REGULATION OF CYTOCHROME C RELEASE IN UV-INDUCED APOPTOSIS

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DEDICATION

I would like to thank the members of my Graduate Committee who have not only provided consistent advice, but also support during my time in lab. I of course also need to extend a special thanks to my mentor Xiaodong Wang, whose enthusiasm and dedication to science is so sincere and heartfelt that he inspires all of those around him. And although there are no awards to commemorate it, Xiaodong is a truly good person who cares about the people in his lab - an achievement that deserves special mention. Deepak Nijhawan, Min Fang, Lai Wang and Xuejun Jiang are other members of the lab who deserve special mention for their contributions to my work, my thinking, and my life. And last, but most importantly, I thank my parents for the years of unrecognized hard work that they poured into their children to give us the opportunity to follow our dreams; my in-laws, who have pulled through brilliantly in all times of need and treated me like part of their family; Jenn, more than just my wife, my true soulmate and friend; and my son Noah, for as much sleep as I've lost and time I've spent worrying about you, I would do it all again, and more.

REGULATION OF CYTOCHROME C RELEASE IN UV-INDUCED APOPTOSIS

by

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Apoptosis, or programmed cell death, is vitally important for maintaining cellular homeostasis. When damaged cells do not appropriately enact the cell death program they have the potential to accumulate genetic mutations and become cancerous. Therefore, a mechanistic understanding of how cells decide to die would provide the foundation for drug discovery to specifically target cancer cells. Current genotoxic chemotherapeutics, and other sources such as ultraviolet (UV) radiation, damage DNA, leading to induction of apoptosis through the mitochondrial pathway. Cytochrome c is released from the mitochondria, binds dATP and Apaf-1, and together they form a structure called the apoptosome. The apoptosome then binds and activates caspase-9, which cleaves caspase-3 and other caspases resulting the morphological changes characteristic to apoptosis. To understand how UV causes apoptosis, an assay was developed to reproduce cytochrome c release *in vitro*. This assay revealed that UV radiation causes a rapid decrease of the anti-apoptotic protein Mcl-1 in HeLa cells. Reduction of Mcl-1 on the mitochondria causes *in vitro* release of cytochrome c from mitochondria three hours before *in vivo* release of cytochrome c is observed, i.e. the mitochondria are primed to release cytochrome c. Removal of Mcl-1 is required for UV-induced apoptosis, but it is not sufficient to induce apoptosis. This means that, in addition to mitochondrial priming, there is another required event, a second hit. It was reasonable to

think that this required second hit might be bound specifically by Mcl-1. To that end, Mcl-1 was found to bind Bim_{EL} with far greater affinity than any other pro-apoptotic Bcl-2 family member. In addition, Bim_{EL} is dephosphorylated after UV in an ERK1/2-dependent manner. Despite perfectly fitting the profile of the second hit, Bim_{EL} and ERK1/2 phosphorylation do not have any affect on induction of caspases after UV. These results have helped gain insight into the regulation of cytochrome c release, which remains the most perplexing and important question in apoptosis.

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BH – Bcl-2 homology

CHX – cycloheximide

ERK – extracellular signal-regulated kinase

MAPK – mitogen activated protein kinase

Mcl-1 – myeloid cell leukemia

MEF – mouse embryonic fibroblast

MEK – MAPK or ERK kinase

zVAD.fmk – benzyloxycarbonyl-Val-Ala-$_{DL}$-Asp-fluoromethylketone

CHAPTER ONE
Introduction

FROM MORPHOLOGY TO MECHANISM

Physiologic Cell Death Gets a Name

The term apoptosis was originally coined from microscopic observation of cells dying in the liver (Kerr et al. 1972). Apoptosis derives from Greek, and refers to leaves falling from a tree. However, this is not a metaphor to autumn, but rather refers to the process where occasionally some leaves fall from a healthy tree -- just as they observed that a small number of cells are always dying, even in a healthy liver. This implies that cell death is a normal aspect of physiology and a regulated, biological process.

Figure 1-1

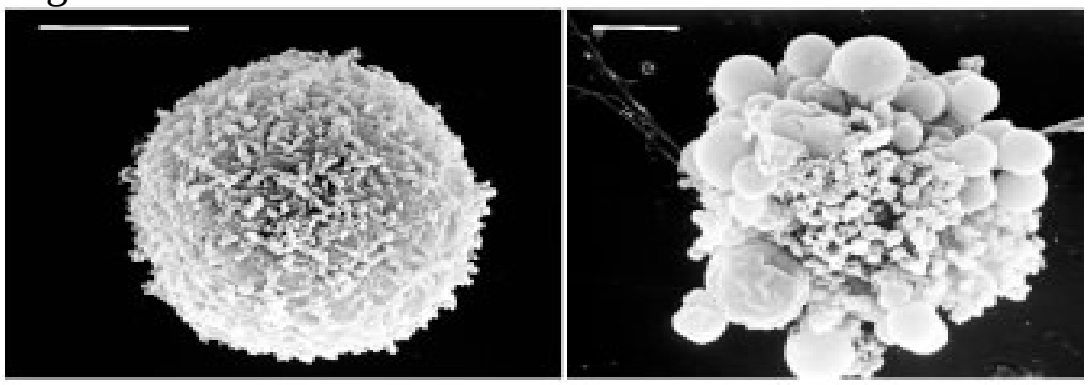

Figure 1-1 Scanning electron micrograph of a cell undergoing apoptosis. The prominent morphological features are: cell shrinkage, chromatin condensation, and membrane blebbing that eventually forms apoptotic bodies.

In their study, they defined the microscopic characteristics of apoptosis as: cell shrinkage, chromatin condensation and membrane blebbing (see figure 1-1) to form apoptotic bodies that are absorbed by the surrounding cells in a process called phagocytosis. Far from traumatic, apoptosis is a very orderly process in which a cell eliminates itself without any inflammation and is absorbed by surrounding cells. This prompted two very important questions: what are the proteins involved in cell death and how does a cell make the decision to die?

C. elegans provides a genetic model to investigate cell death

In the 1970's, Sydney Brenner was promoting the use of the nematode *C. elegans* as a model organism for genetic study. One of the many benefits of this organism for research is that development follows an exact program of cell division and lineage from a single cell to the full-grown organism. Robert Sulston and Robert Horvitz also began working with *C. elegans* in the 1970's and contributed to the complete lineage mapping of every cell division from the single-celled egg to the 1090 cells of the adult (Sulston and Horvitz 1977). It was noted that 131 of these cells are programmed to die during development. Beyond demonstrating how important apoptosis is in development, this provided the perfect genetic model with which to discover genes involved in apoptosis. The Horvitz lab introduced mutations into *C. elegans* and then monitored cell death during development to see which genes affected the 131 cells fated for death. These discoveries led to the basic genetic pathway of cell death. For their pioneering work in *C. elegans* Brenner, Sulston and Horvitz were awarded the Nobel prize in 2002.

egl-1, ced-4, ced-3 and ced-9

Three genes were defined to be required for all somatic cell death to occur: *egl-1*, *ced-4* and *ced-3*. Loss of function mutations in these genes results in the survival of the 131 cells slated for apoptosis (Ellis and Horvitz 1986; Conradt and Horvitz 1998). On the other hand, loss of function of the *ced-9* gene leads to excessive early apoptosis and embryonic lethality (Hengartner et al. 1992). And conversely, overexpression of the *ced-9* gene prevents cell death. Further genetic experiments resulted in the order of activation of genes and the following model. *ced-9* is normally bound to the protein *ced-4*, which prevents *ced-4* from activating *ced-3*. *egl-1* is transcriptionally upregulated during development and binds to *ced-9*, releasing *ced-4* which then activates *ced-3* (see figure 1-2). *ced-3* is a cysteine protease that cleaves at aspartic acid residues, thus named a caspase protein. It is the proteolytic activity of *ced-3* that results in the distinctive morphology of apoptosis: shrinking of the cell, chromatin condensation and membrane blebbing. Once *ced-3* is active, it can cleave more *ced-3*, resulting in a feed-forward cascade that rapidly activates caspases and induces cell death. The crucial step in this process is the inactivation of the protective protein *ced-9*. After *ced-9* protective capacity is gone, the cell rapidly enacts the caspase cascade that results in apoptosis. As it turns out, one of the *ced-9* homologous genes in humans had been found to be upregulated in B cell lymphoma. This gene was named the Bcl-2 gene for the B cell lymphoma it was derived from.

Figure 1-2

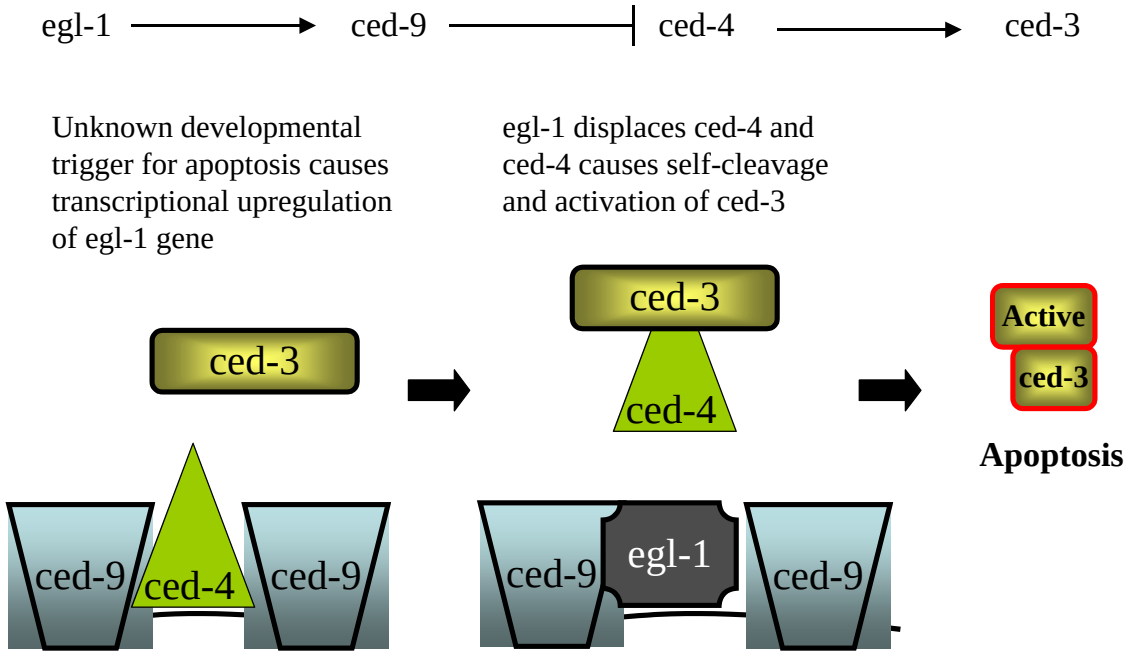

Figure 1-2 C. elegans genetic model of cell death. The egl-1 gene is upregulated by transcription during development in cells fated for apoptosis. Egl-1 binds to ced-9 releasing its inhibition of the ced-4 protein. When ced-4 is released it activates the caspase protein ced-3 which causes apoptosis.

The Bcl-2 gene was originally identified at the chromosomal breakpoint of t(14;18) and is responsible for the resulting follicular B cell lymphoma (Bakhshi et al. 1985; Cleary and Sklar 1985; Tsujimoto et al. 1985). Unlike other oncogenes discovered at the time, Bcl-2 was the first oncogene discovered that didn't promote cell growth, it instead prevented cell death following multiple stimuli (Vaux et al. 1988; McDonnell et al. 1989). This was initially a surprising function for an oncogene. It was easy to understand how genes that affected growth and proliferation, such as ras and myc, could lead to unregulated tumor growth, because it appeared to be a straightforward cause and effect. It was less obvious that blocking

apoptosis could also cause cancer. Over time however, it became clear that in most cancers both events must occur. Put simply, there is both too much growth and too little death.

