Regulation of Cytochrome c Release from Mitochondria during Apoptosis by BCL-2 Family Members

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Abstract

Apoptosis is a stringently regulated programmed cell death pathway by which a cell will kill itself in response to extensive damage, stress or a targeted signal. It is an important process in development, which requires the elimination of superfluous cells, as well as the regulation of mitotically active cells in a fully developed organism. The key regulators at the heart of the apoptotic pathway are a group of proteins known as the BCL-2 family, containing both pro- and anti-apoptotic members. These proteins are intimately involved in mitochondrial and endoplasmic reticulum dysfunction triggered by an apoptotic signal. These dysfunctions include release of cytochrome c from the mitochondria and the efflux of calcium from the ER. It is the BCL-2 family of proteins, their function and regulation, which is the focus of this thesis. We demonstrate that BID, a member of the pro-apoptotic BH3-only subfamily, when processed to its active form, tBID, will induce the integration of another pro-apoptotic protein, BAX, into the outer mitochondrial membrane which leads to the release of cytochrome c from the inter membrane space. There are, however, both BID-dependent and BID-independent pathways that are responsible for the insertion of BAX into the mitochondrial membrane though in either pathway the release of cytochrome c remains dependent on tBID. tBID can also induce the oligomerization of BAK, another pro-apoptotic member constitutively integrated in the outer membrane of the mitochondria and closely related to BAX, which will also lead to cytochrome c release. This oligomerization is a two-step mechanism, first tBID will interact with and activate BAK by inducing a change in conformation of its NH_2-terminus, then the activated form of BAK will initiate an auto-oligomerization cascade with other inactive BAK molecule. Anti-apoptotic BCL-2 will

inhibit the auto-oligomerization of BAK and subsequent release of cytochrome c by binding the active conformer but cannot inhibit tBID-induced activation of BAK. BAD, another BH3-only protein, can co-operate synergistically with tBID by inhibiting BCL-2 function, resulting in the displacement of BAK and the release of cytochrome c. This model supports the hypothesis used in the search for small molecule inhibitors of BCL-2, in the hopes that they can be effect treatments for cancer. Two putative small-molecule inhibitors of BCL-2, BH3I-1 and 2-methoxy Antimycin A_3, were analyzed for their ability to co-operate with an apoptotic signal to induce cytochrome c release form cells over-expressing BCL-2, however, neither showed their intended function *in vivo*. The inhibition of BCL-2 remains a very intriguing target for small molecules, with the intent to induce apoptosis specifically in cancer cells.

Résumé

L'apoptose est un programme de mort cellulaire contrôlé de façon très stricte au cours duquel une cellule se tue suite à une accumulation de dommages, un stress ou un signal spécifique. Ce processus joue un rôle important au cours du développement, qui requiert l'élimination de cellules superflues, ainsi que pour réguler les cellules en division active dans l'organisme adulte. Un groupe de protéines appelé la famille de BCL-2 et contenant des protéines pro et anti-apoptotiques, joue un rôle central dans la régulation de l'apoptose. Ces protéines sont intimement liées aux dérèglements de la mitochondrie et du réticulum endoplasmique observés au cours de l'apoptose, par exemple la sortie du cytochrome c de la mitochondrie et du calcium du réticulum endoplasmique. Cette thèse porte sur la fonction et la régulation de la famille des homologues de BCL-2. Nous démontrons que BID, un membre de la sous-famille pro-apoptotique BH3-seulement, lorsque sous sa forme activée (tBID), induit l'intégration d'une autre protéine pro-apoptotique, BAX, dans la membrane externe de la mitochondrie, ce qui conduit à la sortie du cytochrome c de l'espace inter-membranaire. Cependant, il existe des voies dépendantes et indépendantes de BID qui sont responsable de l'insertion de BAX dans la membrane mitochondriale quoique, quelque soit la voie, la sortie du cytochrome c reste dépendante de tBID. tBID peut également induire l'oligomérisation de BAK, un autre membre pro-apoptotique des homologues de BCL-2 ressemblant beaucoup à BAX qui est constitutivement intégré dans la membrane externe de la mitochondrie et cause également la sortie du cytochrome c. Cette oligomérisation se produit en deux étapes. Premièrement, tBID interagit avec BAK et l'active en induisant un changement de conformation dans son extrémité N-terminale. Ensuite, la forme activée de BAK initie une cascase d'auto-

oligomérisation en interagissant avec des molécules inactives de BAK. La protéine anti-apoptotique BCL-2 inhibe l'auto-oligomérisation de BAK et la sortie du cytochrome c de la mitochondrie en se liant à la forme active de BAK mais ne peut bloquer son activation par tBID. BAD, un autre BH3-seulement, peut coopérer de façon synergistique avec tBID en inhibant l'action de BCL-2, ce qui libère BAK et permet la sortie du cytochrome c. Ce modèle suggère que de petites molécules inhibant BCL-2 pourraient être utilisées dans le traitement du cancer. BH3I-1 et 2-méthoxy Antimicine A_3 sont deux de ces molécules candidates. Elles ont été testées pour leur capacité à coopérer avec un signal apoptotique pour induire la sortie du cytochrome des mitochondries de cellules surexprimant BCL-2. Cependant, aucune n'a induit l'effet voulu *in vivo*. L'inhibition de BCL-2 par de petites molécules afin d'induire spécifiquement l'apoptose dans les cellules cancéreuses reste cependant une avenue de recherche très intéressante.

Acknowledgements

First and foremost in would like to thank my parents, Maria and Luigi Ruffolo, my grandparents, Giuseppina and Luigi Trozzo and Guiseppina Ruffolo, and the rest of my family for their unwavering love and support through the many years of my education. I want to thank Karen Christensen for all of her help, for putting up with me during the writing of my thesis and for saying "yes". I was also very fortunate to have a great supervisor in the person of Dr. Gordon C. Shore who provided strong guidance, support and an environment conducive to explore my own research abilities. A more than great thank you must go to Dr. Mai Nguyen with whom I have had many wonderful conversations that were rarely work related. Thank you, Mai, for being a great friend and your help in making me a better scientist and person. My thanks also go to Dr. Ing Swie Goping who started me off in my project and also for our fun (funny) and interesting discussions, "technically". I must also thank the members of my lab: Mary Sutherland, Dave Breckenridge, Marc Germain, Jaigi Mathai, Bing Wang, Marina Stojanovic, Samar Pai, Stephanie Beuler, and Nhi Nguyen for making the lab intellectually stimulating, relaxed and most of all fun. Many thank you's also go to all of my friends in the Biochemistry department both past and present. There are too many to list but you know who you are. Thank you all for making graduate life a fun time that I will never forget. And finally, I want to thank Maureen Caron for keeping me in line for the past eight plus years and for being a great friend while doing so. I would also like to thank the CIHR and the FRSQ-FCAR Sante for financial support throughout my doctoral research. Nonno Ruffolo and Uncle Charlie, I miss you both very much.

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Abbreviations

aa	amino acid
AIF	Apoptosis Inducing Factor
ANT	Adenine Nucleotide Translocator
ART	Apoptotic Regulation of Targeting
BH	BCL-2 Homology
BIR	Baculoviral IAP Repeat
BMH	Bis-Maleimidohexane
CAD	Caspase-Activated Deoxyribonuclease
CARD	Caspase Activation and Recruitment Domain
cyt c	cytochrome c
cMRM	complete Mitochondria Reconstitution Media
dATP	deoxyribose Adenine Triphospaste
DD	Death Domain
DED	Death Effector Domain
DISC	Death Inducing Signaling Complex
DMSO	Dimethylsulfoxide
DRP-1	Dynein Related Protein - 1
ER	Endoplasmic Reticulum
GFP	Green Fluorescence Protein
HIM	Heart mitochondria Isolation Media
IAP	Inhibitor of Apoptosis Protein
ICAD	Inhibitor of Caspase-Activated Deoxyribonuclease
MAA_3	2-Methoxy Antimycin A_3
MPTP	Mitochondrial Permeability Transition Pore
NAD	Nicotinamide Adenine Dinucleotide
PARP	Poly (ADP-Ribose) Polymerase
PBS	Phosphate Buffered Saline
SDS-PAGE	Sodium Dodecyl Sulfate – Poly-Acrylamide Gel Electrophoresis
TNF	Tumor Necrosis Factor
TRAIL	TNF Related Apoptosis Inducing Ligand
UV	Ultra Violet
VDAC	Voltage-Dependent Anion Channel
wt	wild type
zVAD-fmk	benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone

Contributions of Authors

This thesis includes the text and figures of two previously published research articles, making up Chapters 2 and 3. I am the first author of both of these works. The published works have been re-formatted to fit the overall style of this thesis, and the references of all chapters have been combined into one reference section at the end of the manuscript.

Chapter 2:

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

Most of the experiments performed in this chapter were performed by myself. Dr. Mai Nguyen performed the experiment depicted in Figure 2.1A and Dr. Ing Swie Goping conducted the experiments presented in Figure 2.2A. *Bid null* mouse embryo fibroblasts and the anti-murine BID antibodies were generated and provided by Dr. Atan Gross and Dr. Stanley J. Korsmeyer. The inducible tBID cDNA constructs and the anti-human BID antibodies were generated and provided by Dr. Honglin Li and Dr. Junying Yuan.

Chapter 3:

Ruffolo, S.C., and Shore, G.C. (2003) BCL-2 Selectively Interacts with the BID-induced Open Conformer of BAK, Inhibiting BAK Auto-oligomerization. J. Biol. Chem. **278:** 25039-25045

All the experiments in this chapter were performed by myself. The HA-BCL-2 stably over-expressing cells lines were generated by Dr. Mai Nguyen.

Chapter 4:

Ruffolo, S.C., and Shore, G.C. Work in progress.

This chapter consist of all of my own unpublished data. The BAD BH3 peptide, as well as, the two BCL-2 inhibitors were graciously provided GeminX Biotechnologies.

Chapter 1

General Introduction

1.1 An Overview of Apoptosis

Apoptosis, a form of programmed cell death, is a genetically determined pathway by which a cell will kill itself in response to damage, stress, or a targeted signal. This pathway is completely different from other forms of cellular death, such as necrosis, in that the dying cell will always remain contained and therefore will not elicit an immune response. An apoptotic cell will instead undergo distinct morphological changes; the cell will detach from the extracellular matrix, the chromatin will condense, the DNA will be digested by endonucleases, the cell will shrink and begin to bleb (Hengartner, 2000). Afterwards, the cell will fragment; forming membrane bound apoptotic bodies containing the intracellular remains. During the process, phosphotidyl-serine is flipped from the cytosolic side of the lipid bilayer to the extracellular side. The phosphotidyl-serine on the surface of the apoptotic bodies will then trigger nearby cells to engulf them and after which they are disposed of by endosomal processing (Savill and Fadok, 2000). The entire process occurs very rapidly and, without the aid of the immune system, leaves no trace of the dead cell.

Any dysfunction in the apoptotic process could lead to over-activation, resulting in the clearing of cells that are not destined to die, or inhibition, allowing a damaged cell to survive and possibly lead to aberrant proliferation. Examples of such dysfunctions can be found in many diseases including; the loss of cells in the spinal cord after spinal trauma (Beattie et al., 2002), the loss of neurons in the brain due to neurodegenerative disease (Yuan and Yankner, 2000) and the uncontrolled proliferation of cancer cells (Kaufmann and Gores, 2000; Olopade et al., 1997). Its close ties to so many debilitating diseases makes apoptosis a very intriguing field of study; understanding the mechanism

of regulation of the process will be invaluable in finding new avenues of treatment (Nicholson, 2000).

1.2 Caspases: The Effectors of Apoptosis

Caspases are cysteine proteases that cleave specific 4 amino acid motifs which have an aspartate at position 1 (Nicholson, 1999). The caspases are organized into two separate groups: initiator and effector caspases. These proteases are translated in all cells as inactive pro-enzymes which have three parts: a pro-domain at the NH_2-terminus, which is much longer in initiator caspases and contains protein-protein interaction motifs; followed by an 18kDa portion which contains the active-site cysteine; and ending with a 10kDa fragment. These three parts are separated by one or more caspase cleavage sites and though the pro-enzyme many only have minimal protease activity, if two or more pro-caspases are brought into close proximity, an important mechanism by which the initiator caspases are activated, they have the ability to auto-catalytically process themselves. When apoptosis is induced, the p10 subunit of the holoenzyme is processed, followed by the cleavage of the pro-domains leaving the p18 subunit. The p18 and p10 subunits will then form a tetramer with two other processed fragments to form the active caspase (Hengartner, 2000) (Figure 1.1). The initiator caspases (caspase-1, 8, 9, and 10) are considered apical caspases since they are activated early in the apoptotic pathway. These enzymes, in particular caspase-8, will cleave certain pro- and anti-apoptotic proteins, like BID (Gross et al., 1999b; Li et al., 1998; Luo et al., 1998) and BAP31 (Ng et al., 1997), but they are also responsible for cleaving and activating the effector caspases (caspase-3, 6, and 7) (Nunez et al., 1998). These effector caspases are, as their name would suggest, the downstream effectors of the apoptotic program.

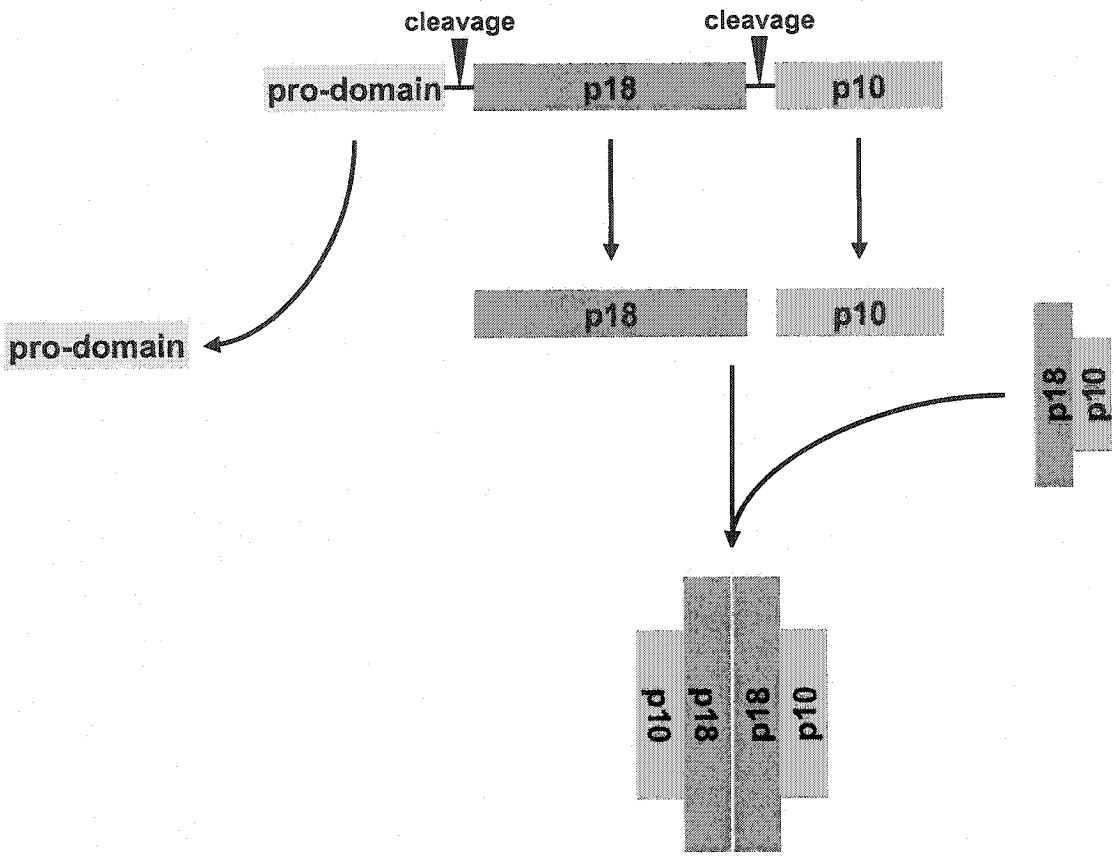

Figure 1.1 Caspase processing and formation of the active holoenzyme

1.2.1 Some Effector Caspase Substrates

They are responsible for inactivating cell survival protein and activating pro-apoptotic proteins, leading to total cellular dysfunction. One notable target is ICAD, which binds to and sequesters caspase-activated deoxyribonuclease (CAD) thus inhibiting its DNase activity. Cleavage of ICAD, by caspase-3, will allow CAD to degrade the DNA within the cell. This is an a common hallmark of apoptosis (Nagata, 2000), and obviously detrimental for the cell, but necessary to eliminated infected or genetically damaged cells. Other targets for the effector caspases are poly (ADP-ribose) polymerase (rendering it non-functional and possibly leading to a loss of RNA stability or preventing

it from using NAD and dATP which are required for apoptotic progression) (Boulares et al., 1999), lamins (causing chromatin condensation) (Ruchaud et al., 2002), as well as the anti-apototic proteins BCL-2 and BCL-X_L (Cheng et al., 1997; Clem et al., 1998). It is also important to note that the effector caspases can also process initiator procaspases, therefore producing a feedback amplification loop.

1.2.2 A Fail-Safe

Since these are such destructive proteins when active, the cells have a fail-safe to prevent death when the caspases misfire; these are the inhibitor of apoptosis proteins (IAPs). IAPs contain baculoviral IAP repeat (BIR) domains, which allow them to bind to the activated caspases thus inhibiting them (LeBlanc, 2003). Therefore, for the apoptotic pathway to continue these IAPs must be inactivated, which will be discussed later.

1.3 The Signals of Apoptosis

1.3.1 Receptor Mediated Apoptosis

Caspases are the downstream effectors of the apoptotic pathway, but what are the signals required to activate them? The most straightforward and best-characterized mechanism for activating caspases is the through the tumor necrosis factor family of death inducing ligands. These ligands, which include TNFα, Fas, and TRAIL, are primarily expressed on the surface of cytotoxic T cells and NK cells. They are used to signal apoptosis, by cells of the immune system, in mature T cells after an immune response, and infected or damaged cells (Krammer, 2000). All of these ligands function by similar mechanisms when they bind their respective receptor (TNFR, Fas/CD95/APO-1, TRAIL-R1/2 respectively) on the target cell, the receptor will trimerize resulting in the

recruitment of an adaptor molecule FADD. This recruitment is usually a direct interaction between the death domain (DD) in the cytosolic portion of the receptor and the DD at the NH_2-terminus of FADD; however, the TNF-R1 receptor does require another protein, TRADD as an adaptor for FADD recruitment. FADD, which also has a death effector domain (DED) at it COOH-terminus, will then recruit procaspase-8, through the DED in its pro-domain (Medema et al., 1997; Muzio et al., 1996). This complex is referred to as the DISC (death-inducing signaling complex) and its formation results in the proximity-induced autocatalytic processing of the three recruited procaspase-8 molecules and the release of active caspase-8 into the cytosol (Muzio et al., 1998).

The amount of procaspase-8 processed at the DISC, however, differs in various cell types: in Type I cells (e.g. SKW6.4 and H9) a large amount of caspase-8 is produced as opposed to Type II cells (e.g. CEM and Jurkat) in which little caspase-8 is generated (Scaffidi et al., 1998). There is enough caspase-8 generated by Fas signaling in Type I cells to directly process and activate caspases 3, 6, and 7, leading to a quick death via apoptosis. However, in Type II cells, the low levels of caspase-8 are insufficient to induce a robust activation of downstream caspases at which point the signal must be amplified which is achieved through mitochondrial dysfunction (Scaffidi et al., 1999) (Figure 1.2).

1.3.2 Activation of Caspases through Mitochondrial Dysfunction

During apoptosis, many changes occur throughout the cell, none are so prominent and as closely linked to apoptosis as the dysfunction which occurs at the level of the mitochondria. Mitochondria undergo morphological changes in an apoptotic cell, such

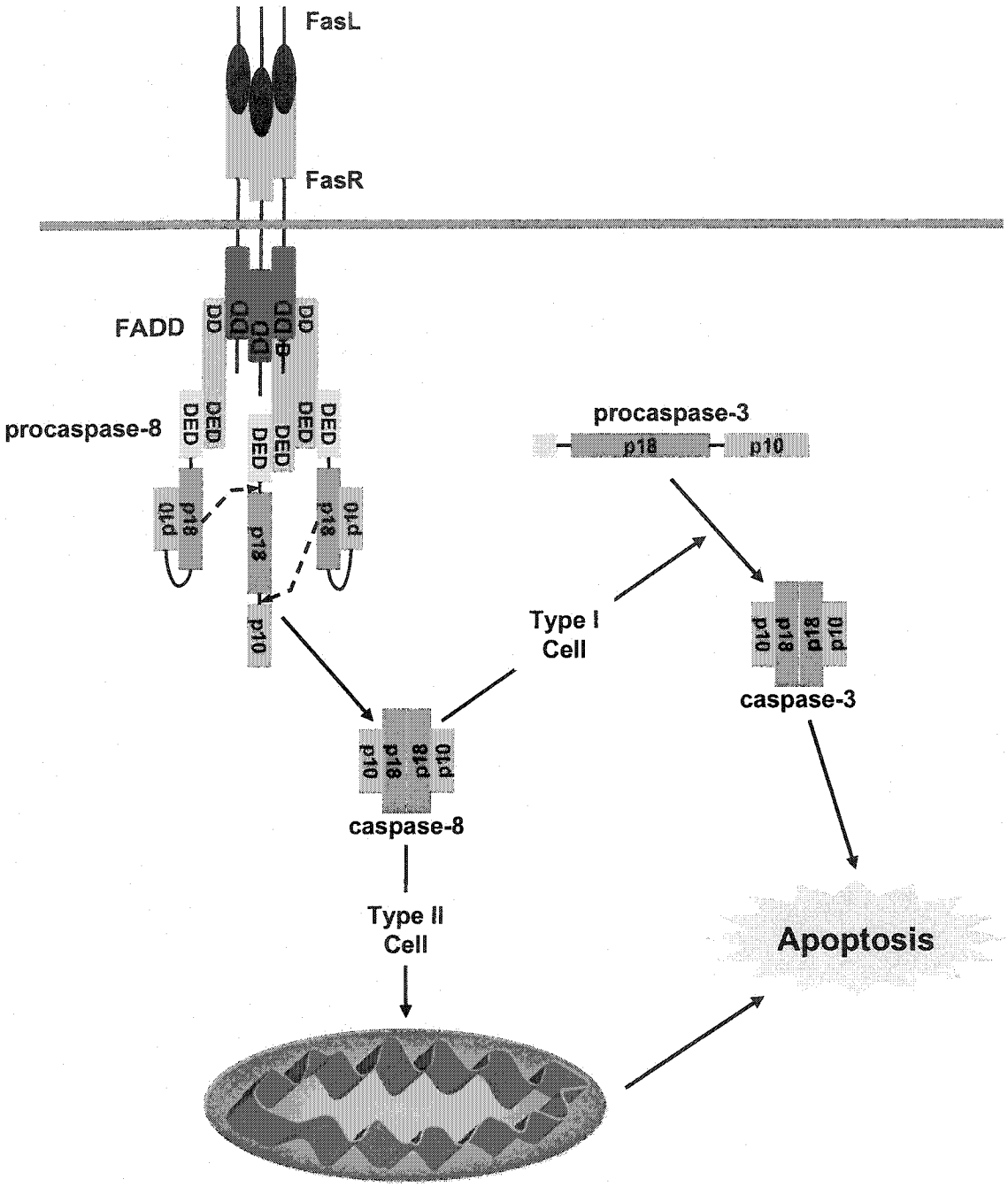

Figure 1.2 Recruitment and activation of procaspase-8 at a Fas ligand-induced DISC, followed by caspase-8 triggering separate apoptotic pathway in Type I and Type II cells

as, remodeling of the cristae within the mitochondria (Scorrano et al., 2002) and the complete alteration of the architecture of the mitochondria by fission (Frank et al., 2001). The mitochondrial membrane becomes depolarized, halting respiration and thus energy

production, reactive oxygen species are expelled, having detrimental effects on other cellular proteins and functions, and many small proteins are released from the inner-membrane space (Green and Reed, 1998).

One such protein is cytochrome c, whose release from the mitochondria will halt respiration and production of ATP. Cytochrome c release has a second, and more important, function in the apoptotic pathway; inducing the processing and activation of procaspase-9. Cytochrome c, along with dATP, will bind an adaptor molecule, APAF-1, which induces it to form an septomer with other activated APAF-1 molecules and recruit procaspase-9 through the caspase recruitment domain (CARD) in its pro-domain. This results in a complex referred to as the apoptosome (Li et al., 1997). At this point, the procaspase-9 molecules can auto-catalytically process themselves, presumably due to proximity induction (Zou et al., 1999), similar to the activation of procaspase-8 at the DISC. Caspase-9 will then cleave procaspase-3 leading to the progression of apoptosis. Recent structural data has revealed that the apoptosome is a wheel-like structure with 7-fold symmetry and also, when the procaspase-9 was substituted for a mutant that could not be processed, the apoptosome was still able to activate pro-caspase-3 (Acehan et al., 2002). That suggests processing of procaspase-9 is not required for subsequent cleavage of procaspase-3 and therefore any processing of procaspase-9 may be the result of the activation of caspase-3 (Figure 1.3).

1.3.2.1 Other Proteins Released from the Mitochondrial Inner Membrane Space

In conjunction with cytochrome c activation of caspases, another protein released from the inner-membrane space during apoptosis will deactivate the active-caspase fail-safe. That protein is Smac/DIABLO which, once released from the mitochondria, will

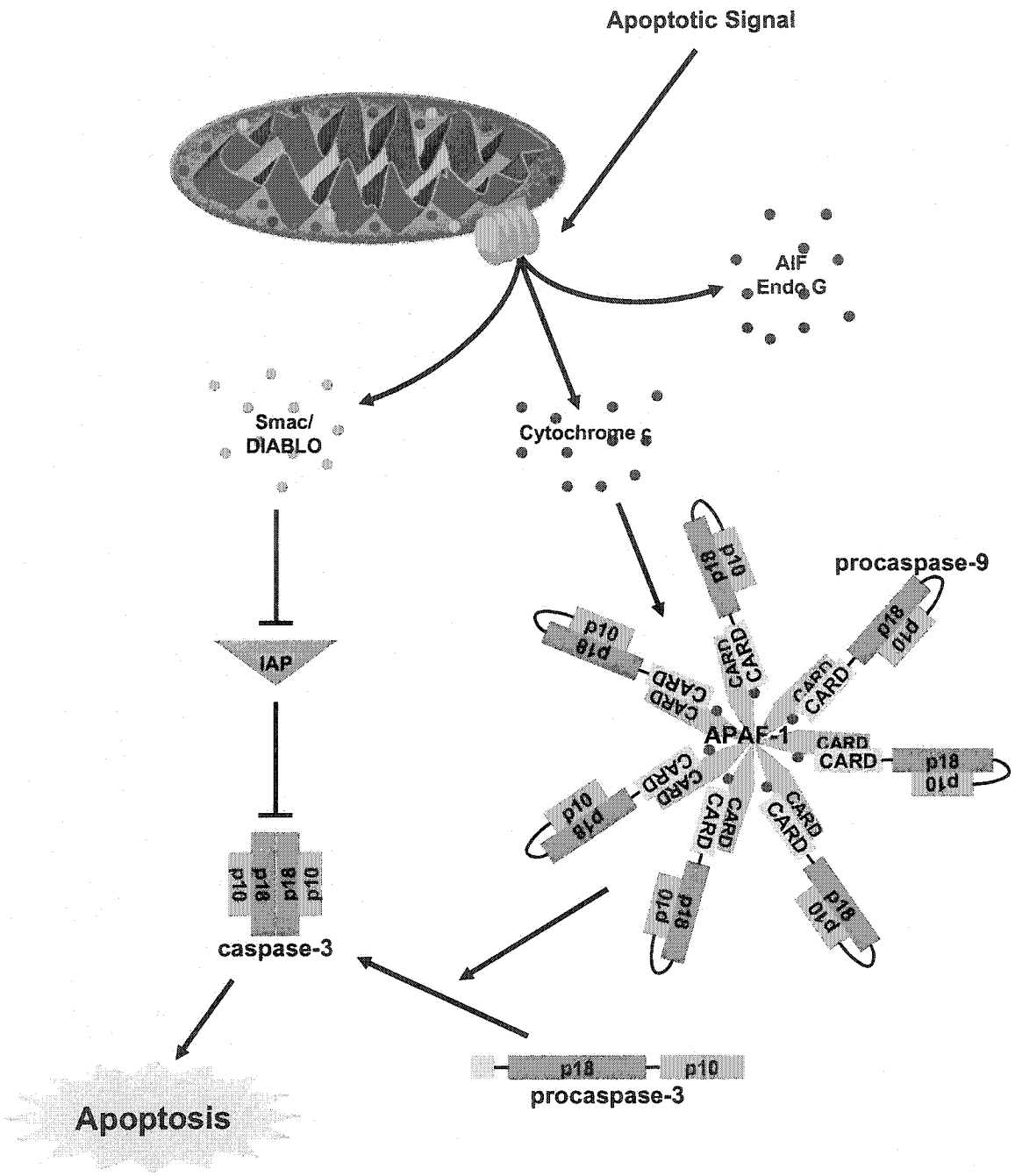

Figure 1.3 Release of mitochondrial inner membrane proteins in response to an apoptotic signal.

bind and sequester the IAPs (Du et al., 2000) releasing the caspase from this inhibitor. Structural data has revealed that Smac/DIABLO interacts with the BIR3 domain of XIAP which is a strong indication of how it blocks IAP function (Liu et al., 2000). Two other

proteins released from the mitochondria during apoptosis, AIF and endonuclease G, have been shown to be involved in induction of DNA degradation (Li et al., 2001; Susin et al., 1999).

