Bcl-2 was cloned as a result of a t(14:18) chromosomal translocation found in human follicular B cell lymphoma which increased Bcl-2 expression (Tsujimoto *et al.* 1985). Functioning to inhibit death, Bcl-2 differed from all other known oncogenes at the time (Hockenbery *et al.* 1990; McDonell 1989; Vaux *et al.* 1988). The first new family members were recognized in 1993 with the cloning of Bcl-2-associated X protein (Bax) and Bcl-x (Boise *et al.* 1993; Oltvai *et al.* 1993). Bcl-2 family proteins can be subdivided into three categories based on the conserved Bcl-2 homology (BH) regions (Figure 1.2, C and D). Pro-apoptotic proteins are divided into two subgroups: BH3-only proteins and multi-region or Bax-like proteins (reviewed in Willis *et al.* 2005). Bim, Bad, Bid, Bik, Bmf, PUMA, Noxa and Hrk are some of the BH3- only proteins and share only the BH3 region (Willis *et al.* 2005). Regulation of BH3-only proteins occurs by phosphorylation or other posttranslational modifications such as proteolytic cleavage, or by transcriptional regulation (Lei *et al.* 2003; Willis *et al.* 2005). The multi-region or Bax-like proteins consist of Bax, Bak, and Bok (Willis *et al.* 2005). These proteins contain the BH1-3 regions. The anti-apoptotic proteins Bcl-2, Bcl-XL, Bcl-w, Mcl-1 and A1, contain all four BH regions and comprise the third category of Bcl-2 family proteins (Willis *et al.* 2005). The BH4 region often has less sequence conservation than the BH1-3 regions but is essential to these proteins' anti-apoptotic activity (Gross *et al.* 1999; Sugioka *et al.* 2003). The multi-domain proteins, pro- and anti-apoptotic, also share a conserved tertiary structure whereby BH regions 1, 2 and 3 form a hydrophobic groove that is involved in heterodimerization within the family of proteins, and is important for function (Figure 1.2) (Willis *et al.* 2005). Three methods of dimerization of this family of proteins have been proposed: heterodimerization at

the BH3 region-binding pocket, domain swapped homodimerization and finally zinc-dependent homodimerization as seen with Bak (Moldoveanu *et al.* 2006; O'Neill *et al.* 2006).

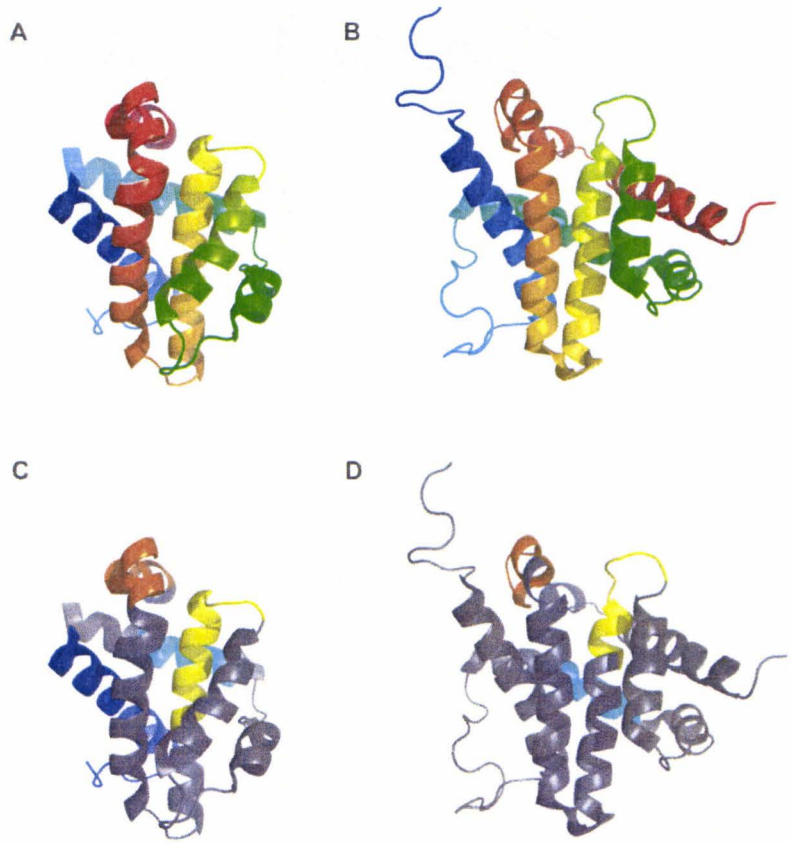

Figure 1.2 Structure of human Bcl-XL and Bax. The crystal structure of human Bcl-XL (A and C) and Bax (B and D) have similar tertiary structures. Bcl-XL and Bax share a similar tertiary structure (A and B) and sequence homology in the BH regions (C and D). BH regions 1 (yellow), 2 (orange) and 3 (cyan) occur in both proteins whereas BH region 4 (blue) is only found in anti-apoptotic proteins such as Bcl-XL.

There is evidence to suggest that Bcl-2 family proteins function in apoptosis through several mechanisms. Bax and Bak are suggested to form pores by oligomerization promoted by the BH3 region through which intermembrane space proteins are released (Gross *et al.* 1999; Kuwana *et al.* 2002; Wei *et al.* 2000). Bax, Bcl-XL and Bcl-2 have been shown to form ion

channels in artificial membranes in acidic pH, supporting an indirect role for these proteins in the release of intermembrane space proteins (Minn *et al.* 1997; O'Neill *et al.* 2006; Schendel *et al.* 1997; Schlesinger *et al.* 1997). The interaction between Bax or Bcl-2 with permeability transition pore protein ANT (adenine nucleotide translocator) suggests that Bcl-2 proteins may interact with and regulate pre-existing pores. Finally, these proteins may interact with lipids to produce lipidic pores (Sharpe *et al.* 2004). Anti-apoptotic proteins may also regulate mitochondrial respiration and COX activity to meet energy needs and promote cell survival without harmful increases in reactive oxygen species (ROS) (Chen *et al.* 2007).

Bax and Bak

Bax and Bak, two multi-domain pro-apoptotic proteins, are important for normal development (Lindsten *et al.* 2000). Bax was found based on its homology to Bcl-2 and is a cytosolic protein (Oltvai *et al.* 1993). Bak, a membrane bound protein, was classified as a Bcl-2 protein based on its homology to Bcl-2 and was initially detected as a binding partner of E1B 19K (Chittenden *et al.* 1995; Farrow *et al.* 1995; Kiefer *et al.* 1995). Mutations in Bax or Bak which confer a survival advantage have been found in some human colorectal carcinomas and hematopoietic tumours, emphasizing the importance of understanding the mechanisms by which these two proteins function (Ionov *et al.* 2000; Kondo *et al.* 2000; Meijerink *et al.* 1998; Rampino *et al.* 1997; Xu *et al.* 2007).

Bax and Bak are redundant in most model systems, such as the knockout mouse model, but there is evidence that some damage signals preferentially utilize Bax or Bak (Dansen 2006; Eischen *et al.* 2001; Gillissen *et al.* 2003; LeBlanc *et al.* 2002; Lindsten *et al.* 2000; Nijhawan *et al.* 2003; Willis *et al.* 2005). Mice and cells deficient in one or both proteins have provided significant information about these two proteins. Bak deficient mice are phenotypically normal whereas Bax deficient mice are viable but male mice are sterile (Lindsten *et al.* 2000). A comparison of Bak-/- and Bak-/+ mice illustrated that Bak function is dependent on the cell type, developmental stage and death stimulus by illustrating that Bak in brain and spinal tissue protect cells from Sindbis virus and excitotoxicity at an early development stage, and that Bak expression decreases over the first few months (Fannjiang *et al.* 2003). Single knockout mice for Bax have increases in some cell types including neurons and lymphocytes, and the loss of Bax accelerated the development of brain tumours and myc-induced

lymphomas in these animals (Eischen *et al.* 2001; Jyotika *et al.* 2007; Knudson *et al.* 1995; Yin *et al.* 1997). Double knockout Bax and Bak mice have severe abnormalities if they survive to birth (Lindsten *et al.* 2000; Lindsten *et al.* 2006). Cells deficient in Bax and Bak are resistant to tBid and Bim and these cells fail to release cytochrome c in response to either protein (Wei *et al.* 2001; Zong *et al.* 2001).

There are four events that occur in Bax activation; exposure of the N-terminus, translocation to the mitochondria, oligomerization and focal clustering on the membrane (reviewed in Sharpe *et al.* 2004). The order of events in Bax activation has yet to be definitively assigned as there is much evidence supporting membrane targeting before oligomerization (Gross *et al.* 1998; Wang *et al.* 1996), but other authors have concluded that oligomerization may occur before insertion through cross-linking studies (Eskes *et al.* 2000; Tan *et al.* 1999). tBid causes a conformational change of Bax resulting in its activation, possibly driven by the N-terminus which has been proposed as a regulatory domain, and may open the hydrophobic groove of Bax in the presence of lipids (Cartron *et al.* 2005; Cartron *et al.* 2003; Desagher *et al.* 1999; George *et al.* 2007; Goping *et al.* 1998; Hsu *et al.* 1998; Juin *et al.* 1995; Lalier *et al.* 2007; Nechushtan *et al.* 1999; Parikh *et al.* 2007; Suzuki *et al.* 2000; Upton *et al.* 2007; Zhou *et al.* 2007). Exposure of the N-terminus of Bax allows the antibody 6A7 to bind and this occurs before membrane insertion (Hsu *et al.* 1998). BH3-peptides from Bid and PUMA bind the N-terminus of Bax, suggesting another mechanism of activation (Cartron *et al.* 2004). Bax is potentially phosphorylated by a threonine-kinase at T167, which may provide another mechanism for Bax regulation (Kim *et al.* 2006). Once activated, Bax auto-activates other Bax molecules shown *in silico* and *in vitro* (Chen *et al.* 2007; Tan *et al.* 2006). The analysis of the mechanism of Bax insertion into membranes suggested Bax inserts at the TOM (translocase of the mitochondrial outer membrane) complex (Bellot *et al.* 2007; Hacker *et al.* 2007; Ott *et al.* 1997). However, Bax undergoes a conformational change and inserts helices 5 and 6 into liposomes and mitochondria similarly suggesting insertion may not be receptor-mediated (Annis *et al.* 2005; Cartron *et al.* 2005; Garcia-Saez *et al.* 2006; Nouraini *et al.* 2000; Schendel *et al.* 1998). Estimates of the size of a Bax pore suggest a diameter of 22-28Å which is large enough for cytochrome c to escape (Saito *et al.* 2000; Schlesinger *et al.* 2006). Larger pore formation may be enhanced by cardiolipin and experiments with liposomes have suggested pore sizes of 10-40nm in a concentration dependent manner (Kuwana *et al.* 2002). Bax activation can be inhibited by anti-apoptotic proteins Bcl-2, Bcl-

w and Bcl-XL by direct binding (Holmgreen *et al.* 1999; Sedlak *et al.* 1995; Yang *et al.* 1995).

Bak is located at the membrane and its activation involves a conformational change at the N-terminus and oligomerization (Griffiths *et al.* 2001; Ihrlund *et al.* 2006; Kinoshita *et al.* 2007; Ruffolo *et al.* 2003; Wei *et al.* 2000). A second conformational change has also been detected. Following exposure of the N-terminus, the BH1 region also becomes available for antibody binding (Bellosillo *et al.* 2002; Griffiths *et al.* 2001; Panaretakis *et al.* 2002; Ruffolo *et al.* 2003). Bak is normally bound to Bcl-XL and Mcl-1 and can be freed from these binding partners by BH3-only proteins such as Noxa (Frey *et al.* 2007; Minet *et al.* 2006; Moldoveanu *et al.* 2006; Uren *et al.* 2007; Willis *et al.* 2005; Zhuang 2006). Bak has also been shown to bind VDAC2 (Cheng *et al.* 2003). Interactions with membrane proteins may be required for Bak stabilization as a decrease in Bak was seen following treatment with siRNA for Mcl-1 (Minet *et al.* 2006). Bak interacts more strongly with Bcl-XL than the Bak/Bcl-2 interaction but its activation can be inhibited by both anti-apoptotic proteins, as well as Bcl-w and A1 (Chittenden *et al.* 1995; Holmgreen *et al.* 1999). Bak shows zinc-dependent homodimerization that does not involve BH1-3 regions, suggesting that dimerization does not affect activation (Moldoveanu *et al.* 2006). As well, the narrower hydrophobic pocket suggests that a BH3 interaction at the membrane may have to be relayed in a different way than Bax (Moldoveanu *et al.* 2006).

Bax and Bak may interact either directly or indirectly with each other at the membrane (Sundararajan *et al.* 2001). Some evidence using cells deficient in Bax or Bak in combination with chemical cross-linking has suggested that Bax may be required for Bak oligomerization, but Bak is not required for Bax oligomerization. In HCT116 and BMK cells that do not have Bax, Bax was required for Bak activation, and an interaction between the two proteins was seen in rat proximal tubule cells (Mikhailov *et al.* 2003). HeLa cells expressing Bif-1 also demonstrated how inhibition of Bax activation by decreased Bif-1 in turn inhibited Bak activation (Takahashi *et al.* 2005). In contrast, a Bax construct lacking the C-terminal insertion sequence is unable to oligomerize without Bak, and oligomerization following some stimuli results in activation of Bak before Bax (Er *et al.* 2007; Ihrlund *et al.* 2006; Neise *et al.* 2007). For example, Bax activation in cisplatin-induced apoptosis requires Bak activation in HeLa and Hep-2 cells (Cartron *et al.* 2003; Kepp *et al.* 2007). This supports a hypothesis that in some systems, these proteins may not be redundant (Mikhailov *et al.*

2003). Taken together, these data indicate that cross-activation of Bax and Bak can occur, but the direction of activation may be cell and stimuli dependent.

tBid

The pro-apoptotic family member tBid is a well-studied model BH3-only protein. By using an assay system with isolated mitochondria, it was found to be a cytosolic component required for cytochrome c release. Bid, the inactive form in apoptosis, is cleaved by caspase-8 or caspase-3 into its p15 and p7 fragments as well as p13 and p11 fragments (Eskes *et al.* 2000; Gross *et al.* 1999; Pei *et al.* 2007; Wei *et al.* 2000; Zhao *et al.* 2001). This may occur before or after apoptosis depending on the cell line and stimulus (Konig *et al.* 2007; Pei *et al.* 2007; Ward *et al.* 2006). Cleavage may also occur before or after translocation to the membrane (Ward *et al.* 2006). This signal transmitted by tBid (the p15 fragment) may then be amplified by cytochrome c released from the mitochondria (Bossy-Wetzel *et al.* 1999; Gross *et al.* 1999). The cleaved p15 tBid fragment can be N-myristoylated, which may be involved in the targeting of tBid to the mitochondria in some cells (Zha *et al.* 2000). tBid may bind and oxidize cardiolipin at contact sites, which disturbs the interaction between cardiolipin and cytochrome c to mobilize cytochrome c, and tBid may regulate remodelling of cristae observed in apoptosis (Esposti *et al.* 2003; Gonzalvez *et al.* 2005; Kim *et al.* 2004; Liu *et al.* 2005; Lutter *et al.* 2000; Ott *et al.* 2002). Cardiolipin may also be responsible for the release of the p15 fragment from the p7 fragment after Bid cleavage as demonstrated by mobilization of tBid from the stationary p7 fragment in a column incubated with pure cardiolipin (Liu *et al.* 2005). Cardiolipin and its hydrolyzed mono-lyso form have similar affinities for tBid (Esposti *et al.* 2003; Liu *et al.* 2005). Full-length Bid is loosely attached to the mitochondria and is therefore not alkali resistant, but active tBid is resistant to alkali extraction and is high salt resistant at the mitochondria, illustrating that it is embedded in the membrane (Eskes *et al.* 2000; Gross *et al.* 1999; Ruffolo *et al.* 2003).

Bid is a critical mediator of death receptor signalling (Luo *et al.* 1998). Bid has been shown to interact with pro-apoptotic protein Bax at Helix 1 or the N-terminus (Cartron *et al.* 2004; Eskes *et al.* 2000). Bid binds and activates both Bax and Bak, initiating oligomerization resulting in cytochrome c release (Cartron *et al.* 2004; Clohessy *et al.* 2006; Eskes *et al.* 2000; Wei *et al.* 2000). In ASTC-a-1 cells, Bid induced the translocation of Bax to the mitochondria by transient interaction with Bax after treatment with TNF-α seen by FRET (fluorescence resonance energy transfer) (Pei *et al.* 2007). Translocation of tBid to

mitochondria is not blocked by Bcl-XL or Bcl-2 in F5.12, HeLa or KB epithelial cells, although these proteins function to inhibit membrane permeabilization, likely by sequestration of membrane-bound tBid (Gross *et al.* 1999; Ruffolo *et al.* 2003). This protein binds the hydrophobic groove of Bcl-XL and Bcl-2, and also Mcl-1, supporting inhibition of apoptosis by sequestration of tBid (Cartron *et al.* 2004; Clohessy *et al.* 2006; Eskes *et al.* 2000; George *et al.* 2007; Wang, K. *et al.* 1996; Wei *et al.* 2000). tBid has been shown to directly interact with Bcl-2 and induces a conformational change (Dlugosz *et al.* 2006; Peng *et al.* 2006).

Aside from binding to and thereby activating Bcl-2 family proteins to induce apoptosis, Bid may also have a Bax/Bak-independent mechanism of inducing membrane permeability. Mutant Bid G94E does not bind Bax or anti-apoptotic proteins but still permeabilized the mitochondrial membrane in Bak/Bax double knockout cells (Desagher *et al.* 1999; Gross *et al.* 1999; Willis 2007). Furthermore tBid may promote negative curvature of the membrane seen in liposome systems allowing for leakage (Epand *et al.* 2002). Bid may also form oligomers without inducing oligomerization of Bax or Bak to trigger apoptosis through the formation of channels similar to Bax (Garcia-Saez *et al.* 2006; Grinberg *et al.* 2002).

Bid knockout animals are viable, suggesting some redundancy with other BH3-only proteins (Kaufmann *et al.* 2007; Yin *et al.* 1999). These animals respond normally to DNA damage and replicative stress or cell cycle arrest and endotoxin-induced liver injury, but are resistant to Fas-induced hepatocellular apoptosis and Bid deficient thymocytes are more resistant to death-receptor mediated apoptosis (Chen *et al.* 2007; Kaufmann *et al.* 2007; Yin *et al.* 1999).

Bcl-XL

Bcl-XL, one of the first family members found after the identification of Bcl-2, was identified by hybridization with a murine *bcl-2* probe in chicken (Boise *et al.* 1993). mRNA was cloned and put into cells where it inhibited cell death in FL5.12 cells (Gonzalez-Garcia *et al.* 1994). Bcl-XL plays an important role in development. Deletion of Bcl-XL results in neuronal degeneration, increased apoptosis in the liver, and decreased survival of immature lymphocytes and erythrocytes in conditional knockouts (Moller *et al.* 2007; Motoyama *et al.* 1995; Orelio *et al.* 2007; Roth *et al.* 1996; Wagner *et al.* 2000). Homozygous deletion of Bcl-XL is lethal (Roset *et al.* 2007). Bcl-XL appears to have housekeeping roles outside of its anti-apoptotic function including inhibition of mitochondrial swelling

in cells treated with apoptotic and necrotic stimuli, regulation of coupled respiration by supporting ATP-ADP exchange across the outer membrane, and inhibition of VDAC gating (Vander Heiden *et al.* 1999; Vander Heiden *et al.* 1997; Vander Heiden *et al.* 2001). Active Bcl-XL targets to the endoplasmic reticulin (ER) as well as mitochondria, and Bcl-XL in pancreatic β cells inhibits mitochondrial bioenergetic responses to glucose, pyruvate methyl ester and α-ketoisocaproate, suggesting a shift from oxidative phosphorylation to aerobic glycolysis with increased expression of Bcl-XL (He *et al.* 2002; Schwartz *et al.* 2007; Zhou *et al.* 2000). Bcl-XL may also have a role in cell cycle as Bcl-XL colocalizes with cdk1 at the G2/M cell cycle checkpoint (Schmitt *et al.* 2007).

The exact mechanism of inhibition of apoptosis by Bcl-XL is unknown; however there is growing evidence that Bcl-XL may prevent cell death via multiple mechanisms. Crosslinking experiments indicate that Bcl-XL exists as a monomer, dimer or trimer depending on the pH (O'Neill *et al.* 2006; Thuduppathy *et al.* 2006). Crystal structures suggest domain swapping occurs during homodimerization but does not involve the hydrophobic groove that mediates binding to pro-apoptotic Bcl-2 family members (Denisov *et al.* 2007; Schmitt *et al.* 2007). Bcl-XL has also been crystallized with BH3 peptides of Bak, Bad and Bim (Liu *et al.* 2003; Sattler *et al.* 1997; Yan *et al.* 2005). Bcl-XL protects against Bax translocation, inhibiting cytochrome c release, and Bcl-XL binds oligomeric Bax (Eskes *et al.* 2000; Poruchynsky *et al.* 1998; Wang *et al.* 2004). Bcl-XL does not inhibit tBid translocation in F5.12 cells but does decrease cytochrome c release (Gross *et al.* 1999). Crystal structures confirm the protein contains two independent regions blocking either homodimerization or heterodimerization that can potentially be targeted for drug development. Bcl-XL may also function "upstream" of mitochondrial events as expression inhibited caspase-8 cleavage and the formation of the DISC complex except at the mitochondria where DISC formation was increased (Wang *et al.* 2004). Bcl-XL may be cleaved by caspase-3 and caspase-1 at D61 into two 18kDa fragments (Clem *et al.* 1998; Fujita, Naoya *et al.* 1998). This cleaved form increases sensitivity to apoptosis (Fujita *et al.* 1998). Bcl-XL inserts into the membrane similarly to other Bcl-2 proteins (Hsu *et al.* 1997; Minn *et al.* 1997). This inserted form may function differently from the soluble protein. It has been suggested that the cytosolic form of Bcl-XL sequesters pro-apoptotic proteins, whereas the membrane-bound protein may form a cationic channel (Minn *et al.* 1997; Sattler *et al.* 1997; Sedlak *et al.* 1995; Vander Heiden *et al.* 2001). This conformational change may require an acidic pH and the presence of negatively charged lipids. Mutations that alter the

putative channel-forming domain also have altered apoptotic activities (Basanez *et al.* 2001; Losonczi *et al.* 2000; Minn *et al.* 1997; Xie *et al.* 1998).