Connecting the genetic discoveries in *C. elegans* and humans was the discovery that the Bcl-2 gene could at least partially complement the *ced-9* deletion (Hengartner and Horvitz 1994) in worms. Functionally then, these genes appeared to be very similar. Additionally, the observation that *ced-9* deletion mutants are embryonic lethal and that BCL-2 overexpression leads to follicular B cell lymphoma implicated anti-apoptotic proteins as important regulators of apoptosis.

Genetically then, the story seemed to fit together nicely. All the components of apoptosis in worms also appeared to be in humans. The Bcl-2 gene was homologous to *ced-9* and known to prevent cell death. There were caspase homologues of *ced-3* in humans and homologues of *egl-1* were soon to be found. How *ced-4* functioned was still quite a mystery however. In the 1990s, some surprising biochemical experiments with the human homolog of *ced-4* not only shed light on how this molecule acted to activate caspases, but also introduced a new player into apoptosis.

Biochemistry defines a mechanism for caspase activation

Caspases had been identified as the executioners of apoptosis. Their activity is responsible for the defining morphology that led to the genetic experiments in *C. elegans*. In worms the responsible caspase is *ced-3*. In humans there are many caspases, but one of the

most abundant is caspase-3. It provides a good marker for caspase activation and was used in Xiaodong Wang's lab to biochemically purify proteins involved in caspase activation.

Apaf-1, cytochrome c and caspase-9 form the apoptosome

Xiaodong Wang discovered that incubating a cytosolic protein preparation (called S100, the supernatant from a 100,000G spin) could cause caspase-3 cleavage. Using this assay, three Apaf proteins (apoptotic protease activating factors) were biochemically purified. Apaf-1 turned out to be the human homolog of *ced-4* (Zou et al. 1997), which was consistent with *C. elegans*. The other two Apaf proteins were cytochrome c and caspase-9 (Liu et al. 1996; Li et al. 1997). The involvement of cytochrome c was met with a great deal of surprise and skepticism. Cytochrome c is an electron transport molecule located in the intermembrane space of the mitochondria and involved in oxidative phosphorylation; its abundance and prominent role in energy production made it an unlikely molecule to regulate cell death. And yet, all three proteins were needed to get caspase activation *in vitro*.

More precisely, the activation of caspases in mammals is as follows. Apaf-1 contains a caspase-recruitment domain (CARD), a nucleotide-binding domain, and multiple WD40 repeats. Cytochrome c first binds to the WD40 repeats of Apaf-1, and this increases Apaf-1's affinity for dATP about 10 fold (Jiang and Wang 2000). The binding of dATP triggers the formation of a seven-spoked helical wheel shape called the apoptosome (Acehan et al. 2002). Apoptosome formation exposes the CARD domains of Apaf-1. Procaspase-9 is then recruited to the exposed CARD domains of the apoptosome. It is thought that this structure can induce

a high local concentration of pro-caspase 9 that auto-activates itself to initiate the caspase cascade (Wang 2001) (see figure 1-3).

Figure 1-3

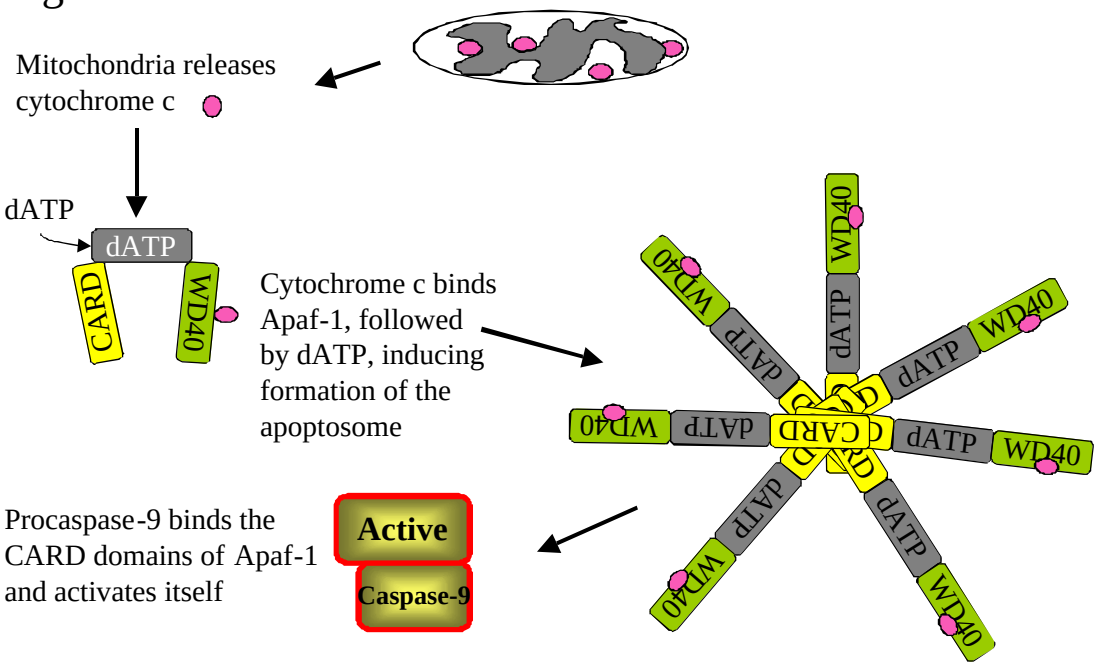

Figure 1-3 Cytochrome c is released from the mitochondria and binds to the WD40 repeats of Apaf-1. Apaf-1 then binds dATP and forms a heptamer called the apoptosome. This configuration places the CARD domains in the center of the apoptosome which can then bind procaspase-9, inducing auto-activation and initiation of the caspase cascade.

The importance of the apoptosome and the linearity of this pathway is underscored by genetic deletions in mice. The deletions of Apaf-1, caspase-9 and caspase-3 are all very similar in their phenotypes (Kuida et al. 1996; Cecconi et al. 1998; Kuida et al. 1998; Yos*hid*a et al. 1998). In all cases there is a profound lack of apoptosis, especially in neural tissues. In many cases, the cranium isn't able to close because of the excessive size of the

brain. Additionally, embryonic stem (ES) cells and fibroblasts (MEF) from these mice failed to activate caspases in response to damage signals such as UV, gamma-irradiation and treatment with chemotherapeutic agents. Although it is impossible to remove cytochrome c from a mouse and get full growth, the embryos do survive to day 8.5 (Li et al. 2000) and cells isolated from these embryos are similarly deficient to apoptotic stimuli. It is important to note however that although all of these deletions are defective in apoptosis, cells still die in response to apoptotic stimuli, death is just delayed. Obviously, once the decision is made to die, there are other events besides the release of cytochrome c and caspase activation that are involved.

These results revealed a key difference in the activation of caspases between *C. elegans* and humans. In *C. elegans*, *ced-9* is thought to directly bind and inhibit *ced-4*, preventing *ced-4* from activating the caspase *ced-3*. In mammals, there doesn't seem to be any direct interaction between Bcl-2 and Apaf-1, and Apaf-1 is activated by cytochrome c. Therefore cytochrome c has to be released from mitochondria in order to activate apoptosome formation with the cytosolic proteins, Apaf-1 and caspase-9. While this model was surprising and the subject of much debate, there was compelling evidence that the mitochondria were important for apoptosis. For one, although *ced-4* does not have WD40 repeats and does not bind cytochrome c, it is localized to the mitochondria by association with the *ced-9* protein. Similarly, Bcl-2 was found to be located on the surface of mitochondria. Once the paradigm of cytochrome c release was established it didn't take long to demonstrate that overexpression of Bcl-2 could prevent cytochrome c release. These

results also demarcated a point of no return for the cell. Once cytochrome c was released, the downstream events of caspase activation were set in motion.

This raised two questions. how is the release of cytochrome c regulated, and are there any other proteins released that affect caspase activation? Studies of other proteins released from the mitochondria revealed some surprising insights into apoptosis in another model organism, Drosophila.

Downstream Regulation of Caspase Activation and Parallels to Drosophila

In most situations, model organisms simplify the processes occurring in mammalian cells. Genetic tools can be easily applied to fewer isoforms of proteins to generate a framework upon which mammalian research can be based. This is true in apoptosis as well, however *C. elegans* and Drosophila unexpectedly revealed dramatic differences in regulation. *C. elegans* and mammalian cells both utilize the Apaf-1/*ced-4* adaptor protein for caspase activation but Apaf-1 was discovered to be activated by the mitochondrial protein cytochrome c. Likewise, the discovery of another protein released from mitochondria, Smac/Diablo (Du et al. 2000; Verhagen et al. 2000), drew parallels to regulation of apoptosis in Drosophila.

rpr, grim and hid Find a Family

Although Drosophila has homologous proteins to all of the proteins discussed thus far, the principle method of caspase regulation appeared to stem from 3 proteins that did not

have any homology to mammalian or *C. elegans* proteins: *rpr, hid* and *grim*. These three genes are encoded in a by a genomic region (H99) that eliminates cell death in response to gamma-irradiation (White et al. 1994; Chen et al. 1996). The most remarkable aspect of these proteins was their apparently complete lack of similarity to any other proteins, apoptotic or otherwise. They do have a region of similarity between them though, an IAP binding motif in the N-terminal region. This N-terminal motif binds and inhibits inhibitors of apoptosis (IAPs), a family of proteins that inhibit active caspases. IAPs were initially discovered as baculovirus-encoded proteins that suppressed apoptosis in insect cells (Clem et al. 1991). But IAPs are more than just inhibitors of active caspases, they are important regulatory molecules in Drosophila. The Drosophila IAP, DIAP, binds to active initiator caspase Dronc -- homologous to *ced-3*/caspase-9 -- through its BIR (baculovirus IAP repeat) domain. Expression of *rpr, grim* or *hid* can displace Dronc from DIAP and allow the active Dronc to initiate the caspase cascade and apoptosis. Mammals also have IAPs, but their role in regulation of apoptosis regulation is not known. Whereas the main level of regulation for *C. elegans* and mammals is at *ced-9*/Bcl-2 preventing activation of caspases, in flies there are already active caspases and regulation stems from removing the DIAP brake holding those active caspases in check. Thus, Drosophila has a distinct way of regulating apoptosis and at the time seemed to have a distinct set of proteins for that purpose. The discovery of Smac in mammalian cells changed that view.

Smac inhibits IAPS in Mammalian cells

Smac is another intermembrane space protein that is released from the mitochondria with cytochrome c (Du et al. 2000; Verhagen et al. 2000). Like other mitochondrial proteins, Smac contains a mitochondrial targeting sequence at its N-terminus that is cleaved upon import into the mitochondria. After cleavage, the first four amino acids are Ala-Val-Pro-Ile (AVPI). When Smac is released from the mitochondria these four amino acids are responsible for binding to the BIR domain of IAPs (Chai et al. 2000). IAPs are thought to serve as a protective buffer to accidental caspase activation because of the feed-forward mechanism of caspase activation. Therefore, even small amounts of accidental caspase activation would have catastrophic consequences without IAPs to block active caspase. During apoptosis, cytochrome c causes caspase activation through Apaf-1 and caspase-9 while Smac acts cooperatively by removing IAP inhibition to those active caspases (see figure 1-4). Functionally, this arrangement makes sense since both molecules are tucked safely away in the mitochondria and their release together allows for full caspase activation.