1.3.3 The Role of the Endoplasmic Reticulum in Mitochondrial Dysfunction

Caspase-8 will induce mitochondrial dysfunction by cleaving, and in effect activating, the pro-apoptotic protein BID which will translocate to the outer mitochondrial membrane where it will initiate dysfunction by a mechanism that will be described in more detail further on. Recent studies have elucidated a mechanism by which caspase-8 can also enhance the release of proteins from the mitochondria via the efflux of calcium stores from the endoplasmic reticulum. One mechanism by which this calcium efflux is initiated is through the cleavage of BAP31 by caspase-8. BAP31 is a transmembrane protein, exclusively located at the ER, that has three membrane spanning alpha-helices and therefore both its NH_2-terminus and longer COOH-terminus are on the cytosolic side of the membrane (Ng et al., 1997). Over-expression of caspase-resistant BAP31, which cannot be cleaved by caspase-8, has been shown to inhibit Fas induced apoptosis (Nguyen et al., 2000) which seems to suggest that full-length BAP31 may act as an inhibitor of apoptosis. During Fas-induced apoptosis, caspase-8 will cleave BAP31 to a 20kDa protein (p20) that is now a very potent transducer of the apoptotic signal (Ng et al., 1997). p20 will trigger the efflux of calcium from the lumen of the ER (Breckenridge et al., 2003), however, it is still unclear how this efflux occurs; the p20 fragment, which can interact with other p20 molecules as well as full-length BAP31, may form a calcium channel or it may modulate an already existing ER pore. Some research has shown that the cytosolic calcium can be taken up by the mitochondria (Duchen, 2000)

resulting in the recruitment of DRP1 to the mitochondria and the subsequent scission of the organelle, which may be involved in the remodeling of the cristae and is purported to enhance cytochrome c release. Another possible role of calcium efflux may be to activate calcineurin, a cytosolic phosphatase activated by free calcium ions. Calcineurin has been shown to dephosphorylate the pro-apoptotic protein BAD which makes it active (Wang et al., 1999), leading to its translocation to the mitochondria where it can initiate mitochondrial dysfunction.

1.3.4 Oncogenic Stimulation of Apoptosis

When a cell's DNA becomes damaged, either chemically or by irradiation (i.e. UV or γ), or an active oncogene is present (such as the adenoviral protein E1A), a cascade of events is initiated leading to the stabilization of the transcriptional activator, p53 (Meek, 1999) (Figure 1.4). Once stabilized, a tetramer of p53 will potentiate the transcription of cell cycle arrest genes, through p21/Waf-1, which will allow the cell time to repair the damaged DNA. However, p53 will also enhance the transcription of pro-apoptotic genes (BIK, PUMA, NOXA, BID, and BAX) (Mathai et al., 2002; Nakano and Vousden, 2001; Oda et al., 2000; Sax et al., 2002; Wu and Deng, 2002).

Some of these pro-apoptotic proteins are targeted to the mitochondria where they will induce the same mitochondrial dysfunction and protein release as the Fas ligand. Other p53-induced pro-apoptotic proteins are targeted to the ER where they can stimulate release of cytochrome c from the mitochondria (Germain et al., 2002), possibly via calcium efflux from the ER. In addition, during apoptosis induced through a p53-dependent mechanism or by chemically stressing the ER (e.g. thapsigargin), BAP31, which also has a DED in its C-terminus, can recruit procaspase-8L, an N- terminal

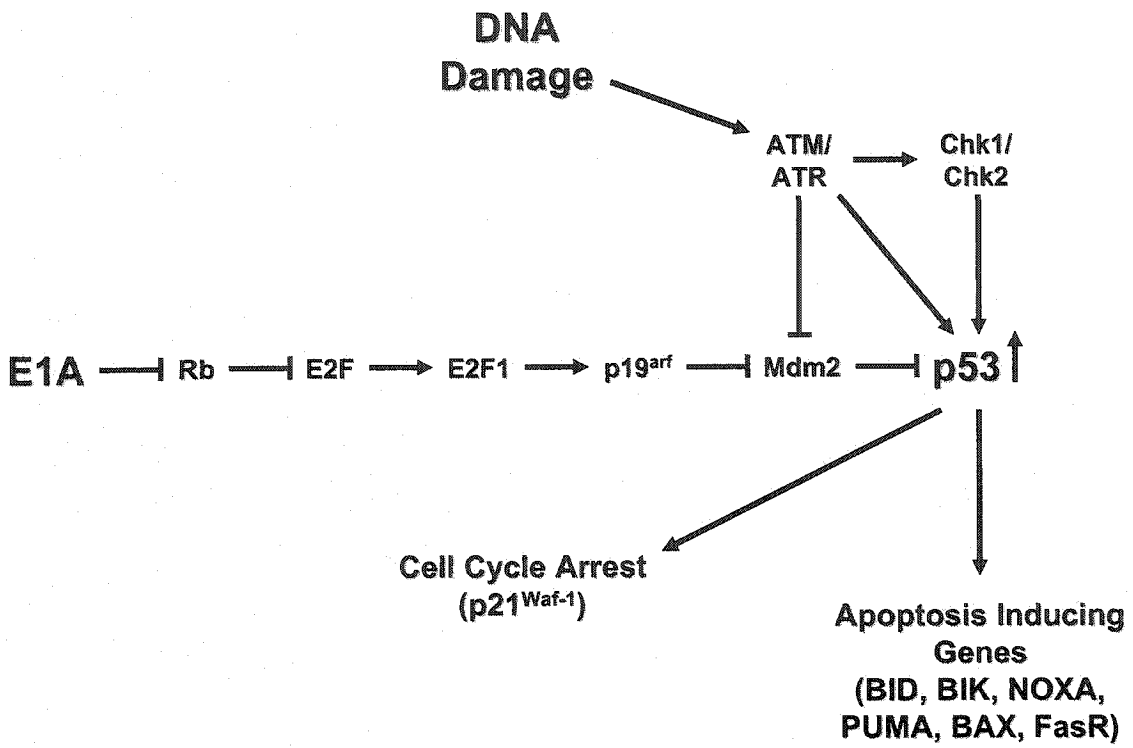

Figure 1.4 Pathways leading to the stabilization and activation of p53 tumor suppressor induced by expression of E1A (an oncogenic stimulus) and DNA damage. Activation of p53 will than lead to the expression of cell cycle arrest genes as well as the expression of apoptosis inducing genes.

extended isoform of procaspase-8, to the ER (Breckenridge et al., 2002). The DED of BAP31 is flanked by two caspase-8 cleavage sites, thus when the pro-caspase-8L is activated (or via the proenzyme's protease activity), it can cleave the COOH-terminus of BAP31 producing the p20 fragment.

p53 can also induce the expression of Fas (Muller et al., 1998), this may make the cell overly sensitive to Fas ligand present on the surface of surrounding cells or even on its own cell surface. This would presumably lead to the formation of more DISCs and activation of more caspase-8. However, in cells lacking the Fas adaptor molecule, FADD, does not prevent activation of caspase-8 in response to E1A-induced apoptosis which functions through p53 (Nguyen et al., 1998).

1.4 The Mediators of Apoptosis

1.4.1 An Overview of the BCL-2 Family Members

The final pieces of the apoptotic puzzle are the signal transduction molecules required to translate the apoptotic trigger to achieve mitochondrial dysfunction or release of ER calcium stores. This task falls onto a large and very diverse group of proteins known as the BCL-2 family. These proteins share regions of homology referred to as BH (BCL-2 Homology) domains and can be divided into pro- and anti-apoptotic submembers. The anti-apoptotic proteins share four distinct regions of homology enumerated BH1, 2, 3, and 4 and all members have a transmembrane domain which targets them to the mitochondrial outer membrane, the ER and the nuclear envelope (Germain and Shore, 2003). These proteins are potent inhibitors of both mitochondrial dysfunction and calcium release from the ER. The pro-apoptotic proteins can be further subdivided into multi-domain (possessing BH domains 1, 2, and 3) and BH3-only proteins. The multi-domain proteins are the pro-apoptotic counterparts of the anti-apoptotic proteins; they are the effectors of mitochondrial dysfunction (Gross et al., 1999a) and have an important, though not clearly determined role in the regulation of ER calcium stores (Scorrano et al., 2003). The BH3-only proteins are smaller molecules that act as sentinels, always on the lookout for an apoptotic signal. An apoptosis trigger will activate these proteins which will in-turn activate the multi-domain pro-apoptotic proteins (Bouillet and Strasser, 2002). The anti-apoptotic proteins can either inhibit the activation or the function of the multi-domain pro-apoptotics (Figure 1.5). This is a very simplistic overview of how this family of proteins is involved in apoptosis and therefore it is necessary to segue into more detail about the function of these proteins and the

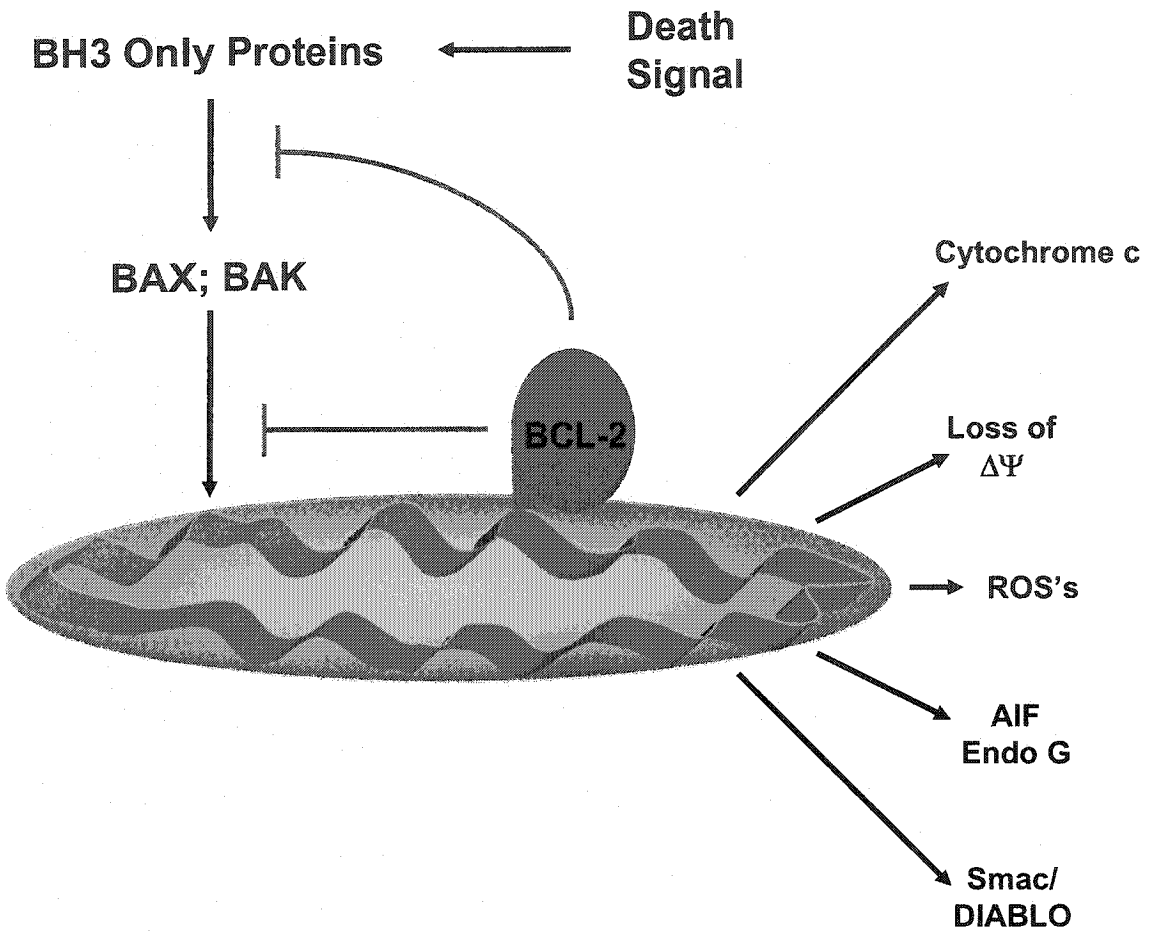

Figure 1.5 Simplified overview of the activation or inhibition functions of the multi-domain pro-apoptotic proteins BAX and BAK and the anti-apoptotic proteins, such as BCL-2, at the level of the mitochondria.

mechanisms by which they act.

1.4.2 Multi-Domain Pro-Apoptotic Proteins

The effectors of mitochondrial dysfunction are the multi-domain pro-apoptotic proteins BAX and BAK. These 23 and 26kDa proteins, respectively, have little sequence homology outside the 3 BCL-2 homology (BH1, BH2, and BH3) domains, however, they are structurally very similar to each other, as well as to their anti-apoptotic counterparts, and bear striking resemblance to the diphtheria toxin (Muchmore et al., 1996). Like the

toxins, BAX and BAK have two central hydrophobic helices surrounded by amphipathic helices (Muchmore et al., 1996; Suzuki et al., 2000) as well as a trans-membrane domain. During apoptosis, both are activated by the BH3-only proteins and will oligomerize in the outer mitochondrial membrane (Eskes et al., 2000; Wei et al., 2000). Reconstitution in liposomes has suggested that these oligomers are in fact pores (presumably using the hydrophobic helices), which would be large enough to cause the release of inner membrane proteins (Epand et al., 2002b; Korsmeyer et al., 2000). BAX has also been shown to interact with ANT (Marzo et al., 1998) and VDAC (Shimizu et al., 1999) which are both part of the large protein complex known as the mitochondrial permeabilization transition pore (MPTP). BAX interaction with the MPTP has been shown to induce the opening of the pore, which is specifically inhibitable by bongkrekic acid and cyclosporin A. The BAX induced opening of the MPTP would preumably also lead to the release of proteins from the mitochondria (Narita et al., 1998).