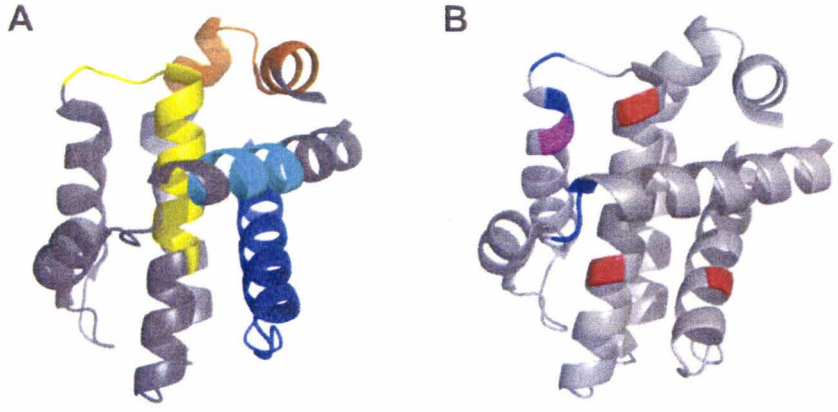

Figure 1.3 Mutational analysis of Bcl-XL. Schematic of the monomer of human Bcl-XL indicating the location of the BH regions: BH regions 1 (yellow), 2 (orange), 3 (cyan) and 4 (blue). The BH3 binding pocket occurs between BH regions 1, 2 and 3 (A). Mutational analysis has revealed mutations resulting in Bcl-XL mutants that do not bind Bax but still inhibited apoptosis (blue), protect cells from apoptosis (magenta), and mutations resulting in an inactive protein (red). These mutations are not localized specifically to any regions but do provide information about residue and areas important to Bcl-XL function (B).

Mutational analysis has been used to tease out inhibition by Bcl-XL (Figure 1.2B). It has been shown that the BH4 region is essential to anti-apoptotic function and is important for binding to Bax (Gross *et al.* 1999). Deletion of the BH1, 2, 3 or 4 regions or the C-terminus of Bcl-XL results in mutants that do not inhibit Bax localization to the mitochondria in GFP-Bax cells (Wang *et al.* 2007; Wolter *et al.* 1997; Zhou *et al.* 2005). Deletion of the C-terminus however did not have an effect on the membrane conformation or insertion assessed by thermal unfolding and tryptophan fluorescence (Thuduppathy *et al.* 2006). Deletion of the N-terminus decreases the helicity of Bcl-XL (Wang *et al.* 2007). Removal of the flexible loop and small deletions in alpha-helices 5 and 6 of Bcl-XL had no affect on Bcl-XL inhibition of Bax localization to the membrane, but these mutant Bcl-XL proteins do not bind Bak or Bax (Zhou *et al.* 2005; Clem *et al.* 1998; Muchmore *et al.* 1996; Liu *et al.* 2006). In another experiment, deletion of the loop increased anti-apoptotic activity of Bcl-XL while not interfering with Bax binding (Chang *et*

al. 1997). Several other point mutations have also been useful in determining important residues in Bcl-XL function. Critical residues involved in hydrophobic interactions with the BH3 domain of pro-apoptotic proteins include Y101, L130 and Y195 (Minn *et al.* 1999). Bcl-XL with mutations G138A, F131V or D133A do not bind Bax but still inhibit cytochrome c release (Desagher *et al.* 1999; Eskes *et al.* 2000; Muchmore *et al.* 1996). These mutants can still be immunoprecipitated with tBid, Bim and Bad (Cheng *et al.* 2001). Minn et al used mutational analysis to show that Bcl-XL mutations Y101K, L130A and Y195G still protected cells from death during IL-3 deprivation, however mutant Y101K does not bind Bax by immunoprecipitation in cell lysates (Minn *et al.* 1999). Mutant Bcl-XL Y101K homodimerizes normally and binds to membranes as determined by immunoprecipitation and pelleting assays (Jeong *et al.* 2004; Minn *et al.* 1999). In contrast, Bcl-XL D61A increases cell survival compared to wild type (Clem *et al.* 1998). A mutant Bcl-xFNK displayed increased function, providing increased resistance to several stimuli (Asoh *et al.* 2000). Finally, mutations G138E, R139L and I149N are not active and do not bind tBid, Bim or Bad using purified mitochondria and immunoprecipitation (Cheng *et al.* 2003). These residues that inactivate Bcl-XL may be important to target in drug development.

Other methods postulated to account for Bcl-XL function include by disrupting the DISC complex, blocking caspase activation and a change in membrane potential, blocking a tBid induced change in the membrane phase, or by forming cation selective pores in the membrane (Basanez *et al.* 2001; Clem *et al.* 1998; Epand 2002; Kim 2005; Lam *et al.* 1998; Minn *et al.* 1999; Wang *et al.* 2004). Deamidation of Bcl-XL at Asn52 and Asn66 has been proposed as a mechanism that negatively regulates the function of Bcl-XL as shown by mutants N52A/N66A, by the conversion of amino acids 52 and 66 to iso-Asp, and by immunoprecipitation following etoposide treatment (Deverman *et al.* 2003; Deverman *et al.* 2002; Grad *et al.* 2000; Zhao *et al.* 2007). Bcl-XL is phosphorylated in cancer cells after treatment with several chemicals that target tubulin in the treatment of malignant cancers and may act as a switch, but is associated with apoptotic death (Basu *et al.* 2003; Poruchynsky *et al.* 1998). However, little is known about the physiologic relevance of any of these mechanisms. Bcl-XL has also been shown to bind cytochrome c, which would prevent the amplification of the apoptotic signal (Kharbranda *et al.* 1997; Li *et al.* 1997). Other proteins that Bcl-XL has been shown to bind include p53 with higher affinity than with Bak, and VDAC (Malia *et al.* 2007; Sot *et al.* 2007; Wang *et al.* 1996). The interaction between Bcl-XL and VDAC may keep VDAC in an open

state as seen in situ (Vander Heiden *et al.* 2001). Finally, Bcl-XL may be involved in tumour angiogenesis by modulation of CXCL8 (Giorgini *et al.* 2007; Karl *et al.* 2007).

Apoptosis at the Mitochondria

Bax and Bak are pro-apoptotic proteins proposed to form pores at the mitochondrial membrane allowing the release of intermembrane space proteins (Garcia-Saez *et al.* 2006; Korsmeyer *et al.* 2000; Wei *et al.* 2001). It is unknown exactly how Bax and Bak are activated and where in the pathway anti-apoptotic proteins such as Bcl-XL prevent Bax/Bak function. There are several theories on how Bax or Bak can be activated to induce the release of cytochrome c and other intermembrane space proteins.

One model of interest is the Displacement model of apoptosis (Figure 1.4A). In this model, activated Bax and Bak are sequestered by anti-apoptotic proteins and can be displaced by BH3-only proteins with varying affinities for anti-apoptotic proteins (Certo *et al.* 2006; Chen *et al.* 2005; Uren *et al.* 2007; Willis *et al.* 2005; Yang *et al.* 1995). Pro-apoptotic protein Bak is bound by Bcl-XL, Mcl-1 and VDAC in healthy cells (Frey *et al.* 2007; Kim *et al.* 2006; Minet *et al.* 2006; Moldoveanu *et al.* 2006; Uren *et al.* 2007; Willis *et al.* 2005; Zhuang *et al.* 2006). Bak is released from Bcl-XL and Mcl-1 using peptides corresponding to the BH3 regions of BH3-only proteins and resulted in the activation of Bak in a temperature sensitive manner (Uren *et al.* 2007). Willis *et al.* also demonstrated binding of Bak with Mcl-1 and Bcl-XL in healthy cells and the release of Bak in the presence of BH3-only proteins (Willis *et al.* 2005). In the absence of Bim and Bid, in combination with a decrease in the expression of Puma, other BH3-only proteins could induce cytochrome c release (Willis *et al.* 2007). Bim, Bid and Puma have been classified previously as activator proteins, proposed to be the only BH3 proteins to cause cytochrome c release by Bax/Bak activation, supporting displacement of pro-apoptotic proteins as the mechanism of pore formation (Letai *et al.* 2002). However, a triple knockout of Bid, Bim and Puma has not been made, which would be required to rigorously test this model (Roset *et al.* 2007). Direct interactions between BH3-only proteins and Bax or Bax have been difficult to detect (Antonsson *et al.* 2001; Nechushtan *et al.* 2001; Wang *et al.* 1996; Wei *et al.* 2000; Willis *et al.* 2007). BH3-only proteins have not been detected in Bax/Bak complexes (Antonsson *et al.* 2001). However, full-length tBid or Bid has been demonstrated to bind to and activate both Bax and Bak to initiate oligomerization and membrane permeability (Cartron *et al.* 2004; Clohessy *et al.*

2006; Eskes *et al.* 2000; Pei *et al.* 2007; Wei *et al.* 2000). This model has several other contradicting publications. Mutational analysis by Kim *et al.* has demonstrated that elimination of interactions between Bax/Bak and anti-apoptotic proteins does not result in apoptosis; both proteins require another signal for activation (Kim *et al.* 2006). As well, while Bax can be sequestered by Bcl-XL, Bcl-2 and Bcl-w, Bax is normally found to be monomeric and cytosolic (Holmgreen *et al.* 1999; Hsu *et al.* 1998; Kuwana *et al.* 2003; Newmeyer *et al.* 2003; Oltvai *et al.* 1993; Sedlak *et al.* 1995; Yang *et al.* 1995). This model may hold true for cancer cells however. Certo *et al.* suggest three states of the cell: unprimed, primed for death and dead such that interactions between Bax or Bak and BH3-only proteins may sometimes not be the significant interaction (Certo *et al.* 2006). This is supported by studies with mitochondria from leukemic cells which have increased expression of Bcl-2. In this setting BH3-only proteins previously categorized as sensitizer proteins induce cytochrome c release if they can bind Bcl-2 (Certo *et al.* 2006). Similarly, Bcl-XL was immunoprecipitated with Bak and Bax in PC3 cells and with Bak in LNCaP cells, two cancer cell lines (Castilla *et al.* 2006).

A second model, the Direct Activation model, proposes that tBid, Bim and Puma interact with and activate Bax or Bak (Figure 1.4B) (Cheng *et al.* 2001; Galonek *et al.* 2006; Kim *et al.* 2006; Kuwana *et al.* 2005; Kuwana *et al.* 2002; Letai *et al.* 2002). Anti-apoptotic proteins sequester these BH3-only proteins known as activator BH3 proteins, to prevent apoptosis (Letai *et al.* 2002). The binding of sensitizer BH3-only proteins, those which do not bind with Bax and Bak, frees activator proteins to interact with Bak and Bax to release cytochrome c (Letai *et al.* 2002). This model is supported by work using full-length proteins and peptides to demonstrate the requirement of activator proteins for membrane permeabilization in the absence of anti-apoptotic proteins (Cartron *et al.* 2004; Clohessy *et al.* 2006; Eskes *et al.* 2000; Pei *et al.* 2007; Ruffolo *et al.* 2003; Wei *et al.* 2000). As well, there is continuing evidence of direct interactions between BH3-only proteins and Bax or Bak, although this interaction may be transient as described in the "hit and run" model (Cartron *et al.* 2004; Clohessy *et al.* 2006; Eskes *et al.* 2000; Kuwana *et al.* 2005; Pei *et al.* 2007; Wei *et al.* 2000). Mutational analysis demonstrated the requirement for activation of Bak and Bax, in that Bax and Bak are not constitutively active and bound by anti-apoptotic proteins, since elimination of this interaction did not increase apoptosis (Kim *et al.* 2006). This model primarily focuses on inhibition of apoptosis by the interaction between activator BH3-only proteins and anti-apoptotic proteins, however

interactions between anti-apoptotic proteins and Bax or Bak have also been detected (Cheng *et al.* 2001; Frey *et al.* 2007; Galonek *et al.* 2006; Holmgreen *et al.* 1999; Kuwana *et al.* 2003; Minet *et al.* 2006; Moldoveanu *et al.* 2006; Newmeyer *et al.* 2003; Sattler *et al.* 1997; Sedlak *et al.* 1995; Uren *et al.* 2007; Willis *et al.* 2005; Yang *et al.* 1995; Zhuang *et al.* 2006). Crystal structures, topology mapping and conformation specific antibodies of both pro- and anti-apoptotic proteins have provided further evidence that these multi-domain proteins require a conformational change, induced by activator BH3-only proteins (Annis *et al.* 2005; Deverman *et al.* 2002; Dlugosz *et al.* 2006; Hinds *et al.* 2003; Hsu *et al.* 1997; Moldoveanu *et al.* 2006; Muchmore *et al.* 1996; Ruffolo *et al.* 2003; Suzuki *et al.* 2000). While this model is not supported by experiments with Bim/Bid knockouts with a decreased expression of Puma, the three activator BH3 proteins, a true triple knockout has not yet been made and would be required to confirm the activator/sensitizer categorization (Roset *et al.* 2007; Willis *et al.* 2007). As mentioned above, this model may not extend to cancer cells, which often have an increase in anti-apoptotic proteins (Amundson *et al.* 2000; Certo *et al.* 2006; Kirkin *et al.* 2004).

The Embedded Together model acknowledges that many interactions are important, but introduces a different perspective (Figure 1.4C) (Leber *et al.* 2007). There are five key points to this model. The first is that Bax and Bak go through regulated conformational changes to become active, as demonstrated in liposomes, mitochondria and cells (Annis *et al.* 2005; Cuddeback *et al.* 2001; Dlugosz *et al.* 2006; Jeong *et al.* 2004; Schinzel *et al.* 2004; Takahashi *et al.* 2005; Zhang *et al.* 2005). The second is that BH3-only proteins activate not only the multi-domain pro-apoptotic proteins Bax and Bak, but also anti-apoptotic proteins by causing conformational changes similarly, and that both binding events may occur according to the kiss-and-run hypothesis as described for tBid activation of Bax (Dlugosz *et al.* 2006; Kim *et al.* 2004; Korsmeyer *et al.* 1999; Tan *et al.* 2006). The third point is that the conformational change that follows activation of Bcl-2 and Bcl-XL allows them to bind to membrane-bound Bax and Bak which prevents further oligomerization. Thus, Bcl-2 and Bcl-XL act as dominant negative with respect to the multi-region pro-apoptotic proteins. This explains the paradox of similarities between multi-region Bcl-2 proteins in their membrane conformation, homodimerization and formation ion-channels in artificial membranes (Annis *et al.* 2005; Jeong *et al.* 2004; Schendel *et al.* 1998; Sharpe *et al.* 2004). This leads to the fourth point, where the dominant negative function arises because Bcl-2 and Bcl-XL heterodimerize with Bax and Bak, but

do not propagate the oligomerization that mediates the formation of membrane-spanning pores. However, mutations that change these proteins into pro-apoptotic family members would do so by allowing this oligomerization (Basanez *et al.* 2001; Cheng *et al.* 1997; Clem *et al.* 1998; Fujita *et al.* 1998; George *et al.* 2007; Hanada *et al.* 1995; Lin *et al.* 2004). The final suggestion of this model is that Bcl-XL and Bcl-2 still bind to BH3-only proteins in solution, but in a different conformation, and therefore with a different affinity. This model provides explanations for the evidence supporting and refuting the Direct Activation and Displacement models but also emphasizes that much of the regulation of the Bcl-2 family proteins occurs at the membrane (Leber *et al.* 2007).

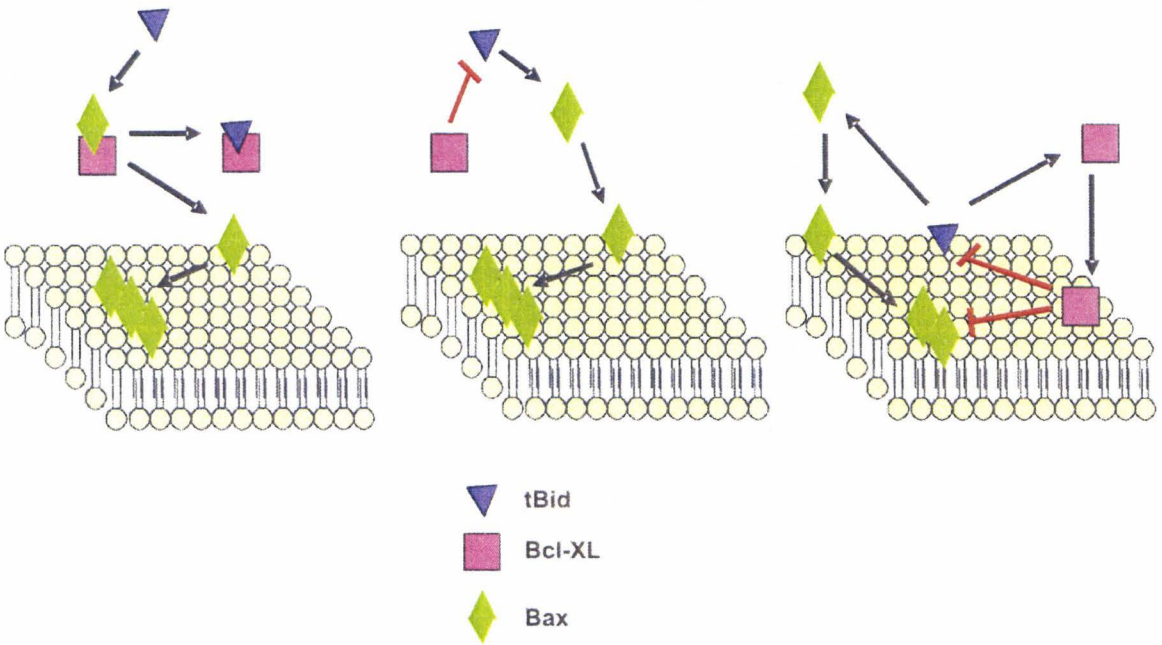

Figure 1.4 Current models of the regulation of apoptosis at the mitochondrial membrane. Models of apoptosis at the mitochondria membrane differ in which interactions are required for apoptosis. (A) The Displacement model proposed Bax is sequestered by anti-apoptotic proteins and release by BH3-only proteins, emphasizing the BH3-only – anti-apoptotic protein interaction. (B) The Direct Activation model suggests BH3-only proteins such as tBid interact with and activate Bax, and are sequestered by anti-apoptotic proteins. (C) The Embedded Together model includes the interaction with the mitochondrial membrane as well as Bax/BH3-only protein interactions and Bax/Bax interactions. Activated anti-apoptotic proteins can inhibit activation or oligomerization of Bax at the membrane.

Bcl-2 proteins as a drug target

Beginning with the discovery of overexpression of Bcl-2 in follicular B cell lymphoma, there is increasing evidence that Bcl-2 family proteins may be an appropriate target for the development of drugs for the treatment of cancer (Adams *et al.* 2007; Labi *et al.* 2006; Tsujimoto *et al.* 1985; Xing *et al.* 2007). Overexpression of anti-apoptotic proteins Bcl-2 and Bcl-XL occur in more than half of all tumours resulting in cells resistant to many apoptotic stimuli and cytotoxic anticancer drugs (Amundson *et al.* 2000; Henry-Mowatt *et al.* 2004). Bcl-XL and Bcl-2 provide protection against M-phase specific chemotherapeutic agents (Simonian *et al.* 1997). Bcl-XL expression provides better protection against etoposide, teniposide, methotrexate, fluorouracil and hydroxyurea in F5.12 cells (Simonian *et al.* 1997). Overexpression of Bcl-2 anti-apoptotic proteins is selective in cancer tissues, for example 53% of head and neck carcinomas had upregulated Bcl-XL or 17% had increased Bcl-2 (Pena *et al.* 1999). Bcl-2 expression remained constant while Bcl-XL was overexpressed in Kaposi's sarcoma (Foreman *et al.* 1996). Gastric and colorectal adenocarcinomas showed an increase in Bcl-w expression (Lee *et al.* 2003; Wilson *et al.* 2000). Bcl-2 family expression in cancer then seems to be tissue specific, as is expression in healthy cells, allowing for selective targeting by BH3 mimetics. Current prospects for drug development include BH3-mimetics that occupy the BH3-binding site on Bcl-2 or Bcl-XL or to activate Bak or Bax using BH3 mimetics of peptides which induce apoptosis (Letai *et al.* 2002). In leukemic cell mitochondria or mitochondria from leukemic cell lines from transgenic mice, sensitizer BH3-only proteins induce cytochrome c release if they can bind Bcl-2 supporting BH3 mimetics as potential cell and protein specific drugs (Certo *et al.* 2006). In such cell lines, a low dose may have a selective effect on activated anti-apoptotic proteins, further supporting the development of BH3 mimetics for cancer treatment. These mimetics can be used in conjunction with current treatments to which cells have become resistant and can be tailored to be specific, as demonstrated using bortezomib in combination with other treatments to target NSCL (Kohl *et al.* 2007; Voortman *et al.* 2007; Wolter *et al.* 1997). Several BH3 mimetics have been screened and binding affinities determined, often by fluorescence polarization. Small molecules currently being studied to inhibit anti-apoptotic proteins include chelerythrine, BH3I compounds, 2-methoxy antimycin, gossypol, apogossypol, purpurogallin and ABT-737 among many others (Chan *et al.* 2003; Konopleva *et al.* 2006; Lee, E. *et al.* 2007; O'Neill *et al.* 2004; Petros *et al.* 2006; van Delft *et al.* 2006; Wang, *et al.* 2006; Zhai *et al.*

2006). Binding affinities for these small molecules vary with the protein of interest. Small molecules that activate pro-apoptotic proteins include a stapled Bid BH3 peptide that activates Bax and Gleevec/Imatinib that indirectly induces Bim expression and prevents degradation, prevents dephosphorylation of Bad and induces transcription of Bmf (Labi *et al.* 2006; Walensky *et al.* 2006). Small molecule GT activates Bak but not Bax to generate ROS and the release of mitochondrial proteins (Pardo *et al.* 2006). One concern of these small activator molecules in that they may target healthy cells in the treatment of cancer as many cancers show an increase in anti-apoptotic protein expression which can sequester activated Bax/Bak (Walensky *et al.* 2006).