Figure 1-4

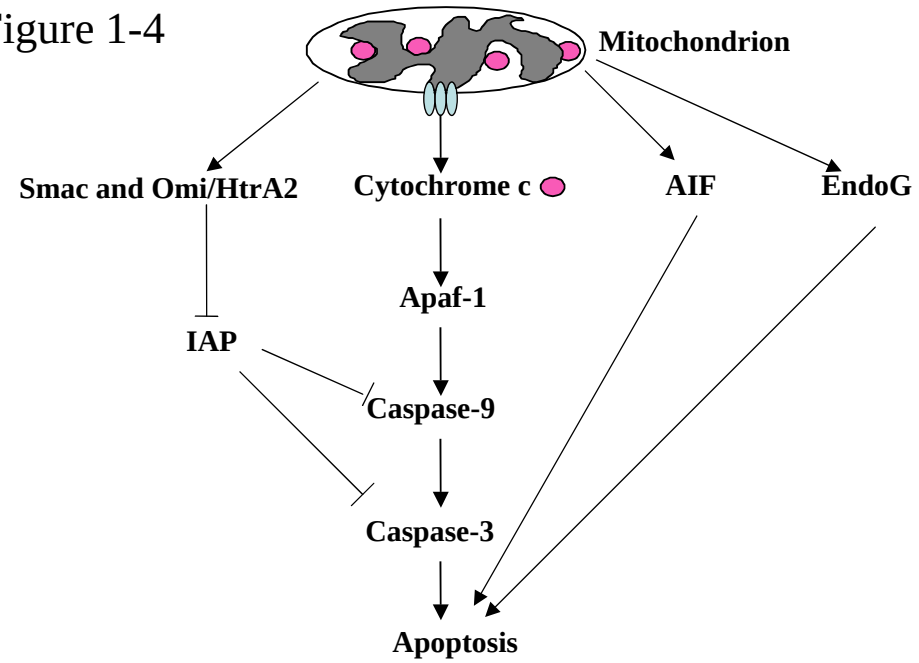

Figure 1-4 Smac is released from mitochondria and works cooperatively with cytochrome c activation of caspases by removing inhibition of IAPs. Other molecules released from mitochondria include Omi/HtrA2, AIF and endoG.

Discovery of Smac also shed light on the enigmatic trio of *rpr*, *grim* and *hid*. These molecules contain conserved sequence at their N-terminus that is critical for binding to DIAP. Functional studies of Smac, however, demonstrated that the first alanine is absolutely required for binding to IAPs, and the AVPI sequence is sufficient (Liu et al. 1998; Chai et al. 2000; Wu et al. 2000). This made it clear the critical portion of the protein is the first few amino acids. *rpr*, *grim* and *hid* all have an N-terminal Ala and conform to the tetrapeptide consensus of A-(V/T/I)-(P/A)-(F/Y/I/V/S). With such a small region of similarity it is easy to see why originally there seemed to be no mammalian homologs to these proteins. Function and homology is only present in the first 4 amino acids. Similarly, active Dronc and caspase-9 contain the tetrapeptide consensus sequence and bind BIR domains of IAPs. When Smac is

released from the mitochondria or *rpr, grim* or *hid* expression is upregulated, they displace active caspases by binding the same site.

Other Molecules Released from Mitochondria

Other molecules that are released from the mitochondria include Omi/HtrA2, AIF and endoG (Susin et al. 1999; Li et al. 2001; Suzuki et al. 2001) (see figure 1-4). Omi/HtrA2 also binds IAPs through a tetrapeptide consensus sequence (Hegde et al. 2002; Martins et al. 2002). AIF is a 57kD flavoprotein that is released from the mitochondrial intermembrane space and translocates to the nucleus where it causes chromatin condensation and large-scale DNA fragmentation (Susin et al. 1999). These effects are independent of caspases, which also cause DNA fragmentation and chromatin condensation. Deficiency of AIF prevents the normal apoptosis necessary for cavitation of embryoid bodies during development (Joza et al. 2001). The exact mechanism of AIF is still unknown. endoG is another molecule released from mitochondria that degrades DNA independently of caspases (Li et al. 2001).

While the function of some of these molecules is still to be determined, they nonetheless reinforce the importance that the mitochondria play in apoptosis. Studies of endoG in *C. elegans* even found that deletion of this molecule results in delayed progression of apoptosis and abnormal DNA fragmentation (Parrish et al. 2001). Although the mitochondria are less important in *C. elegans* apoptosis, they still seem to be involved to an extent. Despite differences in mitochondrial involvement between organisms, all of these mitochondrial proteins function downstream of the decision to die. Once cytochrome c and

Smac are released the cell is still going to die, whether or not caspases are activated. In mammals, the decision to die comes primarily from regulation of the Bcl-2 family.

The Bcl-2 Balance

While the mechanism of caspase activation was being worked out, the Bcl-2 family was beginning to grow, and divide. The first pro-apoptotic Bcl-2 family member, Bax, was discovered based upon its ability to interact with Bcl-2 (Oltvai et al. 1993). Bax and Bcl-2 heterodimerize *in vivo* and antagonize each other. Thus was born the idea of pro and anti-apoptotic molecules balancing each other to create a rheostat of cell health. Tip the balance by increasing the number of pro-apoptotic molecules or reducing the number of anti-apoptotic molecules, and the cell releases cytochrome c, initiating the caspase cascade and apoptosis. Although further research demonstrated that the Bcl-2 family is more complicated than just a balance of pro-death and pro-life molecules, as a general idea it has remained largely viable since 1993.

Two Types of Pro-Apoptotic Molecule

The Bcl-2 family is quite large in mammals. In addition to containing pro and anti-apoptotic members the pro-apoptotic members can be further subdivided into two sub-types based upon structure. The anti-apoptotic molecules include the founding member Bcl-2, Bcl-X_L, Mcl-1, A1 and Bcl-W. They all contain four Bcl-2 Homology domains (BH1-4, see figure 1-5) and have a membrane-spanning segment at their C-terminus.

Figure 1-5

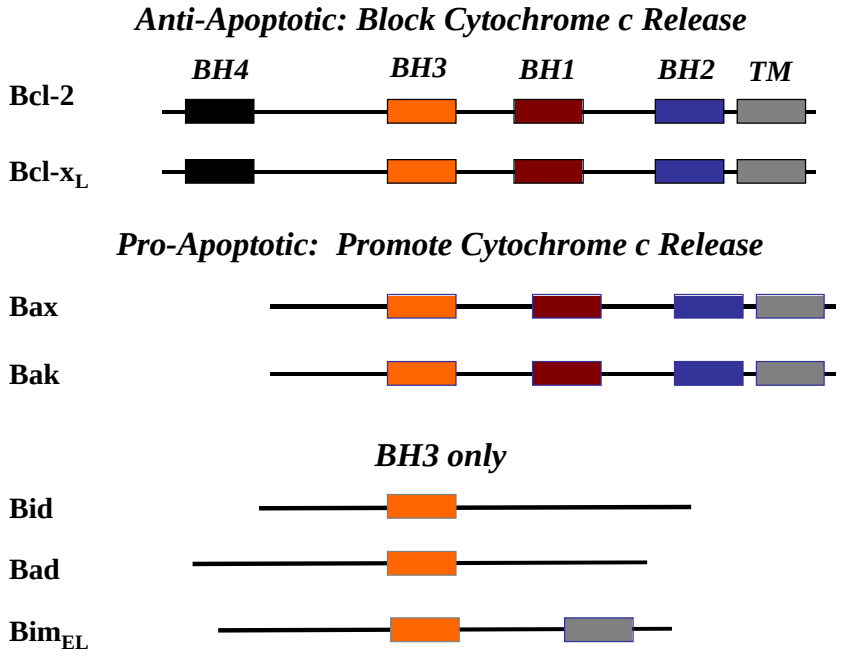

Figure 1-5 The Bcl-2 family is composed of anti-apoptotic and pro-apoptotic members. The pro-apoptotic molecules can be further subdivided into the Bax/Bak type which contain Bcl-2 Homology (BH) domains 1-3, and the BH3-only proteins typified by Bid, Bad and Bim.

Looking at the Bcl-2 family this way it is fairly clear that Bax and Bak are structurally very different from the BH3-only proteins because they have BH1 and BH2

domains in addition to BH3 domains. A functional difference was elegantly proven using the Bax/Bak double null mouse (Wei et al. 2001). Whereas deletion of either gene caused a mild phenotype, the deletion of both caused significant deficiencies of apoptosis. Cell lines derived from the mouse were resistant to multiple DNA-damaging agents that induce apoptosis, and did not release cytochrome c. Clearly, Bax and Bak are required to get cytochrome c release, but the how they release cytochrome c is still not understood.

Although not exclusively membrane bound, a large fraction of anti-apoptotic proteins are inserted into the mitochondrial membrane. Bak too, is almost exclusively located on the mitochondria. Bax however, remains in an inactive form either in the cytosol, or loosely associated with the mitochondrial membrane (Suzuki et al. 2000). Upon apoptotic stimuli, Bax translocates to the mitochondria and inserts into the membrane. Once in the membrane Bax and Bak form oligomers that can be monitored by cross-linking. Presumably, these oligomers create a channel to release cytochrome c from the intermembrane space of the mitochondria. The precise mechanism of this channel formation is still under investigation, however the combined observations that Bax/Bak oligomerization coincides with cytochrome c release (Cheng et al. 2001); genetic deletion of these two molecules blocks cytochrome c release (Wei et al. 2001); and the structural similarity of the BCL-2 family of proteins and the pore-forming helices of bacterial toxins (Muchmore et al. 1996) strongly suggest this that these molecules are at least components of the channel.

So in some form, Bax and Bak are required for the release of cytochrome c from mitochondria. This phenotype is strikingly similar to overexpression of anti-apoptotic Bcl-2 proteins that can block cytochrome c release and Bak and Bax oligomerization. Moreover,

overexpression of anti-apoptotic Bcl-2 family members blocks the formation of oligomers. Thus in order to have Bax/Bak oligomerization and cytochrome c release there either needs to be inactivation of anti-apoptotic proteins, allowing oligomerization to proceed; or, translocation of Bax to the mitochondria overwhelms the ability of the anti-apoptotic proteins to prevent oligomerization. Placing these observations into perspective, even though there are significant differences between *C. elegans* and mammals, the critical regulation of both systems seems to be in antagonizing the activity of the anti-apoptotic proteins (see figure 1-5).

Figure 1-6

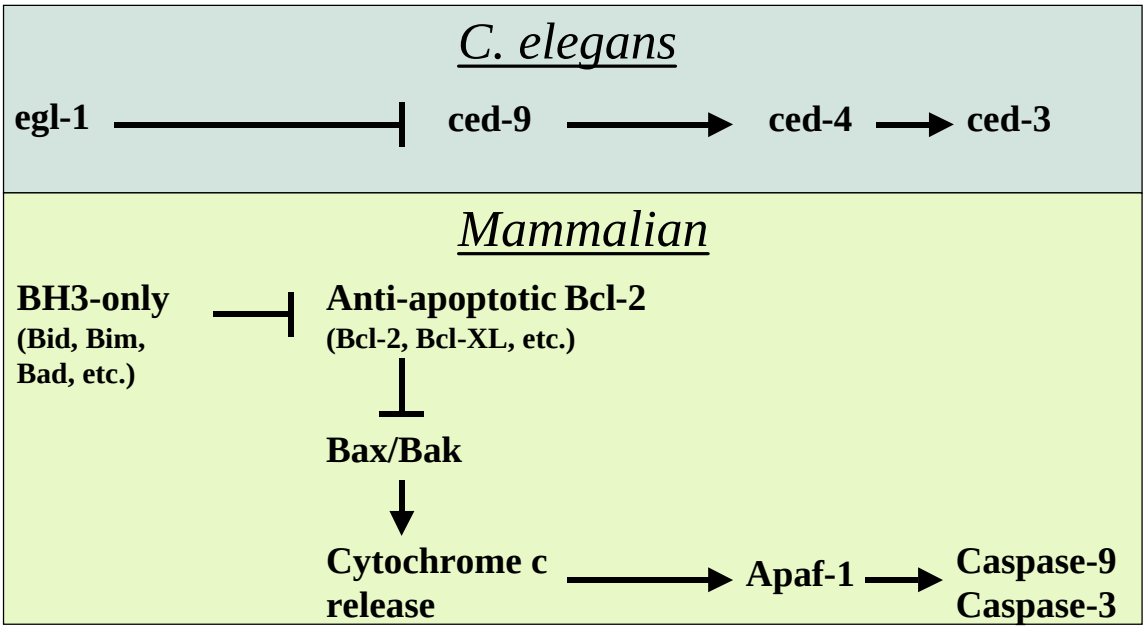

Figure 1-6 Comparison of C. elegans and mammalian pathways of caspase activation.