These functions absolutely require an intact BH3 domain, which is a 9 amino acid helical motif with the following highly conserved residues (highlighted in black):

```
Human BAX aa204 .....  LKRIGDELD
Human BAK aa223 .....  LAIIGDDIN
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Any mutation to these three conserved residues, will completely abrogated their ability to oligomerize and therefore their induction of apoptosis, while mutations to the less conserved, yet similar amino acids (highlighted in grey), have varied effects and seem to be require for binding to other proteins (Wang et al., 1998). The BH3 domain is not only necessary but is also sufficient to induce mitochondrial dysfunction. Constructs that express extended version of the helices which contain the respective BH3 domains have

been shown to bind both BAX and BAK (Simonen et al., 1997) and can induce release of cytochrome c and cell death (Moreau et al., 2003). Despite being similar in structure, function and functional requirements, BAX and BAK differ in their native or un-induced states.

1.4.2.1 Activation of BAX and BAK

BAX, under normal conditions and even with its trans-membrane domain intact, is held in the cytosol or loosely associated to the outer mitochondrial membrane through the action of its N-terminal apoptotic regulation of targeting (ART) domain (Goping et al., 1998). In response to an apoptotic signal, BH3-only proteins induce BAX to undergo a confromational change in its N-terminus (Murphy et al., 2000; Perez and White, 2000), relieving its repression by the ART domain, and allowing BAX to translocate from the cytosol to the mitochondria (Wolter et al., 1997) and integrate into the outer membrane (Goping et al., 1998). There, BAX will oligomerize and induce mitochondrial dysfunction. Although the ART domain seems totally unstructured *in vitro* (Suzuki et al., 2000), its deletion (Goping et al., 1998) or rearrangement by forced dimerization (Gross et al., 1998) can artificially induce BAX integration into the outer mitochondrial membrane and apoptosis. Exchanging the trans-membrane domain of BAX with one from a protein that is constitutively targeted to the mitochondria will also relieve the inhibition by the ART domain (Goping et al., 1998) indicating that the ART domain is a specific inhibitor of the BAX transmembrane domain.

Unlike BAX, BAK is constitutively integrated into the outer mitochondrial membrane therefore; BAK does not require the initial translocation step to effect mitochondrial dysfunction during apoptosis. However, BAK does also undergo a change

in its N-terminal conformation, as assessed by changes in immunoreactivity (Griffiths et al., 1999) and sensitivity to protease digestion (Ruffolo and Shore, 2003). These changes are induced by BH3-only proteins, prior to oligomerization and cytochrome c release. When the N-terminus is deleted, truncated BAK induces unregulated release of cytochrome c (Ruffolo and Shore, 2003) indicating that BAK may also contain an N-terminal domain simlar in inhibitory function to the ART domain of BAX.

As effectors of mitochondrial dysfunction, either BAX or BAK is required for apoptotic signals involving the mitochondria (such Fas, in Type II cells, or p53), since knocking both gene out severely abrogates induced cell death (Wei et al., 2001; Zong et al., 2001). Though the functions of both BAX and BAK seem to be redundant and are interchangeable in many apoptotic signaling pathways, recent studies propose that, in fact, some apoptotic signals selectively enlist one or the other to effect apoptosis (Cartron et al., 2003). This has been supported by findings that certain BH3-only proteins will activate either BAX or BAK but not both.

1.4.2.2 BAX and BAK at the Endoplasmic Reticulum

There is also new and surprising evidence suggesting that BAX and BAK serve a role at the endoplasmic reticulum (ER). Both proteins can translocate to the ER where they are required for the release of calcium stores from the lumen (Scorrano et al., 2003; Zong et al., 2003). However, the mechanism for this translocation is still a mystery. It may be a straight forward integration in the case of BAX, but BAK translocation seems a more complex conundrum which could involve its extraction from the mitochondrial outer membrane and subsequent re-integration into the ER. Also, the exact function of BAX and BAK at the level of the ER, whether it is forming a pore, like at the

mitochondrial membrane, or a modulatory role of an existing pore complex, has not been determined.

BAX and BAK are both potent inducers of apoptosis when over-expressed (McCarthy et al., 1997; Oltvai et al., 1993) and potent signal transducers when activated by an apoptosis trigger. They are kept in check by the anti-apoptotic BCL-2 family members.

1.4.3 Anti-Apoptotic Proteins

The proteins in this sub-group, which include: BCL-2, BCL-X_L, BCL-w, MCL-1, present in mammalian cells and 19K E1B an early adenoviral gene product, are very effective inhibitors of apoptosis and are considered potent proto-oncogenes (Gross et al., 1999a). In many cancers, one or more of these proteins are over-expressed allowing a damaged cell, which would normally self-destruct via the apoptotic pathway, to proliferate unchecked and subsequently lead to tumourgenesis. All have similar three dimensional structures, which, like BAX and BAK, resemble that of diphtheria toxin (Muchmore et al., 1996). All have the four BH domains, and can be found at most intracellular membranes (mitochondrial outer membrane, endoplasmic reticulum and nuclear envelope), suggesting that they all function similarly and thus far there has not been much evidence to dispute that.

1.4.3.1 The BH1/BH2 Cleft

Anti-apoptotic proteins, at the mitochondria, function at the level of BAX and BAK, either inhibiting their activation by sequestering BH3-only proteins (Letai et al., 2002) or inhibiting their function by binding their conformationally altered (activated) form and preventing oligomerization(Ruffolo and Shore, 2003). Both mechanisms are

mediated by the BH1 and BH2 domains of these proteins whose presence and integrity are absolutely required for effective inhibition of apoptosis (Borner et al., 1994; Yin et al., 1994). The solution structure of BCL-X_L, determined by NMR, showed that these two domains form a hydrophobic cleft for the BH3 domains of BAX/BAK or the BH3-only proteins to bind (Petros et al., 2000; Sattler et al., 1997). Through mutagenesis the reverse has also been shown to be true, mutating the leucine at position 211 in BAX (this is not the obligate leucine at position 204), will abrogate binding of BAX to BCL-2 but not its apoptotic function (Wang et al., 1998).

1.4.3.2 The BH4 Domain

Though it may seem obvious that the interaction of an anti-apoptotic protein with BAX or BAK would be sufficient to inhibit the oligomerization of either protein, studies have shown that it is not that simple. Deletion of the BH4 domain in BCL-2 will abrogate its ability to homodimerize; however, the deletion does not affect its interaction with BAX. The deletion also abolishes its anti-apoptotic function, implying that BCL-2 homodimerization also has an important role in the inhibition of apoptosis (Reed et al., 1996b). This role, however, is as of yet, still a mystery.

It is important to note that BCL-2 at the mitochondria is only effective at inhibiting apoptotic pathways that involve the mitochondria such as Fas-induced apoptosis in Type II cells. Moreover, BCL-2 is impotent in preventing Fas-induced apoptosis in Type I cells which do not require a mitochondrial amplification step (Scaffidi et al., 1998). Multi-domain BCL-2 family members constitute a checkpoint at the mitochondria, where the ratio of pro- to anti-apoptotic proteins will determine whether a cell will undergo apoptosis or not. A ratio that is skewed towards the pro-

apoptotics will favor cell death, whereas an abundance of anti-apoptotic proteins will promote cell survival in the face of an apoptotic stimulus. When BAX or BAK are expressed ectopically, cultured cells will undergo apoptosis spontaneously (McCarthy et al., 1997; Oltvai et al., 1993), while, over-expression of anti-apoptotic proteins will render cells resistant to most apoptotic stimuli (Nakasu et al., 1994; Rao et al., 1992; Reynolds et al., 1994).

Under normal conditions, a cell is always teetering on the brink of undergoing apoptosis. All the players required for the process are already present in the cell. What tips the cell into the throws of programmed cell death is the activation of the BH3-only proteins.

1.4.4 BH3-only Proteins

These are a group of smaller proteins which have only one region of homology with other BCL-2 family members, and that is their BH3 domains. Like BAX and BAK, the BH3 domains of these proteins are absolutely required for their ability to induce apoptosis (Wang et al., 1996; Zha et al., 1997) and are, for the most part, also sufficient to induce cell death (Letai et al., 2002; Moreau et al., 2003; Ruffolo and Shore, 2003; Shangary and Johnson, 2002). However, in an un-induced state, these proteins, in cells, are either not transcribed at detectable levels or are present a conformation with their BH3 domain masked. In response to an apoptotic stimulus, these proteins either undergo post-translational modification (BAD, BID, BIM, BMF) or are transcriptionally activated (BID, BIK, NOXA, PUMA). The BH3 domain of all these proteins will then be exposed allowing them to interact with other BCL-2 family members and induce apoptosis. However, recent evidence has suggested that each BH3-only protein has different means

to induce apoptosis, highlighted by differential activation, localization, and affinities to other BCL-2 family members (Letai et al., 2002).

1.4.4.1 Post-Translationally Modified BH3-only Proteins

BID is a 21kDa protein that is cleaved by caspase-8 to produce a 15kDa protein, termed tBID (Gross et al., 1999b; Li et al., 1998; Luo et al., 1998). Once cleaved, the BH3 domain of BID becomes exposed (Chou et al., 1999; McDonnell et al., 1999), tBID becomes myristolated at its cleaved N-terminus (Zha et al., 2000), translocates to the mitochondria and integrates into the outer membrane. tBID will interact with both BAX and BAK inducing a change in the N-terminal conformation of both leading to the translocation and integration of BAX (Desagher et al., 1999; Ruffolo et al., 2000) into the outer mitochondrial membrane followed by oligomerization of both (Ruffolo and Shore, 2003). Anti-apoptotic proteins, such as BCL-2, cannot inhibit the cleavage of BID nor tBID translocation and integration into the outer membrane of the mitochondria (Gross et al., 1999b), though they can inhibit downstream events. At this point, however, there has been recent controversy in the field as to the mehcanism of inhibition by BCL-2 or other anti-apoptotic proteins. One model proposes that tBID will be bound and sequestered by BCL-2 thus inhibiting from activating BAX or BAK (Letai et al., 2002). However, a second model suggests that tBID can interact with either BAX or BAK inducing a change in conformation, irrespective of the presence of excess anti-apoptotic proteins (Ruffolo and Shore, 2003; Sundararajan and White, 2001). According to the latter model, the activated BAX and BAK molecules will be bound by anti-apoptotic proteins, thus inhibiting their subsequent integration, in the case of BAX, and oligomerization (see Figure 3.6).

Unlike BID, BAD does not require cleavage to be activated. BAD becomes phosphorylated at serine 112 and 155 by cAMP-dependent protein kinase A (PKA) and RSK (or MAP kinase activated protein kinase-1) (Harada et al., 1999; Tan et al., 1999; Tan et al., 2000), and at serine 136 by protein kinase B/AKT (Yano et al., 1998) which are all signal transducers of survival pathways. The phosphorylation of BAD at serines 112 and 136 causes it to be bound and sequestered by the protein 14-3-3, whereas the phosphorylation at serine 155, which is at the center of the BAD BH3 domain, disrupts BAD interaction with BCL-X_L (Zhou et al., 2000). In the absence of survival signals, BAD will become dephosphorylated either passively, through the production of more BAD molecules which will not be phosphorylated, or actively, through phosphatases activated in response to a loss of survival signaling. Protein phosphatase 2A has been implicated in the dephosphorylation of serine 112 (Chiang et al., 2003) as well as some *in vitro* data suggesting that protein phosphatase 2C can dephosphorylate serine 155 (Klumpp et al., 2003). Dephosphorylation of BAD by which ever means would render it incapable of being bound by 14-3-3 and would enhance its interaction with BCL-X_L.

In response to other apoptotic triggers, there is some evidence that 14-3-3 is cleaved by caspase-3, rendering it incapable of interacting with BAD (Won et al., 2003), as well as, evidence previously referred to that calcineurin, when activated by calcium, can dephosphorylate BAD (Wang et al., 1999), thus allowing it to translocate to the mitochondria where its exposed BH3 domain will interact with BCL-2 and BCL-X_L with a higher affinity than BAX or BAK. BAD, in itself, is not a very potent inducer of apoptosis; its BH3 domain is not as effective as that of BID in inducing BAK oligomerization or cytochrome c release *in vitro* (Ruffolo and Shore, 2003). However, its

preference for binding anti-apoptotic proteins over pro-apoptotics has led to the discovery of an intriguing mechanism by which BH3-only proteins will co-operate to induce apoptosis. The BH3 domain of BAD has a higher affinity for binding BCL-2 than the BH3 domain of BID or BAK and therefore can displace both BID and BAK from binding BCL-2 allowing them to continue on the apoptotic pathway (Letai et al., 2002; Ruffolo and Shore, 2003).

Two other post-translationally modified BH3-only proteins, BIM and BMF, require phosphorylation to become activate. BIM is sequestered in the dynein motor complex (Puthalakath et al., 1999) while BMF is bound to the myosin V actin motor complex (Puthalakath et al., 2001) in cells that are not undergoing apoptosis. When apoptosis is induced by UV radiation, the c-JUN N-terminal kinase (JNK) will phosphorylate both BIM and BMF thus releasing them from their cytoskeletal attachments (Lei and Davis, 2003). They then induce mitochondrial dysfunction, which seems to be mediated through a BAX dependent pathway, and allow the progression of cell death.

1.4.4.2 Transcriptionally Activated BH3-only Proteins

Then there are those BH3-only proteins (BIK, NOXA, PUMA) that are transcriptionally up regulated when apoptosis is induced (Mathai et al., 2002; Nakano and Vousden, 2001; Oda et al., 2000). They are all up regulated through a p53 dependent mechanism and therefore are responsive to DNA damaging agents as well as viral proteins that cause the stabilization of p53, such as the E1A oncogene. One of these proteins, BIK, is in fact absolutely required for p53 induced apoptosis (unpublished data) and, as opposed to all the aforementioned BH3 only proteins, it targets the endoplasmic

reticulum (ER), not the mitochondria. BIK can interact with BCL-2 present at the ER and will induce a release of ER calcium (unpublished data) through a purportedly BAX-dependent pathway (Gillissen et al., 2003). This mechanism of calcium release may be required to induce morphological changes in the mitochondria, similar to those triggered by caspase cleavage of BAP31 (Breckenridge et al., 2003). BIK can also co-operate with NOXA, another p53-induced BH3 only protein, at the level of the mitochondria to enhance its apoptotic signal (unpublished data), however, the exact mechanism is still unclear. Full length BID is also transcriptionally up regulated in response to elevated levels of p53 (Sax et al., 2002) and may in fact, cause some of the same effect as described previously for tBID, even without being cleaved. However, these effects only occur when full length BID is over-expressed (Eskes et al., 2000).

1.4.5 The Quest for Small Molecule Inhibitors of BCL-2

So what is the importance of deciphering the mechanism of action of all these proteins? This can be answered by the recent paramount finding that the BH3 domains of the BH3-only proteins alone are as functional as the full-length protein (Letai et al., 2002; Moreau et al., 2003; Ruffolo and Shore, 2003; Shangary and Johnson, 2002). Thus, the BH3 domain of BAD will still bind BCL-2 and inhibit it (Letai et al., 2002) while the BH3 domain of BID will bind BAK and activate it (Letai et al., 2002; Ruffolo and Shore, 2003). These motifs are 9-11 amino acids long and bind as an alpha helix with known contact sites, which makes it easier to look for or engineer mimics of these domains. A handful of such mimics have already been discovered, either by chance (Antimycin A_3 and it 2-methoxy analog) (Tzung et al., 2001), by screening compound libraries (BH3I-1 and -2) (Degterev et al., 2001), or by engineering compounds to make the necessary

contacts in the BH1/BH2 cleft (Kutzki et al., 2002). All are able to displace a BAK BH3 peptide from the BH1/BH2 domain cleft of various anti-apoptotic proteins and some have been shown to be able to enhance apoptotic sensitivity in cells over-expressing anti-apoptotic proteins (reviwed in Beauparlant and Shore, 2003). Since many malignant tumour cells have elevated levels of BCL-2, which inhibits the apoptotic pathway, the hope is that an inhibitor of anti-apoptotic proteins will co-operate with an oncogenic stimulus to induce apoptosis selectively in cancer cells.

1.5 Thesis Overview

The following three chapters will show one path in the development of better drugs to modulate apoptosis. It begins by determining the function of a BH3-only protein, BID, which is an activator of BAX translocation and integration into the outer mitochondrial membrane. Although, BID is required for release of cytochrome c from the mitochondria, it is not necessary for the activation of BAX in response to an oncogenic stimulus. The journey continues by detailing the mechanism by which tBID, the caspase-8 cleavage product of BID, functions and how BCL-2 prevents it action. It will be shown that tBID will interact with BAK inducing BAK to undergo a change in conformation (similar to that of BAX) from a closed (inactive) state to an open (active) form. This change in conformation is not inhibitable by BCL-2, however, BCL-2 does prevent the progression of mitochondrial dysfunction by binding the active form of BAK thus not allowing it to oligomerize and form pores. Finally, once a detailed mechanism is determined, targets for modulating the mechanism with small molecules become apparent and so the search begins for the small molecule "key" to fit into the protein "lock". Employing the same system used to elucidate the tBID mechanism, published compounds

as well as, engineered peptides are assayed for their ability to co-operate with a BID BH3 peptide to induce cytochrome c release from mitochondria over expressing BCL-2.

Chapter 2

BID-dependent and BID-independent Pathways for BAX Insertion into Mitochondria

2.1 Rationale

BAX, under non-apoptotic conditions, is prevented from inserting into the outer mitochondrial membrane by NH_2-terminal ART domain (Goping et al., 1998). When apoptosis is induced in cells, BAX will translocate to the mitochondria and integrate in the outer membrane (Hsu et al., 1997; Wolter et al., 1997). In this chapter we explored the regulation of BAX insertion into the outer mitochondrial membrane by *in vitro* reconstitution. We had a well developed and implemented mitochondria import system to study the phenomenon. We began by focusing on caspase induced BAX insertion since the pan-caspase inhibtor, zVAD-fmk, was able to inhibit BAX targeting (Goping et al., 1998). However, there was no evidence that BAX was the target of caspase cleavage, therefore, we concentrated on searching for a cytosolic mediator of BAX insertion into the outer mitochondrial membrane.

2.2 Abstract

In the absence of an apoptotic signal, BAX adopts a conformation that constrains the protein from integrating into mitochondrial membranes. Here, we show that caspases, including caspase-8, can initiate BAX insertion into mitochondria *in vivo* and *in vitro*. The cleavage product of caspase-8, tBID, induced insertion of BAX into mitochondria *in vivo*, and reconstitution *in vitro* showed that tBID, either directly or indirectly, relieved inhibition of the BAX transmembrane signal-anchor by the NH_2-terminal domain, resulting in integration of BAX into mitochondrial membrane. In contrast to these findings, however, *Bid*-null mouse embryo fibroblasts supported Bax insertion into mitochondria in response to death signaling by either TNFα or E1A, despite the fact that cytochrome c release from the organelle was inhibited. We conclude, therefore, that a

parallel Bid-independent pathway exists in these cells for mitochondrial insertion of Bax and that, in the absence of Bid; cytochrome c release can be uncoupled from Bax membrane insertion.

2.3 Introduction

Induction of apoptotic pathways in response to death signals is critically dependent on the status of survival/death regulators within a cell. Prominent among these is the BCL-2 family of anti-apoptotic (BCL-2, BCL-X_L, BCL-w, MCL-1, A-1) and pro-apoptotic (BAX, BAK, BOK) members, whose activities and ability to form heterodimers is influenced by a third subgroup of the BCL-2 family, which includes mammalian BID, BAD, BIK, BIM, BLK, HRK, and *C. elegans* EGL-1 (Adams and Cory, 1998; Gross et al., 1999a). The latter are pro-apoptotic and contain a minimal apoptotic domain, BH3, which targets these proteins for interaction with BCL-2 proteins. Several of these 'BH3 domain only' members, including BID, BAD, and BIM, are themselves influenced by specific signal transduction pathways (Gross et al., 1999b; Li et al., 1998; Luo et al., 1998; Puthalakath et al., 1999; Zha et al., 1996), which serve to link the BCL-2:BAX checkpoint to upstream cell-death initiating events. BCL-2 and BAX each contain a single transmembrane segment at their extreme COOH-terminus, which is responsible for targeting these proteins into membrane sites, including mitochondria (Goping et al., 1998; Nechushtan et al., 1999; Nguyen et al., 1993), where their opposing functions influence organellar integrity and function (Green and Reed, 1998). In situations where anti-apoptotic BCL-2 family members are limiting, mitochondria undergo profound dysfunction in response to most death signals. This includes release of cytochrome c from the intermembrane space (Kluck et al., 1997; Yang et al., 1997), which triggers

activation of downstream caspases (Li et al., 1997), and ultimately induction of permeability transition at the inner membrane, resulting in loss of the electrochemical potential and production of excess reactive oxygen species (Green and Reed, 1998). Recently, mitochondrial transformations have been directly linked to cleavage of cytosolic BID by caspases, including caspase-8, in the CD95/Fas and TNFR1 cell death pathways, at least in certain cell types in culture. The resulting product, tBID, targets the organelle and induces cytochrome c release (Gross et al., 1999b; Li et al., 1998; Luo et al., 1998). This signaling event in the Fas/TNFR1 pathway is likely an important contribution to apoptosis only in type II cells in culture, where upstream induction of the pathway following receptor-mediated activation of caspase-8 may be amplified via mitochondrial transformations (Scaffidi et al., 1998). Further, such amplification by mitochondria may involve additional factors that operate in parallel to BID (Bossy-Wetzel and Green, 1999).