Experiments using knockout cell lines and mice have significantly advanced the field of apoptosis. Mitochondria isolated from wild type and Bak-/- mice provide a system in which to not only study pro-apoptotic Bax or Bak, but also the ability to examine effects of other Bcl-2 family proteins. My project was to use mitochondria from wild type and Bak-/- mouse liver to study Bcl-XL and two potential inhibitors 3e and 3e-D2. Differential centrifugation provided a rapid means of isolating functional membranes that maintained cytochrome c in the membrane fraction. Characterization of these membranes in Chapter 3 indicated the presence of Mcl-1 and Bik at the membrane by immunoblotting. We then examined the sensitivity of both membranes to recombinant tBid and Bax to determine concentrations that induced maximal cytochrome c release in combination. When used together, much less tBid was required to induce membrane permeability in the presence of Bak and Bax, suggesting cross-talk between Bak and Bax. This system was then applied in Chapter 4 to examine interactions required by Bcl-XL to prevent membrane permeabilization and indicated that Bcl-XL can interact with both Bax/Bak and tBid, but also uncovered a mechanism of inhibition independent of its binding to Bax/Bak or tBid. This confirmed data previously demonstrated in liposomes in our laboratory. Taken together, these results are consistent with the Embedded Together model of apoptosis. Using concentrations of tBid and Bax shown in Chapter 3 to maximize cytochrome c release and Bcl-XL determined to inhibit this release, our assay was then applied to examine small molecules 3e and 3e-D2. In Chapter 5, the assay system was used to confirm results obtained by our collaborator with fluorescence polarization that small 3e and 3e-D2 bind Bcl-XL. However, 3e and 3e-D2 were shown here to permeabilize the mitochondrial membrane in the absence of Bcl-2 family proteins, suggesting a method of permeabilization independent of Bcl-XL, Bax and Bak not suspected by the chemical screen used

to identify the molecules. Therefore, the comparison of membranes in the presence and absence of Bak provides a system that can be extensively applied to the study of apoptosis.

Chapter 2: Methods and Materials

2.1 General Materials

The chemical reagents used were obtained from Sigma Chemicals, EMD Science or Bioshop unless otherwise noted. The small molecules monomer and dimer were provided by Chengguo Xing (University of Minnesota).

2.2 Animals

C57BL6 mice (Charles River) and B6.129-Bak1^{tm1Thsn}/J mice (Bak-/- mice) (The Jackson Laboratory) were used as a source of mitochondria. Wild type mice were purchased at 6-8 weeks of age and used within one year. Bak-/- mice (Bak KO) were bred according to AREB protocols and were used within one year. All animals used were at least 8 weeks in age. Animals were euthanized by carbon dioxide as described previously (Hewett *et al.* 1993).

2.3 Antibodies

Western blotting was used to determine whether individual proteins could be detected in mouse liver extracts or membrane fractions. Murine antibodies to detect mouse endogenous proteins included 5C8 (Ex Alpha Biologicals) for tBid (1:8000), Bax antibody 5B7 (1:2,000) (Hsu *et al.* 1997) and anti-Hsp 60 (1:20,000) (a kind gift from Dr. Gupta). Rabbit antibodies were used to detect expression of Bak (1:2,000) (Upstate Cell Signaling Solutions, Cat# 06-536), Bcl-XL (1:10,000) (Cell Signaling, Cat# 2762), Bik (1:1,000) (Cell Signaling, Cat# 4592), A1/Bfl-1 (1:1,000) (Cell Signaling, Cat# 4622), Bad (1:1,000) (Cell Signaling, Cat# 9292), Mcl-1 (1:1,000) (Epitomics, Cat# 1239-1) and Stan, a rabbit anti-Bcl-2 antibody (1:10,000). A sheep antibody against Calreticulin (1:20,000) (Capralogics, Inc) was used to determine endoplasmic reticulum contamination in mitochondrial preparations.

Cytochrome c release was monitored using a purified sheep anti-cytochrome c primary antibody (1:5,000) (Ex Alpha Biologicals).

Monoclonal antibodies were diluted in Monoclonal Antibody Buffer (140mM NaCl, 10mM KPO_4 pH 7.4, 0.1% Triton X-100, 1% BSA) and polyclonal antibodies were diluted in Polyclonal Antibody Buffer (560mM NaCl, 10mM KPO_4 pH 7.4, 0.1% Triton X-100, 1% BSA, 0.02% SDS). Sheep anti-cytochrome c

primary antibody was diluted in TBS-T (10mM Tris, 0.15M NaCl, 0.2% Tween-20, pH 7.4) plus 1%BSA.

All secondary antibodies were raised in donkey and were conjugated to horseradish peroxidase (HRP) (1:10,000) (Jackson Immuno Research Laboratories Inc). Secondary antibodies were diluted in TBS-T plus 1% BSA.

2.4 General Methods

2.4.1 Protein Electrophoresis and Immunoblotting

Samples in Tricene Loading Buffer (2% SDS, 6% glycerol, 25mM Tris pH 6.8, 0.0005% (w/v) bromophenol blue, 0.25M DTT) were run on a Tricene-buffered 10% polyacrylamide gel to separate proteins by SDS-PAGE (Schagger *et al.* 1987). Samples were visualized using Coomassie brilliant blue dye or by immunoblotting. For immunoblotting, samples were transferred onto Nitocellulose (Pall) using the Hoefer semi-dry transfer apparatus (Pharmacia Biotech) for 1 hour at 50mA per membrane. The membranes were blocked at 4°C for a minimum of 30 minutes in Blocking Buffer (140mM NaCl, 10mM KPO_4, 1% azide). Membranes were washed with Wash Buffer (140mM, NaCl 10mM KPO_4 pH 7.4, 0.1% Triton X-100) for 5 minutes before the addition of the primary antibody. The appropriate primary antibody was incubated with the blot overnight at 4°C. The next day, blots were washed for a minimum of three times 15 minutes in TBS-T. Secondary antibodies conjugated to HRP were allowed to bind to the membranes for a minimum of 90 minutes before the blots were washed again using TBS-T. Blots were developed using the Enhance Chemiluminescence (ECL) method (Perkin Elmer).

2.4.2 Protein Sample Quantification

Protein samples were quantified using either the BCA assay (Pierce) or the Bradford assay (Bio-Rad) according to the included protocols. Mitochondrial preparations and whole cell lysate concentrations were determined using the Bradford assay. Concentrations were determined using a standard curve determined using BSA (Pierce).

2.5 Bax Overexpression and Purification

Bax was purified using an intein fusion system. The pBSΔN-VMA-CBD plasmid (Bax-Intein-CBD) (pMAC1572) was transformed into *Escherichia coli* BL21-AI cells by electroporation using Electroporator 2510 (Eppendorf) following the manufacturer's protocol. Cells were grown in LB with 100μg/ml ampicillin.

Bax was purified as reported previously (Yethon *et al.* 2003). Overnight cultures of BL21-AI were inoculated into LB-amp (100μg/ml ampicillin). Cultures were grown at 30°C to an OD_{600} of approximately 0.6. Arabinose at a final concentration 0.2% was used to induce protein expression and cells were grown for 3 hours at 30°C. Cells were harvested by centrifuging 15 minutes in a JA8.100 rotor (Beckman) at 5,000g and stored at –20°C.

Cells were thawed in Bax Lysis Buffer (20mM Tris-HCl pH 8, 500mM NaCl, 0.5mM EDTA, 0.05% Chaps, 2x PIN (approximately 30μM Chymostatin, 30μM Antipain, 30μM Leupeptin, 30μM Pepstatin A, and 60μM Aprotinin) and 1mM PMSF). A small amount of DNaseI and $MgCl_2$ to an approximate concentration of 0.3mM was added to the resuspended cells. The cells were lysed by passing through the French Press twice at 1000psi. The lysate was centrifuged at 15,000rpm in the JA25.5 rotor (Beckman) for 30 minutes at 4°C. The supernatant was removed and 3ml of chitin slurry was added and allowed to bind protein by rotating at 4°C for 2 hours. The slurry was added to a column and the flow through was collected. The column was washed with 30ml of Bax Lysis Buffer followed by 10ml of Bax Cleavage Buffer (10mM HEPES-KOH pH 7.4, 100mM NaCl, 0.2mM EDTA, 0.2% Chaps, 20% Glycerol, 2x PIN and 1mM PMSF). All washes were collected. The Bax was cleaved from the intein fusion by passing 10ml of Bax Cleavage Buffer containing 100mM β-mercaptoethanol into the column and incubating at 4°C for approximately 40 hours. Four 1ml fractions were collected by the addition of cleavage buffer (without PIN or PMSF).

Eluted fractions containing Bax (determined by SDS-PAGE gel and staining with Coomassie brilliant blue; Appendix Figure A1) were pooled and passed through a 0.5ml DEAE-Sepharose (Amersham Biosciences) column equilibrated in Bax Storage Buffer (10mM HEPES-KOH pH 7.4, 100mM NaCl, 0.2mM EDTA). The flow through was dialyzed in 2x1L of Bax Storage Buffer

containing 20% glycerol at 4°C over night. Dialyzed protein was aliquoted and stored at –80°C.

2.6 Overexpression and Purification of Bid

Murine Bid, pCI His-Bid plasmid (pMAC1446), or mutant tBid-mt1 (a pCI His-Bid plasmid coding for protein mutations M97A,D98A; pMAC2144), was transformed into BL21-A1 cells by electroporation as mentioned previously. Bid was purified as reported previously (Yethon *et al.* 2003). Overnight cultures were grown in LB with 100µg/ml ampicillin at 30°C to an OD_{600} of approximately 0.5. Cultures were induced with 0.2% Arabinose and grown three hours at 30°C. Cells were harvested by spinning 5,000g in a JA8.100 rotor for 15 minutes. Cells were stored at –20°C until lysed. For lysis, cells were resuspended in 25ml Bid Lysis Buffer (10mM Phosphate Buffer pH 8, 300mM NaCl, 30mM Imidazol, 1mM PMSF, 1mM DTT). DNAse I and 2 protease inhibitor mini-tablets (Roche) were added before lysing cells with 2 passes through the French press at 1000psi. The lysate was then centrifuged at 15,000rpm in the JA25.5 rotor for 30 minutes at 4°C. The supernatant was added to 2mL nickel-nitrilotriacetic acid-agrose (Qiagen) that had been washed with Bid Lysis Buffer excluding PMSF, DTT, DNAseI and protease inhibitor tablets and allowed to bind protein by rotating at 4°C for 2 hours. The slurry was added to a column and the flow through was collected. The column was washed with Bid Lysis Buffer. Bid was eluted using 10ml Bid Elution Buffer (10mM Phosphate Buffer pH8, 300mM NaCl, 200mM Imidazol, 10% glycerol and 0.2% Chaps) and 6x1ml fractions were collected and stored at –80°C (Appendix Figure A2 A).

2.7 Bid Cleavage and tBid Purification

Fractions from the Bid purification containing the highest concentrations of Bid (Appendix Figure A2 A) were used to purify tBid, as determined by SDS-PAGE and staining with Coomassie brilliant blue. The cleavage reaction contained 100µl Bid protein, 5µl Caspase 8 and 400µl caspase 8 buffer (50mM HEPES pH 7, 100mM NaCl, 0.1% Chaps, 10mM DTT, 10% glycerol). Cleavage was done at room temperature overnight.

To purify tBid, the cleavage reaction was added to 10ml of tBid Wash Buffer (10mM Phosphate buffer pH8, 300mM NaCl) plus 1ml nickel-nitrilotriacetic

acid-agrose. tBid was allowed to bind by rotating for 2 hours at 4°C. The slurry was added to a column and washed with 10ml tBid Wash Buffer. tBid was eluted with tBid Elution Buffer (10mM Phosphate Buffer pH8, 300mM NaCl, 10mM Imidazol, 1.2% Octyglucoside, 10% glycerol) and three 1ml fractions were collected. The column was washed with 2x1ml of Bid Elution Buffer. All fractions were stored at –80°C. Fractions containing tBid determined by SDS-PAGE and staining with Coomassie brilliant blue (Appendix Figure A2 B) were added to 10ml of tBid Storage Buffer (10mM Phosphate Buffer pH 8, 300mM NaCl, 10% glycerol). Protein was concentrated to 1ml with a 10kDa cut-off spin filter (Millipore) and washed with 10ml of tBid Storage Buffer by the addition of buffer followed by concentrating using the spin filter to a final 1ml volume. Samples were quantified using the Bradford assay using a Safire platereader (TECAN), aliquoted and stored at –80°C.

2.8 Overexpression and Purification of Bcl-XL

Plasmids encoding human Bcl-XL, pCyb3 Bcl-XL (Bcl-XL-Intein-CBD) (pMAC1758) or mutant Bcl-XL Y101K (pMAC 2145), were transformed into chemically competent *Escherichia coli* DH5α cells by heat shock. Briefly, 1µg of DNA was incubated on ice with 50µl of cells. Cells were exposed to heat shock at 42°C for 45 seconds, after which the cells were returned to ice for 2 minutes. The cells were allowed to recover in LB for 1 hour at 37°C before plating on LB-amp agar plates. Bcl-XL was purified as per the previously reported protocol (Yethon *et al.* 2003). Overnight cultures were inoculated into 2xYT medium and grown at 30°C to an OD_{600} of approximately 0.5, at which time cells were induced with 500µM IPTG for 3 hours. Cells were harvested by centrifugation at 5,000g in a JA8.100 rotor for 15 minutes. The pellet was stored overnight at -20°C.

Cells were thawed on ice and resuspended in 30ml of Bcl-XL Lysis Buffer (20mM Tris-Cl pH 8, 500mM NaCl, 0.5mM EDTA, 1% CHAPS, 2x PIN, 1mM PMSF). The cells were lysed by two passes through a French Press at 1000psi, and lysates were centrifuged at 15,000rpm for 30 minutes in a JA25.5 rotor to remove debris. The lysate was incubated with 4ml of chitin slurry for 1.5 hours at 4°C to bind the Bcl-XL-Intein-CBD protein. The slurry was added to a column and the flow-through was collected. The column was then washed with 25ml of Bcl-XL Lysis Buffer followed by 10ml of Bcl-XL Cleavage Buffer (20mM Tris-Cl pH 8, 200mM NaCl, 20% glycerol, 0.2% CHAPS, 2x PIN, 1mM PMSF). Bcl-XL

Cleavage Buffer with 100mM β-mercaptoethanol was passed into the column and the cleavage reaction was allowed to occur for approximately 40 hours at 4°C. Approximately 4ml of sample was eluted from the column using Bcl-XL Cleavage Buffer (without PIN or PMSF). These samples were then applied to a column containing 0.5ml phenyl-Sepharose (Amersham Biosciences) equilibrated in Bcl-XL Cleavage Buffer (without PIN or PMSF). The column was washed using the above buffer and 2ml of sample was eluted using Bcl-XL Cleavage Buffer (without PIN, PMSF or NaCl). The eluted protein was dialyzed against 2x1L Bcl-XL Storage Buffer (20mM Tris-Cl pH 8, 20 % glycerol). The protein concentration was determined by BCA and aliquots were stored at -80°C (Appendix Figure A3).

2.9 Preparation of Mouse Liver Mitochondria by Centrifugation on Gradients

Mitochondria were purified from mouse liver and separated from other heavy membranes using sucrose and Histodenz (also reported as Nycodenz) gradients as reported previously (Greenawalt *et al.* 1979; Shore *et al.* 1983). Briefly, two mice were euthanized by carbon dioxide and livers were excised from the abdominal cavity and placed in Buffer A (70mM sucrose, 220mM Mannitol, 2mM Hepes-KOH pH 7.4). The tissue was passed through a tissue press and diluted with 2 volumes equal to the tissue volume of Buffer A before being homogenized with a Potter homogenizer using a glass mortar and Teflon pestle for 4 strokes. Samples were diluted with three times the final volume with Buffer A and centrifuged in a JA25.5 rotor (Beckman) at 1500rpm for 10 minutes at 4°C. The supernatant was transferred to a new tube and centrifuged 12,000rpm in a JA25.5 rotor for 10 minutes at 4°C. The samples were resuspended in 30ml Buffer A and centrifuged at 1700rpm 10 minutes at 4°C. The supernatant was centrifuged at 12,000rpm for 10 minutes at 4°C to pellet mitochondria, which were washed in 30mL Buffer A by resuspending in buffer and centrifuging at 12,000rpm for 10 minutes. The final pellet was resuspended in 5mL Buffer B (250mM sucrose, 10mM HEPES-KOH pH 7.4, 2mM K_2HPO_4, 5mM sodium succinate, 1mM DTT, 1mM ATP and 0.08mM ADP). The protein concentration was determined by A280 and samples were diluted to 2-4mg/mL in Buffer B.

Mitochondria were layered on a two-step sucrose gradient using 2ml of a 1M sucrose buffer and 2ml of a 1.5M sucrose buffer (10mM Tris pH7.5, 2mM EDTA). Samples were centrifuged in an MLS50 rotor (Beckman) at 37,000rpm for 30 minutes at 4°C. A 20 gauge needle was used to pool mitochondria from

the 1.0M/1.5M sucrose interface. Mitochondria were diluted with 10 volumes compared to the pool samples with Buffer A and centrifuged at 12,000rpm for 10 minutes at 4°C in a JA25.5 rotor. Samples were resuspended in 500µL Buffer B and the protein concentration was determined as done above. Mitochondria were layered on a 3-step Histodenz gradient composed of 1.4mL of each step (20%/ 30%/ 40% Histodenz, 250mM sucrose, 10mM Tris pH 7.5, 2mM EDTA). Samples were centrifuged at 32,000rpm for 1 hour at 4°C in an SW50.1 rotor (Beckman). Samples were pooled as done above from the 30%/40% interface. Mitochondria were diluted with 10 volumes of Buffer A and centrifuged 12,000rpm as done above to wash mitochondria. The final pellet was resuspended in 500µl MB-EGTA (210mM mannitol, 10mM HEPES-KOH pH 7.4, 150mM KCl and 0.5mM EGTA) and the concentration of mitochondria was determined using the Bradford assay on a Safire platereader. The sample was diluted to 1mg/ml in MB-EGTA/MCB (210mM mannitol, 150mL KCl, 0.5mM EGTA, 10mM HEPES-KOH pH 7.4, 2mM $MgCl_2$, 2.5mM succinate 2.5mM Na_2PO_4, 2.5uM rotenone).

To purify mitochondria from heavy membranes by centrifugation in self-forming Percoll gradients, heavy membranes in Buffer B were layered over Percoll (30% (v/v) Percoll, 225mM mannitol, 25mM HEPES, 0.5mM EGTA and 0.1% BSA) as described by Luo *et al.* (Luo *et al.* 1998). Samples were centrifuged for 30 minutes at 22,000rpm in a MLS50 rotor (Beckman). The mitochondria were washed in Buffer A and resuspended in MB-EGTA as above. Mitochondria were quantified by Bradford and diluted to 1mg/ml in MB-EGTA/MCB.

2.10 Mitochondria Preparation from Mouse Liver by Differential Centrifugation

Mitochondria used for incubation with exogenous proteins were purified from mouse liver using differential centrifugation as reported previously (Frezza *et al.* 2007). Two livers were removed from euthanized animals and immersed in isolation buffer, IBc (10mM Tris-MOPS, 1mM EGTA/Tris, 0.2M sucrose, pH 7.4). The livers were washed 5 times with 5ml IBc to remove excess blood and minced on ice in IBc. The tissue was then homogenized in a Potter homogenizer using a Teflon pestle for 4 strokes in fresh IBc. The homogenate was centrifuged at 600g for 10 minutes at 4°C in a JA 25.5 rotor (Beckman). The supernatant was then centrifuged at 7,000g for 10 minutes to pellet mitochondria. The pellet was washed in 5ml fresh IBc. The final pellet was resuspended in a small amount of

IBc. Samples were quantified by Bradford and diluted to 1mg/ml in experimental buffer, EBc (125mM KCl, 1mM Tris/MOPS, 0.1mM EGTA/Tris, 0.1mM KH_2PO_4).