BH3-only

The last segment of the Bcl-2 family is the most varied and enigmatic, the BH3-only proteins. They are most similar to *egl-1* and the conserved function is immediately obvious from the above comparison of *C. elegans* and mammalian pathways of caspase activation. However with the exception of some known mechanisms of regulation that will be discussed below, how the BH3-only molecules are regulated remains quite a mystery. The proteins bear little resemblance to one another with the exception of the conserved BH3 domain they share. Additionally, they are located primarily in the cytosol, which means they either physically translocate to the mitochondria or their ability to inactivate anti-apoptotic proteins is somehow enhanced. It is also possible that some of them function in a completely different fashion. However, based upon what is known about the BH-3 proteins these two possibilities seem the most likely.

Transcriptional Regulation

In retrospect, the first BH3-only protein discovered was *egl-1*. Regulation of the *egl-1* gene is through transcription. Transcription is an ideal way to control the synthesis of such a toxic protein and it was no su*rpr*ise to find that transcription is also involved in regulation of BH3-only proteins in mammals. In fact, the best-characterized transcription factor in apoptosis is also the most famous in cancer biology, p53.

p53 has the infamous distinction of being mutated in more than 50% of all cancers (Freedman et al. 1999). Absence of p53 is often a sign of poor prognosis in cancer treatment because of p53's central role in responding to DNA damage -- one of the principal mechanisms of chemotherapeutic agents. In brief, DNA damage induces a signaling cascade

that results in degradation of the protein MDM2. MDM2 is the E3 ubiquitin ligase for p53 and controls its degradation. As the levels of MDM2 decrease, p53 becomes stabilized and increases in quantity. Since p53 is a transcription factor, as its protein level increases, it transcribes its target genes. Two of p53's target genes are PUMA and NOXA (Oda et al. 2000; Nakano and Vousden 2001; Yu et al. 2001), BH3-only genes that can cause apoptosis when present in sufficient quantity.

NOXA and PUMA once again illuminate the intimate link between apoptosis and cancer, but p53 and its downstream targets are clearly not the only players either. There are many cell lines where p53 is absent, and yet these cell lines can still undergo apoptosis in response to chemotherapeutics. In fact, the absence of p53 from tumors does not make them impossible to treat, just more difficult. Moreover, as a potential pathway to target pharmaceutically, it makes more sense to pursue a pathway that does not involve p53 since it is often mutated. So even though the p53 pathway is the textbook example of DNA damage induced apoptosis, it is certainly not the only pathway.

Bim is another molecule that is regulated by transcription, but this mechanism has only been demonstrated in neuronal cells. Bim exists in three forms: Bim_{EL} is the predominant form; Bim_{L}, which is exactly the same as Bim_{EL} with the exception of an alternatively-spliced intron that is removed; and Bim_{S}, which is the smallest, least abundant, and most potent. Two separate groups found that Bim levels dramatically increase in neuronal cell lines that were deprived of nerve growth factor (NGF) (Harris and Johnson 2001; Putcha et al. 2001; Whitfield et al. 2001). The removal of NGF induces apoptosis in a c-jun N-terminal kinase (JNK) dependent manner, and the induction of Bim could be blocked

by expression of dominant negative c-jun. As mentioned previously though, this mechanism has only been shown in neuronal cell lines. In other cells there seems to be a different mode of regulation.

Sequestration and Translocation

Bim, and the related protein Bmf, have also been proposed to function by translocation. In healthy cells Bim and Bmf are sequestered to the cytoskeleton by dynein light chain LC8. Dynein light chain is complexed with intermediate and heavy chains to form the dynein motor complex. The dynein motor complex is involved in retrograde organelle transport and moves along microtubules in an ATP-dependent manner. After a DNA damage stimulus, such as UV light, Bim translocates from the microtubule cytoskeleton to the mitochondria where it antagonizes Bcl-2 and leads to cytochrome c release (Puthalakath et al. 1999; Puthalakath et al. 2001). How this translocation is regulated remains unclear. In their original paper, Strasser and colleagues found that Bim_L and LC8 translocate to the mitochondria together, reasoning there was a disruption of the interaction between the Bim/LC8 complex and the rest of the dynein motor complex. On the other hand, Davis and colleagues found that JNK can phosphorylate Bim_L near the dynein light chain binding motif, disrupting the binding between LC8 and Bim_L (Lei and Davis 2003). Their model not only suggests that JNK regulates Bim in a post-transcriptional manner, but also that Bim_L dissociates from LC8 and moves independently to the mitochondria. Thus, the true nature of Bim regulation remains unclear.

Bad is another BH3-only protein that is regulated by sequestration from the mitochondria. Bad is phosphorylated in response to growth factors, and dephosphorylated in their absence (Zha et al. 1996). In its phosphorylated state, Bad is bound to cytosolic 14-3-3 proteins that sequester it away from the mitochondria. 14-3-3 is an abundant scaffolding protein that binds preferentially to phosphorylated substrates. Dephosporylation of Bad causes it to be released from 14-3-3 and antagonize anti-apoptotic proteins such as Bcl-X_L. This pathway connects Bad to extracellular growth factors, known to be important for promoting cell survival (Raff 1992). On the other hand, genetic deletion of the Bad gene has no real phenotype, and knock-in mouse experiments using alanine and aspartate mutations to mimic phosphorylation has only a small effect on apoptosis (Datta et al. 2002). The major effect seems to be that elimination of Bad affects the threshold for apoptosis, and over time this can lead to development of diffuse large B cell lymphoma (Ranger et al. 2003). However, there is little evidence that Bad is regulated acutely, either by phosphorylation or otherwise.

As mentioned previously, Bax has also been shown to translocate to the mitochondria (Suzuki et al. 2000) from the cytosol. Translocation of Bax has been demonstrated in many systems but its mechanism too is poorly understood. Whether or not another protein is involved in translocation is unknown.

Sequestration and translocation is a popular model for regulation because most apoptosis induced by growth factor withdrawal, DNA damage, or other damaging agents does not cause any appreciable difference in the amount of Bcl-2 family members. Sequestration from the mitochondria is one mechanism that would account for such a

finding. The total levels of the BH3-only protein don't change, but its location and therefore its activity do. On the other hand, genetic deletion of molecules such as Bim, Bad and Bax do not protect cells from apoptosis, they only delay cell death. This either means that multiple BH3-only molecules work in concert (like the Bax/Bak double knock-out) or that there are other signaling pathways involved in regulating apoptosis.

Receptor mediated cell death

Out of all the BH3-only proteins, the mechanism of Bid activation is the best understood. Bid is downstream of the so-called extrinsic pathway, also known as receptor-mediated cell death. The DNA-damaging agents that have mostly been discussed so far activate the intrinsic apoptotic pathway whose signal cascade originates from inside the cell in response to cellular damage. The extrinsic pathway on the other hand involves the immune system and selective removal of cells through molecules that are designed to activate apoptosis.

The immune system is a large set of specialized cells designed to detect invaders in the body. The immune system has to rapidly expand cell populations that are specific for fighting a specific pathogen, and then when the infection has been successfully removed, the expanded set of cells removed by apoptosis. It is disruption of this homeostasis and incomplete removal of immune cells that is defective in follicular B cell lymphoma caused by the Bcl-2 t(14;18) translocation. These cells persist because Bcl-2 prevents them from being appropriately deleted, and in doing so they have an increased chance for mutations

affecting growth in a molecule such as myc. It is the combination that leads to cancer as was elegantly demonstrated by overexpression of Bcl-2 and myc in mice (Strasser et al. 1990).

The immune system also has a more specific method to get rid of individual cells that involves receptor signaling. FasL (short for Fas ligand), TNF-alpha and Apo2L/TRAIL are three secreted molecules that can bind to receptors on cells and induce apoptosis. Fas, which is the receptor for FasL, was discovered to cause apoptosis when a monoclonal antibody that recognized Fas receptor induced cells to die upon addition to the medium (Trauth et al. 1989). Identification of the Fas receptor soon led to the discovery of a disease associated with a Fas mutation. A loss of function Fas mutation was identified in the lymphoproliferative disorder known as *lpr* (Itoh et al. 1991; Watanabe-Fukunaga et al. 1992) indicating that the loss of Fas disrupts immune system homeostasis. Similarly, the ligand for the Fas receptor, FasL, is also associated with a disease called generalized lymphoproliferative disorder *gld* (Takahashi et al. 1994). Together with the cloning of TNF and the TNF receptor (Tartaglia et al. 1993), the extrinsic pathway rapidly began to be illuminated.

Although the following description and figure are oversimplified, they illustrate important aspects of receptor-mediated cell death. The death receptors are activated by trimerized TNF, FasL or Apo2/TRAIL ligands, which induce a conformational change of the cytosolic C-terminal portion of their respective receptors. The activated receptor then assembles a complex on its C-terminus known as the Death Inducing Signaling Complex (DISC) (Muzio et al. 1996). The C-terminus of these receptors contains a death domain (DD) that is very similar to the caspase recruitment domain (CARD) discussed earlier in apoptosome formation (Fesik 2000). The death domains of the receptor bind to the death

domain of the adaptor molecule FADD/MORT (Kischkel et al. 1995). In addition to the death domain that associates with the receptor, FADD/MORT has an additional domain called the death effector domain (DED), which is also very similar to both the death domain and the CARD domain. The death effector domain is responsible for recruiting and activating procaspase-8 in an "induced proximity" model that is believed to be the same as the procaspase-9 auto-activation in the apoptosome (see figure 1-7). Once caspase-8 is cleaved and activated, it is capable of cleaving other downstream caspases such as caspase-3 and caspase-7. This caspase-8 activation is sufficient to kill some cells even in the presence of increased Bcl-2, called type I cells, indicating that the mitochondrial pathway is not involved. However, in type II cells, overexpression of Bcl-2 blocks apoptosis even though caspase-8 is activated indicating that the intrinsic mitochondrial pathway is involved (Scaffidi et al. 1998). This is where the molecule Bid bridges the gap between the extrinsic and the intrinsic pathway.

Figure 1-7

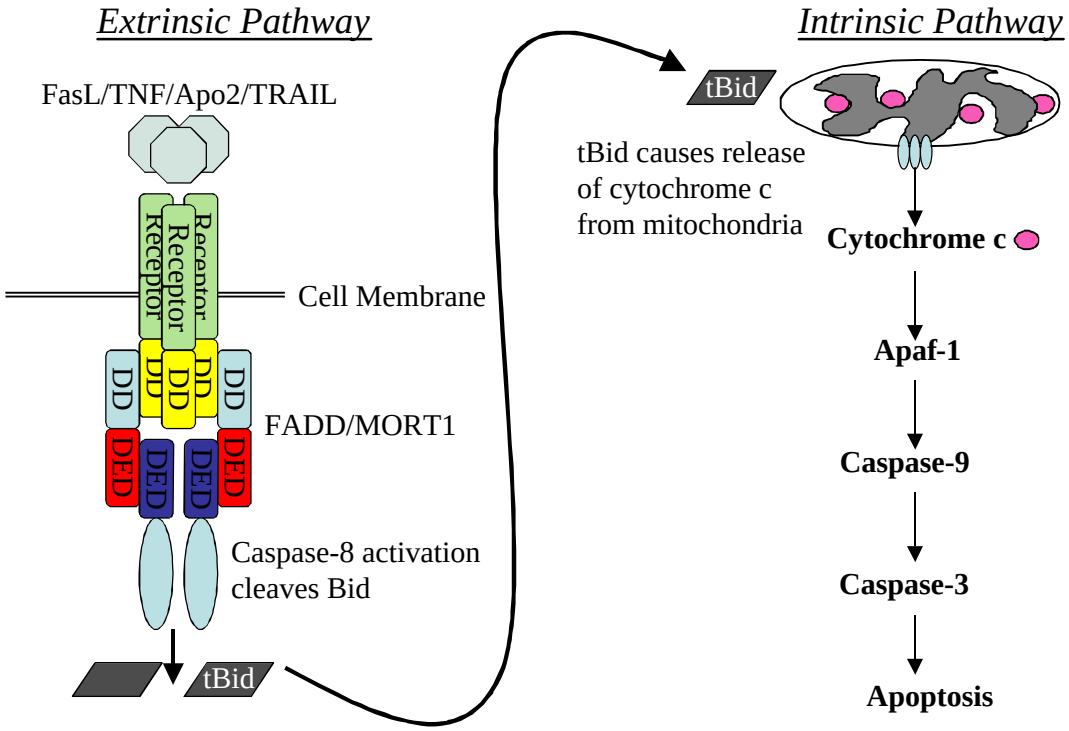

Figure 1-7 Binding of FasL, TNF and Apo2/TRAIL to their receptors induces activation of caspase-8. A conformational change in the C-terminus causes the adaptor protein FADD/MORT1 to associate via its death domain (DD) which then induces binding of procaspase-8 to its death effector domain (DED) and activation. Active caspase-8 cleaves Bid which then translocates to the mitochondria and causes cytochrome c release.