BAX, like BID, is constrained from targeting membrane sites, including mitochondria, until the cell receives a death signal (Goping et al., 1998; Gross et al., 1998; Hsu et al., 1997). In the absence of such a signal, BAX adopts a conformation in which the COOH-terminal transmembrane signal-anchor domain of BAX cannot insert into membranes, and this is dependent at least in part on the NH_2-terminal ART (Apoptotic Regulation of Targeting) domain. This repression by ART is relieved by a death stimulus and the signal-anchor now inserts BAX into mitochondrial membrane (Goping et al., 1998). Membrane insertion is accompanied by a conformational change in the protein, in which the NH_2-terminus of BAX now becomes exposed (Desagher et al., 1999). Alternatively, BAX translocation can be uncoupled from death signals by

forced over-expression (Gross et al., 1998; Rosse et al., 1998; Xiang et al., 1996) or forced dimerization (Gross et al., 1998). Such induced translocation of BAX results in mitochondrial permeability transition (Gross et al., 1998; Wang et al., 1998) and in some contexts causes cytochrome c release (Eskes et al., 1998; Rosse et al., 1998). Moreover, BID and BAX can interact (Desagher et al., 1999; Wang et al., 1996) and both molecules can induce loss of mitochondrial integrity by mechanisms inhibited by BCL-2 proteins (Desagher et al., 1999; Finucane et al., 1999; Gross et al., 1998; Jurgensmeier et al., 1998; Li et al., 1998; Luo et al., 1998).

Here, we have studied the mechanism that stimulates BAX insertion into mitochondria following a death signal. We demonstrate that caspase-generated p15 tBID, either directly or indirectly, releases inhibition of the COOH-terminal signal anchor of BAX by the NH_2-terminal ART domain, and mediates BAX membrane integration. In certain cell types, however, parallel pathway(s) exist to achieve the same end.

2.4 Materials and Methods

2.4.1 General

Earlier studies describe the routine procedures for cell culture and infection with adenovirus type 5 *dl*52OE1B$^-$ expressing only 12S E1A and no E1B products (Boulakia et al., 1996; Ng et al., 1997), and conducting immunocytochemistry by confocal microscopy, synthesizing [^{35}S]-BAX transcription-translation product in reticulocyte lysate, and isolating mitochondria from rat heart and cultured cells (Goping et al., 1998).

2.4.2 Insertion of BAX into mitochondrial membrane in vitro

Apoptotic cell extracts were prepared from HeLa cells exactly as described by Goping et al. 20μL of extract (approximately 10mg protein/mL), either alone or with 5μL of [^{35}S]-methionine-labeled Bax transcription-translation product or 5μL of extract buffer, were incubated in a standard protein import reaction (50μL) containing purified mitochondria from rat heart (1.0mg protein/mL) (Goping et al., 1998). Alternatively, the cell extraction buffer (20μL) alone replaced the extract in control reactions. The reaction mixtures were incubated for 60 minutes at 37°C in the absence of additives, or containing 1mM dATP (extract/dATP), or containing 1mM dATP and 50μM tetrapeptide zVAD-fmk added either at the beginning (extract/dATP + zVAD-fmk) or at the end (pre-activated extract/dATP + zVAD-fmk) of the incubation period. The mitochondria were recovered by centrifugation (Goping et al., 1998) and were analyzed by SDS-PAGE and fluorography to detect [^{35}S]-Bax or by immunoblotting with rabbit anti-BAX N20 antibody (Santa Cruz) to detect BAX derived from the HeLa cell extract. Analysis of rat heart mitochondria alone by immunoblotting revealed negligible Bax associated with the organelle. Alternatively, mitochondria isolated from reaction mixtures were resuspended (0.25mg protein/mL) in freshly prepared 0.1M Na_2CO_3, pH 11.5, and incubated for 30 minutes on ice. The membranes were then collected in an airfuge operating at 30p.s.i. for 10 minutes prior to analysis by fluorography or immunoblotting.

2.4.3 Cytochrome c

Cells (4 X 10^6) were washed in PBS and suspended in 0.1mL HIM buffer (200mM mannitol, 70mM sucrose, 1mM EDTA, 10mM HEPES, pH 7.5). After one cycle of freeze and thaw, the cells were homogenized with 25 strokes in a motorized Teflon-glass homogenizer operating at 500r.p.m. and centrifuged at 800 x g for 10

minutes to remove nuclei and cell debris. The supernatant was centrifuged at 100 000 x g for 10 minutes and aliquots from equivalent numbers of cells were subjected to SDS-PAGE and immunoblotting with a mouse monoclonal 7H8.2C12 anti-cytochrome c antibody.

2.4.4 Bid-null mouse embryo fibroblasts

Mouse embryo fibroblasts were prepared from 9.5 day-old embryos of mice carrying a homozygous deletion in the coding region of *Bid* (Yin et al., 1999). They were cultured in Iscove's modified Dulbecco's medium containing 20% fetal calf serum, and infected with adenovirus type 5 *dl*52OE1B$^-$ (expressing only 12S E1A and no E1B products) or treated with TNFα, as described (Nguyen et al., 1998; Yin et al., 1999).

2.5 Results

Activation of Fas causes recruitment of initiator procaspase-8 into the death-inducing signaling complex (DISC) via the adaptor molecule FADD (Boldin et al., 1996; Kischkel et al., 1995; Muzio et al., 1996). This stimulates auto-activation of procaspase-8 (Medema et al., 1997; Muzio et al., 1996) which then initiates an apoptotic pathway that, in type II cells in culture, involves a mitochondrial-dependent amplification of caspase activation (Boise and Thompson, 1997; Scaffidi et al., 1998). Recent genetic analysis (Juo et al., 1998; Varfolomeev et al., 1998) has revealed that caspase-8 is an obligate and non-redundant constituent at the apex of this pathway. In Figure 2.1A, type II human KB epithelial cells were mock-treated or treated with agonistic anti-Fas antibody in the presence of cyclohexamide (Scaffidi et al., 1999), and BAX in whole cell lysate or in a heavy membrane fraction enriched in mitochondria (Goping et al., 1998) was detected by immunoblotting. Activation of Fas resulted in neither an increase in

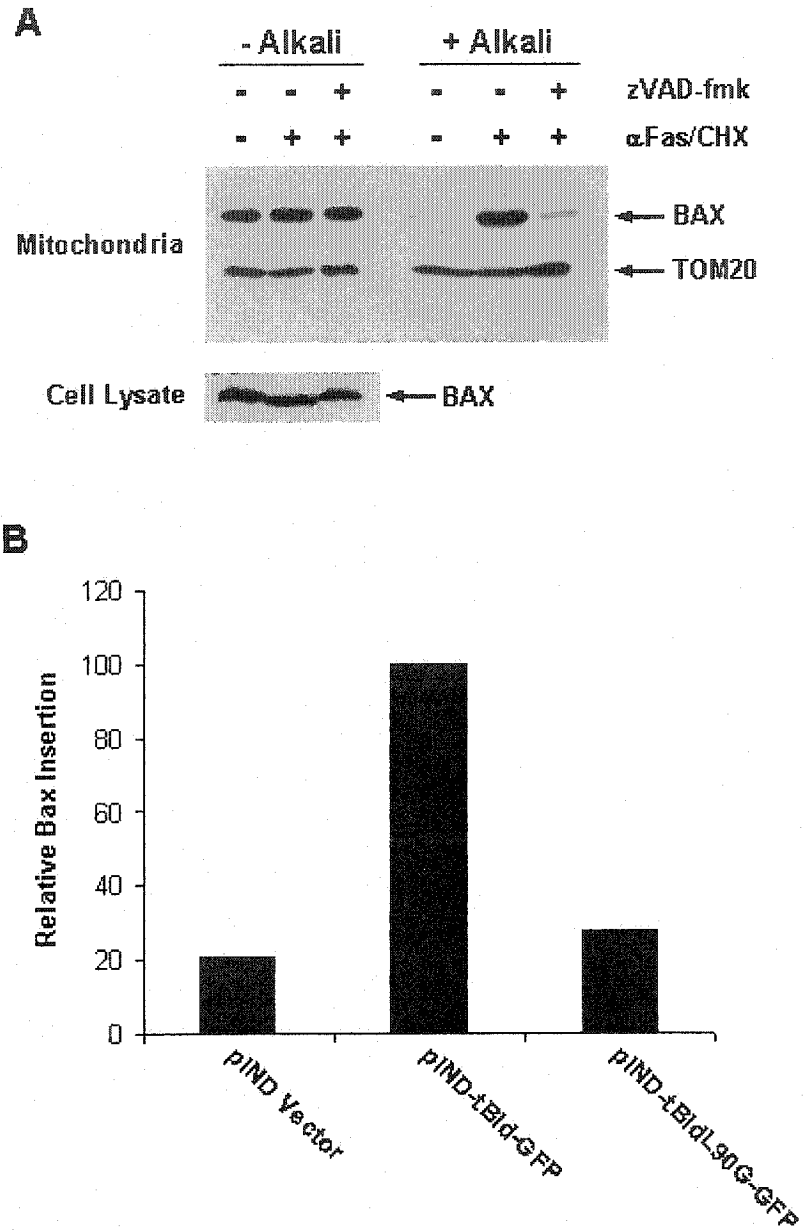

Figure 2.1 Induction of BAX insertion into mitochondrial membrane in human epithelia cells following stimulation of Fas or expression of murine tBid. (**A**) Human KB epithelial cells were mock-treated or treated with 0.5μg/mL mouse monoclonal anti-human Fas (Upstate Biotechnology) and 10μg/mL cyclohexamide (CHX) (Scaffidi et al., 1999) in the presence or absence of 50μM zVAD-fmk for 14h. Cells were homogenized, and heavy membranes enriched in mitochondria recovered from the cell lysates (Goping et al., 1998), subjected to SDS-PAGE either directly (- Alkali) or after extraction with 0.1M Na_2CO_3 (+ Alkali) (from twice the mitochondria as - Alkali) (Goping et al., 1998), and immunoblots developed with rabbit anti-BAX N20 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and chicken anti-TOM20 and visualized by enhanced chemiluminescence. (**B**) Human H1299 epithelial cells were transfected with control vector, pIND-tBid-GFP, or pIND-tBid(L90G)-GFP and after 24h, expression was induced with 5μM ponasterone (Li et al., 1998). After 4h, cells were recovered and the alkali0insoluble mitochondrial protein was analyzed by immunoblotting as in (**A**), and the bands quantified using Power Macintosh 7200/120 and NIH Image version 1.61 image analysis software. BAX expression was normalized by dividing the BAX signal by the TOM20 signal, and setting the maximum value to 100.

levels of total cellular BAX (cell lysate) nor in the amount of BAX recovered with mitochondria. Extraction of these mitochondria with 0.1M Na_2CO_3, pH 11.5, however, which liberates proteins that are peripherally associated with the surface of membranes but retains proteins that are integrated into the lipid bilayer (Fujiki et al., 1982), revealed significant differences. Whereas TOM20, a protein import receptor constitutively integrated into the lipid bilayer of the outer membrane by a single transmembrane domain (McBride et al., 1996), was equally resistant to alkaline extraction in mitochondria obtained from cells with or without Fas stimulation, BAX resisted alkaline extraction only in mitochondria from Fas-stimulated cells. Membrane integration of BAX was abolished, however, when Fas-stimulation was conducted in the presence of the wide-spectrum caspase inhibitor, zVAD-fmk. We conclude, therefore, that upstream caspase-8 in the Fas pathway can initiate a caspase-dependent pathway for BAX integration into mitochondrial membrane. Furthermore, murine tBid, which is generated by cleavage of p22 Bid by caspase-8 in the Fas pathway, also stimulated BAX integration into mitochondrial membrane when expressed ectopically in human H1299 epithelial cells in the absence of Fas stimulation, whereas a BH3-defective mutant of tBid, in which leucine at position 90 within helix 3 was replaced with glycine (Li et al., 1998), did not (Figure 2.1B).

2.5.1 Reconstitution of caspase-dependent insertion of BAX into mitochondria in vitro

Consistent with the findings from Fas-stimulated KB cells, treatment of a control cytosol fraction from human HeLa epithelial cells (Goping et al., 1998) with caspase-8 induced the endogenous BAX in this fraction, when combined with purified rat heart mitochondria, to acquire resistance to alkali extraction, as assessed by immunoblotting

(Figure 2.2B, lanes 1 and 2). Heart mitochondria were employed for these analyses because they can be isolated intact and contain negligible amounts of associated Bax, as determined by immunoblotting (not shown). As well, the treated extracts were capable of subsequently cleaving exogenous PARP (not shown), indicating that caspases were in fact active. A similar amount of alkali-resistant BAX was observed upon dATP-activation of the endogenous caspases in the HeLa extract at 37°C (Goping et al., 1998; Liu et al., 1996), followed by incubation of the extract with mitochondria in the presence of the pan caspase inhibitor, zVAD-fmk (lane 5). In contrast, if zVAD-fmk was added to the extract prior to activation of endogenous caspases with dATP, BAX insertion into mitochondria was ablated (lane 4). Therefore, caspase(s) induce BAX membrane insertion by acting on a pre-existing constituent in the HeLa cell extract. Similar results were obtained for the influence of the zVAD-treated HeLa cell extract on membrane insertion of the [^{35}S]-labeled, full-length BAX translation product (Figure 2.2A). Again, zVAD was inhibitory when added prior to dATP-dependent activation of extract caspases at 37°C (lane 4) but not when added after caspase activation (lane 5). In contrast, generation of the apoptotic 24kDa caspase cleavage product of PARP was inhibited by zVAD-fmk in either circumstance (lanes 4 and 5). Of note, deletion of the NH_2-terminal 19 amino acid ART domain from BAX (Goping et al., 1998) allowed [^{35}S]-BAXΔART to bypass the requirement for the caspase-activated factor, and this was true if the BAXΔART translation product was presented to mitochondria either alone (Figure 2.2C) or together with full-length BAX (Figure 2.2A,C).

Molecular sieve chromatography indicated that most of the [^{35}S]-BAX translation product existed as a monomer (data not shown), consistent with the observations *in vivo*

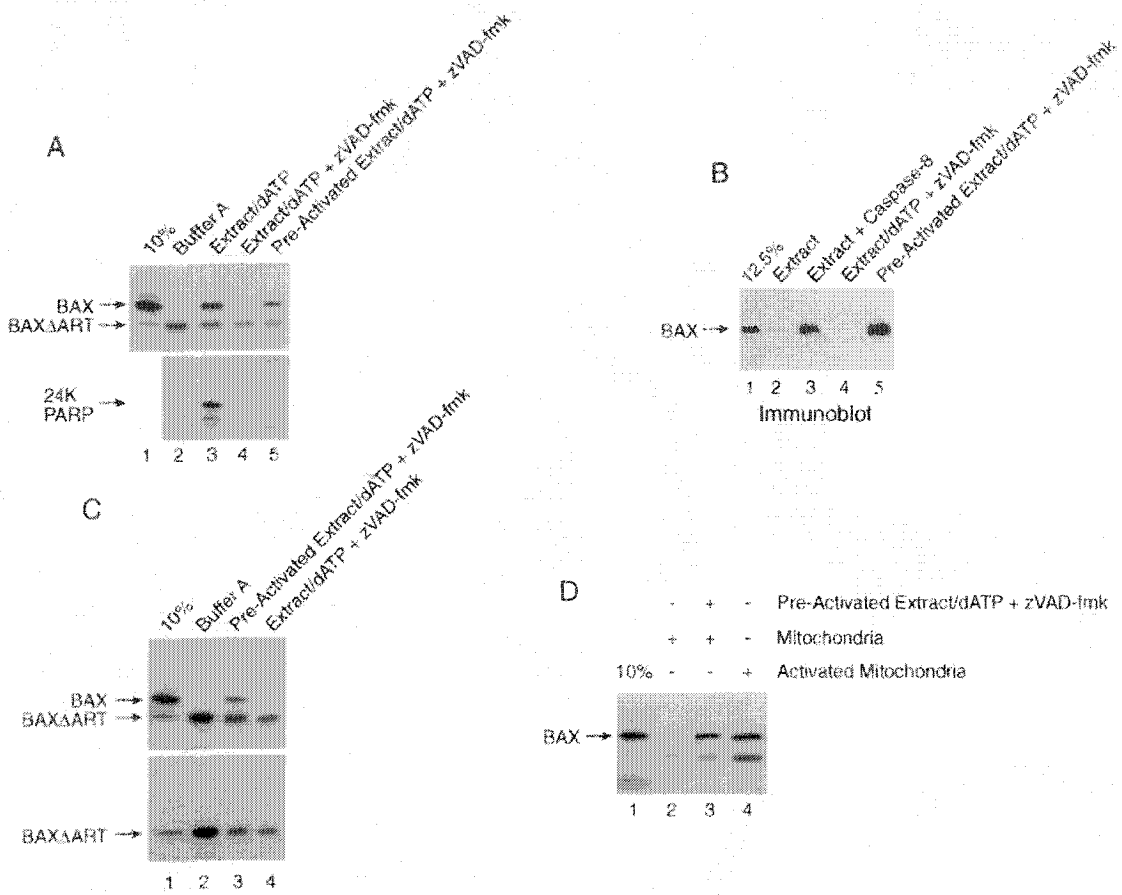

Figure 2.2 Caspase-dependent insertion of BAX into mitochondrial membrane in HeLa cell extracts requires a cytosolic factor(s). (**A**) Influence of caspase activation on BAX membrane insertion and PARP cleavage. Upper panel, [^{35}S]-BAX and [^{35}S]-BAXΔART translation products were incubated with isolated rat heart mitochondria for 60 minutes at 37°C under standard protein import conditions (Goping et al., 1998) in the presence of buffer (lane 2); extract/dATP, in which the caspases in the HeLa extract had been activated by dATP (lane 3); extract/dATP plus zVAD-fmk, in which the zVAD-fmk was added prior to caspase activation by dATP (lane 4); and pre-activated extract/dATP plus zVAD-fmk, in which the zVAD-fmk was added after activation of the caspases by dATP (lane 5). The mitochondria were subsequently collected, extracted with 0.1M Na_2CO_3, pH 11.5 (Goping et al., 1998), and the integral membrane proteins analyzed by SDS-PAGE and fluorography (Goping et al., 1998). Lane 1 represents 10% of input [^{35}S]-BAX. Lower panel, extracts were incubated as above in the absence of mitochondria and subsequently added to [^{35}S]-PARP translation product for 60 minutes at 30°C (lanes 2-5) and the 24kDa apoptotic cleavage product of PARP detected by SDS-PAGE and fluorography (Goping et al., 1998). (**B**) Influence of exogenous caspase-8. Purified mitochondria were incubated with untreated HeLa extract (lane 2); untreated extract plus 4ng/μL caspase-8 (Pharmingen) (lane 3); extract/dATP plus zVAD-fmk (lane 4); and pre-activated extract/dATP plus zVAD-fmk (lane 5). The mitochondria were subsequently treated as in (**A**). Insertion of endogenous BAX in the extract was determined by immunoblotting with rabbit anti-BAX N20 antibody (Santa Cruz), followed by enhanced chemiluminescence. Lane 1, BAX in 12.5% of the input HeLa extract. (**C**) Deletion of the BAX ART domain. As in (**A**) expect that [^{35}S]-BAX and [^{35}S]-BAXΔART (upper panel) or [^{35}S]-BAXΔART (lower panel) translation products were incubated with mitochondria in the presence of buffer (lane 2); pre-activated extract/dATP plus zVAD-fmk (lane 3); and extract/dATP plus zVAD-fmk (lane 4). Lane 1, 10% of input translation product. (**D**) As in (**A**) except that [^{35}S]-BAX translation product was incubated with mitochondria in the presence of buffer (lane 2); pre-activated extract/dATP plus zVAD-fmk (lane 3); or the mitochondria were treated with pre-activated extract/dATP plus zVAD-fmk and the mitochondria re-isolated by centrifugation and resuspended and incubated for 60 minutes in the presence of buffer alone and [^{35}S]-BAX (lane 4).

for the cytosolic form of the protein (Gross et al., 1998; Hsu et al., 1997). Incubation of this translation product with activated HeLa extract did not result in either cleavage of BAX or induction of a higher order structure. Though not conclusive, this suggests that the cytosolic factor influences BAX targeting either indirectly or at the level of the mitochondrion. Consistent with the latter, incubation of mitochondria with activated extract, followed by their re-isolation and subsequent incubation of these activated mitochondria in a standard BAX import reaction, in the absence of HeLa extract, revealed BAX insertion into mitochondrial membrane to a similar extent as for BAX import conducted with control mitochondria in the continued presence of activated extract (Figure 2.2D, lanes 3 and 4). This suggests that the caspase-regulated factor either associates with mitochondria or modifies a constituent of the organelle requisite for BAX membrane insertion, or both.

2.5.2 The caspase-regulated factor is BID

Partial purification of the caspase regulated factor in HeLa cell extracts revealed a BAX mitochondrial-insertion stimulating activity associated with a ~15kDa protein (data not shown). BID's NH_2-terminal domain is removed by caspases, including caspase-8, to yield p15 tBID, which then targets mitochondria and induces mitochondrial dysfunction (Gross et al., 1999b; Li et al., 1998; Luo et al., 1998). When BID was removed from HeLa cell extract by immunodepletion with an antibody against BID prior to activation of caspases (Figure 2.3A, right panel lane 3), the ability of the endogenous BAX in these extracts to insert into mitochondrial membrane was lost upon subsequent caspase activation (left panel, lane 3). Addition of recombinant BID to these BID-depleted extracts reinstated BAX membrane insertion following activation of endogenous caspases

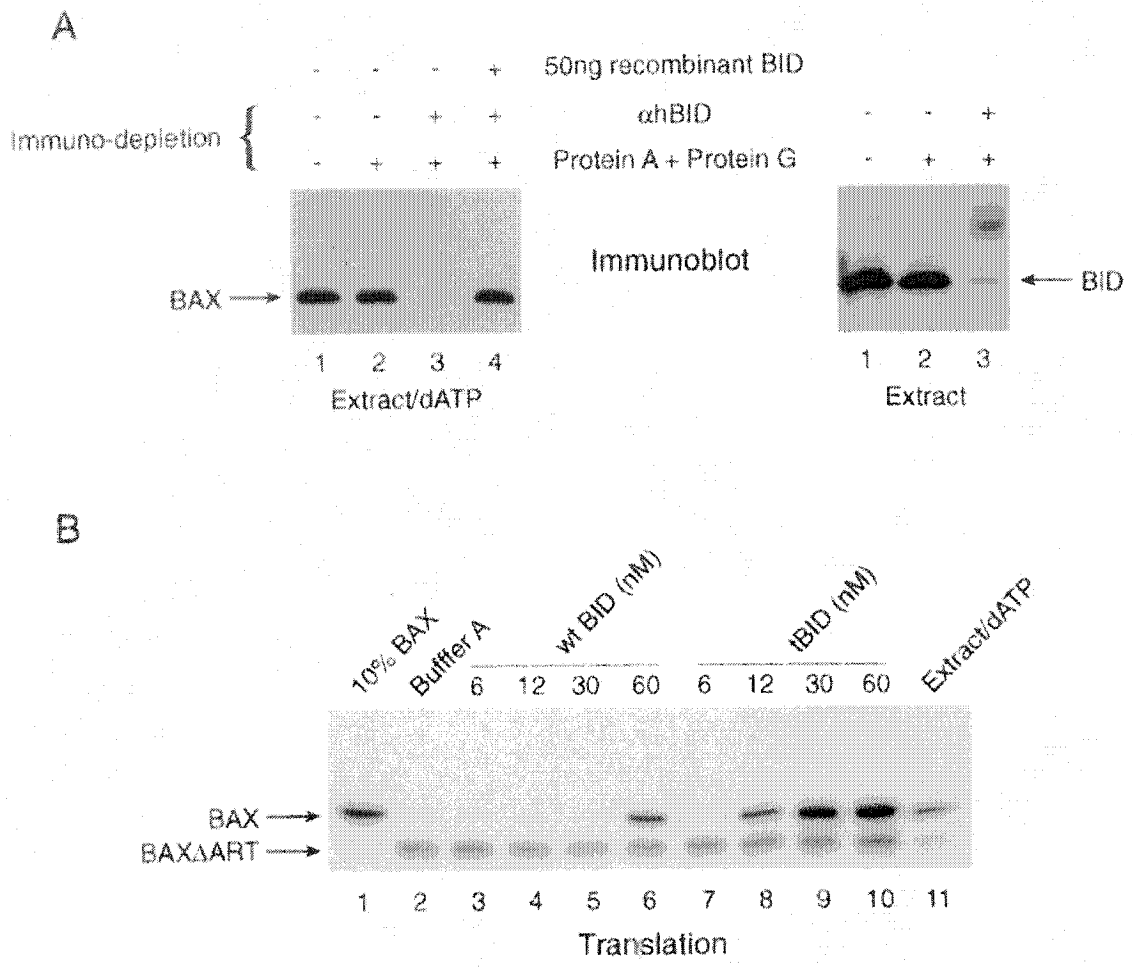

Figure 2.3 BID is required for caspase-dependent insertion of BAX into mitochondrial membrane in HeLa cell extracts. (**A**) Influence on endogenous BAX. Right panel, untreated HeLa extract was incubated with Proteins A and G Sepharose in the presence (lane 3) or absence (lane 2) of rat anti-BID antibody, and immune complexes removed by centrifugation. Subsequently, a fraction of the immunodepleted extracts (lanes 2 and 3) and untreated extract (lane 1) were immunoblotted with the same anti-BID antibody and visualized by enhanced chemiluminescence. Left panel, each of the extracts above was then activated by adding dATP. Rat heart mitochondria were subsequently incubated with extract/dATP (lane 1); mock immunodepleted extract/dATP (minus antibody) (lane 2); BID-immunodepleted extract/dATP (lane 3); and BID-immunodepleted extract/dATP with 50ng of recombinant BID (Li et al., 1998) added to it (lane 4). The mitochondria were then collected, extracted with 0.1M Na_2CO_3, pH 11.5, and the insoluble protein analyzed by immunoblotting with rabbit anti-BAX N20 antibody. (**B**) Translation products. [^{35}S]-BAX and [^{35}S]-BAXΔART translation products were incubated with mitochondria under standard protein import conditions (Goping et al., 1998) in the presence of buffer (lane 2); the indicated concentrations of either full-length BID (lanes 3-6) or p15 tBID, which had been generated by cleavage of BID with caspase-8 (lanes 7-10) (Li et al., 1998); or extract/dATP (lane 11). The mitochondria were subsequently collected and analyzed as in Figure 2.2A.

(left panel, lane 4). Moreover, direct addition of low concentrations (12nM) of recombinant tBID, generated by caspase-8 cleavage of full-length BID (Li et al., 1998), to *in vitro* translated [^{35}S]-BAX could replace the requirement for activated extract and on its own stimulated [^{35}S]-BAX insertion into mitochondrial membrane (Figure 2.3B, lane 8). Full-length BID was also stimulatory, but only at higher concentrations (lane 6). Likewise, full-length BID can stimulate release of cytochrome c from mitochondria, but at concentrations higher than that of p15 tBID (Gross et al., 1999b; Li et al., 1998; Luo et al., 1998). Consistent with the results using cell extracts (Figure 2.2A, C), however, membrane insertion of [^{35}S]-BAXΔART did not depend on and was not further stimulated by either BID or tBID, even at high concentrations of tBID (Figure 2.3B).

2.5.3 Effect of Bid gene deletion in mouse embryo fibroblasts

Previous analysis of embryonic fibroblasts from the *Bid*$^{-/-}$ mouse revealed only a slight delay in cell killing in response to TNFα compared to *Bid*$^{+/+}$ cells, yet a significant inhibition of cytochrome c release from mitochondria in the Bid-null cells was observed (Yin et al., 1999). Similarly, *Bid*$^{-/-}$ mouse embryo fibroblasts were delayed in cell killing following expression of E1A oncoprotein (Figure 2.4A) yet again, there was an inhibition of cytochrome c release from mitochondria as assessed by confocal microscopy, although the organelle did assume a condensed morphology as a consequence of E1A expression (Figure 2.4B). Analysis of the high-speed supernatant fraction from E1A-stimulated cells by immunoblotting likewise showed an inhibition of cytochrome c release to the cytosol in *Bid*$^{-/-}$ cells (Figure 2.4C). Of note, however, E1A expression resulted in Bax insertion into mitochondrial membrane to a similar extent in *Bid*$^{-/-}$ and *Bid*$^{+/+}$ cells over the time course examined (Figure 2.4D). This lack of difference

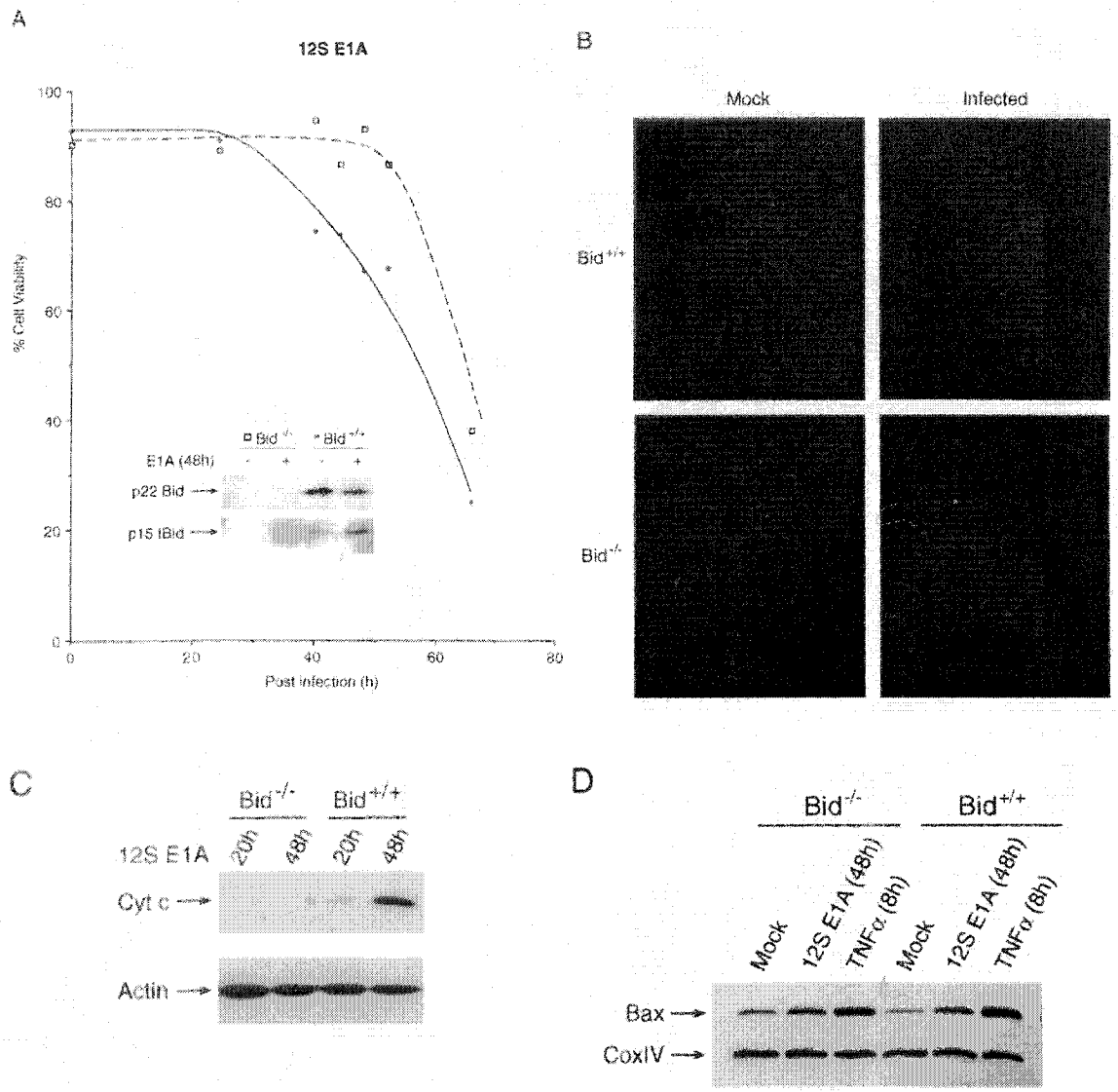

Figure 2.4 *Bid*$^{+/+}$ and *Bid*$^{-/-}$ mouse embryo fibroblasts. (**A**) Influence on cell killing by E1A. Primary embryo fibroblasts from *Bid*$^{+/+}$ and *Bid*$^{-/-}$ mice (Yin et al., 1999) were infected for the indicated times with adenovirus type 5 *dl*53OE1B^{-} (expressing only 12S E1A and no E1B products) (Ng et al., 1997). Cell viability was measured by exclusion of trypan blue. The data are an average of two independent determinations and are representative of multiple killing curves by E1A. After 48h, infected (+) and mock-infected (-) cells were analyzed by immunoblotting with anti-mouse Bid (inset; the lower panel showing tBid was developed by chemiluminescence for twice as long as in the upper panel). (**B**) Influence of E1A expression on cytochrome c distribution. As in (**A**) except that cells infected with or without *dl*52OE1B^{-} were grown on glass cover slips for 48h, fixed, and incubated with mouse monoclonal antibody 2G8.B6 against cytochrome c and anti-mouse IgG coupled to Texas Red, and visualized by immunofluorescence confocal microscopy. Representative images are shown. (**C**) As in (**A**) except that the cells were infected for 20 or 48h with adenovirus type 5 *dl*52OE1B^{-} (expressing only 12S E1A and no E1B products), and high-speed supernatant fractions were generated and analyzed by immunoblotting for cytochrome c and actin. (**D**) As in (**A**) expect that cells infected with adenovirus type 5 *dl*52OE1B^{-} for 48h of with TNFα for 8h (Yin et al., 1999) were homogenized and the heavy membrane fraction containing mitochondria was recovered and extracted with 0.1M Na_2CO_3, pH 11.5 (Goping et al., 1998). The insoluble protein was subjected to SDS-PAGE and developed by immunoblotting with rabbit anti-BAX N20 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse anti-cytochrome c oxidase subunit IV (Cox IV) antibody (Goping et al., 1998).

between the two cell types was evident even though E1A stimulated Bid cleavage in wild-type cells (Figure 2.4A, inset). Likewise, TNFα treatment stimulated Bax insertion into mitochondrial membrane to the same extent in *Bid*$^{-/-}$ and *Bid*$^{+/+}$ cells. Thus, in both cases cell death and Bax insertion into mitochondria can bypass the requirement for Bid in this murine cell type.

2.6 Discussion

Under normal physiological conditions, BAX membrane insertion is regulated and tied to specific signal transduction events (Goping et al., 1998; Gross et al., 1998; Hsu et al., 1997). In the absence of death signals, BAX adopts a conformation in which the COOH-terminal transmembrane signal-anchor is repressed and incapable of targeting the protein to mitochondria (Goping et al., 1998; Nechushtan et al., 1999). We show here that activation of caspases, both *in vivo* and *in vitro*, can override this inhibition and BAX now inserts into mitochondrial membrane. This contribution by caspases may reflect an initiation and/or amplification of regulated BAX targeting. Further, we find that caspase-generated tBID is a direct stimulus of BAX insertion into mitochondrial membrane *in vitro* and can initiate BAX membrane insertion *in vivo* in the absence of other death signals. This dependence of BAX membrane insertion on tBID was bypassed, however, by deleting the BAX NH_2 ART domain. Deletion of the ART domain also enhanced the toxicity of BAX in transfected cells (Goping et al., 1998). Therefore, at least in certain contexts, caspase-generated tBID is an upstream stimulus of BAX targeting that, directly or indirectly, can relieve repression of the COOH-terminal BAX transmembrane signal-anchor segment by the NH_2-terminal ART domain (Goping et al., 1998), permitting BAX integration into mitochondrial membrane (Figure 2.5). A number of point mutations

within different regions in BAX, including the NH_2-terminal (Khaled et al., 1999) and COOH-terminal domains (Nechushtan et al., 1999), and the putative pore-forming helices 5 and 6 (Nouraini et al., 2000), can also bypass the requirement for such regulation and permit constitutive targeting of BAX to mitochondria, as can exposure of the wild-type protein to elevated pH (Khaled et al., 1999). As well, it will be interesting to learn if other BH3 domain-only proteins, such as BAD and BIM, can act like tBID and induce BAX targeting. It may be that the 'closed' inactive conformation of BAX, which correlates with inaccessibility of the NH_2-terminal domain to added protease (Desagher et al., 1999; Goping et al., 1998), can be perturbed or accessed by a variety of both intrinsic and extrinsic factors in addition to tBID, a situation that presumably accounts for the ability of *Bid*$^{-/-}$ mouse embryo fibroblasts to support Bax membrane insertion in response to different death signals. Additionally, however, our findings revealed that, in the absence of Bid, insertion of Bax into mitochondria did not result in release of cytochrome c from the organelle, indicating that the well-documented ability of Bax to stimulate cytochrome c release may depend on co-operation with Bid *in vivo*.