2.11 Liver or Spleen Whole Cell Lysates

For whole cell lysate samples from mouse liver to be used to detect Bcl-2 proteins by Western blotting, two livers were removed and washed as described above in IBc. The livers were homogenized as described and 100µl samples were set aside as whole cell lysates. These samples were quantified using the Bradford assay and diluted to 4mg/ml using TLB. Samples were boiled for 5 minutes, vortexed gently and stored at -20°C until used for Western blotting.

Whole cell lysates from splenocytes were prepared similar to liver tissue whole cell lysates. Five mice were euthanized and spleens were removed and immersed in IBc. The spleen was cut to remove splenocytes, which were homogenized as described above. Preparations were quantified, diluted and stored as above.

2.12 Cytochrome C Release Assay

To measure permeability of the outer mitochondrial membrane, 50µg of mitochondria were incubated with exogenous proteins, potential inhibitors 3e and 3e-D2, or osmotic stressors of interest. Membranes were incubated at 30°C for 1 hour. Samples were then centrifuged for 10 minutes at 13,000g to pellet mitochondria. The supernatant and pellet were separated and 50µl of Tricene Loading Buffer was added to each sample. Samples were boiled for 5 minutes, pellets resuspended by vortexing, and stored at –20°C. Cytochrome c release was determined by immunoblotting and percent release was determined by densitometry using ImageQuant V5.2 (Molecular Dynamics).

2.14 Osmotic Stress and TCA Precipitation

Mitochondria incubated in osmotic stressors to determine permeabilization of the outer mitochondrial membrane were diluted 1:5 after the 1 hour incubation in EBc (Sodium chloride incubations were diluted using EBc without potassium chloride). Samples were centrifuged 10 minutes to pellet the mitochondria as

done in the Cytochrome c Release Assay. To function as a carrier, 2µg of BSA (Pierce) was added to each supernatant. 50µl of Tricene Loading Buffer was added to each pellet. Supernatants were chilled on ice for 2 minutes, after which TCA was added to a final concentration of 20% (v/v). The supernatants were left on ice for 15 minutes. A 10 minute centrifugation at 16,100g was used to pellet samples. The supernatant was removed and pellets were washed with 400µl ice cold ethanol/ether (1:1 v/v) and vortexed to mix well. Samples were pelleted and the ethanol/ether removed by pipetting. The pellets were dried using a Speed Vac Concentrator (Savant) for approximately 15 minutes. To each sample, 50µl of TLB was added. Supernatants and pellets were boiled for 5 minutes and pellets resuspended before being stored at -20°C until analyzed by SDS-PAGE and immunoblotting.

2.15 Statistics and EC_{50}

All statistics were calculated using STATGRAPHICS Centurion XV. Significance was determined using a multifactor ANOVA to determine the F and P values with the Type III Sums of Squares Setting. EC_{50} values were determined using GraFit4.

Chapter 3: Development and Characterization of a Cell Free System to Monitor Functions and Inhibition of Bcl-2 Family Proteins

Introduction

The study of apoptosis and its regulation has benefited tremendously by studies using a variety of model systems including liposomes, isolated membranes, whole cell studies and animal models. While each method of investigation has its limitations, the combination of these studies has tested and validated much information about the process of cell death. *In vitro* systems using liposomes simplifies the system by eliminating many factors and focusing only on components added to the system, but cannot be used to study the effects of other membrane factors such as other proteins. Similarly, the study of Bcl-2 proteins using purified heavy membranes or isolated mitochondria does not include other cellular components that may play a role. Cells and animal models are more natural but also much more complex and therefore it is difficult to manipulate relevant experimental factors.

Studies examining the knockout of single and double genes in mice (and cells derived from them) have provided much insight into the requirement and function of Bcl-2 family proteins with respect to tissue specificity, protein redundancy and the involvement of Bcl-2 family proteins in development and disease (Roset *et al.* 2007). *In vitro* and cell-based assays have shown that elimination of Bax and Bak provides resistance to several apoptotic stimuli including BH3-only proteins (Cheng *et al.* 2001; Lindsten *et al.* 2000; Lindsten *et al.* 2006; Misao *et al.* 1996; Wei *et al.* 2001). Resistance to etoposide was seen in MEF cells that did not express Bax or Bak, but these cells die in response to alkylating agents with similar kinetics to wild type cells by necrosis (Lindsten *et al.* 2006). Other data suggests that double knockout (DKO) cells are only resistant to stimuli which trigger the intrinsic pathway such as UV, DNA-damaging drugs and agents that induce ER stress, but not stimuli which trigger the extrinsic pathway of apoptosis such as the TNF family of proteins or to autophagic death (Misao *et al.* 1996; Shimizu *et al.* 2004; Wei *et al.* 2001). Bax and Bak loss reduces calcium stores in the endoplasmic reticulum possibly by increasing the amount which leaks normally from the organelle (Nutt *et al.* 2002; Oakes *et al.* 2005; Scorrano *et al.* 2003). At a tissue level, Bak and Bax play a role in B cell development where DKO cells display defective cell cycle progression and adult mice develop severe autoimmune disease (Takeuchi *et al.* 2005). The combined loss of Bax and Bak in animals caused accumulation of redundant tissues (Lindsten *et al.* 2000). Specifically, DKO animals demonstrated multiple neuronal abnormalities, the continuance of interdigital webs, severe abnormalities in the hematopoietic

system, gross anatomical and histological abnormalities, unresponsiveness to auditory stimuli, memory cells skewed toward T cells and enlarged brains (Lindsten *et al.* 2000). DKO animals demonstrated resistance to etoposide and radiation-induced cell death but normal Fas-induced cell death. Splenocytes from DKO animals were resistant to death by growth factor withdrawal (Lindsten *et al.* 2000).

Cell-based assays and knockout mice using single knockouts for Bak or Bax demonstrate that there is some redundancy between these two proteins and single knockout animals showed minor abnormalities, with sterility noted in Bax knockout (Bax-/-) males (Lindsten *et al.* 2000). Isolated mitochondria from these mice have been used previously to study apoptosis, but are complicated by the presence of Bak at the membrane. Bak-/- mice provide an abundant source of mitochondria and these animals are phenotypically normal and therefore easily maintained. Isolation of membranes from these animals would provide a source of mitochondria free of Bak and Bax to study regulation of the Bcl-2 family of proteins, as well as an opportunity to examine the function of Bak when compared to wild type membrane. The focus of this section is to determine an appropriate method of mitochondrial isolation and characterization of membranes from wild type and Bak-/- mice sources for the study of apoptosis.

Results and Discussion

Isolation of Mitochondria from Mouse Liver

Mouse liver serves as an easily obtainable, abundant source of mitochondria and isolated mitochondria have been used to study Bcl-2 family proteins and many other proteins (Annis *et al.* 2005; Dlugosz *et al.* 2006; Henderson *et al.* 2007; Hwang *et al.* 2004; Tan *et al.* 2006; Yethon *et al.* 2003). In order to isolate mitochondria free from endoplasmic reticulum and other heavy membranes, gradients such as sucrose, Nycodenz (commercially available as Histodenz) and Percoll have been used (Eskes *et al.* 1998; Gonzalvez *et al.* 2005; Greenawalt *et al.* 1979; Henderson *et al.* 2007; Iaccarino *et al.* 2003; Luo *et al.* 1998; Shore *et al.* 1983). In my initial attempts to purify mitochondria from wild type C57BL6 mouse liver using differential centrifugation with sucrose and Histodenz gradients, cytochrome c was released following incubation in an isotonic buffer (Appendix Figure A4). Release of cytochrome c suggests that the

outer membrane was permeabilized or damaged during purification. This is fatal to our assay system, as we will be using cytochrome c as a marker of apoptosis since its release from the intermembrane space is one of the critical events during apoptosis that is regulated by Bcl-2 family proteins (Acehan *et al.* 2002; Luo *et al.* 1998; Wang 2001; Yang *et al.* 1997). Therefore, the appearance of cytochrome c in the supernatant in the absence of apoptotic stimuli indicates that mitochondria purified in this way are not suitable for the study of apoptosis.

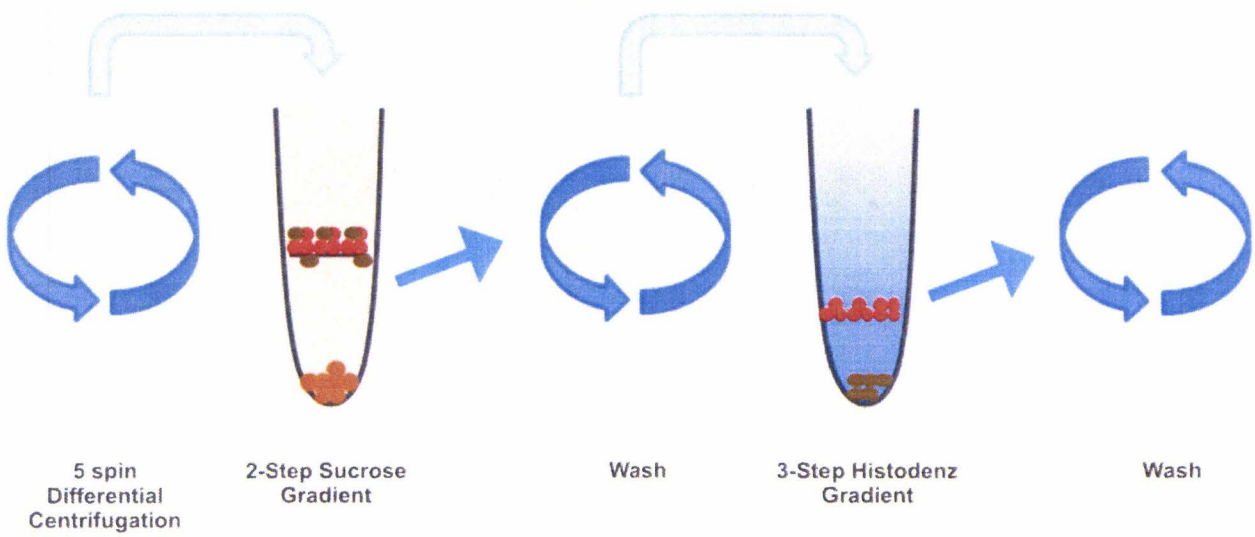

Figure 3.1 Mitochondrial isolation by gradient purification. Purification of mitochondria from mouse liver is performed in three steps. The first step is differential centrifugation comprised of five short spins to isolate heavy membranes. These are then passed through a sucrose gradient and are washed using a high speed spin before layering over a Histodenz gradient. The final sample is washed using a high speed spin before quantifying the amount of total protein by a Bradford assay.

Samples taken at different stages of the purification process demonstrated that membranes purified from wild type mouse liver maintained cytochrome c in the membrane if the sucrose gradient but not the Histodenz gradient was used (Figure 3.2A; Appendix Figure A4). The purification method was then used on mitochondria from Bak-/- mouse liver and these membranes released cytochrome c after the sucrose gradient, but not after differential centrifugation

alone (Figure 3.2A). These initial observations were intriguing because fetal rat liver mitochondria are permeable to sucrose, and this can affect mitochondrial purification by sucrose gradient (Packer *et al.* 1971; Pollak 1975). Percoll gradients however can also be used to isolate mitochondria by density (Lopez-Mediavilla *et al.* 1989). Therefore, the observed difference between wild type and Bak-/- mitochondria with the sucrose gradient from these preliminary experiments suggested that Bak in involved in mitochondrial permeability to osmotic stressors such as sucrose. To examine if the cytochrome c release observed was a result of passing the samples through a gradient or due to the osmotic stress caused by sucrose, samples were purified using differential centrifugation followed by a Percoll gradient. Percoll is an iso-osmotic self-forming gradient that has also been used extensively for the purification of mitochondria (Gonzalvez *et al.* 2005; Iaccarino *et al.* 2003; Luo *et al.* 1998). After incubation in experimental buffer, mitochondria from wild type and Bak-/- mouse liver maintained cytochrome c in the membrane fraction (Figure 3.2B), suggesting that cytochrome c release seen following sucrose gradients is a result of osmotic stress.

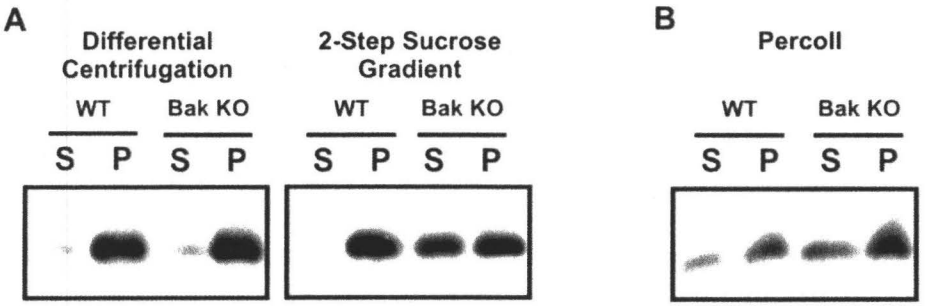

Figure 3.2 Bak-/- mitochondria release cytochrome c during sucrose purification. Mitochondria from wild type C57BL6 (wt) and Bak-/- (Bak KO) C57BL6 mouse liver were purified by differential centrifugation and a sucrose gradient (A). Samples resulting from differential centrifugation and after being passed through a two-step sucrose gradient were incubated for 1 hour at 30°C, and the supernatant (S) and pellet (P) were separated. Mitochondria were also prepared by Percoll gradient following differential centrifugation (B). Samples were immunoblotted to determine cytochrome c release.

Release of mitochondrial intermembrane space proteins has been seen previously when cells were incubated with osmotic stressors such as sorbitol. Criollo *et al.* demonstrated that HeLa cells and HCT116 cells both undergo

osmolyte-induced apoptosis as measured by caspase activation. However, release of intermembrane space proteins is cell type specific (Criollo *et al.* 2007). HCT116 cells release AIF, whereas HeLa cells do not. Cytochrome c was retained in both cell lines, differing from our results with sucrose gradient purification. The release of intermembrane proteins may be initiated by Bcl-2 family proteins and is supported by experiments demonstrating Bcl-2 prevents the loss of membrane potential in response to calcium released from endoplasmic reticulum (Miyawaki *et al.* 1997; Shimizu *et al.* 1998). Other publications indicate that the knockdown of Bax in cells increases resistance to osmotic stress, whereas Bcl-2 and Bcl-XL elimination sensitizes cells to sorbitol-induced apoptosis (Criollo *et al.* 2007). These mitochondria contain Bak and it is unclear if Bak plays a role similar to Bax in osmotic stress. However, Bak is an integral protein of the outer mitochondrial membrane has been reported to bind to VDAC2 and Mcl-1 (Frey *et al.* 2007; Kim *et al.* 2006; Minet *et al.* 2006; Moldoveanu *et al.* 2006; Uren *et al.* 2007; Willis *et al.* 2005; Zhuang 2006). VDAC proteins are involved in mitochondrial permeability (Baines *et al.* 2007) and may open in response to calcium, allowing for the equilibration of ions and water between the matrix and cytoplasm (Bernardi *et al.* 2006; Beutner *et al.* 1998; He *et al.* 2002). Recombinant VDAC incorporated into liposomes showed increased uptake of radio-labelled sucrose in the presence of recombinant Bax or Bak (Shimizu *et al.* 1999). In response to osmotic stress, Bax and Bak were activated downstream of Mcl-1 destabilization, supporting a role for Bak in osmotic stress; note that these upstream signals may be absent in a cell-free system (Fritsch *et al.* 2007). Thus there is physiologic rationale (although no agreement about the mechanism) for the potential role of Bak-induced apoptosis in response to osmotic stressors.

To examine the involvement of Bak in osmotic stress in our system, membranes isolated by rapid differential centrifugation (Frezza *et al.* 2007) were incubated with increasing concentrations of several osmotic stressors including sucrose, sorbitol and sodium chloride. Sorbitol and sodium chloride are often used to induce hypertonic stress in cells and have been used to induce apoptosis (Chen *et al.* 2004; Criollo *et al.* 2007; De Martino *et al.* 2003; Fritsch *et al.* 2007; Huh *et al.* 2002; Michea *et al.* 2002). In contrast to my preliminary results, Bak-/- mitochondria responded similarly to wild type mitochondria following osmotic stress by sodium chloride, sorbitol and sucrose (Figure 3.3, p=0.99, p=0.53, p=p.51 respectively), although Bax-/- membranes consistently released slightly higher amounts of cytochrome c in sucrose titrations. Therefore, the initial

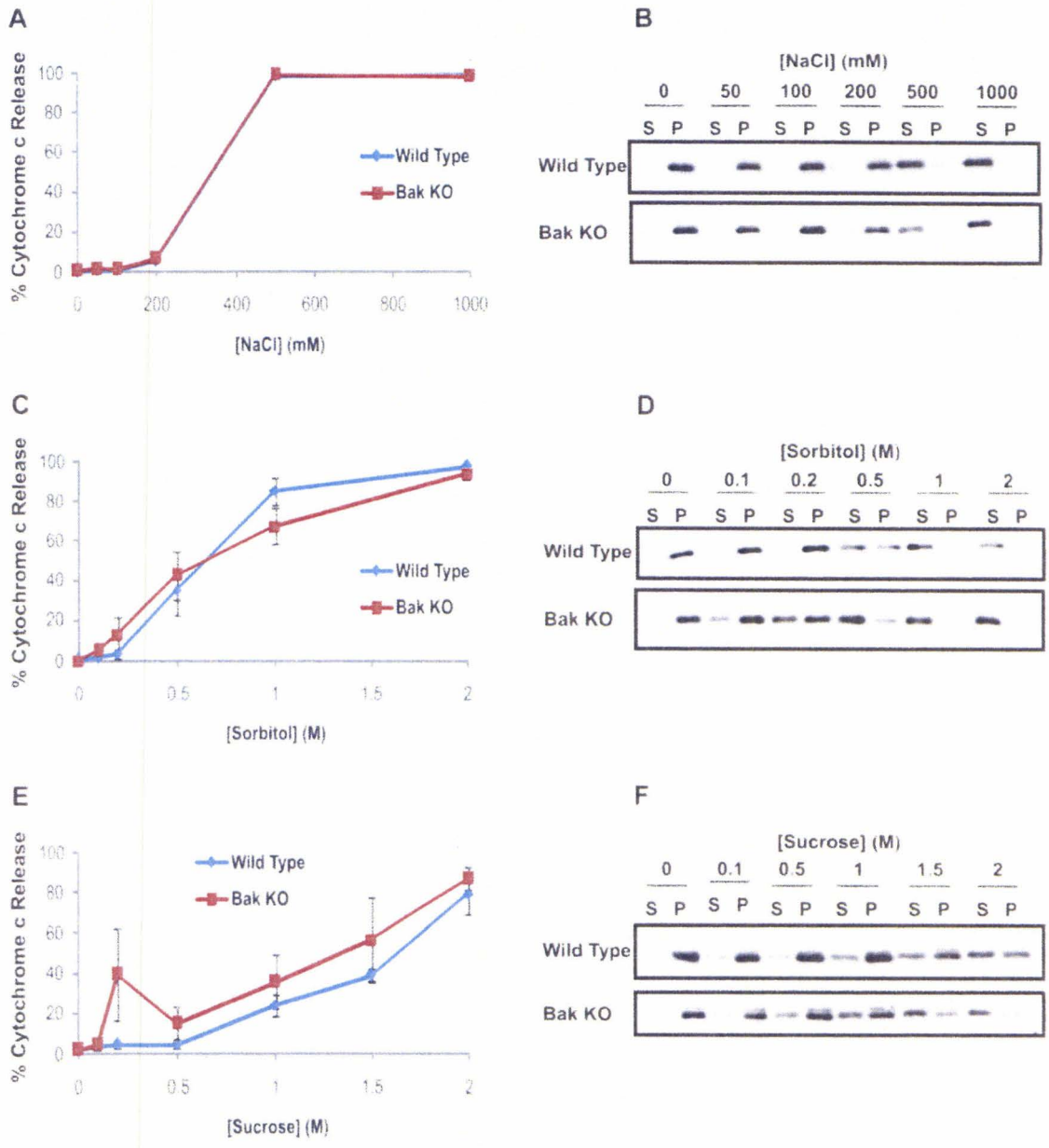

Figure 3.3 Bak-/- mitochondria are not more sensitive to osmotic stressors. Mitochondria purified from wild type and Bak-/- (Bak KO) mouse liver were incubated with increasing concentrations of osmotic stressors sodium chloride (A and B), sorbitol (C and D) or sucrose (E and F). Mitochondria were incubated for 1 hour at 30°C, and the supernatant (S) and pellet (P) were separated. Immunoblotting was used to determine cytochrome c release (B, D and F) and were measured for percent cytochrome c release (A, C and E).

observation was not confirmed and despite the fact that the data suggest that a subtle phenotype might be present in Bak-/- mitochondria, this was not pursued any further.

Recently, Frezza *et al.* have published a simple purification method for the rapid isolation of mitochondria using differential centrifugation; the mitochondria are intact as they maintain respiratory function (Figure 3.4A) (Frezza *et al.* 2007). After incubation in the experimental buffer described by Freeza *et al.*, mitochondria isolated from wild type and Bak-/- mouse liver maintain cytochrome c in the membrane fraction (Figure 3.4B). Even though these enriched mitochondria may be contaminated with other heavy membranes, the simplicity, practicality and reproducibility of this method is perfectly suited to enrich and characterize wild type and Bak-/- mitochondria for extensive examination of the role of Bcl-2 family proteins in apoptosis.