Bid is a BH3-only protein that is present as a soluble molecule in the cytosol. The extrinsic pathway produces active caspase-8 that can cleave Bid into the smaller 22kD form known as tBid (Li et al. 1998; Luo et al. 1998). The newly exposed glycine of tBid is N-myristolated (Zha et al. 2000) and it translocates to the mitochondria, releasing cytochrome c. The cleavage of Bid increases its potency at least 100 fold, which makes it a very effective mediator of apoptosis.

The importance of Bid is underscored by its genetic deletion in mice. Similar to other BH3-only knock out mice there is no gross phenotype, indicating that other BH3-only

proteins most likely can compensate for any role Bid has in development. But hepatocytes from the Bid null mice are resistant to both Fas and TNF (Yin et al. 1999). Hepatocytes are archetypal type II cells that require the mitochondrial pathway for apoptosis induced by the Fas or TNF. Genetic deletion of Bid has the same ability to block Fas-induced apoptosis as overexpression of Bcl-2, indicating both the importance of cross-talk between the extrinsic and intrinsic pathway, and of Bid itself.

The Big Picture

Despite the examples of BH3-only regulation mentioned above, by and large the mechanism by which a cell commits itself to releasing cytochrome c and apoptosis is still a mystery. Genetics has identified and stratified the Bcl-2 family and yet there is still very little known about how they function. Much of this is due to the fact that the Bcl-2 family is largely concentrated on the mitochondrial surface and this makes it very difficult for biochemistry to dissect what is happening. But events upstream of cytochrome c are the most important ones to understand in terms of intervention and disease.

Cancer is a disease in that is intimately intertwined with apoptosis. That tumors are defective in apoptosis is an almost universal, if not defining, feature of cancer. It is thought that this defect in apoptosis allows the cell to ignore regulatory mechanisms that sense the chromosome instability and overgrowth characteristic of tumors. However, it is an overstatement to say that cancer cells do not undergo apoptosis. On the contrary, tumors are constantly in a state of simultaneous growth and apoptosis. It is during this time that

additional adaptive mutations are selected, helping a localized tumor to metastasize and spread.

Chemotherapeutic drugs treat cancer by inducing apoptosis. Many chemotherapeutic drugs affect the integrity of the chromosomes by damaging DNA. For example, etoposide inhibits the enzyme topoisomerase II, which results in double-stranded breaks in DNA. The cell senses the double-stranded breaks and initiates apoptosis because the damage is too much for the cell to fix. However, all cells are susceptible to chemotherapeutics because the mechanism of action is not specific. To be effective they have to kill more cancer cells than healthy cells, but because of the effect on healthy cells the side effects are profoundly debilitating.

Understanding events upstream of cytochrome c release would lead to more specific drug targets, and avoid the toxic side effects of traditional chemotherapeutics. An example of this is the drug Gleevec. Gleevec is a drug that inhibits the bcr-abl fusion protein (Druker et al. 1996) that is responsible for chronic myelogenous leukemia. A chromosomal translocation leads to the bcr-abl fusion protein, which in turn leads to an overactive mutant abl kinase that causes cells to become very resistant to apoptosis by blocking cytochrome c release and caspase activation (Amarante-Mendes et al. 1998). Gleevec can successfully prevent leukemia in these patients without the side effects of traditional chemotherapy -- although the mutation remains (Druker et al. 2001). The biggest drawback is that the bcr-abl fusion represents a very small percentage of all cancers. Finding a more general target would help immensely to both understand and better treat this terrible disease. In order to do this, it is necessary to understand mechanistically how cells make the decision to die.

On the other hand, in many degenerative diseases there is too much apoptosis. Some particularly prevalent and frightening examples are the neurodegenerative diseases such as Alzheimer's, Parkinson's and ALS. Similarly, to better treat these diseases and prevent neurons from undergoing apoptosis, an understanding how the cell makes the decision to die will provide the best pharmaceutical targets to intervene in these diseases and prevent cell death.

How a cell decides to die is basically how a cell decides to release cytochrome c, because in general, once cytochrome c is released it is too late. The Bcl-2 proteins are clearly involved, yet their regulation is poorly understood. Such was the case at one time with the ced proteins in *C. elegans* and caspase activation. Caspase activation was elegantly dissected using a biochemical approach. For this approach to work again, it is first necessary to develop an *in vitro* assay for cytochrome c release and purify the proteins involved.

CHAPTER TWO
An *In vitro* Assay for UV Apoptosis

ULTRAVIOLET IRRADIATION RAPIDLY INDUCES APOPTOSIS

UV Causes Cytochrome c Release *In vivo*

In order to develop an assay for cytochrome c release it is first necessary to find a stimulus for apoptosis. Many chemotherapeutics, such as etoposide, are known to induce apoptosis and provide clinically relevant models, however the cost of using these agents is significant. Ultraviolet light (UV) also rapidly causes apoptosis and it is very inexpensive and reproducible. Moreover, UV is also a physiologically relevant stimulus because it is an ever-present natural DNA-damaging source and there already exists a large body of knowledge of genes involved in responding to UV light. Absent from this knowledge though, is exactly how UV induces apoptosis.

Time Course of Cytochrome c Release and Caspase Activation

Min Fang started working on UV-induced apoptosis in 2000. He found that after a high dose of UV light, 20J/m^2, HeLa cells release cytochrome c within 4 hours and rapidly undergo apoptosis (see figure 2-1). This release of cytochrome c is not dependent on caspases because cytochrome c is still released even in the presence of zVAD, a pan-caspase inhibitor. This insures that cytochrome c release is not downstream of Bid cleavage. Concurrent with cytochrome c release, Bak is oligomerized on the surface of the

mitochondria. All of these events are already well documented but are important in establishing the timeline for HeLa cell death in response to UV. There is some cytochrome c release and caspase activity at 2 hours with robust caspase activation and cytochrome c release at four hours. Even a small amount of caspase activation can become amplified in an *in vitro* incubation and potentially cause Bid cleavage, which causes profound cytochrome c release. Thus in order to study the events upstream of Bak oligomerization and cytochrome c release it was necessary to develop an assay using cells one hour or less after UV treatment.

Figure 2-1

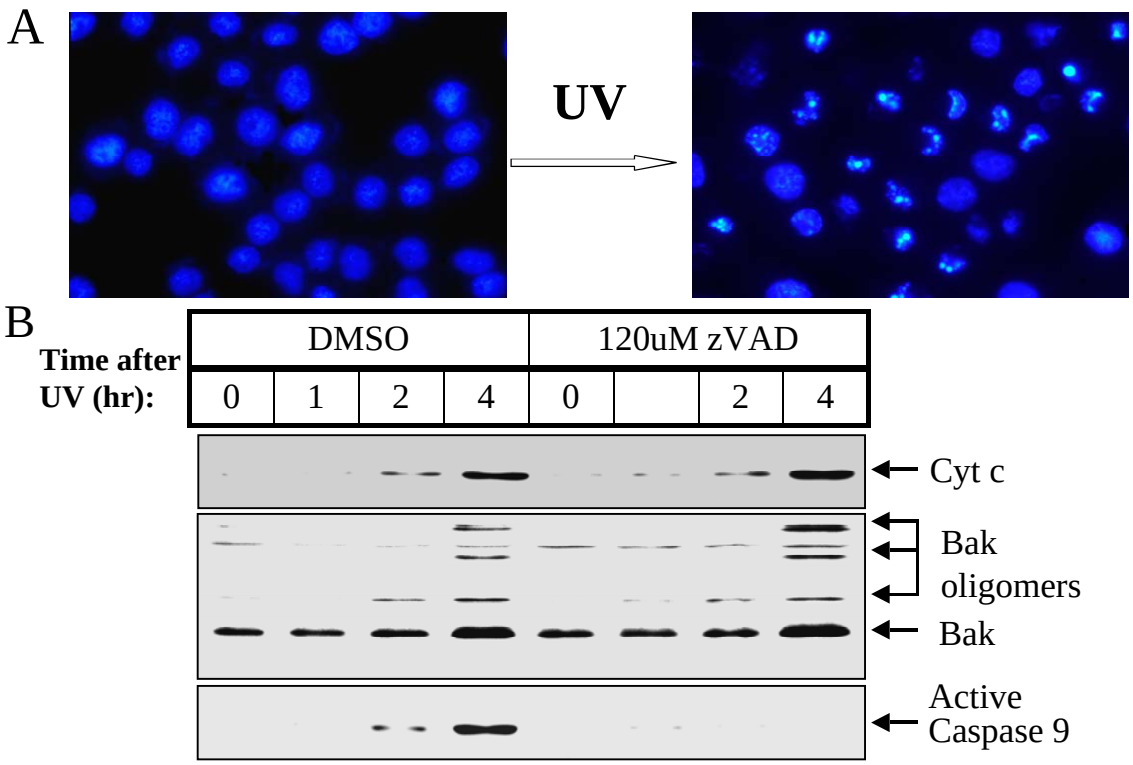

Figure 2-1 UV light induces apoptosis in HeLa cells causing: A) chromatin condensation as evidenced by Hoechst staining of nuclei; and B) Bak oligomerization and release of cytochrome c in four hours. Release of cytochrome c is upstream of caspase activation because the pan-caspase inhibitor has no effect on cytochrome c release or Bak oligomerization. Slide courtesy of Min Fang.

In vitro Assay

Min Fang treated HeLa cells with UV and waited 30 minutes, one hour and two hours before breaking the cells with a needle and syringe. The cells are broken in the presence of sucrose to ensure the integrity of the mitochondria. After breaking apart the cells, the resulting cell soup is incubated at 37 degrees for one hour after which the mitochondria, nuclei and other organelles are pelleted by centrifugation and the remaining supernatant is used for western blot of cytochrome c (see Figure 2-2).

Figure 2-2

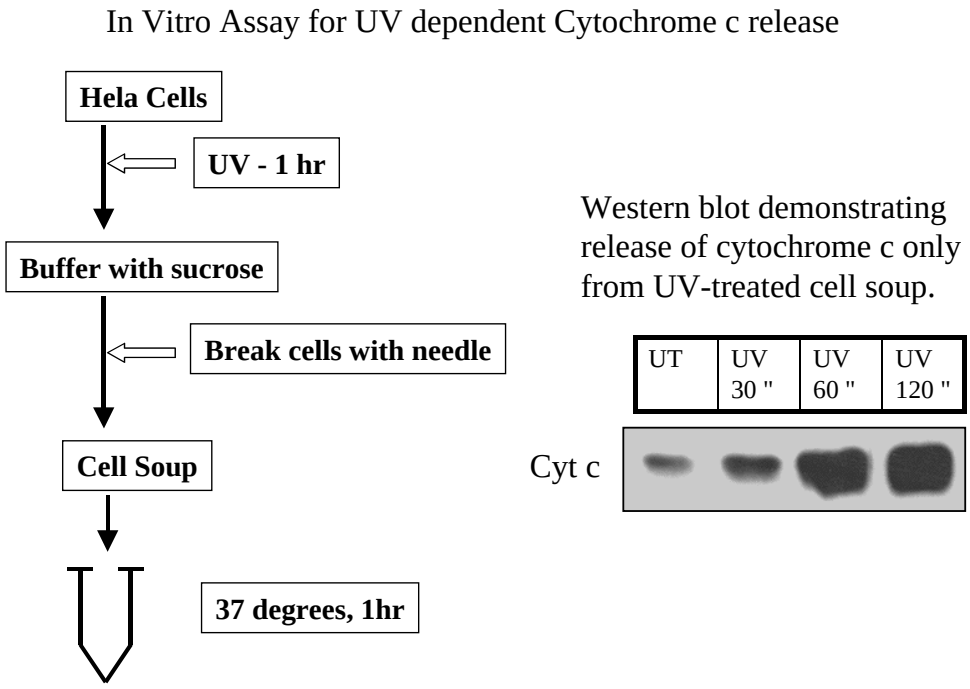

Figure 2-2 The following schematic represents the in vitro assay developed by Min Fang for looking at release of cytochrome c after UV.