The role of BID and other potential regulators in controlling BAX targeting to mitochondria must be interpreted in the context of two conditions that enable BAX to bypass such regulation *in vivo*. Forced over-expression (Rosse et al., 1998; Wang et al., 1998; Xiang et al., 1996) and forced dimerization (Gross et al., 1998) of BAX both result in constitutive mitochondrial integration and cell death. Over-expression might saturate an inhibitory pathway of BAX membrane integration, a role that has been ascribed to anti-apoptotic BCL-2/BCL-X_L family members (Gross et al., 1998). In this context, tBID might inactivate the BCL-2 death suppressors (Gross et al., 1999b; Li et al., 1998; Luo et

al., 1998) (Figure 2.5). Likewise, forced dimerization might preclude the influence of BCL-2 suppressors on BAX distribution, or cause a conformational change in BAX that bypasses regulation of its targeting to mitochondria.

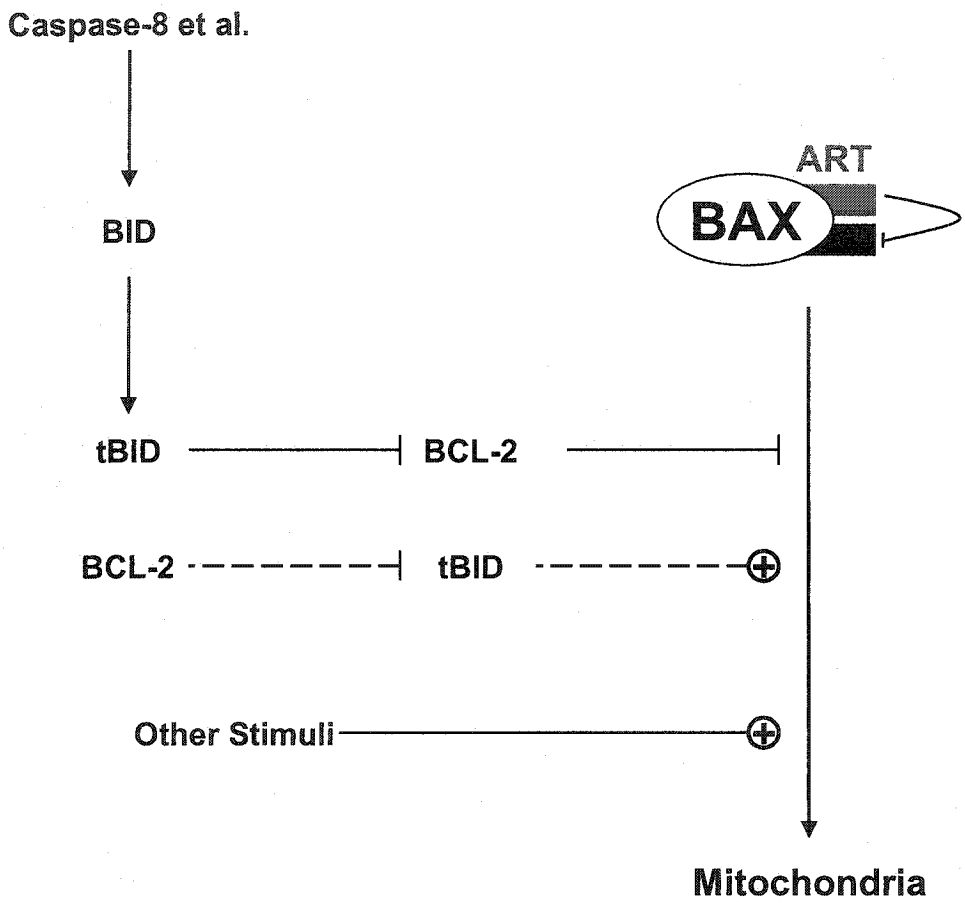

Figure 2.5 Working model for regulation of BAX insertion into mitochondria by BID. See the text for a description. The NH_2-terminal ART and COOH-terminal transmembrane domains of BAX are represented by upper and lower boxes, respectively.

A second possibility is that tBID takes a more direct role in BAX integration into mitochondria (Desagher et al., 1999; Eskes et al., 2000). For example, it may act as a receptor for BAX, inducing a conformational change in BAX and subsequent membrane insertion (Figure 2.5). In this scenario, BCL-2-related suppressors, if in excess, may bind and inactivate tBID. This model is consistent with studies *in vitro* showing that

recombinant full-length BID can interact directly with BAX and support both BAX insertion into mitochondria and subsequent release of cytochrome c from the organelle (Desagher et al., 1999; Eskes et al., 2000). It is not clear, however, how this model reconciles with manipulations to BAX (e.g. over-expression) that allows BAX to bypass the requirement for tBID as receptor or, conversely, with Bax membrane insertion being observed in stimulated Bid-null mouse embryo fibroblasts.

Finally, integration of tBID into mitochondria might exert influences beyond the regulation of other BCL-2 family members. For example, ion channel activity of tBID has been detected *in vitro* (Schendel et al., 1999). Also, the involvement of regulators in the endoplasmic reticulum that influence BAX activity has been recorded (Xu and Reed, 1998). Further insight into the role of tBID in regulating BAX membrane insertion will undoubtedly emerge by elucidating the structural basis for this influence by tBID and by assessing the potential requirement for other factor(s) in this pathway.

2.7 Acknowledgements

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Chapter 3

BCL-2 Selectively Interacts with the BID-induced Open Conformer of BAK, Inhibiting BAK Auto-Oligomerization

3.1 Rationale

Finding that tBID could induce BAX integration into the outer mitochondrial membrane, we were interested in determining whether this was an effect of the BH3 domain alone or if other regions of the protein were required. We therefore decided to generate synthetic BID BH3 peptides. Also, given that BAX, once inserted into the mitochondrial outer membrane, formed oligomers as did BAK, and that both could be induced by tBID (Korsmeyer et al., 2000), we decided to assay BAK oligomerization and cytochrome c release, since they are simpler than assaying BAX membrane insertion, and as informative. The second goal in this chapter was to elucidate the actual mechanism of inhibition by BCL-2 and, using the peptides to determine interactions between proteins, we were determined to distinguish between the two possible mechanisms proposed in Chapter 2.

3.2 Abstract

Caspase-8 cleaves BID to tBID, which targets mitochondria and induces oligomerization of BAX and BAK within the outer membrane, resulting in release of cytochrome c from the organelle. Here, we have initiated these steps in isolated mitochondria derived from control and BCL-2 over-expresssing cells, using synthetic BH3 peptides, and subsequently analysed the BCL members by chemical cross-linking. The results show that the BH3 domain of BID interacts with and induces an "open" conformation of BAK, exposing the BAK N-terminus. This open (activated) conformer of BAK potently induces oligomerization of non-activated ("closed") conformers, causing a cascade of BAK auto-oligomerization. Induction of the open conformation of BAK occurs even in the presence of excess BCL-2 but BCL-2 selectively interacts with

this open conformer and blocks BAK oligomerization and cytochrome c release, dependent on the ratio of BID BH3 and BCL-2. This mechanism of inhibition by BCL-2 also occurs in intact cells stimulated with Fas or expressing tBID. Although BID BH3 interacts with both BCL-2 and BAK, the results indicate that when BCL-2 is in excess it can sequester the BID BH3-induced activated conformer of BAK, effectively blocking downstream events.

3.3 Introduction

Recent genetic analysis of mammalian programmed cell death has indicated that many death signals depend on the multi-domain pro-apoptoptic members of the BCL-2 family, BAX and BAK, to effect the mitochondrial apoptotic pathway, including release of the Apaf-1 co-factor, cytochrome c, to the cytosol, initiating a caspase cascade (Wei et al., 2001). BAX and BAK, however, differ in their initial cellular location, with BAK primarily constitutively integrated into the lipid bilayer of membranes including the mitochondrial outer membrane. BAX, on the other hand, typically resides in the cytosol or is loosely associated with membrane surfaces (Goping et al., 1998; Wolter et al., 1997). Following a death stimulus, BAX undergoes conformational changes including exposure of its N-terminus and functional activation of its C-terminal membrane anchor, which correlate with integration of the protein into the mitochondrial outer membrane (Desagher et al., 1999; Goping et al., 1998; Hsu and Youle, 1998; Nechushtan et al., 1999; Sundararajan and White, 2001; Suzuki et al., 2000). In this location, stimulated BAX assumes higher order oligomeric structures, resulting in cytochrome c release from the organelle (Eskes et al., 2000). While lacking the initial step of regulated insertion into mitochondrial membrane, constitutively integrated BAK likewise responds to

multiple death stimuli by forming oligomers in the membrane (Wei et al., 2000). BAX and BAK likely cooperate with lipid components of the outer membrane to effect protein egress from the organelle (Epand et al., 2002a; Esposti, 2002; Kuwana et al., 2002).

A second pro-apoptotic sub-group of the BCL-2 family, the BH3 domain-only members, couples upstream death signals to downstream activation of BAX/BAK (Puthalakath and Strasser, 2002). Genes for some of these BH3 only proteins respond to transcriptional cues, yielding the active entity (e.g., Noxa, PUMA, and BIK) (Mathai et al., 2002; Nakano and Vousden, 2001; Oda et al., 2000; Yu et al., 2001). Others, however, are constitutively expressed and undergo an activating conformational change in response to a death stimulus (e.g., BAD, BIM, and BID) (Gross et al., 1999b; Harada et al., 1999; Li et al., 1998; Luo et al., 1998; Puthalakath et al., 1999), which is predicted to make available the BH3 helix. BID is cleaved by caspase-8 to tBID following death receptor stimulation and moves to mitochondria where it interacts with and induces oligomerization of BAX and BAK (Eskes et al., 2000; Perez and White, 2000; Wei et al., 2001). Although direct interactions between most other BH3 domain-only members and either BAX or BAK have not been reported, BH3 only members such as BAD also stimulate BAX/BAK as the death effectors (Cheng et al., 2001; Zong et al., 2001), by binding BCL-2 and sensitizing BAX/BAK to other stimuli, including tBID itself (Letai et al., 2002). Multi-domain BCL proteins contain a deep groove formed by BH1 and BH2, which accepts the BH3 domain of a binding partner (Minn et al., 1999). In instances where anti-apoptotic BCL-2/BCL-X_L are in excess, one model holds that these members bind to and sequester BH3 only proteins via their exposed BH3 helix, preventing them from stimulating the BAX/BAK effectors (Cheng et al., 2001). Another model suggests

that BH3-only proteins bind anti-apoptotic BCL-2 proteins, preventing them from inhibiting the BAX/BAK effectors (Zong et al., 2001). A third model, proposed on the basis of the anti-apoptotic function of adenovirus E1B 19 kDa protein, argues that 19K preferentially binds and inhibits a conformationally altered form of BAX in cells after receipt of a death stimulus (Perez and White, 2000). Which of these pathways dominates may in fact depend on the 3-way relative expression levels of anti-apoptotic BCL members, pro-apoptotic BAX, BAK, and the active BH3-only entities that initiate mitochondrial apoptosis.

A recent study has suggested a two step mechanism for mitochondrial release of cytochrome c in response to tBID: BH3-independent mobilization of intra-cristae cytochrome c stores and a BH3-dependent egress of cytochrome c from the organelle, associated with BH3-stimulated oligomerization of BAK (Scorrano et al., 2002). Documentation of this latter step, accompanied by findings from mutagenesis in which the tBID mitochondrial targeting sequence was replaced with that of BCL-2, have argued that simple concentration of the tBID BH3 domain at the surface of mitochondria is sufficient to initiate BAK oligomerization in the outer membrane (Wei et al., 2000). Further, generation of tBID by caspase cleavage involves exposure of its BH3 domain (Chou et al., 1999; McDonnell et al., 1999) and a freely diffusing synthetic peptide corresponding to the tBID BH3 domain stimulates BAK oligomerization in the mitochondrial outer membrane and release of cytochrome c (Letai et al., 2002). Use of the isolated BH3 domain in the form of a pure oligo-peptide offers certain advantages, such as biotinylation and directional cross linking, for probing directly the molecular interactions that mediate these BH3-dependent events. Outstanding issues for example

relate to the nature of the BAK oligomerization pathway itself and the opposing roles of tBID and BCL-2 in its regulation.

Here, we have utilized derivatized oligo-peptides corresponding to the entire helix 3 of BID to study these questions and find that this BH3-containing peptide binds BAK, resulting in exposure of the BAK N-terminus. This open conformer of BAK itself potently induces oligomerization of non-activated (closed) BAK members in the outer membrane, indicative of an amplification cascade. BCL-2, at levels that block cytochrome c egress from mitochondria, does not interfere with binding of BID BH3 to BAK or induction of the open conformer of BAK, but it selectively interacts with the tBID BH3-stimulated (open) BAK conformer, inhibiting BAK oligomerization and cytochrome c release.

3.4 Materials and methods

3.4.1 General

Isolation of cytosol and mitochondria, SDS PAGE of whole cell lysates or cell fractions, transfer of proteins to nitrocellulose filters, development of blots with antibodies and detection by enhanced chemoluminescence, and assays to measure release of cytochrome c from mitochondria, have been documented in earlier publications (Goping et al., 1998; Nguyen et al., 2000; Ruffolo et al., 2000). Alkali treatment of mitochondria was performed as described in Ruffolo et al. (2000).

3.4.2 Peptides

The wt and mt BID BH3 peptides were generated by the Alberta Peptide Institute (University of Alberta, Edmonton, Alberta, Canada), and represent amino acids 79-101 of

BID. Both are biotinylated at their N-termini, contain an arg to lys substitution at position 88 and an amide in place of a carboxyl at the C-terminus. The mutant peptide also contains a leu to gly substitution at position 90. The corresponding wild-type BAD and BIK BH3 peptides were also synthesized. All peptides were purified (> 95%) by HPLC.

3.4.3 BAK translation products

Two separate BAK constructs were engineered for *in vitro* assays, both of which contain a Flag epitope at their N-termini. Flag-BAK-ΔN-C to A has a deletion of the N-terminal 36 amino acids and a substitution of its single cys to ala at position 166. Flag-BAK-C to A comprises full length BAK with substitutions of both of its cys residues at positions 14 and 166, to ala. The constructs were cloned into pcDNA3.1 and *in vitro* transcribed and translated using the TNT® Rabbit Reticulocyte Lysate System (Promega) as per the manufacturer's protocol for non-radiolabelled translations.

3.4.4 Release of cytochrome c from mitochondria

Mitochondria (50 μg protein in 25 μl cMRM) isolated from control (- HA-BCL-2) KB or from KB cells over-expressing HA-BCL-2 (Nguyen et al., 1994) were incubated with 20μl HIM (200 mM mannitol, 70 mM sucrose, 10 mM HEPES-KOH, 1 mM EGTA, pH 7.5) or 20μl HeLa cytosolic extract in HIM (4 mg protein/ml), as described (Goping et al., 1998; Ruffolo et al., 2000) plus or minus recombinant caspase-8 (100ng in 1μl Tris-HCl). Alternatively, the mitochondria were incubated with recombinant tBID (in 5μl PBS) (Li et al., 1998), or synthetic BH3 peptides (in 5μl 0.5mM Pipes, pH6), or BAK translation product, or control buffer (0.5mM Pipes, pH6) and adjusted to a final volume

of 50µl with HIM and incubated for 1 hour at 37°C (Goping et al., 1998; Ruffolo et al., 2000). The reaction mixtures were then centrifuged at 9000 rpm (Sorvall MC 12V), and equivalent aliquots of both the supernatants and pellets (resuspended to the same volume as the supernatant) analyzed by SDS PAGE and immunoblot using the mouse monoclonal anti-pigeon cytochrome c antibody 7H8.2C12 (Pharmingen).

3.4.5 BAK oligomerization

Reactions were conducted as for release of cytochrome c from mitochondria, except that the mitochondria (50µg) which were recovered at the end of the reaction were resuspended in 47.5µl HIM. Subsequently, 2.5µl of DMSO (control) or 2.5µl of a solution of bis-maleimidohexane (BMH) (Pierce) in DMSO were added and incubated at room temperature for 30 minutes. The reactions were centrifuged at 12000 rpm (Sorvall MC 12V) and the pellets analyzed by SDS PAGE and immunoblot using a rabbit polyclonal antibody directed against amino acids 23-37 of human BAK (Upstate).

3.4.6 Treatment of BAK with trypsin

5 µl MRM (250mM sucrose, 10mM HEPES, pH 7.5) containing trypsin (0.125mg/ml, Sigma) and 5 µl MRM with (mock) or without chymotrypsin-trypsin inhibitor (1.25mg/ml, Sigma) were added directly to reaction mixtures, which were then incubated on ice for 20 minutes. The trypsin digestion was stopped by subsequent addition of 5µl of the inhibitor and the mitochondria recovered at 9000 rpm (Sorvall MC 12V) for 5min through a cushion of MRM,and BAK analyzed by SDS PAGE and immunoblot as above.

3.4.7 Directional Cross-linking

Biotinylated peptides were incubated in the presence of excess succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxy-(6-amidocaproate) (LC-SMCC, 10mM stock in DMSO, Pierce), an amine/sulfhydryl hetero-bifunctional cross-linker, for 1 hour at room temperature The amine reactive group on any excess cross-linker was then quenched by addition of Tris-HCL (pH8) to a final concentration of 350mM followed by incubation at room temperature for 15 minutes. The derivatized peptides were then added to 50µl standard reaction mixtures containing mitochondria. After 1 hour at 37°C, the mitochondria were recovered and solubilized in 200µl RIPA buffer (10mM Tris, pH 7.4, 150mM NaCl, 5mM EDTA, 1% Triton X-100, 1% deoxycholate, and 0.1% SDS). 20 µl of a 50% slurry of Immunopure® immobilized streptavidin beads (Pierce) were then added and the mixtures incubated overnight at 4°C. The beads were recovered, washed and resuspended in SDS PAGE loading buffer and analyzed for BAK or BCL-2 by SDS PAGE and immunoblot using anti-BAK or the monoclonal hamster anti-human BCL-2 antibody 6C8 (Biomol).

3.4.8 Immunoprecipitation

Mitochondria from HA-BCL-2 over-expressing cells were incubated in standard cross-linking reactions in the absence of detergent, recovered, and solubilized in 200 µl RIPA buffer. The monoclonal mouse anti-HA antibody 16B12 (BAbCO) was then added and incubated overnight at 4°C. 20 µl of a 50% slurry of protein G Sepharose (Amersham Pharmacia) were added to the samples and incubated at 4°C for 1 hour. The beads were washed and analyzed for the presence of BAK and BCL-2 as above.

3.4.9 Analysis of BCL-2 and BAK interaction in Fas-stimulated KB cells

Human KB epithelial cells over-expressing HA-BCL-2 were treated with Fas ligating anti-Fas antibody (0.5μg protein / ml; Upstate, CH11), as described (Nguyen et al., 2000). At the indicated times, mitochondria were isolated, aliquots (100μg protein) treated with the cross-linker BMH, and HA-Bcl-2 recovered following protein dissolution in RIPA buffer with anti-HA and precipitates analyzed with antibodies against BCL-2 and BAK as described above.

3.4.10 Transfection and Immunofluorescence

Control and HA-Bcl-2 over-expressing KB cells were plated to 50 % confluency on 12mm coverslips in 24-well plates and transfected over night with a combination of pEGFP, pIND-tBID-GFP, and pVgRXR (Li et al., 1998) in a 2:4:4 ratio using LipofectAMINE PLUS™ (Life Technologies) as per the manufacturer's protocol. The expression of tBID was then induced by treating the cells with 1μM Ponasterone A (Invitrogen) in growth media. At the indicated times, the cells were fixed and immunofluorescence performed as described in Ruffolo et al. (2000) using either a mouse monoclonal anti-cytochrome C antibody (BD Biosciences, 6H2.B4) or a mouse monoclonal, BAK conformation specific antibody directed to the N-terminus of BAK (Oncogene, TC100), as the primary antibody. Alexa Fluor® 594 goat anti-mouse secondary antibody (Molecular Probes) was used to visualize cytochrome c and the reactive BAK conformation. Transfected cells expressing EGFP were scored for the release of cytochrome c from mitochondria to cytosol and for staining of the conformation-specific, exposed BAK N-terminus

3.5 Results and Discussion

BAK is a 211 amino acid protein that contains a predicted membrane-anchor sequence towards its COOH-terminus and three of the four BH domains that define membership in the BCL family of apoptosis regulators, BH1-BH3 (Figure 3.1A). The protein is constitutively integrated in the outer membrane of mitochondria, facing the cytoplasm, and undergoes oligomerization in response to tBID, which correlates with tBID-induced release of cytochrome c from the organelle (Wei et al., 2000). As a baseline for establishing an in vitro system for studying the responses of mitochondrial BAK to a synthetic BID BH3 peptide (Figure 3.1B), we first combined a high speed cytosolic fraction derived from HeLa cells with a heavy membrane fraction enriched in intact mitochondria isolated from KB epithelial cells (Goping et al., 1998). Treatment of the cytosol with caspase-8 generated p15 tBID, which was recovered in the alkaline-insoluble mitochondrial pellet fraction (Figure 3.1C). As previously noted (Gross et al., 1999), BCL-2/BCL-X_L did not influence the association of tBID with mitochondria and therefore the amount of tBID that binds to mitochondria that were derived from cells that do or do not over-express BCL-2 (see insert, Figure 3.1E) was the same (Figure 3.1C).

3.5.1 BID BH3 peptide recapitulates tBID

When the combined cytosol-mitochondria reaction mixtures were subsequently centrifuged to pellet the organelle, cytochrome c was observed in the supernatant only if the cytosol had been treated with caspase-8 (Figure 3.1D, lanes 3 and 4). Mitochondria isolated from KB cells over-expressing HA-BCL-2, on the other hand, resisted this caspase-8-induced release of cytochrome c (Figure 3.1D, lanes 1 and 2). As expected, supplementing the HeLa cell cytosol with 0.06 μM recombinant tBID (Li et al., 1998;

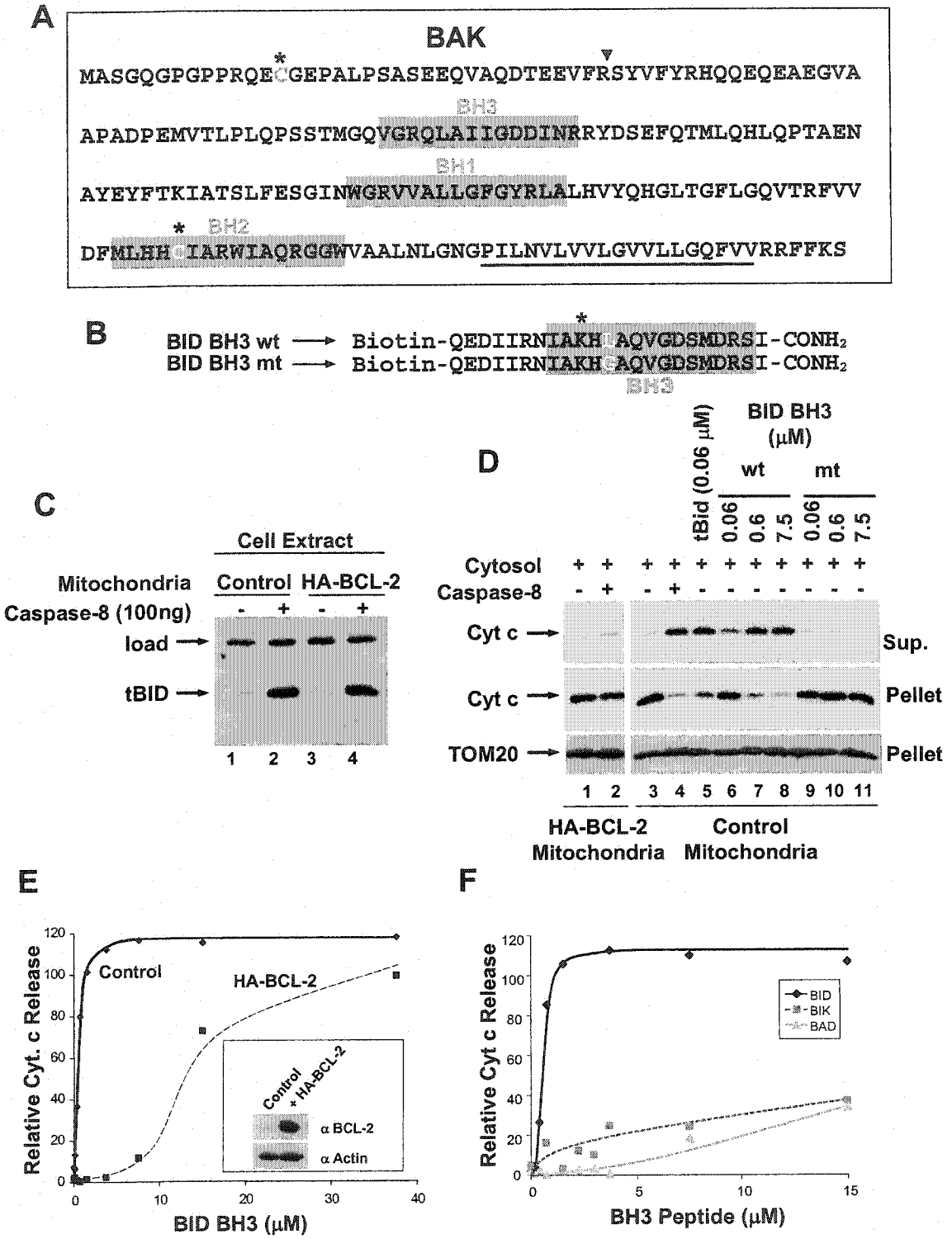


Figure 3.1 tBID-initiated release of cytochrome c from mitochondria are mimicked by an engineered peptide representing the BID BH3 domain. (A) Amino acid sequence of BAK with BH1, 2, and 3 domains highlighted, the two cys residues at positions 14 and 166 indicated with asterisks, deletion of aa 1-36 represented by the arrowhead, and the predicted transmembrane segment underlined. (B) Sequence of biotinylated wt and mt BID BH3 peptides, with the BH3 domain (highlighted), loss of function leu to gly point mutation (white), and silent arg to lys substitution (asterisk) indicated. (C) HeLa cell cytosolic extracts were incubated with or without recombinant caspase-8 (100 ng) and then combined with isolated mitochondria from control or BCL-2 over-expressing KB cells. The mitochondria were recovered and the

presence of the alkaline-resistant cleaved product of BID (tBID) determined by SDS PAGE and immunoblot. (D) Mitochondria were isolated as in (C) and incubated with either recombinant caspase-8 (100 ng), or recombinant tBID (0.06 μM), or the indicated concentrations of wt or mt BID BH3 domain peptides in the presence of HeLa cell cytosolic extract. After centrifugation, the supernatants and mitochondrial pellets, derived from an equivalent cell number, were analysed for cytochrome c by SDS PAGE immunoblot. Pellets were also probed with antibody against the outer membrane marker, TOM20. (E) Mitochondria isolated from control and HA-BCL-2 over-expressing KB cells were incubated with increasing amounts of the BID BH3 peptide in the absence of cytosol. Cytochrome c release to the supernatant was analysed by immunoblotting, the bands quantified by NIH Image version 1.61, and plotted as relative cytochrome c release versus peptide concentration. Insert: SDS PAGE and immunoblot of extracts from control (minus HA-BCL-2) and HA-BCL-2 over-expressing KB cells probed with anti-BCL-2 and anti-γ-actin (Nguyen et al., 2000). (F) As in (E), except that BID, BAD, and BIK BH3 peptides were analyzed with mitochondria derived from control KB cells.

Ruffolo et al., 2000) by-passed the requirement for treatment of the cytosol with caspase-8, and the control mitochondria (- HA-BCL-2) effectively released cytochrome c (lane 5). An equivalent 0.06 μM concentration of synthetic peptide corresponding to the 23 amino acid helix 3 of BID, which contains the 15 aa BH3 domain (Figure 3.1B), also stimulated release of cytochrome c from control mitochondria (lane 6), but concentrations of peptide that were 5-10 fold higher than the equivalent concentration of recombinant tBID were required for a comparable amount of cytochrome c release (compare lane 5 with lanes 6 and 7). This differential is consistent with a membrane targeting sequence present in tBID, but lacking in the synthetic peptide, that serves to concentrate tBID at the mitochondria (Wei et al., 2000). Of note, a mutant peptide, in which the highly conserved leu present in all BH3 domains examined to date (amino acid 90 in BID) was changed to gly, completely abrogated the ability of the BID BH3 peptide to cause cytochrome c release from the organelle (Figure 3.1D, lanes 9-11). Also, as expected, cytosol had negligible effect on the ability of the wt BID BH3 peptide to induce cytochrome c release in this assay (not shown).

As expected, mitochondria from HA-BCL-2 over-expressing cells (inset, Figure 3.1E) resisted the induction of cytochrome c release from the organelle both by

recombinant tBID (not shown) and by the synthetic BH3 peptide (Figure 3.1E, conducted in the absence of cytosol). This protection by HA-BCL-2, however, could be overcome by increasing the concentration of BH3 peptide (a representative experimental result is shown in Figure 3.1E). The ratio of BCL-2 and BH3 peptide, therefore, appears to determine the sensitivity of the mitochondria to cytochrome c release (see also Letai et al., 2002). Mitochondria from cells over-expressing HA-BCL-2 were able to resist the effects of BID BH3 up to about 10 μM peptide (Figure 3.1E) and of tBID up to about 1 μM (not shown). A corresponding resistance was also observed toward the ability of the BH3 peptide to induce BAK oligomerization in the mitochondrial outer membrane (Figure 3.2A, compare lanes 3-5 with lanes 10-12), which was visualized as high molecular weight anti-BAK immuno-reactive products detected by SDS PAGE following chemical cross-linking of mitochondria with the sulfhydryl specific homo-bifunctional *bis*-maleimidohexane (BMH) (Wei et al., 2000). Interestingly, at the peptide concentrations used, both BAK oligomerization (Figure 3.2A) and cytochrome c release from mitochondria (Figure 3.1F) were much more responsive to the BID BH3 peptide compared to BH3 peptides of similar size and design derived from BAD and BIK (see also Letai et al., 2002). This may reflect the observation that among BH3 only proteins, tBID is unique in that it yields detectable interactions with pro-apoptotic BAX and BAK (Korsmeyer et al., 2000).