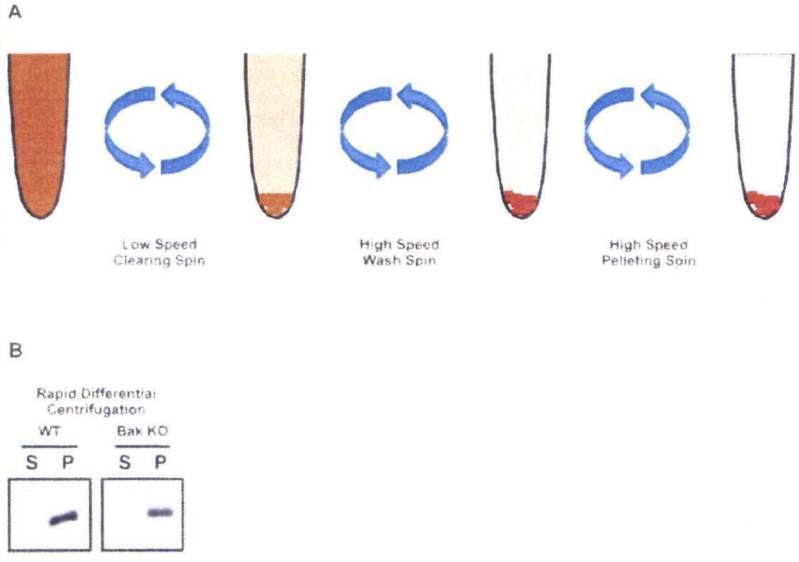

Figure 3.4 Isolation of mitochondria by rapid differential centrifugation. Mitochondria from mouse liver can be isolated in three short spins; the first to clear debris and whole cells, and higher speed spins to remove light membranes (A). Mitochondria from wild type and Bak-/- animals (Bak KO) maintain membrane integrity as illustrated by the presence of cytochrome c in the pellet (P) but not the supernatant (S) detected by immunoblotting.

Mitochondria Isolated from Wild Type and Bak-/- Mouse Liver Contain Few Bcl-2 Family Proteins

One advantage in the use of liposomes is that all the components are known and quantified, ensuring that no compounding factors affect the results. The benefit of using isolated mitochondria is that it provides a system that more closely resembles what occurs in live cells. However, mitochondria may contain other Bcl-2 family members and potentially other proteins that affect the results. Therefore, to characterize this system, we determined whether other Bcl-2 family proteins are present in these heavy membrane fractions.

Western blotting was used to detect several Bcl-2 proteins that have been reported to be expressed in hepatocytes and are detected at the mitochondria (Table 3.1). Based on the publication by Chen *et al.*, Bcl-2 proteins whose mRNA levels increase in response to apoptotic stimuli include bad, bax, bcl-10, bcl-2, bcl-w, bcl-x, bfl-1, bid, bik/blk, bim, bok/mtd, hrk and mcl-1 (Chen *et al.* 2005). Therefore we screened for the presence of Bak, Bax, tBid, Bim, Bad, Bik, Bcl-XL, Bfl-1/A1 and Mcl-1 as these proteins may be present in liver mitochondria or will be used in our assay (D'Sa-Eipper *et al.* 1998; Danial *et al.* 2003; Hegde *et al.* 1998; Ko *et al.* 2003; O'Connor *et al.* 1998; Puthalakath *et al.* 1999; Rodriguez *et al.* 1996; Zhang *et al.* 2000; Zhou *et al.* 2005). Bcl-2, Hsp60 and calreticulin were run as controls for different organelles. Even though mRNA for Bcl-2 was present according to Chen *et al.*, in mice Bcl-2 protein is not highly expressed in the liver (Chen *et al.* 2005; Negrini *et al.* 1987). Hsp60 is a mitochondrial matrix protein and calreticulin is an endoplasmic reticulin (ER) protein which serves as an indicator of ER contamination. It would be interesting to screen for Hrk also as it is present in mitochondria and binds Bcl-XL, but there is currently no antibody which recognizes the mouse variant (Cuconati *et al.* 2003; Harada *et al.* 2000; Inohara *et al.* 1998; Sunayama *et al.* 2004). Bok was not assayed as its expression in mice is limited to the testis, ovaries and uterus (Hsu *et al.* 1997; Inohara *et al.* 1998).

Lysates and enriched mitochondria were used from wild type C57BL6 mice and Bak-/- mice to determine the presence of any of these proteins as assessed by immunoblotting (Figure 3.5). As expected, Bak is present in mitochondrial membranes in the wild type liver and not in the Bak-/- samples. Bid, Bcl-XL and Bcl-2 are proteins of interest for use with these purified mitochondria and samples were analyzed with controls to determine the Table

Table 3.1 Tissue expression and localization of Bcl-2 family proteins. Bcl-2 family proteins shown to have increased mRNA expression were reviewed for protein expression in hepatocytes, localization to the mitochondrial membrane and binding to Bax, Bak, tBid and Bcl-XL.

	Expression in Liver	Localization	Interacts with Bax/Bak, tBid, Bcl-XL	References
Bad	Yes	Cytosol	Bcl-XL; Bcl-2	Danial *et al.* 2003; Rodriguez *et al.* 1996; Yang *et al.* 1995; Zha *et al.* 1997; Zhou *et al.* 2005
Bak	Yes	Mitochondria	Bcl-XL; tBid; Bax	Kiefer *et al.* 1995; Rodriguez *et al.* 1996; Sattler *et al.* 1997; Sundararajan *et al.* 2001; Wei *et al.* 2000
Bax	Yes	cytosol	tBid; Bak	Cartron *et al.* 2004; Eskes *et al.* 2000; Hsu *et al.* 1998; Oltvai *et al.* 1993; Rodriguez *et al.* 1996; Sedlak *et al.* 1995; Sundararajan *et al.* 2001; Tan *et al.* 2006
Bcl-10	Yes	Cytosol; nucleus	None	Cheng *et al.* 2001; Thome *et al.* 2007; Willis 1999; Yeh *et al.* 2000
Bcl-2	No			Rodriguez *et al.* 1996
Bcl-w	No			O'Reilly *et al.* 2001
Bcl-x	Yes	Mitochondria; Cytosol	Bax; Bak; tBid	Cartron *et al.* 2004; Eskes *et al.* 2000; George *et al.* 2007; Gonzalez-Garcia *et al.* 1994; Poruchynsky *et al.* 1998; Rodriguez *et al.* 1996; Sattler *et al.* 1997; Uren *et al.* 2007; Wang, K. *et al.* 1996; Wang *et al.* 2004

Bfl-1/A1	Yes	Mitochondria	Bax; Bak; tBid	D'Sa-Eipper *et al.* 1998; Sedlak *et al.* 1995; Simmons *et al.* 2007; Werner *et al.* 2002; Zhang *et al.* 2000
Bid	Yes	cytosol	Bax; Bak; Bcl-XL	Cartron *et al.* 2004; Eskes *et al.* 2000; George *et al.* 2007; Wang, K. *et al.* 1996; Wei *et al.* 2000; Yin *et al.* 1999
Bik/Blk	Yes	Mitochondria	Bcl-XL; Bcl-2	Hegde *et al.* 1998
Bim	Yes	Nucleus; cytosol	Bcl-2; Bcl-XL	O'Connor *et al.* 1998; O'Reilly *et al.* 2000; Puthalakath *et al.* 1999
Bok	No			Hsu *et al.* 1997; Inohara *et al.* 1998
Hrk	Minimal mRNA expression	Mito	Bcl-XL; Bcl-2	Harada *et al.* 2000; Sunayama *et al.* 2004
Mcl-1	Yes	Mito	tBid; Bak	Cuconati 2003; Harada *et al.* 2000; Uren *et al.* 2007

presence of these proteins. By immunoblotting, Bid and Bcl-XL were not detected in whole cell lysates or isolated heavy membranes. As has been noted previously, Bcl-2 was detected in splenocytes but not in liver samples (Negrini *et al.* 1987; Orelio *et al.* 2007). Bax was also absent in these samples by immunoblotting and neither Bad nor A1 was detected. There was a band of the expected size for Bik that is more prominent in the isolated membranes, suggesting Bik may be present in these samples. Mcl-1 immunoblots showed a faint band at the expected size for Mcl-1 in the whole cell lysates and the heavy membranes for wild type and Bak-/- tissue. The controls, Hsp60 and calreticulin, were seen in wild type and Bak-/- mitochondria. Calreticulin appears only as a faint band at the expected size. For all the samples, the banding patterns and intensities were similar for wild type and Bak-/- samples (with the expected exception of Bak), suggesting there is no change in expression of other proteins. Our results are consistent with those previously published claiming that mouse

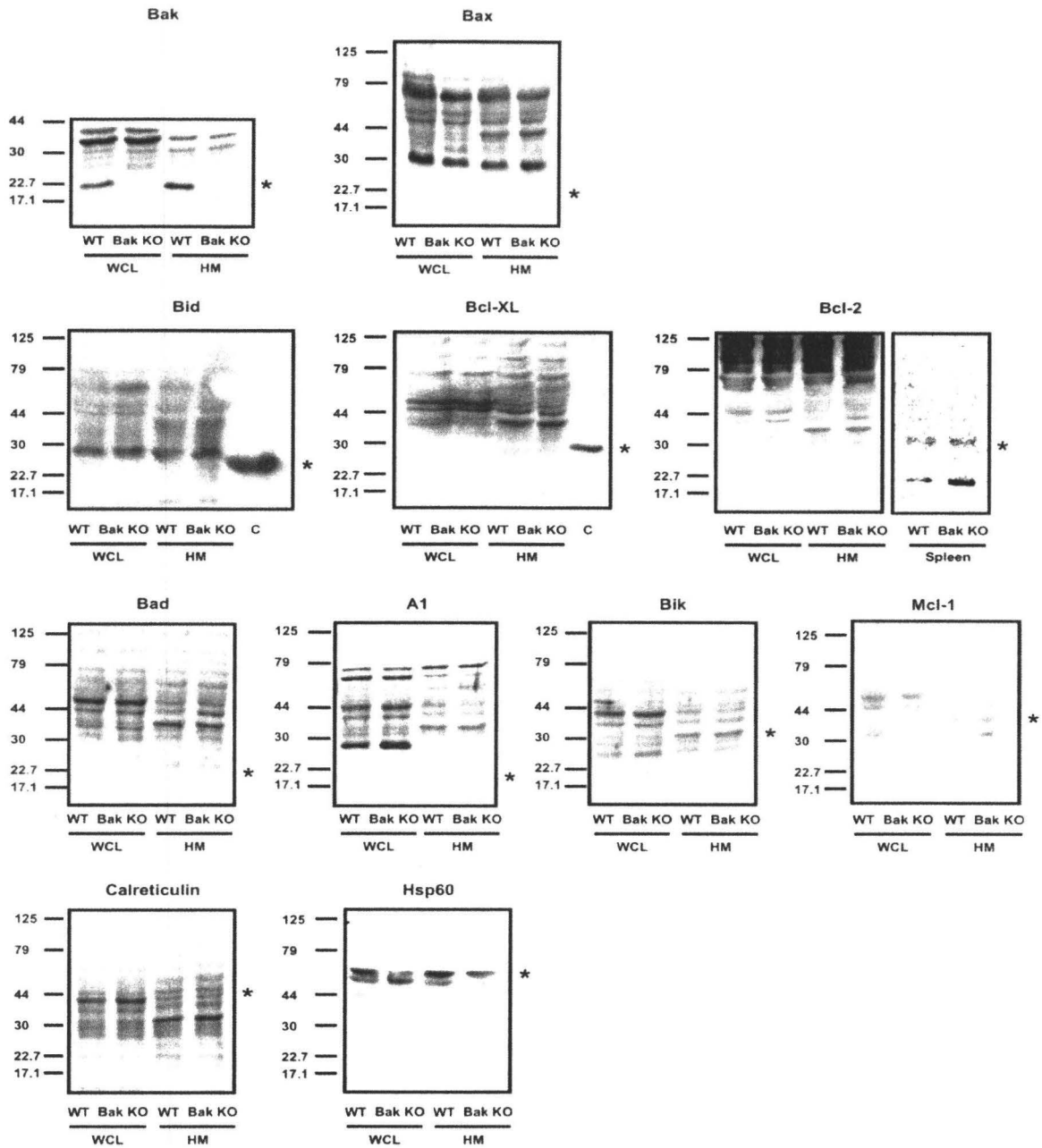

Figure 3.5 Bcl-2 family proteins in mouse mitochondrial membranes. Whole cell lysates (WCL) and heavy membrane fractions (HM) were screened for Bcl-2 family proteins and for control proteins calreticulin and Hsp60. Samples were taken from wild type (WT) and Bak-/- (Bak KO) tissue. Recombinant protein for Bid and Bcl-XL were used as a control (C), as well as samples from splenocytes (spleen) to determine the expected size. The predicted molecular weight for each protein (*) is labelled in kDa, as are the molecular markers.

liver mitochondria do not contain Bcl-2, Bax, Bak, Bim, Bid/tBid or PUMA when up to 125µg of total protein are loaded per well to be assayed by immunoblotting (Uren *et al.* 2007). This publication did report trace amounts of Bcl-XL and Mcl-1 present in mouse liver mitochondria, although Bcl-XL was not detected in our samples when less total protein is used (Uren *et al.* 2007).

The relative absence of Bcl-2 family proteins provides a physiologically relevant membrane surface to examine the role of added recombinant full length Bcl-2 proteins. Specifically, the main proteins of interest (Bax, Bid and Bcl-XL) are absent in the isolated membranes and Bak is only present in wild type. The presence of Bik and Mcl-1 in the membranes may complicate the system as these proteins can interact with tBid, Bcl-XL, Bax and Bak, but did not confound the experimental results, as discussed in Chapters 4 and 5 (Boyd *et al.* 1995; Chen *et al.* 2005; Kim *et al.* 2006; Uren *et al.* 2007; Verma *et al.* 2001; Zhuang 2006). The release of cytochrome c resulting from membrane permeabilization in our system is therefore due to the added components.

Mitochondria Isolated from Bak-/- Mouse Liver are Less Sensitive to tBid than Wild Type but are Equally Sensitive to High Bax Concentrations

It has been shown previously that tBid activates both Bax and Bak, after which membrane permeabilization occurs with the release of cytochrome c. The exact concentration of the Bcl-2 family proteins that will affect this process in our system is unknown, and therefore it was necessary to determine appropriate concentrations of Bax and tBid that would not permeabilize the membrane alone, but would induce the release of cytochrome c when used in combination. Titrations of tBid and Bax alone were done in mitochondria isolated from wild type and Bak-/- mouse liver.

As seen in Figure 3.6, tBid induced cytochrome c release in wild type mitochondria as the concentration was increased, likely due to the presence of Bak that becomes activated (Figure 3.6, A and B) (Cartron *et al.* 2004; Clohessy *et al.* 2006; Eskes *et al.* 2000; Harada *et al.* 2000; Marani *et al.* 2002; O'Connor *et al.* 1998; Opferman *et al.* 2003; Wei *et al.* 2000). Although there is a minority view that Bak requires Bax for full activation (Mikhailov *et al.* 2003; Takahashi *et al.* 2005), our results are not consistent with this conclusion. Higher concentrations of purified tBid up to 1nM do not induce cytochrome c release from Bak-/- mitochondria, consistent with previous reports using Bak/Bax double

knockout cells where tBid and Bim did not induce cytochrome c release or apoptosis (Wei *et al.* 2001; Zong *et al.* 2001). Previously, it was reported that tBid released cytochrome c from purified Bak-/- MEF and FDM mitochondria suggesting Bax is present in these mitochondrial preparations (Pardo *et al.* 2006). However, cytochrome c release was observed in these experiments in which mitochondria were isolated by differential centrifugation and incubated with 40nM recombinant tBid, much higher concentrations than used in our experiment. It is unclear if the same mechanisms are involved, or if tBid at such concentrations permeabilized membranes by interacting with other substrates.

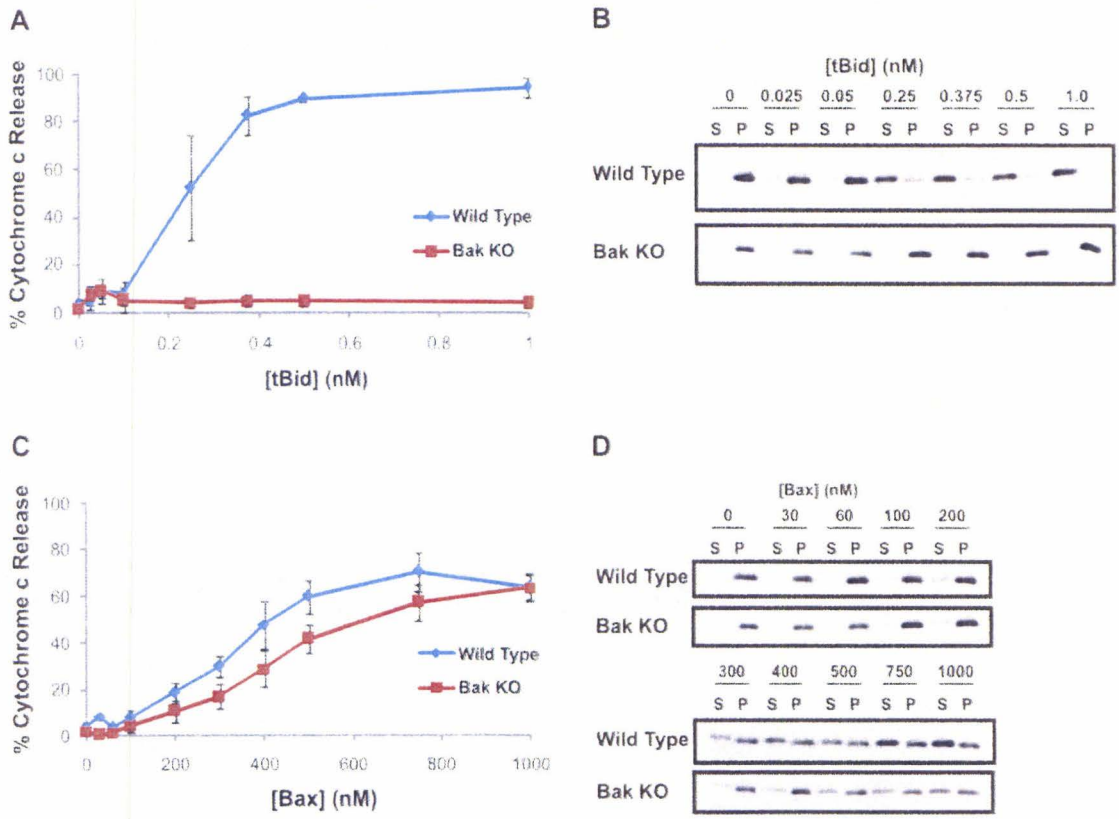

Figure 3.6 Mitochondria are less sensitive to permeabilization by tBid but not Bax in the absence of Bak. Mitochondria isolated by differential centrifugation from wild type and Bak-/- mouse liver were incubated with purified recombinant tBid (A and B) or Bax (C and D). The supernatant (S) and pellet (P) were separated. Samples were immunoblotted for cytochrome c (B and D) and percent cytochrome c release was determined (A and C).

Addition of Bax resulted in similar effects in wild type and Bak-/- mitochondria as Bax induced cytochrome c release in both membranes equally at relatively high concentrations (Figure 3.6, C and D; p=0.74). Polster *et al.* estimated that Bax concentration in cells is 0.7-1.1µg per milligram of mitochondria in unstressed cells (Polster *et al.* 2003). The concentrations used here which release cytochrome c are in this range or above. Our results suggest that Bax causes membrane permeabilization at high concentrations, possibly due to the presence of some small fraction of activated protein that is a consequence of our purification process. Other proteins such as p53 and Bif-1 not screened in this system have been reported to activate Bax, but these proteins are normally cytosolic and should not be present following subcellular fractionation (Chipuk *et al.* 2004; Cuddeback *et al.* 2001; Leu *et al.* 2004; Takahashi *et al.* 2005). The release of cytochrome c in the absence of added tBid is at variance with models in which tBid-induced remodelling of cristae is required for efficient mobilization of the sequestered cytochrome c (Ott *et al.* 2002). Based on these considerations, the working concentration for Bax was chosen to be 200nM for wild type and Bak-/- mitochondria as this concentration did not induce cytochrome c release.

To choose a concentration of tBid for use in combination with Bax, tBid was titrated with Bax in incubations containing wild type or Bak-/- mitochondria. The range chosen was based on the titration curves for tBid alone. Concentrations of tBid that induced almost 100% cytochrome c release in combination with 200nM Bax were 0.025nM for wild type mitochondria and 0.25nM for Bak-/- mitochondria (Figure 3.7). The release of cytochrome c after addition of increasing amounts of tBid with 200nM Bax differed between the two sources of mitochondria (p=0.009). This difference is caused by the presence of Bak, which can be activated by both tBid and Bax (Gross *et al.* 1999; Kuwana *et al.* 2002; Wei *et al.* 2000). The permeabilization by tBid and Bax in Bak-/- mitochondria indicates that for mouse liver mitochondria Bak is not necessary for this process, in contrast to observations in some cell types (Er *et al.* 2007; Neise *et al.* 2007).