Even as early as 30 minutes after UV treatment, the UV cell soup releases more cytochrome c than untreated cells. More importantly, this difference in cytochrome c release

between untreated and UV cell soup reproduces what happens three hours later *in vivo*. This is the starting point from which the *in vitro* assay was developed.

Cytoplasmic Inhibitor and Primed Mitochondria

To further understand which components of the cell are involved in release of cytochrome c, a further fractionation was necessary. The cell soup contains mitochondria, nuclei and all other organelles in a mix with cytosolic proteins. The first obvious fractionation involved separating the mitochondria -- where the cytochrome c is released from -- from the rest of the cellular components. When the mitochondria were isolated and incubated alone in sucrose buffer, there was increased cytochrome c release in the mitochondria derived from UV-treated cells (see lanes 1-5, figure 2-3) as well as Bak oligomerization. Mitochondria after UV treatment were somehow "primed" by the cells to release cytochrome c. They did not however release cytochrome c *in vivo* until a much later time point (four hours, see figure 2-1), but the *in vitro* release of cytochrome c reproduced the later *in vivo* release.

Figure 2-3

Only mitochondria from UV-treated cells exhibit Bak oligomerization and cytochrome c when incubate in vitro

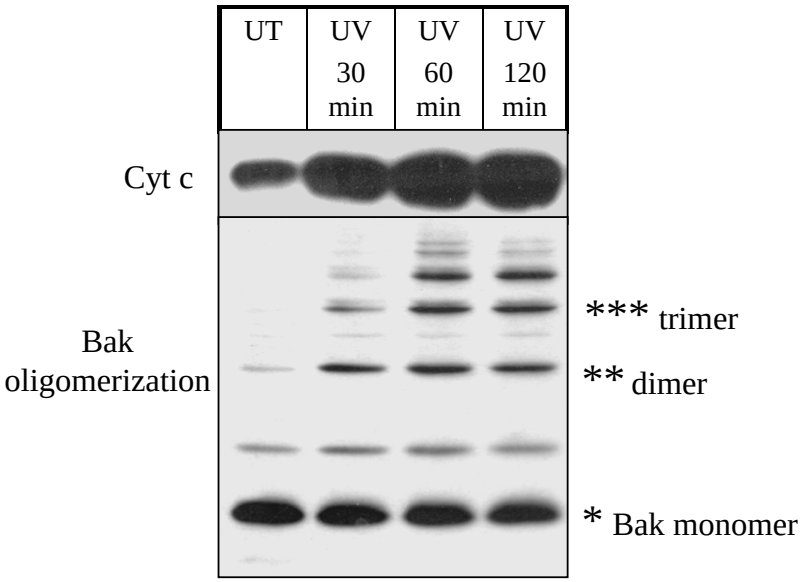

Figure 2-3 Mitochondria are primed to release cytochrome c after UV treatment. Mitochondria isolated after no treatment, or the indicated times after UV treatment only have Bak oligomerization and cytochrome c release with UV. This activity precedes in vivo release of cytochrome c by three hours, hence they are primed to release cytochrome c but do not. Slide courtesy of Deepak Nijhawan.

Next the cytosol was investigated. Primed mitochondria, defined henceforth as mitochondria isolated one hour after UV treatment, oligomerize Bak and release cytochrome c when incubated in sucrose buffer alone. If cytosol from untreated HeLa cells is added to primed mitochondria, Bak oligomerization and cytochrome c release are blocked (see lanes 1 and 2, figure 2-4). Thus there is an inhibitor present in the cytosol that is capable of blocking cytochrome c release from primed mitochondria. Further, this inhibitor is removed after UV treatment as evidenced by the addition of UV-treated cytosol to primed mitochondria having no effect on Bak oligomerization and cytochrome c release (see lanes 3-5, figure 2-4).

Figure 2-4

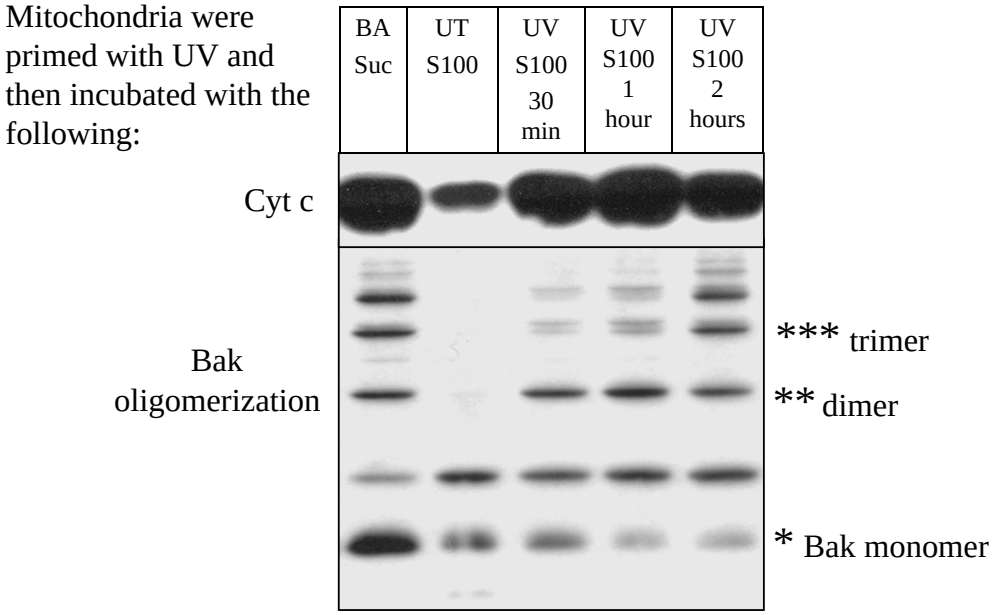

Figure 2-4 Cytosolic inhibitor is removed after UV. Primed mitochondria were either incubated alone, first lane, or with cytosol (S100) from untreated cells and UV-treated cells, lanes 2-5. An inhibitor present in the cytosol of untreated cells blocks Bak oligomerization and cytochrome c release and this inhibitor is removed by UV. Slide courtesy of Deepak Nijhawan.

Thus it seemed likely that there were two antagonizing activities present in the *in vitro* assay. A cytosolic inhibitor that prevents cytochrome c is rapidly removed from the cytosol over a short time period of about 30 minutes to 1 hour. And on the other hand, something was activating the mitochondria that allowed them to release cytochrome c (see figure 2-5). Deepak Nijhawan began working on purification of the inhibitory protein in the cytosol. However, biochemical purification of primed mitochondria was impossible because we couldn't separate out the activating factor from the mitochondria without destroying the mitochondria and releasing cytochrome c. In order to study priming we needed another approach.

Figure 2-5

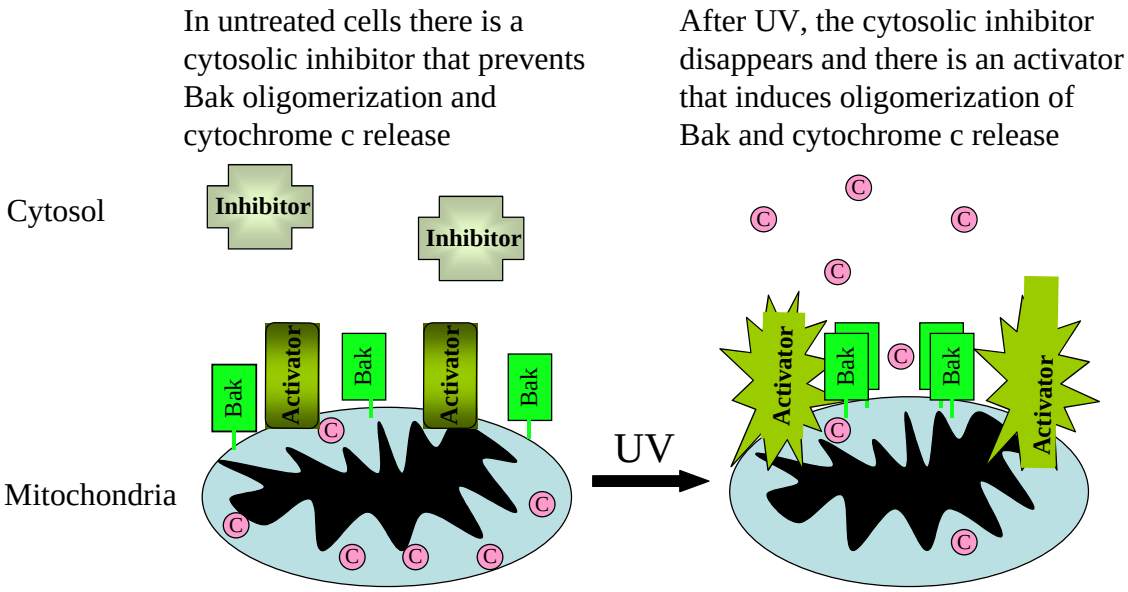

Figure 2-5 Model for cytosolic inhibitor and primed mitochondria. After UV, the cytosolic inhibitor rapidly disappears and something happens to the mitochondria that causes them to release cytochrome c when incubated in vitro.

2-Dimensional Gel Analysis of Primed Mitochondria

Functionally there was a clear difference between untreated and UV primed mitochondria reflected in Bak oligomerization and the release of cytochrome c. The method of isolating the mitochondria was the same, so there had to be some change in either the amount of proteins or the activity of proteins could be altered by phosphorylation or some other modification. A good way of investigating both of these possibilities, without making any assumptions as to what might be happening on the mitochondria, is to use 2-Dimensional (2-D) gels.

The actual process of running 2-D gels involves separating proteins first by their isoelectric point on a pH gradient, and then by size on an SDS-PAGE gel (see figure 2-6). The separation by pH gradient serves two purposes: first, it separates proteins of similar size so that they all run on top of each other in the SDS-PAGE gel; and second, proteins that are modified by the addition of phosphate groups or other modifications can be separated by pH gradient into multiple spots on a 2-D gel. The proteins on the SDS-PAGE gel can then be stained with silver and the pattern of protein spots inter*pr*eted.

Differential Labeling of Samples

One of the problems with comparing 2-D gels for untreated and primed mitochondria is that small variations between gels and isoelectric focusing can make it hard to align identical protein spots from two different gels. This makes it hard to inter*pr*et whether or not a protein has been modified in any way, a serious problem for analyzing 2-D gels. A new technique for labeling proteins overcame that problem though. Using two different fluorescently labeled maleimide dyes, it is possible to label untreated mitochondria with one dye and primed mitochondria with another, focus them on the same strip, and then run the two samples together on the same gel. This way the variation in focusing and SDS-PAGE gels is removed, and the pattern of spots can be analyzed both more easily and quantitatively.

This approach seemed like an ideal way to uncover the difference between untreated and primed mitochondria. An example of the results is shown in figures 2-6 through 2-8.