3.5.2 BID BH3 interacts with BAK and induces an open conformation, exposing the BAK N-terminus

Inspection of the BAK sequence reveals the presence of two cysteine residues, the first lying toward the N-terminus at amino acid position 14 and the second, at position 166,

within the BH2 domain (Figure 3.1A). As noted by Wei et al. (2000), treatment of non-induced mitochondria with BMH results in intra-molecular cross linking of these two cysteine moieties in BAK, generating a BAK species that migrates in SDS PAGE with a faster mobility than unmodified BAK (*double asterisk* in Figure 3.2A). This intra-molecular cross-linked species was lost in mitochondria that were stimulated with the BID BH3 peptide whereas high molecular weight cross-linked BAK oligomers appeared (Figure 3.2A, lanes 3-5). A similar pattern was observed in intact cells following Fas stimulation (not shown). Although one explanation is that the cysteine residues in BH3-stimulated BAK preferentially engage in inter-molecular cross linking, this explanation does not account for the observation that in the presence of excess BCL-2, BAK oligomerization is inhibited yet the loss of intra-molecular cross-linking is still observed following treatment with the BID BH3 peptide (Figure 3.2A, lanes 10-12). Another explanation, therefore, is that BH3-stimulated BAK undergoes a change that precludes intra-molecular cross linking between cys14 and cys166, even in the presence of BCL-2.

As shown in Figure 3.2B, addition of wt but not mt BID BH3 peptide to mitochondria caused BAK to undergo a conformational change, in which trypsin-resistant BAK became susceptible to the protease and resulted in loss of reactivity of BAK toward an antibody directed toward the BAK N-terminus (aa 27-35). This indicates that in its unstimulated form, BAK is in a closed conformation with its N-terminus inaccessible (Griffiths et al., 1999; Sundararajan et al., 2001), and this corresponds to the form of BAK whose N-terminal cys is susceptible to intra-molecular cross-linking by BMH to the cys residue within the BH2 helix (Figure 3.1A, 3.2A). Importantly, HA- BCL-2 in excess did not inhibit the ability of either 3.75 μM BID BH3 peptide or 0.15

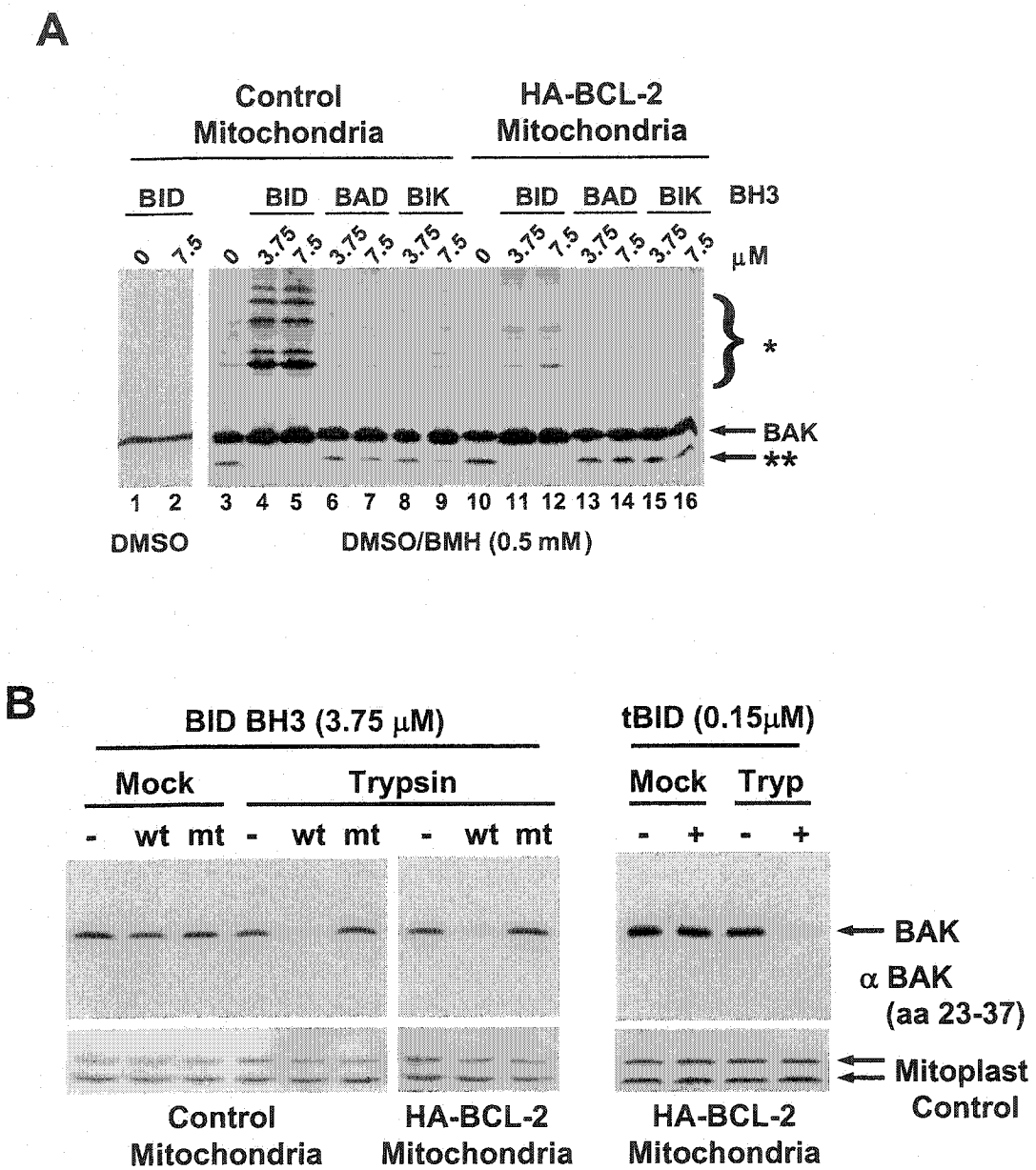


Figure 3.2 BID BH3 domain peptide induces a conformational change in BAK. (A) Mitochondria isolated from control and HA-BCL-2 over-expressing cells were incubated (minus cytosol) in the presence of the indicated peptides. The mitochondria were isolated, subsequently treated with 0.5mM BMH, and then analyzed by SDS PAGE and immunoblotting with anti-BAK. The positions of monomeric BAK (arrow), monomeric BAK harboring an intra-molecular cross-link (double asterisk), and BAK oligomers (bracket and asterisk) are indicated. (B) Mitochondria from control cells and cells over-expressing HA-BCL-2 were incubated in the absence (-) or presence of 3.75 μM wt or mt BID BH3 peptide or with 0.15 μM recombinant tBID, treated with trypsin or trypsin plus trypsin inhibitor (mock), and analyzed for BAK by SDS PAGE and immunoblot with antibody directed against aa 23-37 of BAK (top panel). The immunoblot membrane was also probed with antibody recognizing mitoplast (inner membrane + matrix) proteins (bottom panel).

μM recombinant tBID to induce exposure of the BAK N-terminus (Figure 3.2B), whereas BCL-2 did inhibit both reagents from inducing BAK oligomerization (Figure 3.2A, lanes 10-12; not shown) and cytochrome c release (Figure 3.1E; not shown). Therefore, BCL-2 blocks tBID-induced cytochrome c release from mitochondria downstream of tBID induction of the BAK open (trypsin-sensitive) conformation.

The BID BH3-dependent change at the N-terminus of BAK correlated with a direct interaction between BAK and the BID BH3 peptide. Such interaction was demonstrated by first covalently attaching the hetero-bifunctional (amino / sulfhydryl) cross-linker, LC-SMCC, to the single lys residue in the peptide (Figure 3.3A). This lys was introduced in place of arg88 that normally resides at this position in the wt sequence. This change did not affect the ability of tBID to induce apoptosis in transfected cells (not shown) and therefore was considered to be a silent change. 3.75 μM of the biotinylated wt or mt (L to G) peptide-LC-SMCC complex was incubated with mitochondria in the absence of detergent, and covalent adducts to one of the two cys residues in BAK (Figure 3.1A) were recovered following solubilization and vigorous washing in RIPA buffer containing Streptavidin beads. Only the wt biotinylated BH3 peptide formed a covalent complex with BAK (Figure 3.3B, upper panel). Of note, BAK-BH3 cross-linked adducts were generated in mitochondria lacking or harboring excess HA-BCL-2 (Figure 3.3B, lower panel), indicating that BCL-2 does not interfere with the specific interactions of wt BID BH3 with BAK, despite the fact that HA-BCL-2 strongly inhibited BID BH3-induced BAK oligomerization (Figure 3.2A) and release of cytochrome c from the organelle (Figure 3.1D,E). Furthermore, this inability of HA-BCL-2 to block interactions of BID BH3 with BAK occurred even though the peptide also interacted with HA-BCL-2

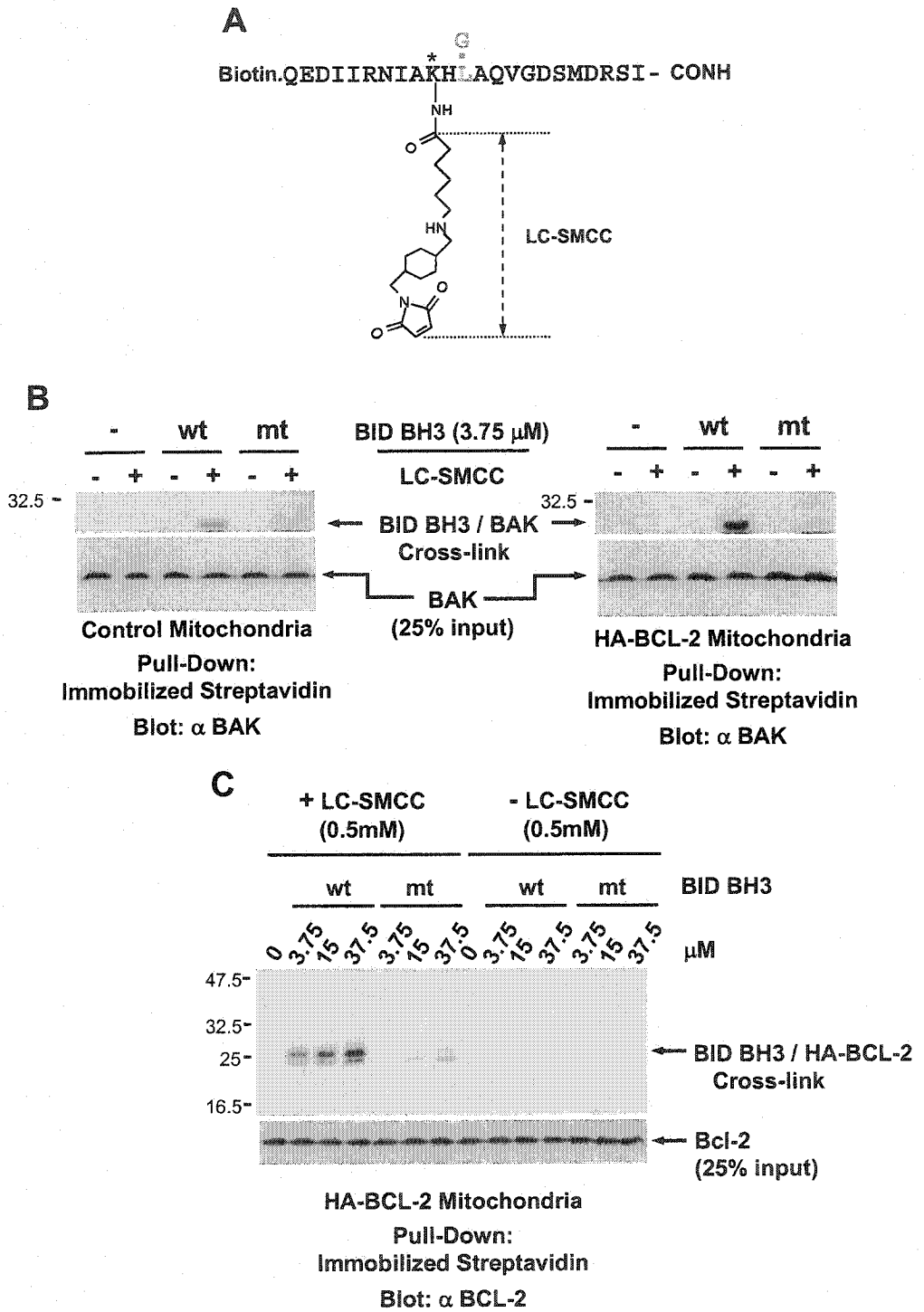


Figure 3.3 BID BH3 interacts with BAK and BCL-2. (A) Biotinylated wt and mt BID BH3 peptides were pre-conjugated with LC-SMCC. (B) The conjugated peptides were incubated with mitochondria isolated from control and HA-BCL-2 over-expressing cells. After directional chemical cross-linking, the mitochondria were then isolated, solubilized in RIPA buffer, the peptides pulled-down using Streptavidin immobilized on Sepharose beads, and analyzed for the presence of peptide-BAK cross-links by SDS PAGE and immunoblot with antibody against BAK. The input BAK (25% total) for each lane is also shown. (C) As in (B) except that blots were developed with anti-BCL-2.

(Figure 3.3C). This binding to BCL-2, therefore, does not result in functional sequestration of BID BH3 from BAK, and suggests that the binding equilibria at the mitochondrial surface do not favor BH3-BCL-2 interactions.

3.5.3 BCL-2 preferentially interacts with BID BH3-stimulated BAK

Interactions between BAK and BCL-2 in mitochondria isolated from HA-BCL-2 over-expressing KB cells were assessed by chemical cross-linking with BMH, followed by protein dissolution, precipitation with anti-HA and immuno-blotting of precipitates with anti-BAK. It is particularly noteworthy that cross-linking between HA-BCL-2 and BAK was not observed in the absence of the BID BH3 stimulation (Figure 3.4A). Specific covalent adducts between HA-BCL-2 and BAK were observed, on the other hand, following stimulation with wt but not mt BH3 peptide (Figure 3.4A, upper panel). Collectively, therefore, the results show that BID BH3 physically interacts with BAK at the surface of mitochondria (Figure 3.3B) and induces a conformational change in which the BAK-N-terminus becomes accessible to trypsin (Figure 3.2B); BCL-2 preferentially interacts with this BH3-stimulated open conformation of BAK (Figure 3.4A) and inhibits BH3-induced BAK oligomerization (Figure 3.2A) and cytochrome c release, dependent on the ratio of BID BH3 and BCL-2 (Figure 3.1E).

3.5.4 A cascade of BAK auto-oligomerization

Although tBID initiates BAK oligomerization, the mechanism for how this is accomplished is not known. The fact that BID BH3 caused BAK to adopt an open (i.e., trypsin accessible) conformation even in the presence of excess BCL-2 (Figure 3.2B) suggests that BCL-2 functions downstream of this step to inhibit both BAK oligomerization (Figure 3.2A) and release of cytochrome c from mitochondria (Figure

3.1D,E). One possibility is that the unstimulated (closed) conformation of BAK is incompetent for auto-oligomerization and that BID BH3, by inducing exposure of the N-terminus, relieves this barrier. To test this idea, a BAK construct was generated that lacks amino acids 1-36 (Flag-ΔN-BAK, see Figure 3.1A) and its ability to induce oligomerization of endogenous BAK in isolated mitochondria was assessed. Since oligomerization is assayed by cross-linking with the cys-specific homo-bifunctional BMH cross-linker, both the full length and ΔN-BAK constructs that were used as the "donor" proteins were further modified to change their constituent cys residues to ala (Figure 3.1A). In this way, the donor BAK molecules cannot be visualized as part of the oligomeric complex, whereas oligomerization of endogenous "acceptor" BAK molecules can. As documented in Figure 3.4B, the transcription-translation product of Flag-ΔN-BAK(C to A) stimulated oligomerization of endogenous mitochondrial BAK and release of cytochrome c from the organelle whereas full length Flag-BAK(C to A) did not. The latter construct, as expected, was resistant to degradation of its N-terminus by trypsin (Figure 3.4B, right panel). Further, mitochondria containing excess HA-BCL-2 (Figure 3.1C-E, 2A) resisted BAK oligomerization and cytochrome c release in response to Flag-ΔN-BAK(C to A) (not shown). Since the antibody that was used to detect BAK oligomers recognizes the BAK N-terminus, which is lacking in the Flag-ΔN-BAK(C to A) translation product, the observed oligomers must have derived from the endogenous population of mitochondrial BAK.

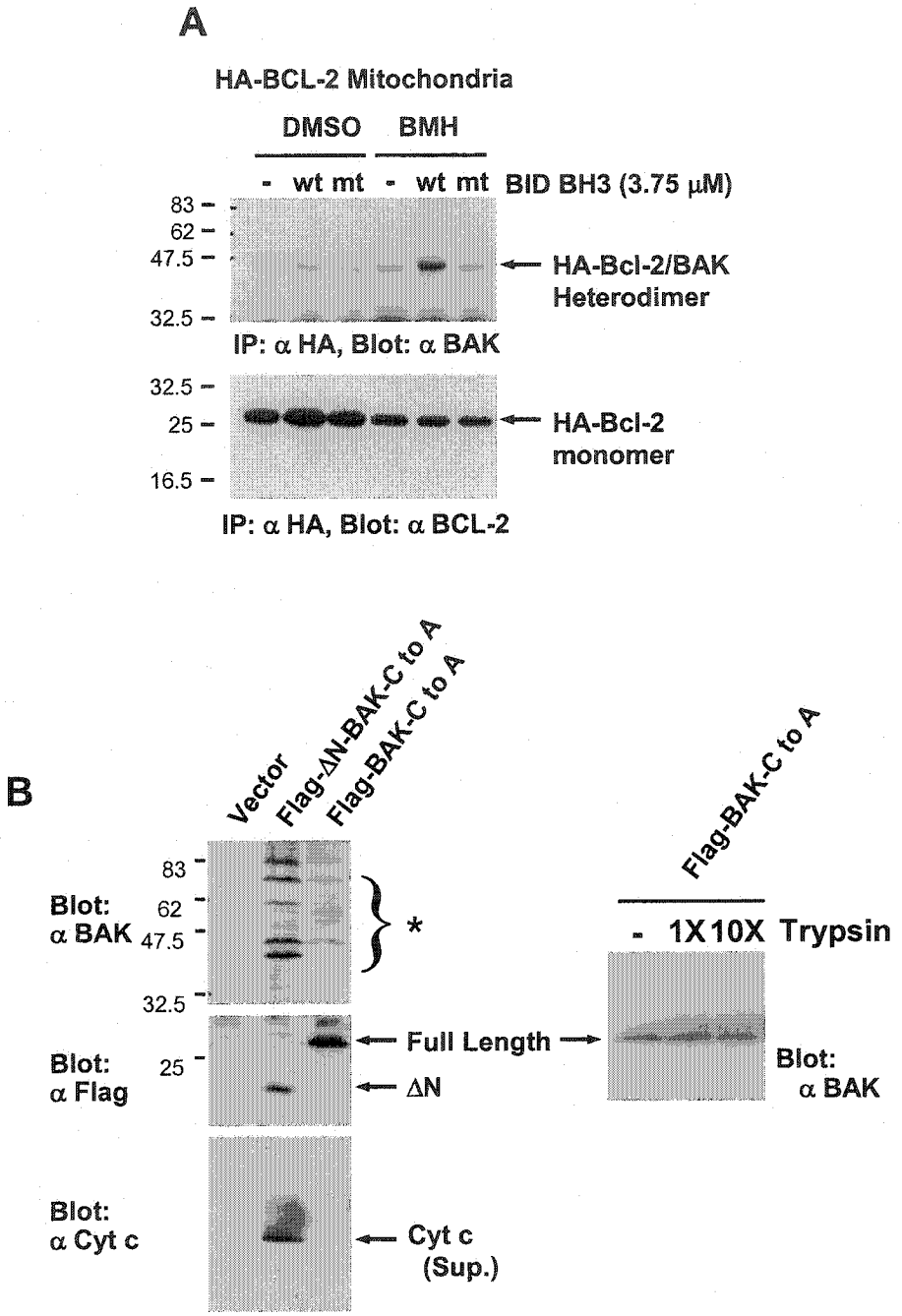
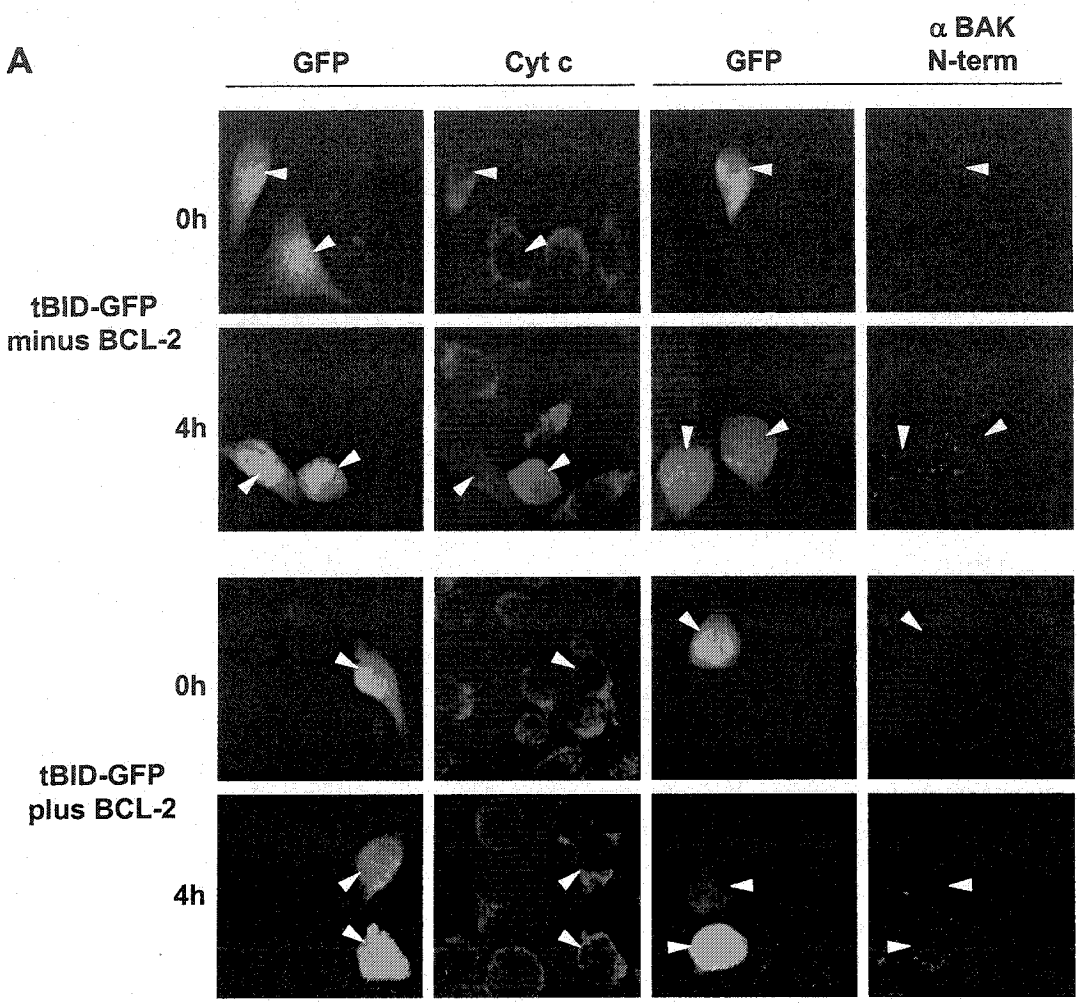
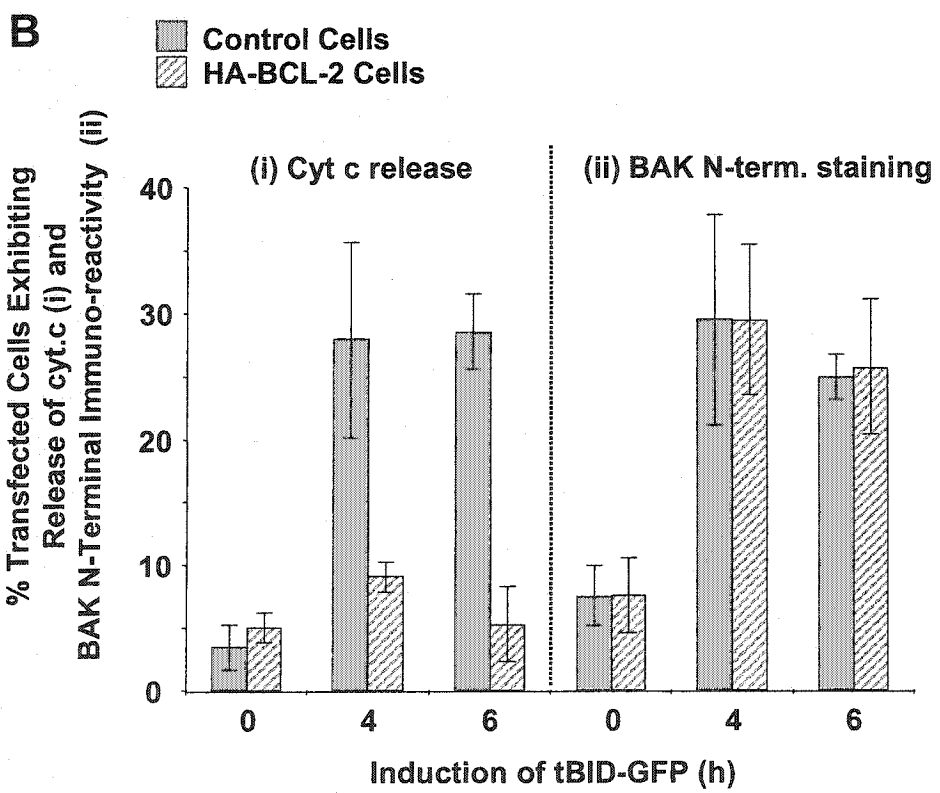


Figure 3.4 BID BH3 promotes binding of BCL-2 to BAK, which otherwise has the intrinsic capability of auto-oligomerization. (A) Mitochondria from HA-BCL-2 over-expressing KB cells were incubated in the absence (-) or presence of 3.75 μM of the indicated peptide and either vehicle (DMSO) or vehicle plus 0.1 mM BMH. After cross-linking, the mitochondria were solubilized in RIPA buffer, and subjected to immunoprecipitation with anti-HA. The precipitates were resolved by SDS PAGE and probed with anti-BAK to detect HA-BCL-2/Bak heterodimers (upper panel). Input monomeric HA-BCL-2 after cross-linking was detected by immunoblotting with anti-BCL-2 (lower panel). (B) Flag-tagged in vitro translated wt and ΔN-BAK containing cys to ala mutations were incubated with mitochondria isolated from KB cells. After centrifugation, the supernatants were analyzed for cytochrome c by immunoblot (bottom panel), while the mitochondrial pellet was treated with BMH, subjected to SDS PAGE and immunoblotted with anti-BAK to visualize cross-linked BAK oligomers (upper panel, bracket and asterisk) or with anti-Flag to visualize input translation product (middle panel). The full length translation product was subjected to trypsin digestion at concentrations of the protease 1-times and 10-times that used in Fig. 2B (right panel).

3.5.5 BCL-2 selectively interacts with death stimulated BAK in intact cells

The results presented in Figure 3.5 show that the key findings presented above utilizing isolated mitochondria in vitro can be recapitulated in intact human KB epithelial cells stimulated with tBID or Fas ligation. tBID-initiated changes in BAK conformation and cytochrome c distribution was achieved by co-transfecting a vector expressing EGFP and a vector conditionally expressing tBID under the control of an inducible promoter (Li et al., 1998; Ruffolo et al., 2000). Following induction of tBID expression with Ponasterone A, EGFP-positive cells were probed by immunofluorescence using an antibody against cytochrome c and an antibody directed to the N-terminus of BAK; the latter selectively recognizes a specific conformation of BAK with an exposed N-terminus (Griffiths et al., 1999). As shown in Figure 3.5A and 3.5B (left panel), tBID induced the release of cytochrome c in control cells but not in cells over-expressing HA-BCL-2 (see insert, Figure 3.1E). Excess BCL-2 has no effect, however, on the ability of tBID to activate the conformationally altered BAK with exposed N-terminus (Figure 3.5A and 3.5B, right panel), consistent with the findings in vitro that BCL-2 functions downstream of the tBID-induced activated conformer of BAK to restrict loss of cytochrome c from the organelle (Figure 3.2B). Moreover, the previous finding that BID BH3 peptide induces BAK / BCL-2 interaction in isolated miotchondria (Figure 3.4A) argue that these interactions occur in the presence but not absence of cell death stimuli that are coupled to BID. To test this, KB cells over-expressing HA-BCL-2 were stimulated for 0, 2, or 3 h with agonistic anti-Fas antibody, the mitochondria isolated and subjected to cross-linking with BMH, after which proteins were dissolved in RIPA buffer, subjected to BCL-2 pull down with anti-HA, and the precipitates probed by immunoblot with antibody against


A
GFP
Cyt c
GFP
α BAK
N-term
0h
tBID-GFP
minus BCL-2
4h
0h
tBID-GFP
plus BCL-2
4h


B
Control Cells
HA-BCL-2 Cells
(i) Cyt c release
(ii) BAK N-term. staining
% Transfected Cells Exhibiting
Release of cyt.c (i) and
BAK N-Terminal Immuno-reactivity (ii)
40
30
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6
Induction of tBID-GFP (h)

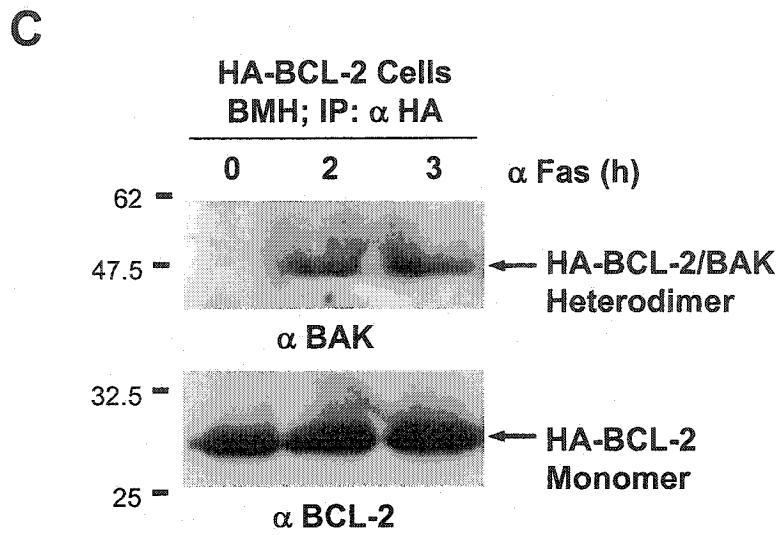

Figure 3.5 BAK conformational change and interaction with BCL-2 in intact cells over-expressing HA-BCL-2. (A and B) Control and HA-BCL-2-expressing cells were transiently co-transfected with EGFP and tBID, under the control of an inducible promoter. After 4 and 6 hours of treatment with the inducer, Ponasterone A, the cells were fixed to coverslips and cytochrome c release to the cytosol (i) and selective reactivity with antibody recognizing BAK with an exposed N-terminus (ii) were scored in EGFP positive (green) cells, as described in Materials and methods. The averages from 3 independent determinations, with standard deviations, are presented in (B). (C) KB cells stably over-expressing HA-BCL-2 were treated with an activating anti-Fas antibody for the indicated times. After treatment, the cells were harvested, mitochondria isolated and treated with 0.1 mM BMH, and after cross-linking HA-Bcl-2 was recovered by immunoprecipitation following dissolution in RIPA buffer. The precipitates were analyzed by immunoblot using anti-BCL-2 and anti-BAK antibodies, as described in Fig. 4.

BCL-2 or BAK. HA-BCL-2/BAK hetero-dimers were observed after but not before stimulation of the cells by Fas ligation (Figure 3.5C).

3.6 Concluding Remarks

Altogether, the findings presented here suggest a model (Figure 3.6) in which the BAK N-terminus represses the ability of unstimulated BAK to undergo spontaneous self-oligomerization in the mitochondrial outer membrane. By binding to BAK, the BID BH3 domain overcomes this barrier. Further, the resulting open (N-terminus exposed) BAK conformer can induce oligomerization of closed BAK conformers, indicative of a BID BH3-initiated cascade of BAK auto-oligomerization and cytochrome c release from

mitochondria. BCL-2 preferentially interacts with the BID BH3-induced open conformer of BAK, a finding consistent with the observation that BCL-2 (Figure 3.5) and BCL-X_L (Griffiths et al., 1999, 2001) do not prevent stimulus-induced exposure of the BAK N-terminus. This model might also extend to BAX since the adenovirus BCL-2 member, E1B 19K, appears to selectively bind the BAX open conformer (Cuconati et al., 2002; Perez and White, 2000; Sundararajan et al., 2001; Sundararajan and White, 2001). By interacting with the BID BH3-stimulated open BAK conformer, BCL-2 can intercede and inhibit BID BH3 signaling by selectively targeting the initiation step of the BAK oligomerization cascade, consistent with the model proposed for 19K (Perez and White, 2000; Sundarajan and White, 2001). Excess BCL-2, at levels that can inhibit cytochrome c release from mitochondria, does not prevent BID BH3 from binding BAK, indicating

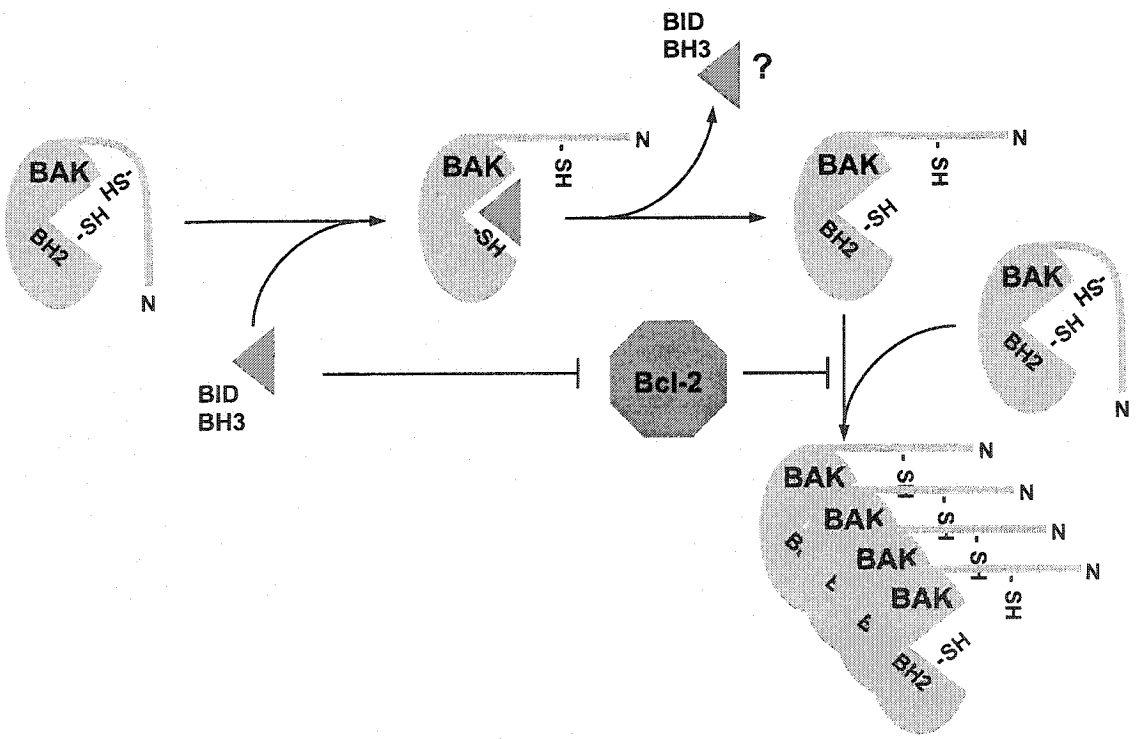

Figure 3.6 Working model for stimulation of BAK oligomerization by BID BH3 in the outer membrane of mitochondria. See text.

that BCL-2, at levels that can inhibit cytochrome c release from mitochondria, does not sequester BID BH3 nor prevent it from directly activating BAK. Sequestration of BID BH3 by BCL-2, of course, would occur at further elevated BCL-2, where BCL-2 is sufficiently in excess relative to BAK that it represents an over-whelming sink for tBID (Cheng et al., 2001). Conversely, the fact that BID BH3 in excess can bind to both BAK and BCL-2, however, suggests a two step mechanism for BID BH3-induced oligomerization of BAK: interaction of BID BH3 with BAK to induce the open conformation and interaction with BCL-2 to prevent BCL-2 from binding and inhibiting this active BAK conformer (Figure 3.6). Consistent with this idea, subsequent BAK oligomerization and release of cytochrome c from mitochondria is dependent on the ratio of pro-apoptotic BID BH3 and anti-apoptotic BCL-2. Moreover, this model suggests that BH3-only proteins that interact with BCL-2 but not with BAK may require a co-stimulus to induce the open BAK conformer, in order to initiate the mitochondrial pathway of apoptosis (Letai et al., 2002).

Finally, recent studies (Kuwana et al., 2002) suggest that the critical path for BAX-dependent egress of macromolecules such as cytochrome c across a membrane lipid bilayer can be reconstituted by combining just BAX and tBID in the appropriate lipid environment. Likewise, mitochondria isolated from cellular homogenates, such as those described here, that in intact cells undergoing apoptotic transitions, barriers that help to maintain the integrity of the mitochondrial network are probably removed by parallel pathways. For example, important transformations have been identified that sensitize mitochondria to a BH3-only hit, such as tBID, including mitochondrial fission and

fragmentation induced by endoplasmic reticulum calcium-mediated pathways (Breckenridge et al., 2003).

3.7 Acknowledgements

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Chapter 4

Co-operation Analysis of Purported BCL-2 Inhibitors with a tBID-dependent Apoptotic Stimulus

4.1 Rationale

Having deciphered a model for tBID activation of BAK and inhibition by BCL-2 different from that proposed by Letai et al., we were interested in determining whether a BAD BH3 peptide could co-operate with the BID BH3 peptide in our system. The hypothesis was that the BAD BH3 peptide could interfere with the interaction of activated BAK with BCL-2, similar to the displacement of a BID BH3 peptide induced by the BAD BH3 peptide seen by Letai et al. We then wanted to systematically study several small molecules purported to be BCL-2 inhibitors. Our goal was to determine whether they could co-operate with a BID BH3 peptide to overcome the over-expression of BCL-2 in our *in vitro* system. Results from this system would serve as a filter to find true synergistic compounds which would then be analyzed for their ability to co-operate with an apoptotic stimulus to induce death in cells over-expressing BCL-2.

4.2 Abstract

As most know, cancers arise from a block in the apoptotic pathway that will allow a cell to propagate unregulated even though it is being signaled to die. Two major blocks which can occur are due to either mutations in p53, making it inactive and therefore unable to signal apoptosis (Morgan and Kastan, 1997) or over-expression of anti-apoptotic proteins, in particular BCL-2, which can block apoptosis at many levels (Reed et al., 1996a). The discovery that BCL-2 is a very potent proto-oncogene is not a new one and has led many labs on the search for small molecules that can inhibit BCL-2, in the hopes that inhibiting BCL-2 will specifically derepress apoptosis specifically in cancer cells. Since BCL-2 has been shown to inhibit the effects of pro-apoptotic proteins by binding their BH3 domains within its BH1/BH2 cleft (Petros et al., 2000; Sattler et al.,

1997), research groups are focusing on small molecules that can disrupt this interaction. Many of these molecules have recently been published however; no extensive research has gone into determining their mechanism of action. Here we look at two such molecules, BH3I-1 discovered by Degterev et al. and 2-methoxy Antimycin A_3 (MAA_3) studied by Tzung et al. The following results will show that *in vitro* and *in vivo*, BH3I-1 and MAA_3 both have different effects on the release of cytochrome c from the mitochondria of control and BCL-2 over-expressing cells, alone and in combination with a second stimulus. BH3I-1 seems to mimic BID *in vitro* in that it cause efflux of cytochrome c from control mitochondria at low concentrations, however, *in vivo* it has very negligible effects. Whereas, MAA_3 mimic BAD *in vitro* by co-operating with a BID BH3 peptide to induce robust release of cytochrome c from mitochondria over expressing BCL-2, though, *in vivo*, it reverts to a BID-like compound.

4.3 Introduction

Much study has recently gone into discerning the function of the pro-apoptotic BH3-only proteins in inducing cytochrome c release form the mitochondria and mitochondrial dysfunction. A general consensus has emerged from all this research suggesting the BH3-only proteins act as sentinels within the cell poised to translate an apoptotic signal to the multi-domain pro-apoptotic proteins which will effectuate changes at the level of the mitochondria (Scorrano and Korsmeyer, 2003). In response to an apoptotic signal, the BH3-only proteins will be either post-translationally modified, (e.g. BID, BAD, and BIM) or transcriptionally up-regulated (e.g. BIK, NOXA, and PUMA), both presumably resulting in the exposure of their highly apoptogenic BH3 domains, a prime example being the exposure of the BID BH3 peptide after cleavage by caspase-

8(Chou et al., 1999; McDonnell et al., 1999). Peptides engineered to represent the BH3 domains of these proteins bear the same functions and work via the same mechanisms as their full-length counterparts(Letai et al., 2002; Moreau et al., 2003; Ruffolo and Shore, 2003; Shangary and Johnson, 2002). Using these peptides as research tools, two models have emerged describing the mechanism of BAK activation by tBID, the caspase-8 cleavage product of BID, and inhibition by BCL-2.

One of the models, derived from research in our lab, suggests that tBID will interact with BAK and induce an N-terminal conformational change that renders BAK active and able to auto-oligomerize with inactive BAK molecules. BCL-2, which is unable to inhibit tBID-induced activation of BAK, will only bind the activated form of BAK and thus inhibit its oligomerization. When tBID is in excess, it will then interact with BCL-2 relieving the inhibition of BAK and allowing oligomerization to progress resulting in the release of cytochrome c from the inner membrane space of the mitochondria (Ruffolo and Shore, 2003). In opposition, a second model, proposed by Letai et al 2002, indicates that tBID will be bound and sequestered by BCL-2, preventing it from activating BAK. They go on to suggest that a second activated BH3-only protein, BAD, which is rather ineffective at inducing apoptosis on its own, at low concentrations, will bind BCL-2 and displace the BH3 domain of tBID allowing it to then interact and activate BAK. Though quite different, both models have profound implications in the search for drugs that will induce apoptosis in tumour cells.

Cancers arise from damaged cells which are able to survive due to aberrations in the apoptotic pathway though they are receiving signals to undergo apoptosis. The most common chemical treatments, in recent years, have targeted DNA (Cisplatinum, a DNA