One concern using an *in vitro* system with enriched mitochondria is that cytochrome c release observed is only an acceleration of the spontaneous cytochrome c release that occurs in isolated mitochondria (Antignani *et al.* 2006). However, control experiments indicate that for our incubation period chosen no cytochrome c release is observed. Furthermore, as noted above, mitochondria purified by differential centrifugation as described here maintain normal oxygen consumption suggesting these mitochondria remain coupled in the experimental

buffer which mitigates one cause of cytochrome c release (Frezza *et al.* 2007). Further evidence of the physiological relevance of the mitochondrial system is that the Bcl-2 conformational change seen in cells following treatment with doxorubicin and etoposide can be mimicked in isolated mitochondria that are treated directly with tBid and Bax (Dlugosz *et al.* 2006). This conformational change is also reproduced in liposomes using Bcl-2 tethered to the membrane (Peng *et al.* 2006). Therefore, a comparison of wild type and Bak-/- enriched mitochondria will provide a relevant system to examine the interactions of proteins and for testing inhibitors monitoring of cytochrome c release.

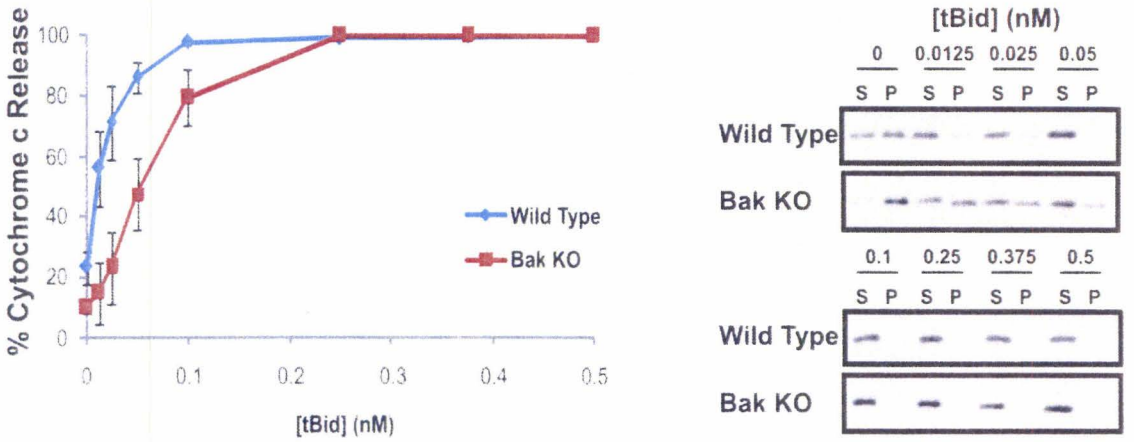

Figure 3.7 Bak-/- mitochondria are less sensitive to tBid in the presence of Bax. Wild type and Bak-/- mouse liver was used to purify mitochondria. Heavy membranes were incubated in excess Bax (200nM) and increasing amounts of tBid. Samples of the supernatant (S) and pellet (P) were used to immunoblot for cytochrome c (B) and percent cytochrome c release was determined (A).

Conclusions

The field of apoptosis has been quickly advanced by use of both *in vitro* and *in vivo* assays. To examine mechanisms and functions of Bcl-2 family proteins, wild type and Bak-/- membranes can be used to study this group of proteins. Differential centrifugation provides a rapid means of isolating functional mitochondria and these mitochondria contain minimal Bik and Mcl-1 but no other confounding Bcl-2 proteins. A comparison of wild type and Bak-/- mitochondria provides an opportunity to look at interactions of Bak with Bcl-2 family proteins tBid, Bax and Bcl-XL. Addition of tBid and Bax demonstrated Bak activation by tBid as well as permeabilization of mitochondria by Bax alone in higher

concentrations independent of Bak and tBid. With the well characterized system, it is possible examine closely the direct effects of Bax and its inhibition by Bcl-XL, and to examine Bcl-2 proteins as targets for small molecule inhibitors.

Chapter 4: Apoptosis at the Membrane

Introduction

Since the anti-apoptotic proteins of the Bcl-2 family have no catalytic activity to explain their pro-survival function, one area of particular interest is how these proteins interact with the pro-apoptotic Bcl-2 family members at the mitochondria. Current models of regulation are the Direct Activation model, the Displacement model and the Embedded Together model. There is evidence to both support and refute the Direct Activation and Displacement models, which are mutually exclusive in their conclusions regarding which protein interactions are required for regulation. The Direct Activation model states that the interactions between the BH3-only proteins and the multi-domain pro-apoptotic proteins Bax and Bak are critical (Cartron *et al.* 2004; Certo *et al.* 2006; Kuwana *et al.* 2005; Letai *et al.* 2002; Takahashi *et al.* 2005; Yang *et al.* 1995). In contrast, the Displacement model proposes that the interaction between anti-apoptotic proteins and BH3-only proteins frees active Bax and Bak resulting in membrane permeabilization (Certo *et al.* 2006; Chen *et al.* 2005; Kuwana *et al.* 2005; Willis *et al.* 2005).

The final model, the Embedded Together model, acknowledges that many interactions are important but introduces a different perspective (Leber *et al.* 2007). It concludes that Bax and Bak go through regulated conformational changes, and most controversial is the concept that BH3-only proteins activate both multi-domain pro- and anti-apoptotic proteins by causing similar conformational changes in each at the mitochondrial membrane, and that these conformational changes allow Bcl-2 and Bcl-XL to bind to membrane-bound Bax and Bak, preventing further oligomerization.

Bcl-XL has been linked to several diseases such as cancer as its expression correlates with resistance to chemotherapeutic agents in the 60 cancer cell lines held the NCI repository (O'Neill *et al.* 2004). It has been demonstrated to have a large effect on cell survival with a small increase in expression as compared to Bcl-2 in the human MCF7 breast cancer cell line (Fiebig *et al.* 2006). However, the exact mechanism of inhibition by Bcl-XL is unknown. Crystal structures suggest domain swapping occurs during homodimerization but does not involve the hydrophobic groove that mediates binding to pro-apoptotic Bcl-2 family members (O'Neill *et al.* 2006). As well, crystallization of Bcl-XL with BH3 peptides of Bak, Bad and Bim suggest Bcl-XL may inhibit apoptosis by sequestering other Bcl-2 proteins, but this has not yet

been investigated with full length untagged proteins (Yan *et al.* 2005). Nevertheless, the implication of these crystal structures is that there may be multiple independent functional regions of Bcl-XL that can be targeted for drug development.

Mutational analysis has been used to investigate the mechanism of apoptosis inhibition by Bcl-XL by eliminating specific protein interactions, changing localization or removing cytochrome c release inhibition (Cheng *et al.* 2003; Clem *et al.* 1998; Desagher *et al.* 1999; Eskes *et al.* 2000; Ottilie *et al.* 1997; Sugioka *et al.* 2003; Zhou *et al.* 2005). Mutant Bcl-XL Y101K is one of many mutants that can be used to study inhibition of apoptosis by anti-apoptotic proteins. This mutant Bcl-XL homodimerized and bound to membranes as determined by immunoprecipitation and pelleting assays, but did not bind Bax by immunoprecipitation in cell lysates (Minn *et al.* 1999). Residues that inactivate or change Bcl-XL function may be important in defining regions of interest of the protein to target in drug development.

There are several other methods by which Bcl-XL may function at the membrane such as blocking a tBid induced change in the membrane phase (Clem *et al.* 1998; Epand *et al.* 2002; Kim 2005; Minn *et al.* 1999; Wang *et al.* 2004). However, focus for the development of protein and cell specific drugs has been on interactions between Bcl-XL and other Bcl-2 proteins at the mitochondria. Improvement in the design of these small molecules for the treatment of cancer is limited to the current knowledge of regulation and inhibition of apoptosis at the mitochondria. The development of effective and specific inhibitors of Bcl-XL will be greatly aided by investigation of the functional relevance the protein-protein interactions suggested by crystal structures and mutational analysis in a reliable, robust *in vitro* system that recapitulates but simplifies what happens in cells. Therefore, this chapter examines the significant interactions required for Bcl-XL function in the mitochondrial assay described in the previous chapter.

Results and Discussion

The Embedded Together model emphasizes the importance of the interaction between Bcl-2 family proteins at the membrane. The significance of the membrane interaction in mediating the inhibitory effects of Bcl-XL has been demonstrated in our laboratory using a liposome system (Billen *et al.* Submitted

November 6, 2007). To confirm these observations in a more physiologic membrane system, we used enriched mitochondria prepared by the method described in Chapter 3 incubated with purified recombinant Bcl-XL, Bax and tBid, using both wild type proteins and mutants that eliminated specific protein-protein interactions.

Using concentrations of tBid and Bax that release cytochrome c from wild type or Bak-/- mitochondria (Figure 3.7), we determined the minimal concentrations of wild type Bcl-XL that inhibits cytochrome c release. In both wild type and Bak deficient mitochondria, 0.5nM Bcl-XL inhibited cytochrome c release as completely as the negative control with no tBid/Bax added (Figure 3.6). The concentrations of tBid (0.25nM) and Bax (200nM) that are inhibited by Bcl-XL in the Bak deficient mitochondria suggests that complete inhibition by 0.5nM Bcl-XL prevented Bax activation by binding to tBid in a stoichiometric fashion (Figure 4.1). Consistent with this mechanism, Bcl-XL has been shown by immunoprecipitation to interact with tBid (Cheng *et al.* 2003). Wild type mitochondria (which contain Bak) required ten times less tBid to induce cytochrome c release. Significantly, inhibition of cytochrome c release in this system required the same amount of Bcl-XL as used for Bak-/- mitochondria. As both conditions contained an equal amount of Bax, this suggests that tBid activated Bak contributed to membrane permeabilization through forming pores itself and/or by activating Bax, and that these steps were also inhibited by Bcl-XL. There is evidence that Bak and Bax cross-activate, however the exact mechanism may be cell dependent as different studies support either direction of activation (Er *et al.* 2007; Mikhailov *et al.* 2003) and Bcl-XL binds both Bax and Bak. Bcl-XL interacts with Bak through the BH3 region and, similarly to Bcl-2, binds and sequesters the open conformer of Bak thereby preventing cytochrome c release (Chittenden *et al.* 1995; Ruffolo *et al.* 2003; Sattler *et al.* 1997).

The Direct Activation and Displacement models propose different protein-protein interactions as critical for the inhibition of apoptosis by Bcl-XL/Bcl-2 (Figure 1.4 A and B) (Cartron *et al.* 2004; Certo *et al.* 2006; Chen *et al.* 2005; Kuwana *et al.* 2005; Letai *et al.* 2002). To test the predications of these models in our *in vitro* system, we used mutants that selectively eliminate one or the other of these interactions; a mutant tBid-mt1 (M97A,D98A) that does not bind to Bcl-2 and Bcl-XL (Desagher *et al.* 1999; Minn *et al.* 1999; Wang *et al.* 1996), and mutant Bcl-XL Y101K that does not stably bind Bax but can both bind tBid and target to membranes (Jeong *et al.* 2004; Sattler *et al.* 1997). Using these tBid or Bcl-XL mutants either individually or in combination, we can eliminate Bcl-XL

binding to either or both tBid and Bax. When one of the protein-protein interactions was eliminated, Bcl-XL inhibited cytochrome c release in wild type (Bak containing) and Bak-/- mitochondria (Figure 4.1). More Bcl-XL was required for complete inhibition of cytochrome c release when Bcl-XL could not interact with either Bax or tBid, suggesting that Bcl-XL was most efficient when it interacts with both proteins. However, neither interaction was essential for Bcl-XL mediated regulation of cytochrome c release from mitochondria.

These data indicate that contrary to the Direct Activation model, the interaction between tBid and Bcl-XL was not required for inhibition of membrane permeabilization. In this case, it is plausible that Bcl-XL inhibits apoptosis by binding to Bax. In fact, immunoprecipitation studies using non-ionic detergents such as NP-40 that cause a conformational change in Bax demonstrated that Bcl-XL binds to the activated conformation of Bax (Hsu *et al.* 1998; Tan *et al.* 2006). Thus, when Bcl-XL did not bind and sequester tBid, free tBid activated Bax but Bax in turn was bound and sequestered by Bcl-XL. These results are consistent with other experiments using mutant tBid-mt1, in which Bax changed the conformation of Bcl-2, which prevented pore formation by Bax in liposomes (Peng *et al.* 2006). Furthermore, chemical cross-linking studies with membranes has demonstrated that Bcl-XL prevents oligomerization of Bax and Bak in the membrane (Reed *et al.* 2006; Ruffolo *et al.* 2003).

However, our data are also incompatible with aspects of the Displacement model, as the removal of Bax/Bcl-XL binding by using the mutant Bcl-XL Y101K decreased but did not eliminate Bcl-XL function. This mutation also did not induce membrane permeabilization by Bax (Figure 4.1) (Chen *et al.* 2005; Cheng *et al.* 2003; Cuconati *et al.* 2003; Willis *et al.* 2005). The increase in Bcl-XL Y101K concentrations required for complete inhibition of cytochrome c release may be required to ensure all tBid is sequestered to prevent the activation of Bak or Bax, as subsequent inhibition of activated Bax would not be possible. This scenario is plausible as both Bax and Bak have been shown to auto-activate and therefore in time a small amount of protein activated by tBid may cause cytochrome c release. Inhibition of apoptosis by Bcl-XL without Bax/Bak interaction has been previously demonstrated in insect, mammalian and yeast cells (Cheng *et al.* 1996; Jeong *et al.* 2004; Liu *et al.* 2006; Minn *et al.* 1999; Sattler *et al.* 1997).

It was interesting to note than when both protein-protein interactions are eliminated by using both mutants together, Bcl-XL still inhibited cytochrome c

release. However, in the absence of binding to tBid and Bax, the mutant Bcl-XL was sixteen times less effective as 8nM Bcl-XL was required to decrease cytochrome c release to control levels (Figure 4.1, B and D). This suggests an additional mechanism of Bcl-XL function that is less potent than that conferred by stable binding to Bax/Bak or tBid. The Bcl-XL Y101K mutant spontaneously targets to the membranes whereas a C-terminal deletion mutant Bcl-XL that does not target to membranes is almost completely impaired demonstrated in our laboratory by Lieven Billen (Billen *et al.* Submitted November 6, 2007). In combination with the Bcl-XL Y101K and tBid-mt1 mutations, this C-terminal deleted mutant of Bcl-XL Y101K had no remaining function (Billen *et al.* Submitted November 6, 2007). These data suggest that Bcl-XL must be membrane-bound to inhibit permeabilization as described in the Embedded Together model (Figure 1.4 C) (Leber *et al.* 2007).

Comparing the results obtained from wild type and Bak deficient mitochondria, a similar pattern appears whereby elimination of one protein-protein interaction moderately decreased the efficiency of inhibition by Bcl-XL, whereas elimination of both interactions severely decreased its efficiency. However, in Bak-/- mitochondria, removing the interaction between tBid and Bcl-XL resulted in a greater loss of Bcl-XL function than the elimination of the Bax/Bcl-XL interaction (Figure 4.1 C and D). The concentration of wild type Bcl-XL required to inhibit cytochrome c release in this system was greater than the molar concentration of tBid-mt1 present as it must prevent oligomerization of the large amount of activated Bax present, and elimination of this interaction demonstrates the importance of the tBid/Bcl-XL interaction (Figure 4.1 A and B). In the presence of excess Bax in the system, if Bcl-XL was unable to interact with tBid it must prevent oligomerization of the large amount of Bax, requiring more Bcl-XL. The importance of this interaction is underlined by the observation that even though a larger amount of Bcl-XL is required, complete inhibition of cytochrome c release is still possible by Bcl-XL binding to Bax alone in this system. As well, in the absence of a tBid/Bcl-XL interaction, Bcl-XL is still activated, suggesting Bax can also activate Bcl-XL.

With wild type mitochondria (which contain Bak in the membrane), removing the interaction between Bcl-XL and Bax (as well as Bak), has a greater effect on Bcl-XL function than the removal of the tBid/Bcl-XL interaction, opposite to results noted with Bak-/- mitochondria (Figure 4.1 Compare A with D, $p<0.05$). Thus when both of these pore forming proteins were simultaneously present (as is likely the case in many physiological systems), the interaction between

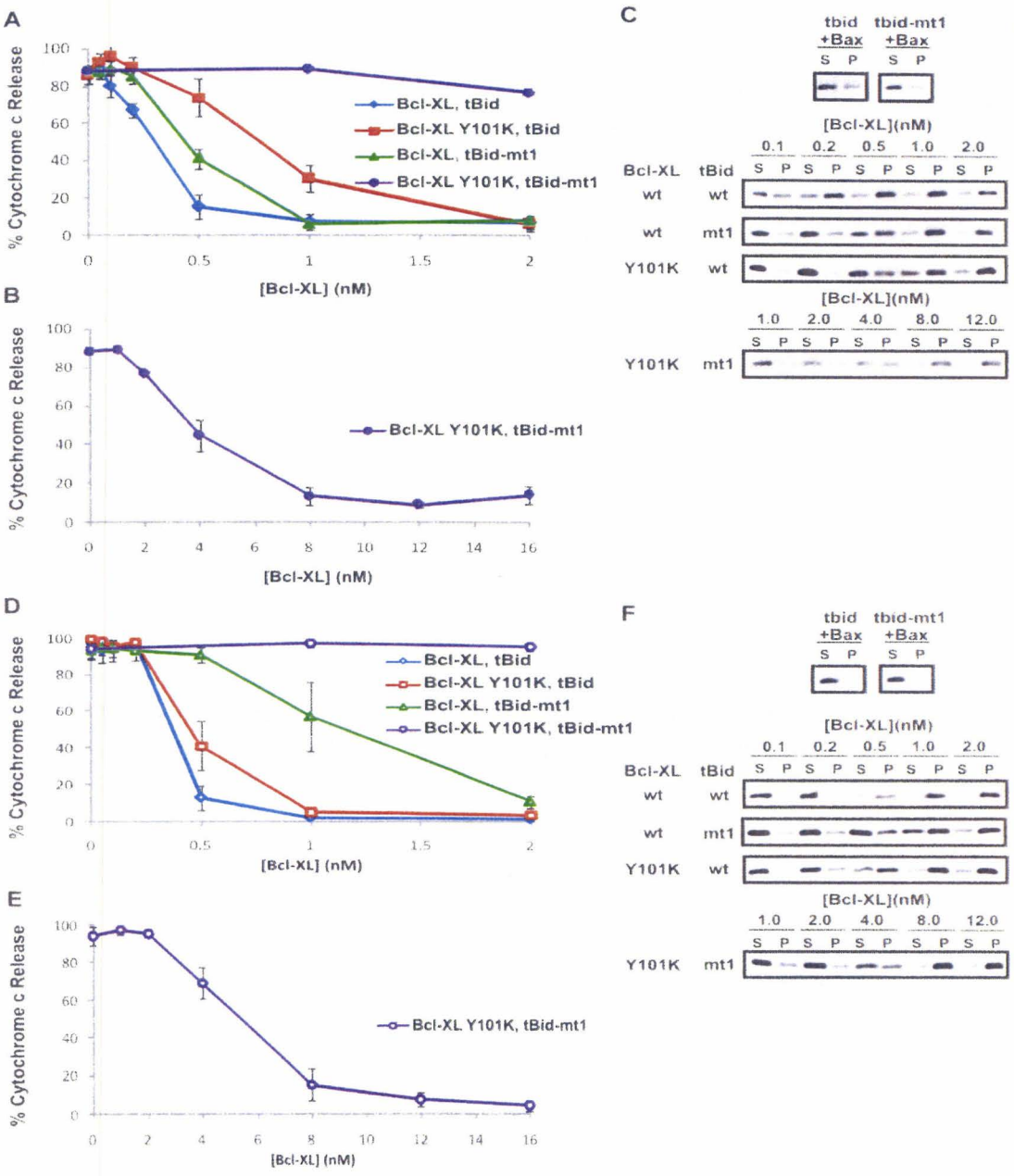

Figure 4.1 Bcl-XL inhibits cytochrome c release by several mechanisms. Mitochondria isolated from the liver of wild type C57BL6 mice (A-C) and Bak-/- mice (D-F) were incubated with wild type tBid or mutant tBid-mt1, 200nM Bax and increasing concentration of wild type Bcl-XL or mutant Bcl-XL Y101K. Concentrations of tBid or mutant tBid were 0.025nM for membranes from wild type liver and 0.25nM for Bak-/- membranes. Immunoblotting for cytochrome c was performed on the supernatant (S) and pellet (P) fractions (C and F) to determine percent cytochrome c release (A, B, D, and E).

Bax/Bak and Bcl-XL may play the more significant role in the anti-apoptotic function of Bcl-XL. However, a full understanding of this system would require knowledge of the relative affinity of tBid for membrane bound Bcl-XL, Bax and Bak.