Figure 2-6

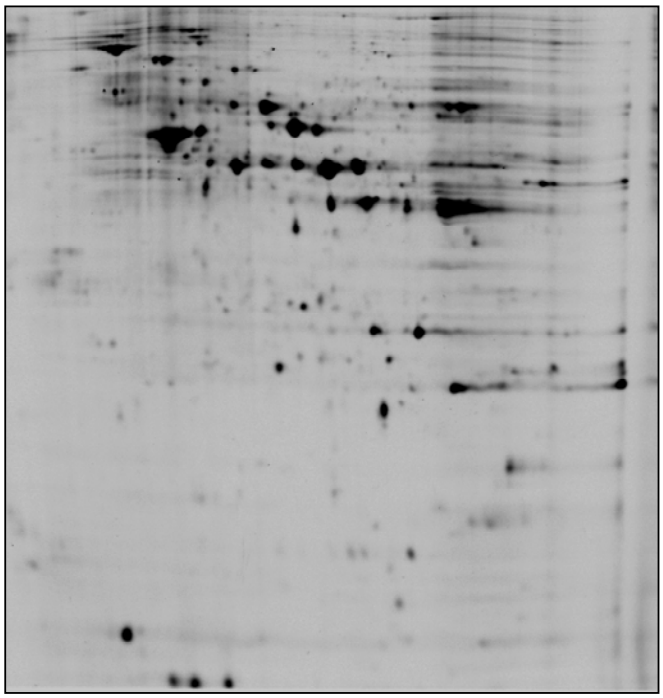

Figure 2-6 2-D gel of untreated mitochondria labeled with Cy3 dye.

Figure 2-7

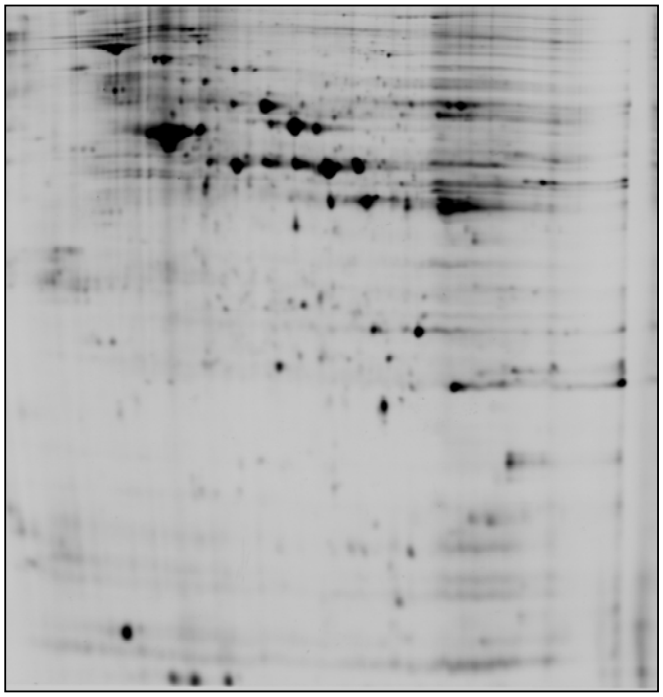

Figure 2-7 2-D gel of UV-treated mitochondria labeled with Cy5 dye.

Figure 2-8

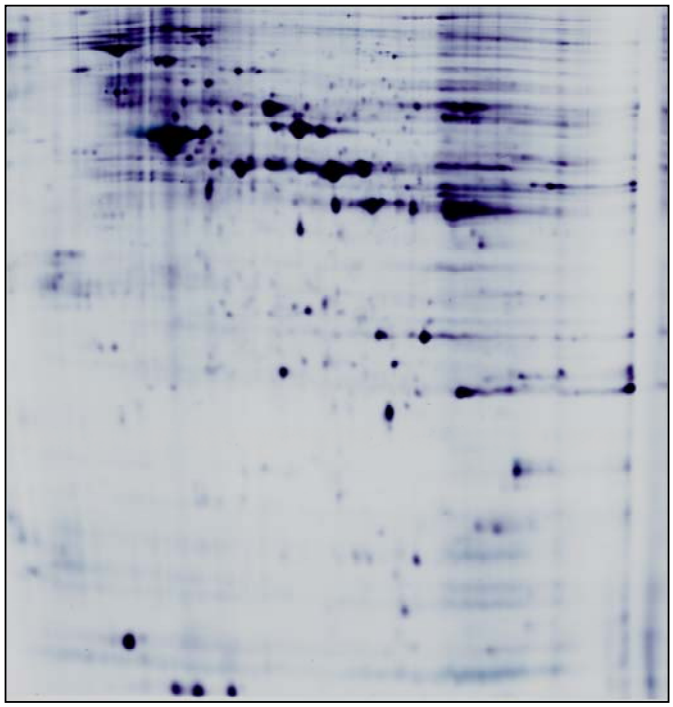

Figure 2-8 Overlay of untreated (blue) and UV-treated (red) 2-D gel images. For the most part the images are identical. No difference was determined using 2-D Analyzer software.

Although there should be some difference between untreated and primed mitochondria, there was no significant difference detected using this technique. This may be due to lack of sensitivity if the activating factor is in low abundance. Or, there may be precipitation of the activating factor during focusing. In either case, 2-D gels were not able to determine what causes priming of mitochondria. Another approach was needed.

CHAPTER THREE
An *In vitro* Assay for Priming

A NEW APPROACH

Developing a Biochemical Assay for Priming

2-D gels initially appeared to be an ideal way to study mitochondria. Biochemical fractionation of mitochondria is impossible without destroying the integrity of the organelle, which makes it impossible to then measure cytochrome c release. One potential way around this dilemma was to convert untreated mitochondria into primed mitochondria *in vitro*, and then look for cytochrome c release. Such an assay would provide an approach to purify proteins involved in priming. A schematic for such an approach is diagrammed below in figure 3-1.

Figure 3-1

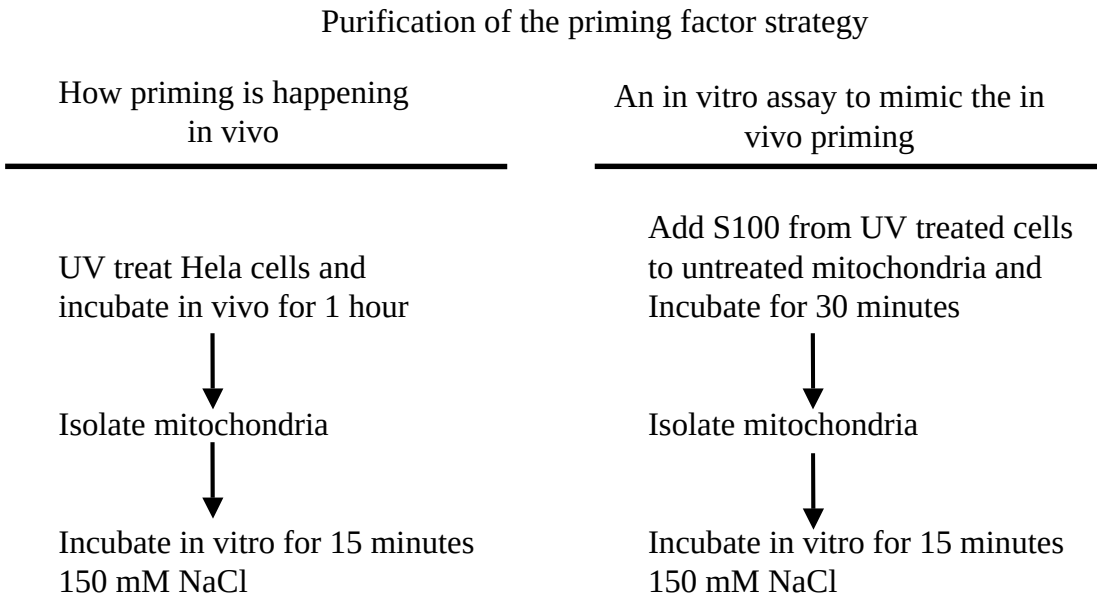

Figure 3-1 Schematic for development of an in vitro priming assay.

UV-Treated Cytosol is Capable of Priming Untreated Mitochondria

Both untreated and UV-treated mitochondria were incubated at 37 degrees with either untreated or UV cytosol for one hour. This step reproduces the hour that primed mitochondria spend in UV-treated cytosol *in vivo*. The mitochondria were then pelleted again, washed once, resuspended in sucrose buffer, and incubated at 37 degrees to assay for cytochrome c release, i.e. priming. As figure 3-1 outlines above, this is basically an *in vitro* duplication of what is happening to mitochondria *in vivo*. The results of this assay are displayed in figure 3-2 below. Control mitochondria were kept on ice while untreated and UV-primed mitochondrial samples were incubated with either untreated or UV cytosol. The mitochondria were then pelleted, washed and assayed for Bak oligomerization and

cytochrome c release. Lanes 1 and 4 are controls, demonstrating the difference in cytochrome c release between untreated and UV-primed mitochondria. Untreated mitochondria incubated in untreated cytosol don't have any cytochrome c release, as would be expected; however, when untreated mitochondria are incubated with UV cytosol, they become primed in the sense that they release cytochrome c in a manner similar to UV treated mitochondria (see lanes 3 and 4). And as discussed in the previous chapter, UV-primed mitochondria incubated with untreated cytosol do not exhibit cytochrome c release because of the inhibitor present in untreated cytosol.

Figure 3-2

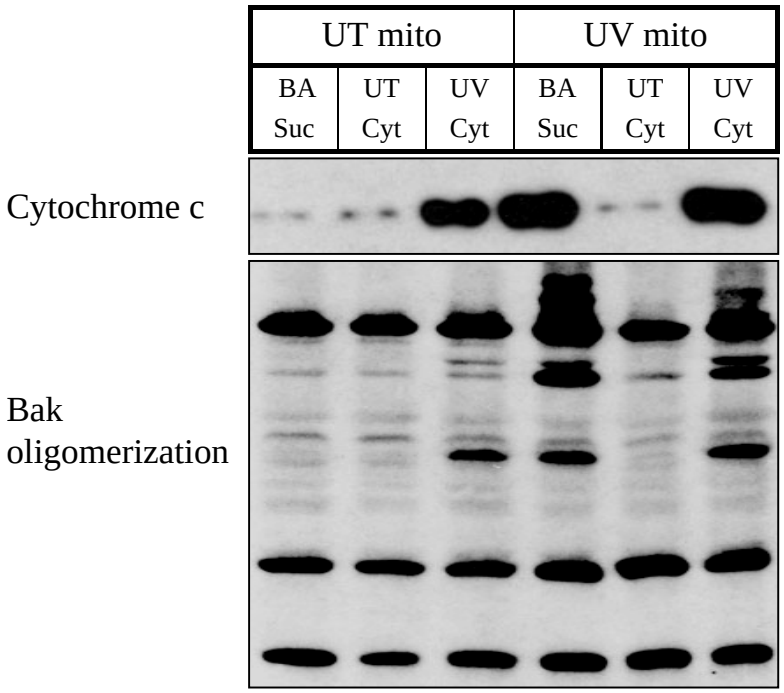

Figure 3-2 In vitro priming assay. Untreated and UV mitochondria were kept on ice in Buffer A Sucrose or pre-incubated with cytosol from untreated and UV-treated cells. The mitochondria were then pelleted, washed once and assayed for Bak oligomerization and cytochrome c release.

Purification of Activating Factor from UV-treated Cytosol

Based upon the original set of experiments by Min Fang, the model for primed mitochondria (see figure 2-5) placed the activating factor on the surface of the mitochondria. Now that there was a way to prime untreated mitochondria with UV-treated cytosol, it meant that activating factor is present in the cytosol, either exclusively or perhaps located in both cytosol and the mitochondria. Additionally, the activating factor may cause cytochrome c release solely due to the absence of inhibitor, or may become even more active after UV (see figure 3-3).