alkylating agent) and/or the DNA replication machinery (topoisomerase inhibitors such as etoposide) in an attempt to kill rapidly dividing cells, as cancer cells are. These chemotherapeutics induce cell death by activating the tumor suppressor p53 (reviewed in (Beauparlant and Shore, 2003). However, other normal cells in the body are actively replicating as well and are also affected by these chemotherapeutics leading to quite uncomfortable and deleterious side effects. The line between an effective concentration of a commonly used anti-cancer drug and a concentration that will begin to kill normal cells is very thin; there is very little selectivity in terms of the drug's target. Hence, much research has gone into searching for drugs that are more selective and as such more effective at treating cancer. The two previously describe models present mechanistic data to explain how a new category of cancer therapeutics, termed BCL-2 inhibitors, would function to selectively promote apoptosis in cancer cells.

In many cancers, BCL-2 is over-expressed leading to the inhibition of apoptosis (Reed et al., 1996a), presumably via one of the two mechanisms described previously. Therefore many groups have been on the hunt for small molecules that will mimic the BAD peptide, in that they will not readily induce apoptosis in a normal cell, as a BID BH3 peptide would, but will be able to bind the BH1/BH2 cleft of BCL-2. The hope is that these small molecules will co-operate with an apoptotic signal and displace any pro-apoptotic proteins bound to BCL-2 thus allowing apoptosis to progress. These compounds would also act specifically on cancer cells since inhibiting anti-apoptotic proteins in normal cells would presumably not cause an induction of apoptosis. Numerous compounds have been discovered that are able to displace a BAK BH3 peptide from the BH1/BH2 cleft of BCL-2 or BCL-X_L. In Degterev et al., the screening of a

compound library yielded BH3I-1, a small molecule with a 7.8μM affinity to BCL-X_L and able to displace a BAK BH3 peptide from its interaction. Tzung et al. stumbled upon a possible BCL-2 inhibitor when analyzing the effects of the mitochondrial respiration inhibitor Antimycin A_3 (AA_3). They determined that both AA_3 and its inactive counterpart 2-methoxy-Antimycin A_3 (MAA_3) where able to bind BCL-X_L, with an affinity of 2.5μM, and induce apoptosis specifically in cells over-expressing the anti-apoptotic protein.

Our lab devised an assay to quickly determine if candidate compounds would be able to co-operate with another apoptotic stimulus, like a BID BH3 peptide, at low concentrations *in vitro*. Assaying the aforementioned published compounds, it was determined that *in vitro* they fall into two distinct categories: BH3I-1 acted as a BID-like compound in that it was able to induce cytochrome c release from control mitochondria, while MAA_3 was a BAD-like compound inducing minimal cytochrome c release from control mitochondria but able to co-operate with a BID BH3 peptide to induce release of cytochrome c from mitochondria over-expressing BCL-2. However, moving to an *in vivo* assay and using an activating anti-Fas antibody as a co-stimulus, BH3I-1 was unable to induce cytochrome c release in either control or BCL-2 over-expressing cells, in contrast to what was expected from *in vitro* results. Whereas MAA_3, *in vivo*, readily induced cytochrome c release in both cells types.

4.4 Materials and Methods

4.4.1 General

Isolation of cytosol and mitochondria, SDS PAGE of cell fractions, transfer of proteins to nitrocellulose filters, development of blots with antibodies and detection by enhanced chemiluminescence have been documented in earlier publications (Goping et al., 1998; Ruffolo et al., 2000). Generation of the peptides used was described in Ruffolo and Shore, 2003.

4.4.2 Release of cytochrome c from mitochondria

Mitochondria (50 μg protein in 25 μl cMRM) isolated from control (- HA-BCL-2) KB or from KB cells over-expressing HA-BCL-2 (Nguyen et al., 1994) were incubated with 20μl HIM (200mM mannitol, 70mM sucrose, 10mM HEPES-KOH, 1mM EGTA, pH 7.5) plus or minus synthetic BH3 peptides (in 0.5mM Pipes, pH6), or plus or minus 2-methoxy Antimycin A_3 (in ethanol, Biomol) or BH3I-1 (in H_2O, provided by GeminX Biotechnologies) to the indicated concentrations and adjusted to a final volume of 50μl with 0.5mM Pipes (pH6) and incubated for 1 hour at 37°C (Goping et al., 1998; Ruffolo et al., 2000). The reaction mixtures were then centrifuged at 9000 rpm (Sorvall MC 12V), and 1μL of the supernatants were analyzed by human cytochrome c specific ELISA (Chemicon Cat# APT200).

4.4.3 Cell culture and α-Fas treatment

Human KB epithelial cells and KB cells stably over-expressing HA-BCL-2 were treated with Fas ligating anti-Fas antibody (0.5μg protein / ml; Upstate, CH11) plus cycloheximide (10μg/mL), as described (Nguyen et al., 2000) plus or minus 2-methoxy Antimycin A_3 or BH3I-1 at the indicated concentrations in supplemented growth media described in Ruffolo et al., 2000. After 4 hours of treatment, cell cytosols were isolated

and equivalent aliquots cytosols were analyzed by SDS PAGE and immunoblot using the mouse monoclonal anti-pigeon cytochrome c antibody 7H8.2C12 (Pharmingen).

4.5 Results and Discussion

As described in Ruffolo and Shore, 2003, a peptide representing the BAD BH3 domain was inefficient at inducing BAK oligomerization at the mitochondrial outer membrane and the release of cytochrome c, in comparison to a BID BH3 peptide of the same size. Evidence put forth by Letai et al 2002, suggested that a BAD BH3 peptide could co-operate with a BID BH3 peptide to induce cytochrome c release from isolated mitochondria, over-expressing BCL-2. Since the two mechanistic models conflicted, it was necessary to determine whether BAD could co-operate with BID in our system.

4.5.1 BAD will synergize with BID to induce the release of cytochrome c

When a BID or BAD BH3 peptide are incubated in low concentrations, 2 and 3μM, with mitochondria isolated from cells over-expressing BCL-2, neither will induce any significant release of cytochrome c (Figure 4.1). However, when the two peptides, at the same concentrations, are combined prior to incubation with the mitochondria, there is almost a ten fold increase in the amount of cytochrome c release from the isolated mitochondria. This release is well above the additive effect of the two separate peptides suggesting they are acting in synergy. This effect is also not the result of over-loading the mitochondria with synthetic peptide since incubating with the BAD peptide at a concentration of 5μM, the total peptide concentration of the two combined peptides, resulted in some cytochrome c release but nowhere near the levels of the peptide combination.

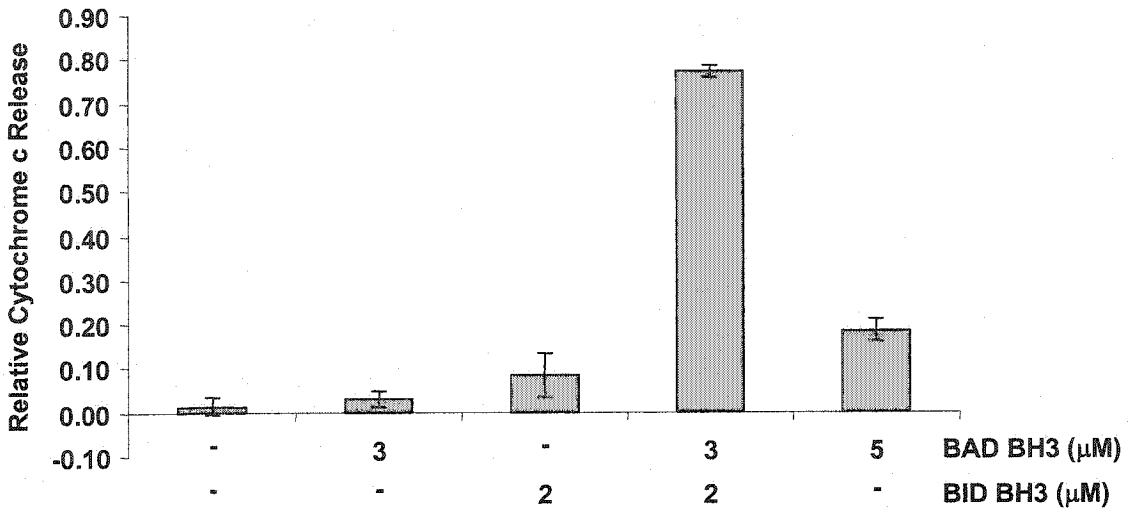


Figure 4.1 Co-operation of BID and BAD BH3 peptides. Mitochondria isolated from cells stably over-expressing HA-BCL-2 were incubated with either buffer alone or engineered peptide representing the BH3 domains of BID or BAD at the indicated concentrations. After one hour at 37°C, the reactions were centrifuged at 10xg to pellet the mitochondria and 1μL of the supernatant was assayed for cytochrome c in triplicate by ELISA (Chemicon) as per the manufacturer's protocol.

Since a BAD BH3 peptide does have a higher affinity than a BAK BH3 peptide from the BH1/BH2 bind cleft of BCL-2 (Petros et al., 2000), it is presumed that the BAD peptide in this system is interacting with BCL-2 and disrupting its binding to the activated form of BAK, allowing oligomerization and cytochrome c release to progress. Therefore, the BAD peptide is displaying perfect BCL-2 inhibitor attributes; at the concentrations used, it does not readily induce cytochrome c release from control mitochondria but can co-operate with another apoptotic stimulus to overcome inhibition by BCL-2. BID, on the other hand, though it can inhibit BCL-2 (Ruffolo and Shore, 2003), will only do so at higher concentrations, well above those able to induce cytochrome c release from control mitochondria. When searching for BCL-2 inhibitors, they may fall into two very different categories: those that are BID-like; which would

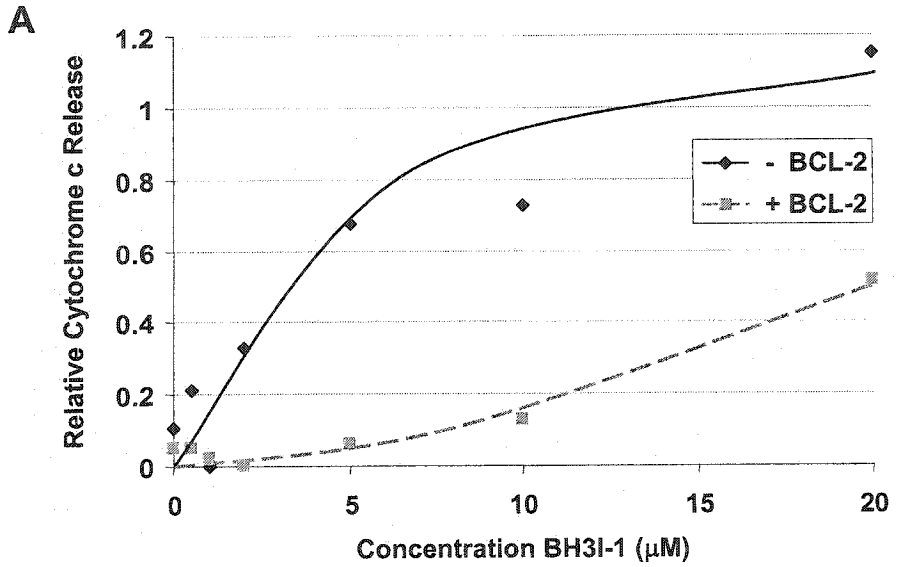
probably be highly toxic, and those that are BAD-like; which would hopefully co-operate with an already existing apoptotic signal to induce apoptosis specifically in cancer cells.

4.5.2 BH3I-1 is a BID-like compound

Having a co-operation model for cytochrome c release from isolated mitochondria, our focus turned to published compounds that were categorized as BCL-2 inhibitor. The goal was to determine if these compounds could effectively replace the BAD BH3 peptide and co-operate with a secondary signal to induce cytochrome c release from isolated mitochondria. However, prior to determining whether a given compound will fit into the co-operation model, a dosage curve of the compound is required to determine what concentration range should be tested. That range would be one in which the compound, on its own, will not cause the release of cytochrome c from mitochondria isolated from BCL-2 over-expressing cells nor from those isolated from control cells.

When the dosage curve was performed for the Degterev et al. compound, BH3I-1, it was clear that there was a small yet significant release of cytochrome c at concentrations as low as 3μM in control mitochondria (Figure 4.2A). BCL-2 is an effective inhibitor of BH3I-1 when the compound is at low concentration, but, like the BID BH3 peptide, increasing the concentration of the compound, to 20μM and above, will overcome BCL-2 inhibition (Figure 4.2A). This indicates that BH3I-1 may functions similarly to BID to induce changes to the mitochondria.

This is supported by results from co-operation experiments shown in figure 4.2B. Again, it is evident that BH3I-1 on its own can overcome the inhibition by BCL-2 and will induce significant release of cytochrome c. BID, in these experiments, is being used at a concentration (2μM) low enough so as not to overcome the inhibition by BCL-2, as



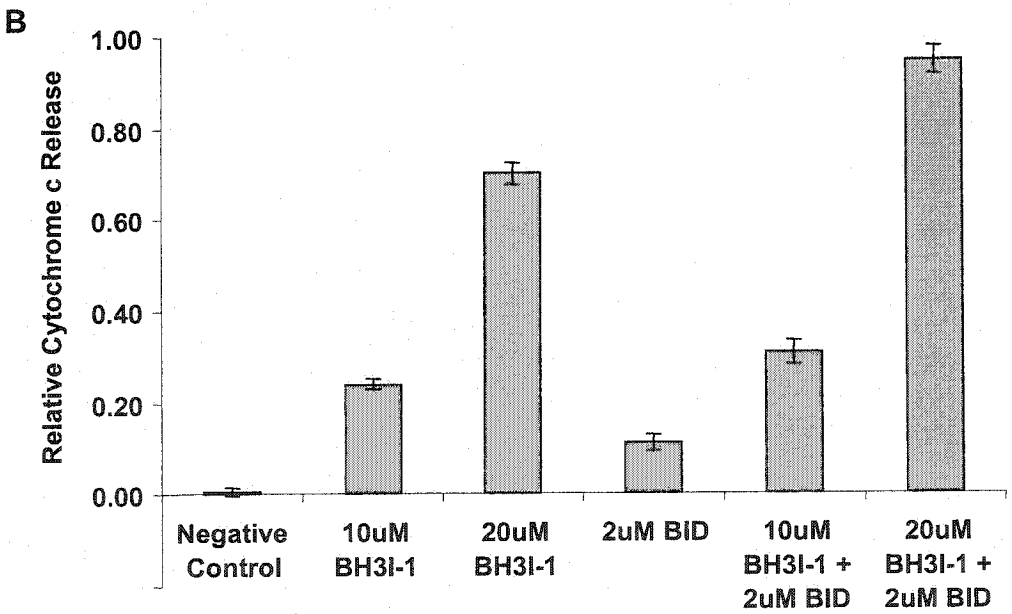


Figure 4.2 BH3I-1 has BID-like characteristics. (A) Mitochondria isolated from control (- BCL-2) and HA-BCL-2 (+ BCL-2) over-expressing cells were incubated with increasing concentrations of BH3I-1 (provided by GeminX Biotechnologies) to a maximum of 20μM. After one hour at 37°C, the reactions were centrifuged at 10xg and 1μL of the supernatant was assayed for cytochrome c by ELISA as per the manufacturer's protocol. (B) Mitochondria isolated from cells over-expressing HA-BCL-2 were incubated with either buffer, BH3I-1 (10 or 20μM), or a BID BH3 peptide (2μM), alone or in combination as indicated. Reactions were assayed for cytochrome c release as described in Figure 4.1

shown, and therefore, any release of cytochrome c is due to either synergy between the peptide and the compound or the compound on its own. When the compound, at concentrations of 10 and 20μM, and the BID BH3 peptide are combined in the presence of isolated mitochondria, there is a minimal increase in the amount of cytochrome c released. The total amount of cytochrome c released at either concentration of BH3I-1 in the co-operation experiment is approximately equal to the sum of the amount released by each component separately, indicating that the compound only has an additive effect. This additive effect suggests that the compound is inducing the release of cytochrome c either at the same point at which the BID BH3 peptide is acting, therefore leading to a combined total effect on release, or it is inducing a parallel pathway, which is also inhibitable by BCL-2, leading to release of cytochrome c.

No matter which mechanism BH3I-1 is using to induce cytochrome c release, these results imply that this compound would be unsuitable for the treatment of cancers since it would likely be toxic to normal cells. However, to be certain of these effects, *in vivo* studies must be performed.

4.5.3 MAA_3 is a BAD-like compound

Unlike BH3I-1, 2-methoxy antimycin A_3 is unable to induce any significant release of cytochrome c from isolated mitochondria, from either control or BCL-2 over-expressing cells, on its own even at concentrations as high as 50μM (data not shown, Figure 4.3, line graph). However, due to its ability to displace a BAK peptide from the BH1/BH2 cleft of BCL-X_L, as described by Tzung et al., there was interest in determining if the compound could co-operate with the BID peptide by disrupting the interaction of BID BH3-activated BAK and BCL-2. Therefore, a combination of the BID

BH3 peptide (at 2μM) and MAA_3 (at 50μM) was incubated with mitochondria isolated from cells over-expressing BCL-2 and the release of cytochrome c was assayed by ELISA (Chemicon). The combination of the two factors induced a substantial release of cytochrome c well above the additive effects of each, and though it seems to account for only half of maximal release, obtained by overwhelming the mitochondria with 15μM BID BH3 peptide, it is a very significant increase (Figure 4.3, histogram).

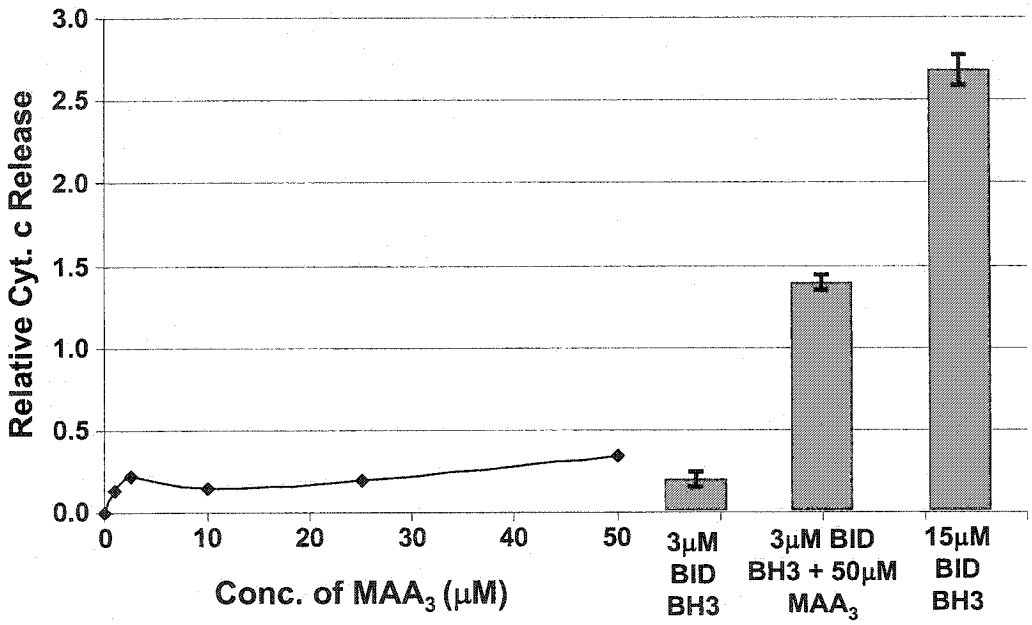


Figure 4.3 MAA_3 has BAD-like characteristics. Mitochondria from cells over-expressing HA-BCL-2 were incubated with increasing concentrations of 2-methoxy Antimycin A_3, 2 or 15μM BID BH3 peptide, and a combination of MAA_3 and BID. Reactions were assayed for cytochrome c release as described in Figure 4.1.

These results indicate that MAA_3, like the BAD peptide, is acting at a separate point, from the BID peptide, along the BID-induced pathway, making their combined effect more than simply additive. It could be inferred that MAA_3 is interfering with the interaction between BCL-2 and the open-conformer of BAK, therefore, allowing the activated BAK to auto-oligomerize at lower levels of the BID BH3 peptide, which would

normally be inhibited by BCL-2. This model is also supported by the finding of Tzung et al that MAA_3 has the ability to displace BAK from an anti-apoptotic partner.

The very different effects of these two compounds suggests that, at least *in vitro*, that BCL-2 inhibitors will fall into at least two separate categories: BID-like and BAD-like inhibitors. This becomes a very important distinction since finding a compound that is simply an inhibitor of BCL-2/BAK peptide interaction does not necessarily make it a good candidate for treatment. It would therefore be best to determine which category the new inhibitor would fall under or whether there are other categories of BCL-2 inhibitors. Rather than start testing on a complex, whole-cell system, we show that compounds can be preliminarily categorized with a simple and fast *in vitro* method, using the cytochrome c release from isolated mitochondria a quantifiable output. This, however, does not negate the necessity of performing experiments on *in vivo* systems, but can act as a selection tool to avoid testing compounds that do not have the desired effects *in vitro*. As such, the next step was to determine whether these two compounds showed the same effects in a cell culture system using cells that over-express BCL-2 and an activating anti-Fas antibody as a secondary stimulus in determining synergy.

4.5.4 Cell Culture Results Show Different Inhibitor Effects

Control cells and cells over-expressing BCL-2 were treated with an activating anti-Fas antibody (Upstate). The Fas antibody triggers the formation of the DISC in these cells and therefore results in the activation of procaspase-8 and the processing of BID to it highly apoptotic form, tBID. The over-expressed BCL-2 does not inhibit the processing of either caspase-8 or BID but blocks the downstream effects of tBID: BAK oligomerization, cytochrome c release and apoptosis (data not shown). These

observations agree with results published for type II cells undergoing Fas induced apoptosis in which BID cleavage and translocation is not inhibited by BCL-X_L (Gross et al., 1999b). This was therefore used as a model for a cancer cell, one which is receiving a death stimulus but is prevented from undergoing apoptosis by the over-expression of an anti-apoptotic protein. As expected, control cells treated with the activating antibody show a large release of cytochrome c to the cytosol after only four hours of treatment, which is inhibited by BCL-2 (Figure 4.4, Buffer). This represented a system which could be used to test the BH3I-1 and MAA_3 *in vivo* for their ability to co-operate with the Fas signal to induce cytochrome c release from cells over-expressing BCL-2.

Cells were simultaneously treated with the Fas antibody and either compound at two different concentrations; however, both BH3I-1 and MAA_3 demonstrated different effects than were expected from the *in vitro* results. *In vitro* data obtained for BH3I-1 suggested that it would induce the release of cytochrome c from control cells, which would be inhibited by BCL-2, and show additivity when combined with the Fas signal. In fact, BH3I-1 induced minimal release of cytochrome c, in control cells, at a concentration of 10μM which even dropped below control levels at 20μM. What was even more intriguing was that there was a decrease in the amount of cytochrome c released after combined treatment in cells over-expressing BCL-2. This demonstrates that BH3I-1 did not have an additive effect in combination with the Fas antibody, but rather it seemed to enhance the inhibition of cytochrome c release (Figure 4.4, BCl-2 + Fas). MAA_3, on the other hand, showed a lot of promise after *in vitro* testing and was expected to co-operate well with the anti-Fas treatment, as it showed a very robust release of cytochrome c when isolated mitochondria were treated in combination with the BID

peptide. However, at the concentrations used, MAA_3 was able to induce some release of cytochrome c on its own, which there was no evidence of *in vitro*, and that release was inhibitable by BCL-2 over-expression. When combined with the anti-Fas treatment, there was minimal increase, well below even the additive effects of the two separate treatments (Figure 4.4).

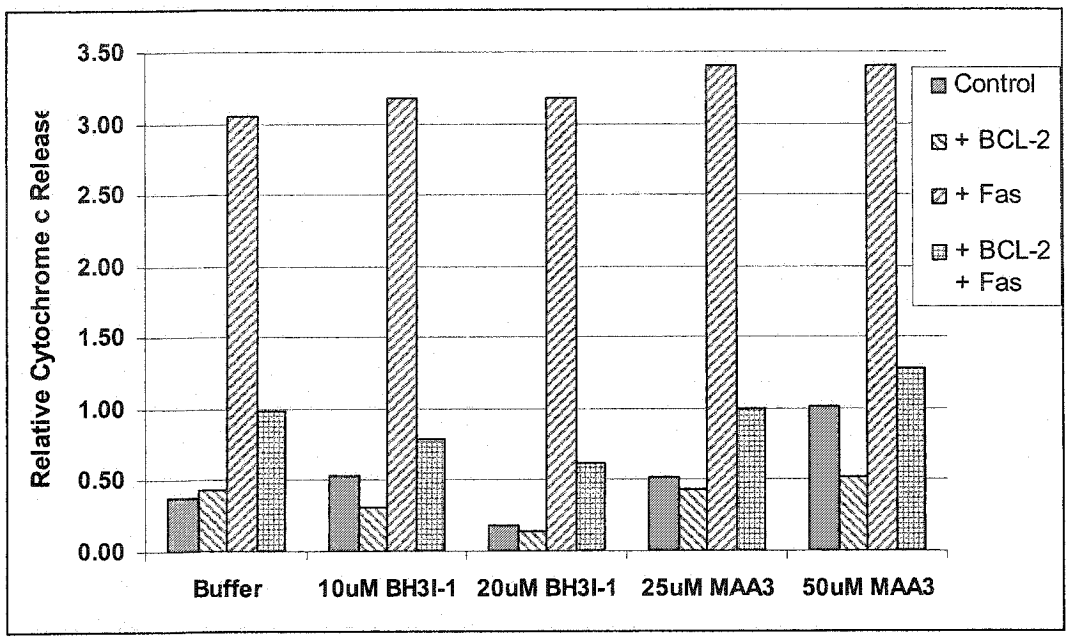


Figure 4.4 Co-operation of MAA_3 and BH3I-1 with an activating anti-Fas antibody in a cell culture system. Control and HA-BCL-2 over-expressing cells (BCL-2) were treated with various concentrations of MAA_3 and BH3I-1 (as indicated) alone or in combination with an activating anti-Fas antibody (0.5μg protein/mL, Upstate (CH11)) plus cyclohexamide (10μg/mL) (+ Fas) in normal growth media for 4 hours. After treatment, cells were harvest, washed, and homogenized to isolate their cytosols which were assayed for cytochrome c by western blotting with a mouse monoclonal anti-human cytochrome c antibody (7H8.2C12; Pharmingen). Densitometry of the resulting western blot was preformed using NIH Image version 1.61 to obtain relative value for the amount of cytochrome c released.

In the work published by Tzung et al, it is demonstrated that MAA_3 can effectively induce cell death in cells stably over-expressing BCL-X_L. That being said, the control cells used in our research have relatively large amounts of BCL-X_L present endogenously (data not shown) and the stable cell lines over-expressing BCL-2 were created on this high BCL-X_L background. Therefore, it is reasonable to infer that the

control cell lines may have been more sensitive to MAA_3 due to their high levels of BCL-X_L, but the effect was inhibited by over-expressing BCL-2, an intriguing difference between the two anti-apoptotic proteins. From this, one would have expected the *in vitro* experiments to also be affected by the presence of high levels of BCL-X_L on the mitochondrial outer membrane, but, BCL-X_L is not solely present at the mitochondria, it is also present at the endoplasmic reticulum and the nuclear envelope. Therefore, since MAA_3 was unable to induce any significant release of cytochrome c from control mitochondria, it may be possible that in a whole cell system the compound is inducing apoptosis through BCL-X_L at another intracellular compartment.

4.6 Conclusions

It has been a well known and described fact that *in vitro* results do not necessarily translate into the same results *in vivo*, and this was yet another example of that. However, to understand what these compounds are doing mechanistically, it is imperative to study them in a controlled system. Up to now there has been solid evidence to elucidate the mechanisms by which tBID and BAD can co-operate to induce cytochrome c release from the mitochondria (Letai et al., 2002). From those mechanisms it is clear that any novel compounds purported to be BCL-2 inhibitors should function as a BAD mimic and not a BID mimic. We implemented a simple and quick system to determine the characteristics of two such BCL-2 inhibitors in a tBID-induced apoptotic pathway. Even though, these characteristics were not confirmed *in vivo*, it allowed us to eliminate BH3I-1 as an effective candidate prior to performing any *in vivo* studies.

There are many other published compounds described as BCL-2 inhibitors, all of which must be well tested to determine their true potential as treatments. Only one of the

many BH3I compounds discovered by Degterev et al was analyzed in this report. There are even more intriguing compounds such as those developed by Kutzki et al which have a terphenyl moiety as a structural scaffold and modified side groups to make similar contacts to those determined by the structural studies of the interaction between BCL-2 and BAK. However, the focus of these compounds may no be limited to those that are BAD mimics. As more is understood about the mechanisms of other BH3-only proteins, other targets, not only at the level of the mitochondria, may emerge. A number of BH3-only proteins target the ER as well, such as BIK and Spike (Germain et al., 2002; Mathai et al., 2002; Mund et al., 2003), and once their mechanisms are elucidated, mimics of their BH3 domains may prove even more useful. This notion of co-operation between BH3-only family members may also not be limited to BAD and BID. BIM and BMF are both sequestered to the cytoskeleton(Puthalakath et al., 1999; Puthalakath et al., 2001) and are activated by JNK phosphorylation in a UV-irradiation response (Lei and Davis, 2003), therefore, are possible partners in inducing apoptosis. It is entirely possible that the whole group of BH3-only proteins could be paired off into partners which when activated together function most effectively to induce apoptosis. Only more time and more research will determine whether that is in fact the case.

As for now, there is only the activator and inhibitor pair of BID and BAD that is a clear model to use as a basis for finding more BCL-2 inhibitors, hopefully leading to the ultimate goal of developing better treatments for cancer.

Chapter 5

General Discussion

The main focus of this thesis has been the pro-apoptotic BH3-only protein BID, specifically the determination of its function in apoptosis, the mechanism which it uses to propagate an apoptotic signal and finally, the application of this knowledge in the search for novel cancer treatments.

5.1 BID Function

5.1.1 BH3-only proteins as apoptotic signal transducers

It is clear that BID, when activated by caspase processing, can activate both BAX and BAK inducing the former to insert into the outer mitochondrial membrane and causing both to form oligomers (Korsmeyer et al., 2000) that act as channels to allow the release of proteins from the mitochondrial intermembrane space. *In vitro* data from the BID depletion experiments would suggest that BID, or more accurately tBID, is required for insertion of BAX into the outer mitochondrial membrane. This has become a very common theme for most BH3-only proteins, many of which are present in the cell as either in an inactive form or sequestered in an inactive state, in which their BH3 domains are masked. When an apoptotic signal is detected, BH3-only proteins become activated by some form of post-translational modification exposing their BH3 domains, or they are transcriptionally upregulated in a presumably already active form. These BH3 only proteins act as the link between an apoptotic signal and the apoptotic effectors at the mitochondria and the endoplasmic reticulum.