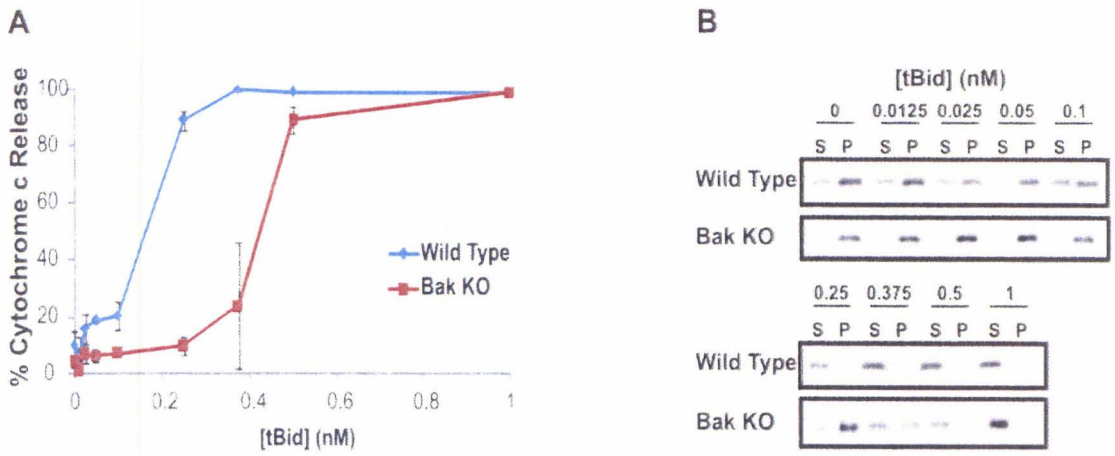

Figure 4.2 Bak-/- Mitochondria are less sensitive to tBid and Bax. Bax (200nM), Bcl-XL (0.5nM) and increasing concentrations of tBid were incubated with wild type or Bak-/- mitochondria. Samples of the supernatant (S) and pellet (P) were used to immunoblot for cytochrome c release (B). Percent cytochrome c release was determined for each tBid concentration (A).

To further investigate the role of Bak activation in this system, tBid was added in increasing amounts to wild type or Bak-/- mitochondria in the presence of excess Bax (200nM) and 0.5nM Bcl-XL, which is the minimal required by both membranes to prevent cytochrome c release. Note that the starting concentrations of tBid for this titration series differed for wild type and Bak-/- mitochondria, as described in Chapter 3 (Figure 3.7). In the Bak-/- membranes, Bcl-XL inhibited cytochrome c release when less than stoichiometric amounts of tBid were added, after which tBid induced maximal cytochrome c release (Figure 4.2). However, in the presence of membrane-bound Bak, the threshold for tBid that induced cytochrome c release was drastically decreased (Figure 4.2, $p<0.05$) and concentrations higher than 0.1nM tBid (5-fold less than the molar amount of Bcl-XL present) overcame Bcl-XL inhibition. Wild type membranes

samples contain both Bak and Bax and these proteins out-compete Bcl-XL for tBid binding and subsequent activation at increased tBid concentrations. Activation of Bax in this setting may occur by either free tBid or Bak and Bcl-XL must inhibit both interactions to be effective. The binding of Bcl-XL to multiple targets in the same system has been demonstrated in our laboratory with liposomes. Bcl-XL activated by tBid at the membrane did not interact with inactivate soluble Bax as assessed by cross-linking (Billen *et al.* Submitted November 6, 2007). However, if less Bcl-XL was present such that there was sufficient free tBid to target some Bax to membranes, the activated Bax was then bound by Bcl-XL. These results suggest that multiple functionally significant interactions occur at the membrane, and that the Bcl-XL/tBid interaction prevents the targeting of Bax to the membrane.

Conclusions

The examination of interactions that are sufficient for Bcl-XL function in our mitochondrial assay system indicates that the major predications of the Displacement and Direct Activation models are incomplete, in that Bcl-XL binding to both tBid and Bax/Bak is important. Experiments with liposomes and the Bcl-XL mutant lacking the C-terminal tail by Lieven Billen indicate that all the relevant interactions take place after membrane targeting of Bcl-XL, consistent with the Embedded Together model. An important implication is that this critical membrane binding step represents a novel target for the development of an effective inhibitor of Bcl-XL as a chemotherapeutic agent, in addition to the important protein-protein interactions that we have elucidated here.

Chapter 5: Isolated Mitochondria as a System for the Screening of Bcl-2 Family Inhibitors

Introduction

Bcl-2 family protein expression and activity has been related to many diseases including neurodegenerative diseases, autoimmune disease and cancer (Grodzicky *et al.* 2002). As a result, there is increasing research into the development of activators and inhibitors of this family of proteins, including BH3-mimetics that occupy the BH3-binding site on Bcl-2 and Bcl-XL or other compounds that activate Bax or Bak to induce apoptosis (Letai *et al.* 2002). In mitochondria from leukemic cell lines or leukemic cells from transgenic mice sensitizer BH3-only proteins induce cytochrome c release if they can bind Bcl-2, supporting the concept that BH3 mimetics may be cell and protein specific drugs (Certo *et al.* 2006). Work by Certo *et al.* has suggested that cancer cells are "addicted" to anti-apoptotic proteins, having activated Bax and Bak sequestered Bcl-2/Bcl-XL/Mcl-1 (Certo *et al.* 2006). Small molecules that free Bax and Bak would allow cancer cells to undergo apoptosis, whereas healthy cells that do not have activated Bax and Bak may remain unaffected.

One particular target of interest for cancer cells is Bcl-XL. Expression of Bcl-XL strongly correlates with a decrease in sensitivity to diverse chemotherapeutic agents in 60 cell lines held at the NCI repository (O'Neill *et al.* 2004). Bcl-XL is more abundant in prostate carcinomas of higher Gleason grades and is significantly associated with the onset of hormone-refractory disease (Castilla *et al.* 2006). In breast cancer, Bcl-XL increases metastatic activity and inhibits apoptosis induced by the chemotherapeutic drugs etoposide, teniposide, florouracil, hydroxyure and cisplatin (Espana *et al.* 2005). In response to doxorubicin treatment, Bcl-XL prevents PARP degradation, externalization of phosphatidyl serine and condensation of nuclei, all markers of apoptosis, more potently than the related anti-apoptotic protein Bcl-2 in MCF7 cells (Fiebig *et al.* 2006). Due to its potent effects and the lack of sensitivity of its detection in routine clinical samples, the clinical significance of Bcl-XL expression is likely under-estimated. As such, Bcl-XL is an appropriate target for cancer research.

Bcl-XL may inhibit apoptosis by several mechanisms. The primary focus for the development of inhibitors has been on BH3-mimetics that bind the hydrophobic groove (O'Neill *et al.* 2004). Inhibitors of anti-apoptotic proteins have been found using virtual and chemical screens (Chan *et al.* 2003; Degterev *et al.* 2001; Enyedy *et al.* 2001; Kitada *et al.* 2003; Wang *et al.* 2000) and through modeling and structure based design (Fu *et al.* 2007; Rega *et al.* 2007).

Independent of the method of discovery, most small molecule inhibitors are screened by fluorescence polarization to determine the IC_{50} or binding efficiency, NMR or crystal structures to determine where the inhibitor binds the target, and cell based assays to ensure that the small molecule increases cell death (Kitada *et al.* 2003; Kline *et al.* 2007; Lee *et al.* 2007; Oltersdorf *et al.* 2005; Rega *et al.* 2007; Schwartz *et al.* 2007). Most screens are done with truncated Bcl-XL or Bcl-2 purified proteins or with the modelled structure (Baell *et al.* 2002). There are currently several inhibitors of Bcl-XL in various stages of clinical development such as ABT-737 and gossypol (Kim 2005; Kitada *et al.* 2003; Oltersdorf *et al.* 2005; Tanabe *et al.* 2003; Wang *et al.* 2000).

Confirming results noted with fluorescence polarization in our *in vitro* mitochondrial assay system provides an opportunity to ensure that potential inhibitors function as expected at the mitochondria and do not have other effects that may be missed when studied in cells. The reported inhibitors of Bcl-XL 3e and 3e-D2 have been tested for their ability to bind Bcl-XL by fluorescence polarization experiments (Wang *et al.* 2007). To determine if these inhibitors function similarly in organelles, 3e and 3e-D2 were tested using our assay system. We found that both compounds induced cytochrome c release from mitochondria, but the mechanism appears to be unrelated to inhibition of Bcl-XL.

Results and Discussion

Two small molecule inhibitors, 3e and 3e-D2 (Figure 5.1), were designed to inhibit Bcl-XL. We examined 3e and 3e-D2 inhibition Bcl-XL in our mitochondrial assay system to determine the effect of these small molecules on mitochondrial permeability by themselves, as well as any modification of Bax or Bcl-XL activity. Furthermore, we studied 3e and 3e-D2 with wild type and Bak-/- mitochondria to determine if 3e and 3e-D2 had an affect on Bak.

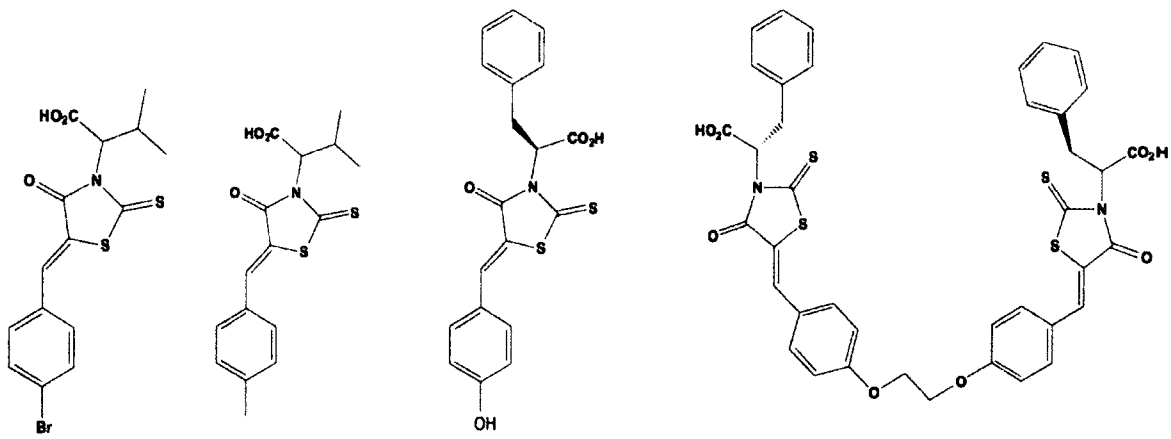

Figure 5.1 Small molecules 3e and 3e-D2 are similar to current Bcl-XL inhibitors. Small molecules 3e and 3e-D2 were designed as derivatives of BH3I compounds such as BH3I-1 and BH3I-1".

The small molecule 3e is a BH3 mimetic which has a similar structure to several BH3I-1 compounds that bind to Bcl-XL (Figure 5.1) (Degterev *et al.* 2001; Xing *et al.* 2007). BH3I-1" has a Ki of 12.5μM for Bcl-XL (Degterev *et al.* 2001). The affinity is increased to a K_i of 2.4μM by the addition of bromine to the benzene ring, resulting in BH3I-1 (Degterev *et al.* 2001). Xing *et al.* examined the efficacy of these small molecules in an assay based on the displacement of a fluorescent Bak BH3 peptide and found BH3I-1 had a K_i of 133μM for Bcl-XL and was more specific for Bcl-2 and Bcl-w (Xing *et al.* 2007). By replacing the bromine with a hydroxyl group and the methylbutanoic acid with phenylpropanoic acid, the K_i for Bcl-XL was decreased 5.35μM (Xing *et al.* 2007). The small molecule 3e-D2 is also similar in structure to BH3I-1. 3e-D2 is a dimerized form of the small molecule 3e, joined by a four atom ethylene glycol linker (Figure 5.1) (Wang *et al.* 2007). 3e-D2 has increased affinity for Bcl-XL *in vitro* and therefore it is a good candidate to test for activity in our assay system.

When 3e was added to Bak-/- mitochondria, cytochrome c release occurred at concentrations higher than 20μM and maximal cytochrome c release was observed at approximately 100μM (Figure 5.2 B). For mitochondria with no added recombinant proteins, the EC_{50} for 3e is 61.0 +/- 0.8μM (Table 5.1), indicating that 3e has intrinsic mitochondrial permeabilization activity. The addition of Bax does not cause a significant change in cytochrome c release in

these membranes, nor does the addition of recombinant proteins in combination tBid, Bax and Bcl-XL (Figure 5.2 B; p=0.46).

The addition of 3e to wild type membranes (containing Bak) without recombinant proteins yields a similar curve, where 3e at concentrations higher than 20μM causes cytochrome c release (Figure 5.2 A; p=0.73). Addition of Bax to this system results in an increase in cytochrome c release (p=0.12). The EC_{50} of 3e decreases in the presence of Bax and Bak to ~36.6μM. One possible explanation for these results is that 3e activated Bak, but only in the presence of Bax. Several studies suggests that Bax may be required for Bak oligomerization or visa versa (Er *et al.* 2007; Mikhailov *et al.* 2003; Sugioka *et al.* 2003; Sundararajan *et al.* 2001). During tBid titrations, tBid induced cytochrome c release from membranes containing only Bak or Bax demonstrating that in this particular system, neither protein required the other protein for membrane permeabilization (Figure 3.6 and 3.7). Membrane permeability with 3e in the absence of tBid as an activator, but with Bax and Bak present suggests that a small amount of activated Bak may be amplified by excess of Bax (Sundararajan *et al.* 2001). 3e was designed to bind the hydrophobic groove of Bcl-XL, which is found in most multi-domain Bcl-2 family proteins (reviewed in Willis *et al.* 2005). Bax has a larger BH3 binding pocket than Bak, which may be related to the specificity of 3e, if 3e acts by binding the hydrophobic groove as demonstrated with Bcl-XL (Moldoveanu *et al.* 2006; Xing *et al.* 2007). When tBid, Bax and Bcl-XL are added to wild type membranes at concentrations that release minimal cytochrome c (0.025nM, 200nM and 0.5nM respectively; Figure 5.2 A), the effective concentration of 3e is similar to that seen in wild type membranes alone. If 3e inhibited Bcl-XL the titration curve expected would be similar to that seen with Bax alone since in this case, Bcl-XL would not inhibit Bax/Bak. Instead, Bcl-XL inhibited 3e-activated Bax/Bak and decrease cytochrome c release to that seen with membranes in the presence of 3e without exogenous recombinant proteins. This suggests that 3e may indeed function as a BH3 mimetic, but as an activator of Bax or Bak and therefore functions similar to tBid. This approach was proposed by Letai et al, however the focus of drug development has been on mimetics that target anti-apoptotic proteins (Letai *et al.* 2002). A stapled Bid BH3 peptide activates Bax, but this molecule may target healthy cells better than cancer cells (Walensky *et al.* 2006). This prediction is based on the increase in anti-apoptotic proteins illustrated in many cancer cell lines that may sequester activated Bax and Bak compared to what is observed in non-cancerous cells (Amundson *et al.* 2000; Certo *et al.* 2006; Walensky *et al.* 2006).

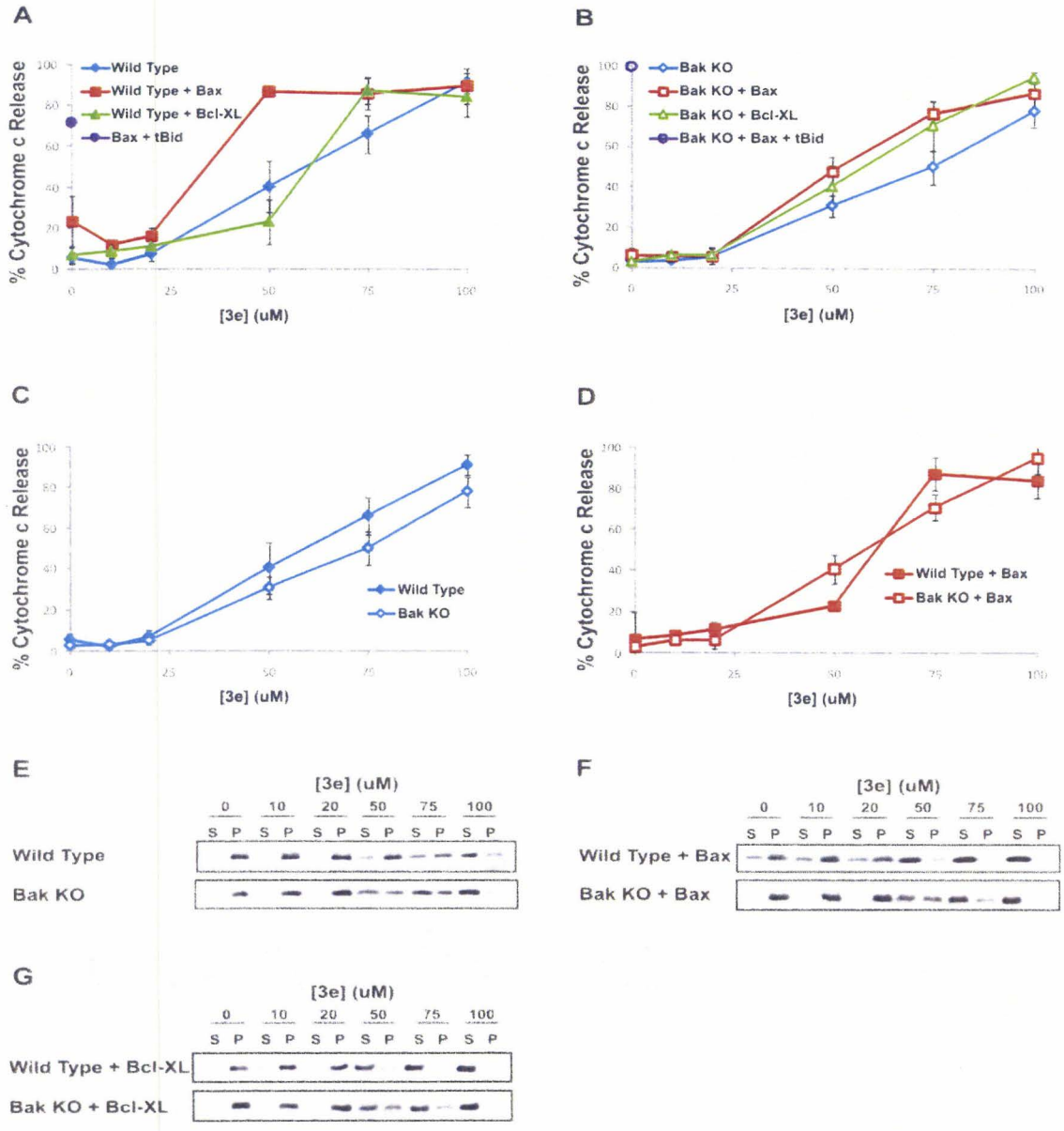

Figure 5.2 Cytochrome c release by small molecule 3e is enhanced in the presence of Bak and Bax. Mitochondria from wild type (A, C-G) or Bak-/- (B, C-G) mouse liver were used to determine the mechanism of cell death by the small molecule 3e. Mitochondria were incubated with increasing concentrations of 3e alone (A-C and E), in the presence of 200nM Bax (A, B, D and F) or in the presence of 0.5nM Bcl-XL, 200nM Bax and either 0.025nM tBid for wild type mitochondria or 0.25nM tBid for Bak-/- mitochondria (A, B and G). Samples from the supernatant (S) and pellet (P) were used to immunoblot for cytochrome c release (E- G) and percent cytochrome c release was determined (A-D).

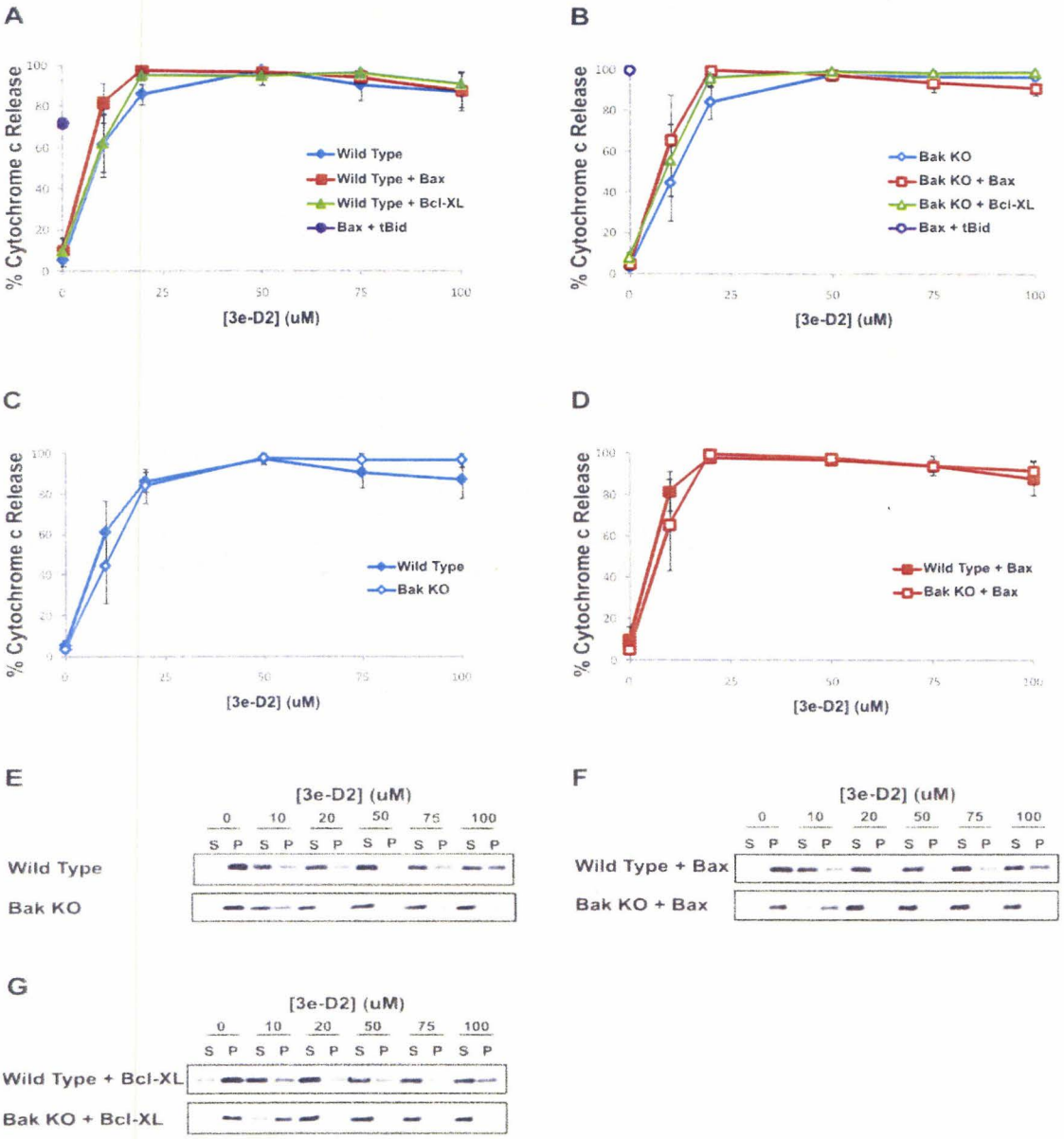

Figure 5.3 Cytochrome c release by small molecule 3e-D2 is independent of Bak, Bax, tBid or Bcl-XL. Mitochondria from wild type (A, C-G) or Bak-/- (B, C-G) mouse liver used to determine the mechanism of cell death by the small molecule 3e-D2. Mitochondria were incubated with increasing concentrations of 3e-D2 alone (A-C and E), in presence of 200nM Bax (A, B, D and F) or in the presence of 0.5nM Bcl-XL, 200nM Bax and either 0.025nM tBid for wild type mitochondria or 0.25nM tBid for Bak-/- mitochondria (A, B and G). Samples from the supernatant (S) and pellet (P) were used to immunoblot for cytochrome c release (E- G) and percent cytochrome c release was determined (A-D).