5.1.2 Uncoupling Bax insertion and Cytochrome c Release

When inducing apoptosis *in vivo*, by TNFα or the oncogene E1A, in mouse embryo fibroblasts devoid of Bid, the requirement of tBid for Bax integration is lost.

However, insertion of Bax is not sufficient for cytochrome c release, which suggests that either Bax, in the absence of tBid, can not form an oligomeric pore or that tBid is a necessary component of the oligomer and therefore cytochrome c is not released from the mitochondria. Data obtained using the BID BH3 peptide shows that it can induce insertion and oligomerization of BAX (unpublished data) as well as the oligomerization of BAK and release of cytochrome c. This seems to preclude the hypothesis that tBid is a necessary component of the Bax oligomer pore since the peptide is neither targeted to nor inserted into the outer mitochondrial membrane. Our evidences shows that insertion of Bax into the outer mitochondria membrane can be uncoupled from cytochrome c release in the absence of tBid, therefore Bax-induced cytochrome c release, is likely to be at least a two step process similar BAK oligomerization.

5.1.2.1 Step 1: Insertion of BAX, Is it Dependent on tBID?

The results presented in Chapter 2 indicate that BAX insertion into the outer mitochondrial membrane in an *in vitro* reconstituted system is absolutely dependent on presence of tBID. However, the system implemented to study BAX insertion *in vitro* is devoid of many other BH3-only proteins; there is likely no BIM or BMF present since they are associated with the plasma membrane and eliminated in the production of the cytosolic extracts and there are none of the transcriptionally activated proteins, like BIK, NOXA, and PUMA, since the extracts are prepared from healthy, uninduced cells.

This could explain the results obtained *in vivo* using E1A as an apoptotic stimulus. E1A expression will lead to the stabilization of p53 which can in turn induce the expression of BIK, NOXA, PUMA (Mathai et al., 2002; Nakano and Vousden, 2001; Oda et al., 2000) and some research has even suggested that BAX is upregulated by p53

(Wu and Deng, 2002). Any of these proteins could induce BAX insertion into the outer mitochondrial membrane, not to mention the plethora of other proteins that are transcriptionally activated by E1A that have yet to be functionally characterized.

The ability of TNFα to induce Bax insertion is not so clear. TNFα, like Fas, propagates its apoptotic signal through the activation of caspase-8 (Muzio et al., 1997) whose substrate is Bid. In the absence of Bid, the active caspase-8 may be implementing a more indirect pathway to trigger Bax insertion. Caspase-8, in the absense of Bid, will still cleave Bap31 producing the p20 fragment which will induce the release of calcium from the endoplasmic reticulum (Breckenridge et al., 2003). The efflux of calcium can lead to the activation of calcineurin which can dephosphorylate and activate Bad (Wang et al., 1999), this active Bad could then induce Bax insertion. Another possibility is that the mitochondrial fission triggered by the release of calcium (Breckenridge et al., 2003) may also induce insertion of Bax. It has been shown recently that DRP-1 and BAX co-localize to scission sites at the mitochondria (Karbowski et al., 2002), it is possible that the DRP-1 targeted to the mitochondria in response to its uptake of calcium may pull BAX with it and cause it to insert into the membrane. This is also another plausible explanation for the E1A induced insertion of Bax, since we do, in fact, see the fission of the mitochondria after treatment with the oncogene in the absence of Bid (Figure 2.4B, bottom right panel).

5.1.2.1 Step 2: Release of Cytochrome c (Bax Oligomerization?) is Dependent on Bid

There has been no evidence to suggest that cytochrome c can be release from the mitochondria during apoptosis in the absence of BAX and/or BAK oligomerization, and conversely, no groups, including ours, have observed no cytochrome c release when BAX

and/or BAK are oligomerized. In other words there is, as of yet, no evidence that uncouples BAX and BAK oligomerization from cytochrome c release. It is then plausible to infer that, when we do not observe cytochrome c release from $Bid^{-/-}$ cells after induction of apoptosis with E1A, the Bax that is inserted into the membrane may not be oligomerized. Therefore tBid may be required to drive the oligomerization of Bax, similar to its effects on BAK. There is, however, also no evidence to support this either and may simply be a fanciful extrapolation.

5.2 The Mechanism of BID Function

Though tBID may not be necessary for BAX insertion into the mitochondrial outer membrane, it is sufficient to induce the translocation (Figure 2.3B) and oligomerization, as it can induce the oligomerization of BAK at the mitochondria (Korsmeyer et al., 2000). As evidenced in type II cells, BCL-2 can inhibit both integration and oligomerization induced by tBID and our research was focused on discerning between the two possible inhibition mechanisms previously depicted (Figure 1.6). In the course of trying to determine how BCL-2 inhibits BAX insertion and BAK oligomerization, we were able to determine the mechanism of tBID-induced BAK oligomerization as well, and discovered neither of the hypothesized mechanisms was totally correct.

5.2.1 The Two Steps of BAK Oligomerization

5.2.1.1 Step 1: Conformational Change

BAK in uninduced cells takes on a conformation in which its NH_2-terminus is in close proximity to its BH2 domain, determined by chemical cross-linking, suggesting that

the NH_2-terminus is masking the BH1/BH2 cleft of BAK. The evidence presented shows that a BID BH3 peptide will interact with BAK and it ,as well as tBID, can induce a change in conformation of its NH_2-terminus. This is parallel to what occurs with BAX which requires the displacement of its NH_2-terminal ART domain to allow the COOH-terminal transmembrane domain to insert into the outer mitochondrial membrane (Desagher et al., 1999; Goping et al., 1998). Unlike BAX, BAK is constitutively integrated into the outer mitochondrial membrane and does not require translocation to the mitochondria. The change in conformation of BAK is important for the next step in tBID-induced BAK oligomerization, however, it still remains unclear as to whether it is the unmasking of the BH1/BH2 cleft that drives auto-oligomerization or, like the BH3-only proteins, the change in conformation leads to the exposure of the BAK BH3 domain.

5.2.1.2 Step 2: Auto-oligomerization

When the ART domain of BAX is deleted, insertion of BAX is unregulated and will induce the release of cytochrome c without any further stimulus (Goping et al., 1998). Similarly, when the NH_2-terminus of BAK is deleted it becomes constitutively active and no longer requires tBID to induce oligomerization. It will, in fact, induce the oligomerization of inactive molecules of BAK which does not require any further tBID (Figure 3.4B). This would suggest that BAK, like BAX, also has an NH_2-terminal inhibitory domain which lends credence to the theory that these two proteins, which have similar structures and functions, though located to different areas of the cell, have similar mechanisms of activation as well. If true, this would in turn support the notion that BAX insertion and oligomerization may be two separable steps, akin to BAK activation and oligomerization.

5.2.2 BCL-2 Inhibition

BCL-2, at levels that will inhibit cytochrome c release, will not inhibit the interaction of tBID with BAK nor the activation of BAK, however, BCL-2 will bind the activated form of BAK and, presumably in so doing, will inhibit the formation of oligomers. Excessive amount of the BID BH3 peptide will in turn relieve the inhibition of BCL-2 by interacting with it and presumably displacing the activated BAK allowing it to auto-oligomerize. Of note, is that the BID BH3 peptide seems to interact more strongly with BAK in the presence of BCL-2 as opposed to in its absence (Figure 3.3B). This may indicate that BCL-2 is trapping tBID while it binds BAK and also suggests that the interaction between tBID and BAK is very transient making tBID a catalyst for BAK oligomerization. tBID will activate one molecule of BAK by inducing a change in conformation and then continue on to find other inactive molecules to interact with, making it another mechanism for amplifying the original signal. Maybe BCL-2 is inhibiting BAX by a similar mechanism, where it will interact with BAX prior to its insertion into the membrane but after an activating change in conformation.

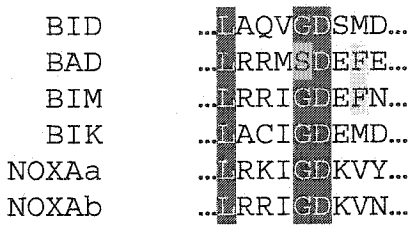
This model of BCL-2 inhibition is in obvious conflict with the model proposed by Letai et al. which suggests that BCL-2 binds and sequesters tBID, preventing it from interacting with and activating BAK. Presumably, excess amounts of tBID would saturate the BCL-2 and move on to activate BAK. It is likely that the preferred mechanism would depend on the amount of BCL-2 present in the cell. When there are extremely high levels of BCL-2, it may act as a sink for tBID even though the affinity of tBID for BCL-2 may not be as strong as its affinity for BAK. At BCL-2 levels that are lower but which still prevent the release of cytochrome c, the affinity of tBID for BAK

will lead to the activation of BAK. The activated BAK will then be bound and sequester by BCL-2. This difference in BCL-2 levels may also be reflected in the BID/BAD co-operation findings from both groups. Where we required a relatively small amount of BAD BH3 peptide to effectively inhibit BCL-2 and allow the progression of BID-induced cytochrome c release, the group of Letai et al. utilized concentration almost one hundred times higher to achieve similar result. The greater amount of BCL-2 present may have required a much greater amount of BAD peptide to neutralize its effects.

5.2.3 BAD Co-operation

Letai et al. also demonstrate that a BAD BH3 peptide can displace tBID from the BH1/BH2 cleft of BCL-2 allowing it to activate BAK. They go on to show that *in vitro*, the BAD BH3 peptide has a higher affinity for BCL-2 than the BID BH3 peptide, 41nM for BAD compared to 220nM for BID. Our results show a similar displacement though in this case, a BAD BH3 peptide is presumably displacing the activated BAK from its interaction with BCL-2, leading to its auto-oligomerization. Therefore, the BAD BH3 domain must have a higher affinity for BCL-2 than BAK as well, which was calculated by Sattler et al. to be approximately 200-320nM for BCL-X_L.

The BAD BH3 peptide, on its own, is not a very potent inducer of BAK oligomerization or cytochrome c release. So if all BH3 domains have the same required amino acids, where do their affinities and potencies lie? The most likely answer would be the other amino acids within the BH3 domains. The following is an alignment of the BH3 domains of some BH3-only proteins:



The BAD BH3 domain is, in fact, the only BH3 domain that diverges from the LXXXGD motif common to all other pro-apoptotic proteins in the family. Instead of the small side chain of glycine, there is the large, polar ethoxy group of serine which could easily account for it low apoptotic activity and may form a different and/or stronger contact with BCL-2. There is a definite function of this serine in interactions with BCL-X_L since, when this serine (position 155) becomes phosphorylated, the interaction is lost (Tan et al., 2000). It could just as well be a combination of the serine and the phenylalanine at the COOH-terminus of the BH3 domain since the BIM BH3 domain also has a high affinity for BCL-2 (74nM) though just better than half that of BAD (Letai et al., 2002). It is at these positions of variance where the differences between the effects of each BH3 domain must lie.

5.3 The Co-operation Model put to Practical Use

5.3.1 Small Molecule Inhibitors of BCL-2

The obvious implications of this work have been clearly stated. Anti-apoptotic BCL-2 family members are commonly over-expressed in cancer cells and in many of these cells it is the blockade at which the apoptotic pathway is being inhibited. Though these cells are presumably being pressured to undergo apoptosis by some oncogenic stimulus or other signal, they are surviving due to the ability of BCL-2 to prevent: oligomerization of BAX and BAK, release of cytochrome c at the mitochondria, calcium

efflux from the endoplasmic reticulum, and possibly other apoptotic pathways that have yet to be elucidated. Discovering that a BAD BH3 peptide, though not a potent inducer of apoptosis on its own, can inhibit BCL-2 and allow low levels of a BID BH3 peptide or tBID to drive BAK oligomerization and cytochrome c release (Letai et al., 2002) is invaluable in terms of find new treatments for cancers. The rationale would follow that a small molecule that could mimic the BAD BH3 peptide may be able to co-operate with an oncogenic stimulus that is being inhibited by over-expressed BCL-2, to promote apoptosis selectively in these cancer cells. Though the BID BH3 peptide was also able to inhibit BCL-2, it is not a practical model for BCL-2 inhibitor; it requires much higher concentrations to inhibit BCL-2 and it is a potent inducer of apoptosis in normal cells. Therefore, an optimal small molecule BCL-2 inhibitor should be able to displace either BAK or tBID from the BH1/BH2 cleft of anti-apoptotic proteins at relatively low concentrations but should not induce apoptosis in the absence of a second apoptotic stimulus (i.e. not kill normal cells) (Figure 5.1).

5.3.1.1 The Candidates

Of the two candidate BCL-2 inhibitors studied, 2-methoxy Antimycin A_3 showed the most promise after *in vitro* studies, however, failed to come through on that promise when tested in cultured cells. There are many more candidates in the literature: (Degterev et al., 2001) discovered a whole set of fluoroforms of BH3I-1 as well as another subset of compounds labeled BH3I-2; the group of (Kutzki et al., 2002) has modified a terphenyl scaffold to make BAK-like contacts with the BH1/BH2 cleft of BCL-X_L; the first small molecule inhibitor of BCL-2 to be published is HA14-1 identified by (Wang et al., 2000); and *in silico* screening of virtual compound libraries led

(Enyedy et al., 2001) to identify 2,5,6,9-tetramethoxy-11,12-dihydro-dibenzo[c,g][1,2]diazocine which is able to bind both BCL-2 and BCL-X_L. All these compounds have been shown to displace BH3 peptides from the BH1/BH2 cleft of either BCL-2 or BCL-X_L and most have shown some promise *in vivo* (Beauparlant and Shore, 2003), however, the mechanism of their actions must be addressed to make certain they are indeed dealing with compounds that have BAD BH3-like properties.

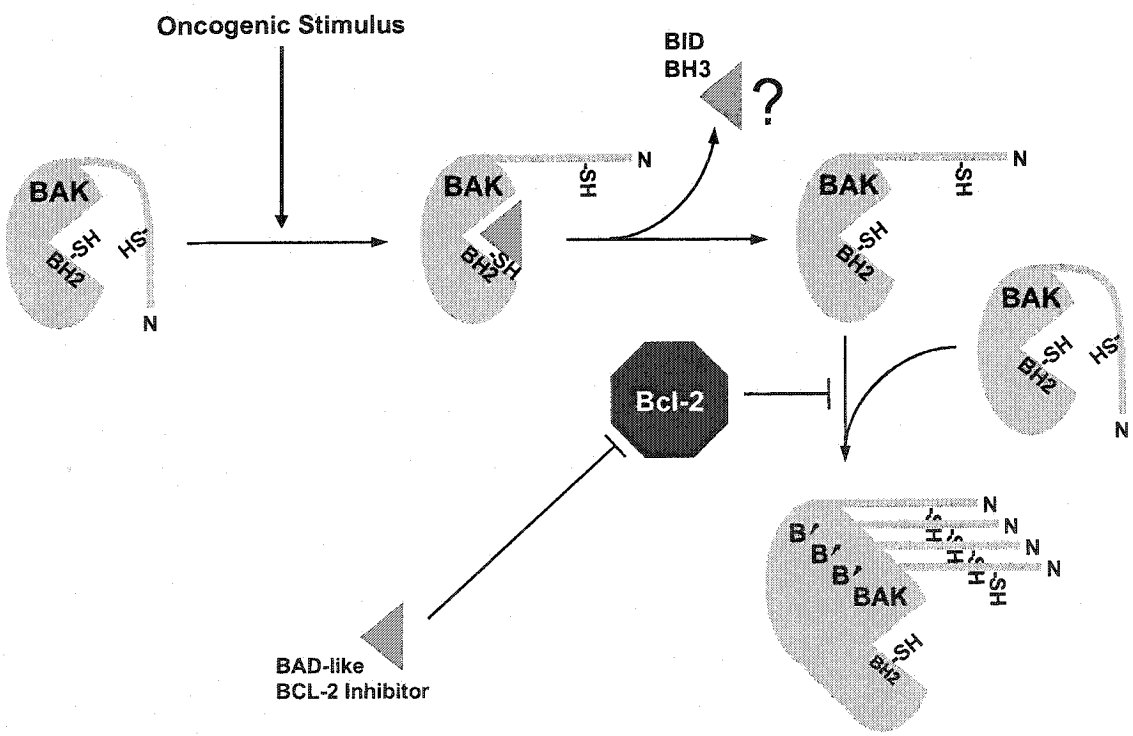


Figure 5.1 Model of co-operation between a BAD-like BCL-2 inhibitor and an oncogenic stimulus

5.3.2 The Other Side of the Mechanism

From the elucidation of the mechanism we discern BCL-2 as the obvious target for cancer treatments and the BAD BH3 domain as a model for small molecule, but what about the other side of apoptosis? Can we obtain any insight on how to maybe inhibit apoptosis chemically at the level of the BCL-2 proteins? Most research on inhibiting apoptosis chemically has focused on the inhibition of activated caspases (Garcia-Calvo et al., 1998; Thornberry et al., 1994). There has been a plethora of small tripeptide compounds, of which zVAD-fmk is a member of, which mimic the caspase recognition and cleavage site and therefore, bind the active site of caspases but are not processed and some even chemically alter the caspases to permanently inactivate them. However, due to the number of caspases and many having different cleavage site specificities makes it quite troublesome to inhibit all caspases with a single compound. The reverse strategy of administering a multitude of caspase-specific compounds seems cumbersome and could give rise to that many more side effects. With the discovery of the BIR domains in the IAP family of caspase inhibitors (Hozak et al., 2000), many groups are now focusing on finding or engineering compounds that will mimic this small domain. However, the presence of active caspases, especially if they are the effector caspases, would indicate that the cell is very close to death and this seems like quite a late step to be inhibiting them though it may be very effective.

From the models of both BAX and BAK activation there may be a means of inhibiting apoptosis by preventing the activation of downstream caspases. The possibility may lie within the NH_2-terminal regions of both BAX and BAK. From double knock-out studies, BAX and/or BAK have been shown to be absolutely required for the progression

of apoptosis induced through a variety of signals (Wei et al., 2001; Zong et al., 2001). Results have clearly shown that the ART domain of BAX is required to inhibit BAX from targeting itself to the mitochondria (Goping et al., 1998). The NH_2-terminus of BAK is also obviously important since its deletion prompts BAK to form oligomers spontaneously.

From flow-cytometry and cross-linking experiments, we have found no evidence to suggest that there are other proteins bound to BAX or BAK prior to activation, indicating that it is unlikely that these regions are interacting with some inhibitory protein. Presumably then, these NH_2-terminal regions are themselves having inhibitory effects on BAX and BAK, keeping them inactive until apoptosis is induced in the cell. Therefore, like the BH3 and BIR domains, searching for compounds that mimic these NH_2-terminal regions may lead to chemical means of inhibiting the activation of both BAX and BAK. Such a compound would have to have a higher affinity for BAX or BAK than the BID BH3 domain but should not induce any activation of either protein. As a consequence of inhibiting activation, it is likely to inhibit oligomerization, cytochrome c release and the activation of downstream caspases. This type of treatment, however, would have the shortfall of inhibiting only the apoptotic pathways that require a mitochondrial amplification step and would be completely ineffectual in preventing apoptosis in Fas treated type I cells, for example.

Should there be an inhibitory protein bound to the NH_2-terminus of either protein, then chemical mimics of the NH_2-terminus may have the opposite effect, they may, in fact, displace the inhibitory protein and lead to activation of BAX or BAK.

5.4 Apoptosis and the BCL-2 Proteins: A Tale of *Two* Organelles

A lot of research has gone into the role of the BCL-2 proteins at the level of the mitochondria and for good reason: cytochrome c release, as well as the release of Smac/DIABLO, from the inner membrane space is a key amplification step for many apoptotic signals. However, more and more evidence is surfacing showing that most apoptotic signals affect both the mitochondria and the endoplasmic reticulum. The evidence also suggests that there is a certain amount of cross-talk or co-operation between the two organelles in propagating an apoptotic signal. The TNFR family of receptors (TNFR, FasR, TRAIL-R1/2) all induce the activation of procaspase-8 which will process BID, leading to mitochondrial dysfunction, and BAP31, leading to the release of calcium from the ER. Both of these processes have been shown to be important in the progression of apoptosis (Nguyen et al., 2000). Oncogenic stress will lead to apoptosis via p53-dependent induction of the *bik* gene, whose product will target the endoplasmic reticulum (Germain et al., 2002; Mathai et al., 2002), but will also induce the expression of BID, BAX and can trigger the processing of procaspase-8L at the ER to its active form (Breckenridge et al., 2002), which can cleave BID and BAP31, leading to calcium efflux (Breckenridge et al., 2003). As groups continue studying other apoptotic signaling pathways chances are this pattern of signal transduction through both the ER and the mitochondria will keep repeating.

In addition to BIK, Spike, and PUMA, other BCL-2 family members also play a role at the level of the endoplasmic reticulum. Both BAX and BAK translocate to the ER in response to certain apoptotic signals, though not in large amounts (Scorrano et al., 2003; Zong et al., 2003). Do they create pores in the ER to facilitate the efflux of

calcium or do they modulate an existing pore? Either way, the two proteins play a role in maintaining calcium levels in the ER since knocking both out effectively reduces the amount of steady-state calcium present in the lumen (Scorrano et al., 2003). BCL-2 is also present at the endoplasmic reticulum where it interacts with BAP31; does it play a role in inhibiting p20-induced calcium release or is there to inhibit BAX and BAK translocation and/or function? Can BIK or BAD or any other BH3 only protein interact with BCL-2 at the endoplasmic reticulum to propagate an apoptotic pathway? There are still too many questions to be answered in relation to the functions of BCL-2 proteins even at the level of the mitochondria. There are only theories as to how cytochrome c and Smac/DIABLO are released from the inner membrane space whether it is through BAX/BAK pores or the BAX/BAK-induced opening of the MPTP or maybe some yet undefined mechanism. Is insertion of BAX into the outer mitochondrial membrane spontaneous, or is it dependent on mitochondrial import protein? There has been some suggestion that the lipid compositon of the mitochondrial outer membrane may have some influence on the targeting and possibly the insertion of various pro-apoptotic protein (Kuwana et al., 2002; Lutter et al., 2000; Lutter et al., 2001). Unpublished preliminary data we have generated indicates that there is a trypsin sensitive component on the surface of the mitochondria that is required for insertion of BAX into the outer membrane. What that protein or proteins could be, however, is still unknown.

To date, research on BCL-2 family members has led to very interesting avenues for treatments of aberrant apoptotic pathways. With the discovery of new pathways involving the function of BCL-2 family members at the ER, it is entirely possible that new targets will emerge. And who knows, maybe the key in the battle against cancer

may not be a single miracle drug but may lie in a combination of drugs which targets both the mitochondria and the endoplasmic reticulum to induce apoptosis.

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Original Contributions to Knowledge

1. Identified BID is the caspase-activated factor required for BAX insertion into the outer mitochondrial membrane.
2. Documented that tBID alone can induce BAX translocation to the mitochondria and integration in the absence of any other cytosolic factor.
3. Demonstrated that the requirement for Bid in Bax integration can be bypassed in mouse embryo fibroblasts in response to TNFα or E1A, though Bid is still required for cytochrome c release.
4. Documented that Bax insertion into the mitochondrial outer membrane and cytochrome c release can be uncoupled in those same cells.
5. Demonstrated that a synthetic peptide representing the BH3 domain of BID can mimic the effects of tBID in releasing cytochrome c from mitochondria.
6. Demonstrated that tBID induces a change in conformation of the NH_2-terminus of BAK which is not inhibitable by BCL-2
7. Demonstrated that the BH3 domain of BID will interact with BAK in the presence or absence of BCL-2.
8. Demonstrated that BCL-2 preferentially binds the BID BH3-induced open (active) conformer of BAK and inhibits the formation of oligomers.
9. Demonstrated that the open conformer of BAK can induce the oligomerization of closed BAK molecules.
10. Documented that a BAD BH3 peptide can synergize with a BID BH3 peptide to relieve the inhibition of BAK by BCL-2 and induce release of cytochrome c.

11. Determined that small molecule inhibitors of BCL-2 can be classified into at least two separate categories: BID-like inhibitors and BAD-like inhibitors.

Appendix