Titrations of 3e-D2 were also performed in wild type and Bak-/- mitochondria with and without recombinant proteins. In contrast to 3e, 3e-D2 is more potent such that the EC_{50} for cytochrome c release without any added proteins is approximately 8.3 +/- 1.2μM (Table 5.1). However, there is no difference between wild type and Bak-/- mitochondria or with the addition of Bax, tBid or Bcl-XL (p=1.0) (Figure 5.3). Therefore, the dimeric form of the small molecule is more potent than 3e, but its activity is not dependent upon Bax/Bak, tBid or Bcl-XL.

Table 5.1 EC_{50} for small molecules 3e and 3e-D2. EC_{50} values were calculated for wild type (Wt) and Bak-/- (Bak KO) mitochondrial membrane for membranes alone, in the presence of Bax, or with the addition of Bax, tBid and Bcl-XL (+ Bcl-XL).

	3e EC_{50} (μM)	3e-D2 EC_{50} (μM)
Wt	55.7 +/- 1.7	8.3 +/- 1.3
Wt + Bax	36.6 +/- 27.3	7.5 +/- 1.5
Wt + Bcl-XL	51.9 +/- 21.0	9.7 +/- 1.9
Bak KO	61.0 +/- 0.8	10.9 +/- 0.2
Bak KO + Bax	49.3 +/- 0.5	9.7 +/- 7.7
Bak KO + Bcl-XL	56.1 +/- 1.2	9.8 +/- 0.1

It is possible that 3e and 3e-D2 mediate effects on mitochondria through other Bcl-2 family proteins present. As described in Chapter 3 (Figure 3.5), wild type and Bak-/- mitochondria were screened for the presence of other Bcl-2 proteins. Mcl-1 and Bik were potentially present as detected by immunoblotting, but the relevant bands were not distinct from cross-reacting bands, and there is no detectable Bax or Bcl-XL, although some studies have found a small amount of Bcl-XL in mouse liver mitochondria (Uren *et al.* 2007). Bik is a BH3-only protein and therefore is not a good candidate to interact with either inhibitor and give the observed phenotype if the interaction is inhibitory. Furthermore, Bik does not induce membrane permeability by itself but rather is considered to act as a sensitizer, binding anti-apoptotic proteins (Cartron *et al.* 2005; Certo *et al.* 2006; Kim *et al.* 2006; Kuwana *et al.* 2005; Letai *et al.* 2002). Mcl-1 binds to Bak (Shimazu *et al.* 2007; Uren *et al.* 2007; Willis *et al.* 2005). Mcl-1 has a smaller

binding pocket than Bcl-2, Bcl-XL and Bcl-w and often is not targeted by chemicals designed for other anti-apoptotic proteins due to the difference in the structure of the hydrophobic groove (Czabotar *et al.* 2007; Lee *et al.* 2007; Zhai *et al.* 2006). Moreover, if 3e and 3e-D2 targeted Mcl-1, then one would expect a difference in activity between wild type and Bak-/- mitochondria as an inhibitor of Mcl-1 would be active in the presence of Bak, but not in its absence. The absence of potential Bcl-2 family proteins as targets for 3e and 3e-D2 further supports the proposal that both compounds either cause membrane permeabilization themselves or target another membrane protein not examined. Therefore, further work is needed to determine the mechanism of membrane permeabilization by 3e and 3e-D2.

Conclusion

Structure based design has been a useful approach to the development of inhibitors of the Bcl-2 family of proteins. 3e and 3e-D2 were designed to inhibit Bcl-XL by binding at the hydrophobic groove and can displace a Bak-BH3 peptide as demonstrated using fluorescence polarization. Fluorescence polarization showed dimerization of 3e increased its affinity for Bcl-XL. However, in our cell free system of membranes enriched in mitochondria, both 3e and 3e-D2 induce cytochrome c release from membranes without detectable Bcl-XL or added exogenous proteins. The addition of Bax, tBid and Bcl-XL had no affect on the effective concentration of the dimerized product 3e-D2. However, the presence of Bak and Bax increased cytochrome c release from the baseline of membranes alone during titrations of 3e, suggesting 3e is not an inhibitor of Bcl-XL in this more physiologic system but instead functions as an activator of the multi-domain pro-apoptotic proteins.

Chapter 6: Discussion

The field of apoptosis, inaugurated in 1972 with a new term, is still young in many ways. While the field has grown substantially, examining pathways initiated from external and internal signals until the final stages of cell death, much remains unknown about the regulation of the intermediate steps in the commitment to cell death. These steps occur at the mitochondria, where many signals converge and a "point of no return" is reached with the release of intermembrane space proteins. The main factor responsible for the cellular decision to die at the mitochondria is the Bcl-2 family of proteins, which consists of pro- and anti-apoptotic family members. There are several theories of apoptotic regulation by Bcl-2 proteins: the Direct Activation model, the Displacement model, and the Embedded Together model. A new arsenal of drugs, in the form of small molecules that bind to this family of proteins is being explored as over- or under-expression and inappropriate activation of Bcl-2 proteins has been linked to many diseases. The development of effective small molecules that activate or inhibit Bcl-2 family proteins requires two developments. First, it is necessary to understand how Bcl-2 family proteins function in order to target the most appropriate protein-protein interactions for any particular disease state. Second, an appropriate method of testing must be designed. It is important to ensure that compounds identified by screening using *in vitro* methods with purified proteins or peptides function as expected in a cellular setting. My project focused on the design and characterization of a cell-free system to study Bcl-2 family proteins in a robust, quantitative and reproducible manner. The cell-free system offers many benefits compared to *in vitro* liposome and cell-based assays. However, to develop a full understanding of apoptosis and potential drug treatments, testing in all three systems yields important complementary information.

Preliminary work to isolate mitochondria that maintain membrane integrity was a challenging and critical step in the development of this cell-free system. Initial methods of purification suggested that Bak is involved in membrane integrity. The interaction between Bak and VDAC, in combination with evidence that Bak deficient mitochondria are sensitive to sucrose (Figure 3.2), suggested a role for Bak in the movement of osmolytes and maintenance of membrane integrity during osmotic stress. It had been noted previously in the purification of rat mitochondria that sucrose is moved into the mitochondria that disturbs the separation of mitochondria from other heavy membranes (Packer *et al.* 1971; Pollak 1975). VDAC has been postulated to increase sucrose uptake in the

presence of recombinant Bax and Bak in a liposome system, and is regulated by calcium levels to equilibrate of ions and water across the membrane (Bernardi *et al.* 2006; Beutner *et al.* 1998; He *et al.* 2002; Shimizu *et al.* 1999). By testing two other osmolytes used to induce osmotic stress in cells, sodium chloride and sorbitol, it was it was noted that all three cause cytochrome c release with increased concentrations (Figure 3.3). There was a subtle but not statistically significant difference in cytochrome c release between wild type and Bak-/- mitochondria when sucrose was used as the osmolyte. Therefore small molecules and ions, such as sorbitol and sodium chloride may pass through the membrane or other channels to equilibrium whereas uptake of sucrose may require Bak. However other methods of measuring membrane integrity and examination of other small and large osmolytes would be required to test this hypothesis and were not pursued at this time.

Differential centrifugation proved an efficient means of isolation for mitochondrial membranes. An examination of endogenous proteins in this preparation was used to determine contamination with endoplasmic reticulum (ER) and other Bcl-2 family proteins present which may complicate the interpretation of experiments with added proteins (Figure 3.5). Immunoblotting revealed the presence of calreticulin, an endoplasmic reticulum protein. It is important to acknowledge the presence of this heavy membrane as Bcl-2 family proteins target the ER and there is interest in the role ER plays in apoptosis (reviewed in Hetz 2007). Calcium release from the ER has been shown to be one component of the regulation of apoptosis (Shimizu *et al.* 1998). The presence of ER in the heavy membranes did not confound the results as seen in Chapter 3 and 4, and further purification of mitochondria was not essential for the proposed experiments as the assay was designed to function as an intermediate step between liposome and cell-based assays. Bcl-2 family proteins detected in this membrane preparation included Bik and Mcl-1. Bik has been characterized as a sensitizer protein which binds to Bcl-XL and Bcl-2 (Kuwana *et al.* 2005; Letai *et al.* 2002). Mcl-1 is an anti-apoptotic protein that binds Bak and Bax at the membrane, inhibits Bim and tBid-induced cytochrome c release, and can be inhibited by Puma, Bmf, Bik and Noxa (Chen *et al.* 2005; Clohessy *et al.* 2006; Kuwana *et al.* 2005; Opferman *et al.* 2003; Shimazu *et al.* 2007). The expression of these two proteins did not change in the presence or absence of Bak. However, Mcl-1 may be more available to sequester tBid in the absence of Bak. This has not yet been examined in this system.

One application of this cell-free assay was to examine the mechanism of regulation of Bcl-2 family proteins at the membrane. The first requirement was to determine concentrations of tBid, a representative BH3-only protein, and Bax required to induce cytochrome c release. Titrations of tBid alone in the presence and absence of Bak demonstrated that Bax is not required for Bak-induced permeabilization in our system (Figure 3.6), as reported previously in other contexts (Mikhailov *et al.* 2003; Takahashi *et al.* 2005). Titrations of tBid and Bax in the absence of Bak illustrates that Bax permeabilization also does not require Bak (Figure 3.7). The combination of Bak and Bax required much less tBid in order to release maximal amounts of cytochrome c, supporting previous observations of cross-activation between Bak and Bax (Figure 3.7) (Mikhailov *et al.* 2003; Yamaguchi *et al.* 2002). Thus for the study of activator proteins and the multi-domain pro-apoptotic proteins Bak and Bax, the use of wild type and Bak-/- mitochondria provides an opportunity to tease out each interaction.

The Direct Activation and Displacement models emphasize opposing protein-protein interactions for the regulation of apoptosis by Bcl-2 family proteins. The Direct Activation model proposes that Bax and Bak are directly activated by BH3-only proteins, and that BH3-only proteins can be sequestered by anti-apoptotic proteins to prevent cell death (Cartron *et al.* 2005; Certo *et al.* 2006; Kuwana *et al.* 2005; Letai *et al.* 2002). The Displacement model proposes that anti-apoptotic proteins sequester active multi-domain pro-apoptotic proteins that can be freed through the binding of BH3-only proteins (Certo *et al.* 2006; Chen *et al.* 2005; Kuwana *et al.* 2005; Wang *et al.* 1996). The Embedded Together model acknowledges that both protein-protein interactions are important and that regulation occurs at the membrane (Leber *et al.* 2007). The focus of Chapter 4 was on the role of Bcl-XL in the regulation of apoptosis. Mutations were used to illustrate that Bcl-XL can inhibit cell death by interacting with Bax/Bak or tBid, but also that there is a Bax- and tBid-independent method of inhibition. Experiments done with liposomes demonstrate that a critical requirement is the ability to bind the membrane. A C-terminal deletion mutant that eliminates Bcl-XL membrane targeting has decreased inhibitory function, with no inhibitory function if protein interactions are also removed (Billen *et al.* Submitted November 6, 2007), providing further evidence for the Embedded Together model. However, removal of the interaction between tBid and Bcl-XL also suggests that Bax/Bak may activate Bcl-XL to target the membrane, analogous to Bax/Bak cross-activation. Bax activation of Bcl-2 was demonstrated previously, suggesting Bax and Bak can activate anti-apoptotic proteins (Peng *et al.* 2006).

This together suggests that anti-apoptotic proteins may function as defective pro-apoptotic proteins.

Targeting and immunoprecipitation assays demonstrate similar activities between pro- and anti-apoptotic proteins. As well, replacement of helix 5 of Bcl-XL with Bax helix 5 converts Bcl-XL into a pro-apoptotic protein in MEF Bax/Bak DKO cells (George *et al.* 2007). Similarly, cleavage by caspase-1 removes the BH4 region and converts Bcl-XL into a pro-apoptotic protein (Basanez *et al.* 2001; Clem *et al.* 1998; Fujita *et al.* 1998; Hanada *et al.* 1995).This system can assess other aspects of regulation of apoptosis such as the proposed activator and sensitizer categorization of BH3-only proteins in the Direct Activation model since both wild type and Bak-/- membranes are free of Bid, Bim and Puma (Figure 3.5) (Letai *et al.* 2002). A screen for the presence of Puma would be required to ensure its absence in these membranes, confirming previous observations that Puma is not found in mouse liver mitochondria samples (Uren *et al.* 2007).

This system was further used to study two potential inhibitors of Bcl-XL discovered using fluorescence polarization, 3e and 3e-D2. Incubation with wild type or Bak-/- membranes demonstrated that similarly to *in vitro* experiments, 3e-D2 is more potent than the monomeric form in that it induced cytochrome c at lower concentrations. As demonstrated in the endogenous protein screen, Bcl-XL is not detected by immunoblotting in either membrane source, suggesting the primary target of 3e and 3e-D2 is not Bcl-XL. Incubation with recombinant proteins Bax and Bak decreased the effective concentration of 3e, suggesting 3e is an activator BH3 mimetic. The requirement for both Bax and Bak to increase the efficacy of 3e suggests that cross-activation likely occurs. The direction of cross-activation can be determined in this system as both proteins go through several measureable stages of activation, including translocation of Bax and conformational changes detectable by antibodies for both proteins before or concomitant with oligomerization. Bak has two stages of conformational change, the exposure of the N-terminus followed by the exposure of the BH1 region (Bellosillo *et al.* 2002; Griffiths *et al.* 2001; Panaretakis *et al.* 2002; Ruffolo *et al.* 2003). Specific inhibitors of Bak can also be used to clarify Bak and Bax permeabilization of the membrane using A1 or the vaccinia virus protein F1L that bind to Bak but not Bax (Postigo *et al.* 2006; Simmons *et al.* 2007; Taylor *et al.* 2006; Wasilenko *et al.* 2005).

This work has developed and characterized a system that has been used to study Bcl-XL and potential inhibitors. Characterization of these membranes provides an assay system in which Bcl-2 family proteins can be examined in a controlled setting. The interactions required for inhibition by Bcl-XL recapitulated those observed in liposomes. This system therefore functions as an intermediate between liposome and other *in vitro* assays and cell-based assays. Finally, enriched mitochondria have been demonstrated as a useful tool in the study of small molecules designed to target Bcl-2 family proteins to examine the mechanism of their activity in a way not possible in cells.

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Appendix

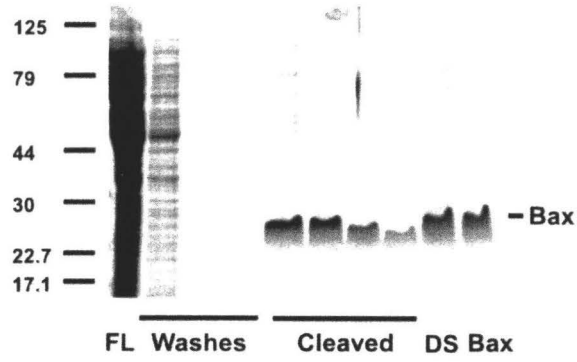

Figure A1 Purification of recombinant Bax. BL21-AI cell lysates containing recombinant Bax were incubated with chitin beads. The flow-through (FL) was collected and the column was washed with 30ml Lysis Buffer (Washes). Bax was cleavage from the intein fusion using Cleavage Buffer (Cleaved) and samples were loaded onto a DEAE-Sepharose column (DS). Eluted Bax was dialyzed and quantified (Bax). Samples were run on an SDS-PAGE gel and stained using Coomassie blue.

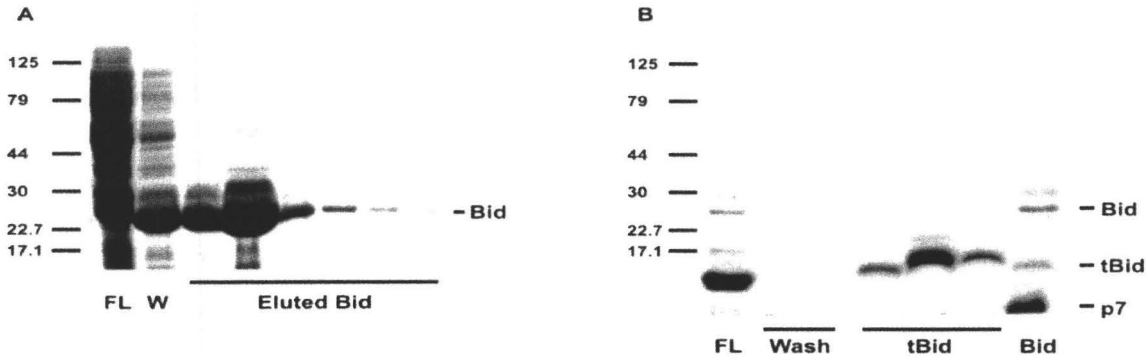

Figure A2 Purification of recombinant Bid and cleavage and purification of tBid. BL21-AI cell lysates containing recombinant Bid were added to nickel-nitrilotriacetic acid-agarose. The flow-through was collected (FL) and the column was washed with Bid Lysis Buffer (W). Bid was eluted using Bid Elution Buffer (Eluted Bid) (A). The first four fractions containing Bid were cleaved using caspase-8 and the products were added to nickel-nitrilotriacetic acid-agarose (B). The flow-through (FL) was collected and the column was washed using tBid Wash Buffer (Wash). tBid was eluted using tBid Elution Buffer (tBid) and remaining protein, including uncleaved Bid, tBid and the p7 fragment, was removed from the column using Bid Elution Buffer (Bid). All samples were run on a SDS-PAGE gel and stained with Coomassie Blue.

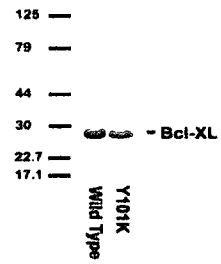

Figure A3 Purification of recombinant Bcl-XL. DH5μ cells containing recombinant wild type Bcl-XL or mutant Bcl-XL Y101K were incubated with chitin and the flow-through was collected. Samples were washed with Bcl-XL Lysis Buffer and the proteins were cleaved from the intein fusion protein using Bcl-XL Cleavage Buffer. These samples were applied to a phenyl-Sepharose column and eluted Bcl-XL was dialyzed. The final samples were run on a SDS-PAGE gel and stained with Coomassie blue.

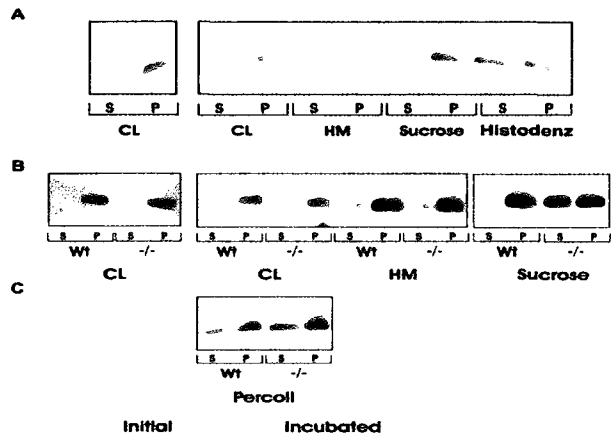

Figure A4 Isolation of mitochondria by density gradients. Isolation of mitochondria using gradients as described previously includes four stages: cell lysis to produce whole cell lysates (CL), differential centrifugation to purify heavy membranes (HM), a sucrose gradient (Sucrose), and a Histodenz gradient (Histodenz). Samples from wild type mitochondria at each stage of purification revealed that following the Histodenz gradient, mitochondria released cytochrome c whereas mitochondria remained in tact following a sucrose gradient (A). However, when wild type (Wt) and Bak-/- (-/-) membranes were isolated using differential centrifugation and sucrose gradients, Bak-/- mitochondria released cytochrome c (B). Cytochrome c release did not occur following isolating by an iso-osmotic Percoll gradient (C). Cytochrome c release was determined by comparing cytochrome c release in the supernatant (S) to the pellet (P) of the reaction immediately after purification (Initial) or following a 1 hour incubation at 30°C.