Figure 3-3

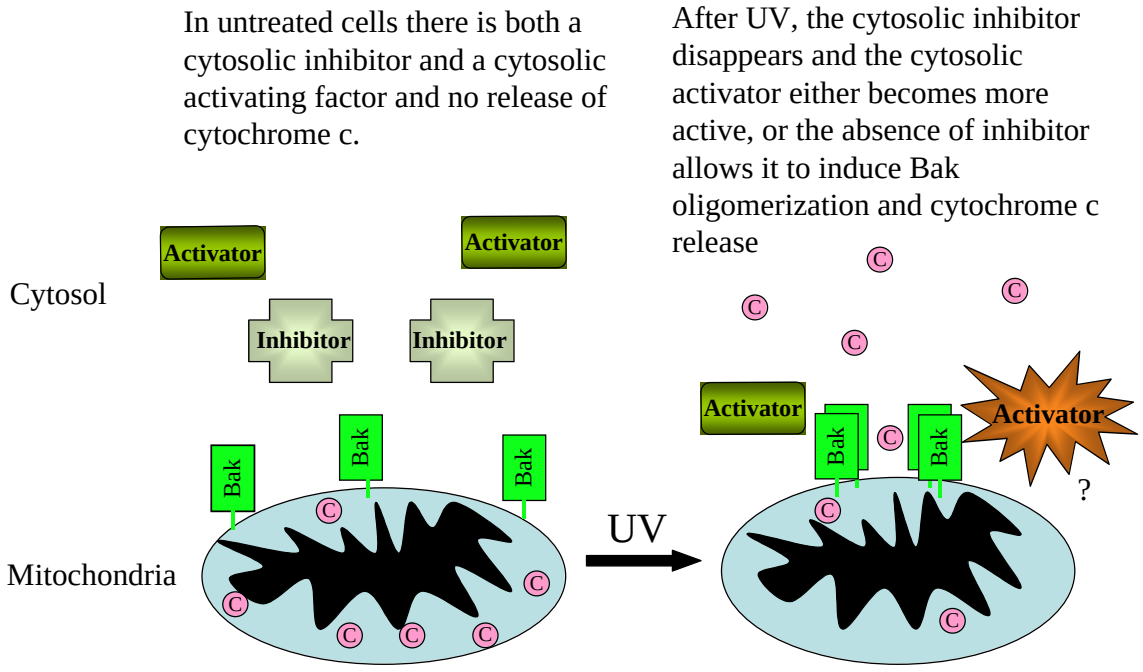

Figure 3-3 Revised model for priming based upon in vitro assay.

The combination of a cytosolic activating factor and an *in vitro* assay to prime mitochondria meant that it was possible to biochemically purify the activating factor. The only drawback to this approach was that the starting material was UV cytosol (UVS100). Since large amounts of starting material are needed for purification, the in-house production of UVS100 became the rate-limiting factor. Another time-consuming factor was doing westerns for both cytochrome c release and Bak oligomerization. Since Bak oligomerization is more straightforward and robust, that became the assay of choice.

Before starting purification, it was important to establish that it is a protein (or proteins) responsible for *in vitro* priming. To test this, untreated and UV-treated S100 were incubated at 55 degrees and 65 degrees Celsius. Many proteins denature at these temperatures, whereas salt and molecules such as NADH do not. The results of the experiment were quite interesting. Whereas control untreated and UV-treated cytosol (UTS100 and UVS100 kept on ice) have a clear difference in Bak oligomerization (lanes 1 and 2), once untreated cytosol is incubated at 55 degrees, it starts to behave like UV-treated cytosol (see figure 3-4 lanes 2 and 3). During this time, Deepak Nijhawan was purifying the cytosolic inhibitor and had determined that the inhibitor is heat-sensitive at 55 degrees. Thus it was reasonable to think that a 55 degree Celsius incubation destroys inhibitor in untreated cytosol which then allows activator to induce Bak oligomerization. This data implies that removal of the inhibitor is sufficient for priming. However, after the 55 degree incubation the UV cytosol has more activity than untreated (lanes 3 and 4). Additionally, incubation at 55 degrees appears to increase the activity of UV cytosol (lanes 2 and 4). Together, these

indicate that the activity of the activator likely increases after UV. A 65 degree incubation removes both the inhibitor and the difference between untreated and UV cytosol.

Figure 3-4

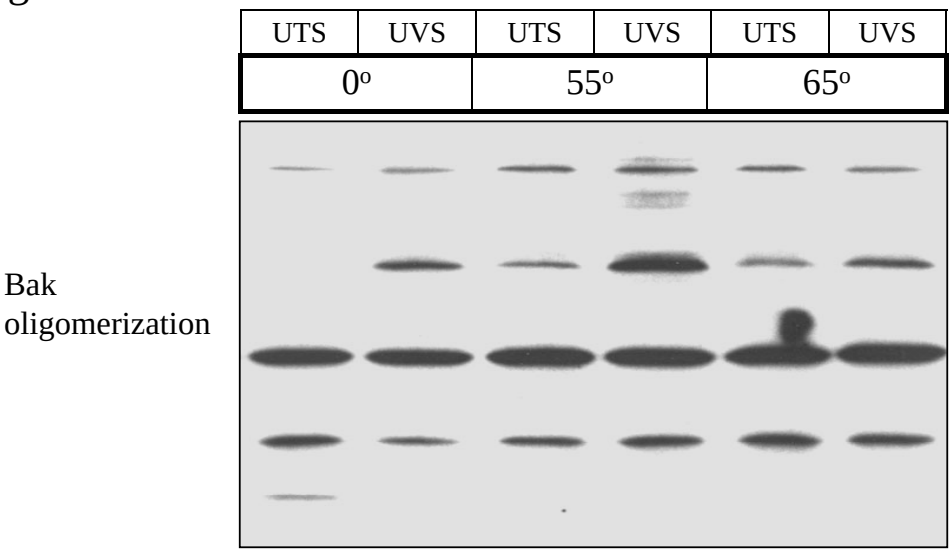

Figure 3-4 Untreated and UV-treated S100 was either incubated on ice, at 55° C or 65° C for 15 minutes. Afterwards the samples were incubated with untreated mitochondria and assayed for Bak oligomerization.

Thus, the activating factor is not heat sensitive at 55 degrees, and incubation at this temperature preserves UV specificity. The next step was to investigate the ammonium sulfate profile.

Addition of ammonium sulfate to cytosol exposes hydrophobic protein domains that results in protein precipitation. Ammonium sulfate can thus be used both to separate and concentrate protein. UV cytosol was incubated with differing amounts of ammonium sulfate and the precipitated proteins separated by centrifugation. Both the pellets and the supernatant were then dialyzed overnight to remove ammonium sulfate and assayed. The activating

factor is soluble at 30% ammonium sulfate but precipitates at 50% ammonium sulfate (see figure 3-5, lanes 4 and 5).

Figure 3-5

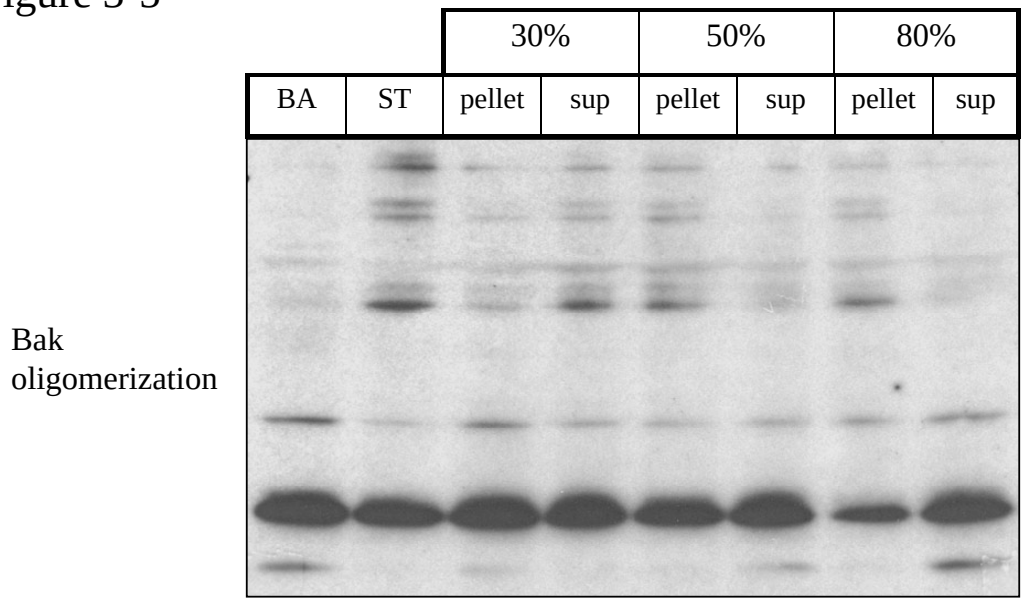

Figure 3-5 Ammonium sulfate profile of activating factor. Ammonium sulfate was added to UV cytosol at the indicated percentages and centrifuged to separate the pellet and supernatant. The pellets were resuspended in Buffer A and both pellets and supernatant dialyzed to remove ammonium sulfate. The samples were then incubated with untreated mitochondria and assayed for Bak oligomerization. As negative and positive controls, mitochondria were also incubated with Buffer A and sucrose alone (BA) and with UV cytosol starting material (ST).

To both purify and concentrate activity, ammonium sulfate was added to 30% and centrifuged. The supernatant was removed and more ammonium sulfate added to achieve a level of 50% ammonium sulfate. Centrifugation then concentrated the activating factor in this pellet, which was then run on a Superdex 200 gel filtration column. Gel filtration columns separate proteins by size and in the case of the activating factor the activity came off the column just before salt ion peak (see figure 3-6 -- the pink line represents salt and the blue line protein). This is consistent with a protein in the size range of 20-40 kD.

Figure 3-6

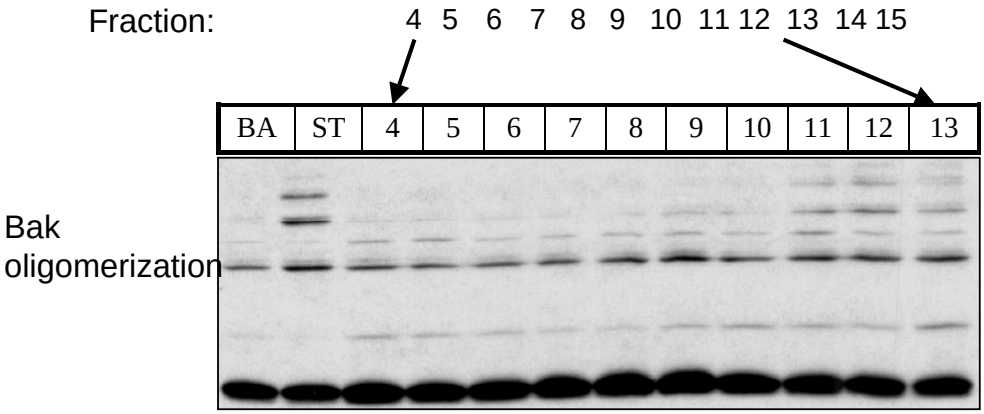

Figure 3-6 Gel filtration of activating factor. The activating factor was concentrated from U cytosol with a 30-50 ammonium sulfate cut and then run on a Superdex 200 gel filtration column. The trace in the top panel represents amount of protein (as measured by UV) as line, and salt ions as the lighter pink line (fractions 14 and 15). Activity of the fractions is demonstrated in the lower panel by Bak oligomerization. As negative and positive control mitochondria were also incubated with Buffer A and sucrose alone (BA) and with UV cyto starting material (ST).

Fractions 11, 12 and 13 were pooled, dialyzed and run on a mono Q column. The Q column binds negatively charged protein, and the interaction between protein and the column is disrupted by salt. The activating factor bound to Q and eluted from the column at about 200 mM NaCl (see figure 3-7). In this experiment the starting material did not have much activity (activity was observed to decrease with extended periods on ice or at 4 degrees, data not shown) but the Q column concentrated the activity such that the peak was in fractions 3-5 with fraction 4 having the most activity (see figure 3-7).

Figure 3-7

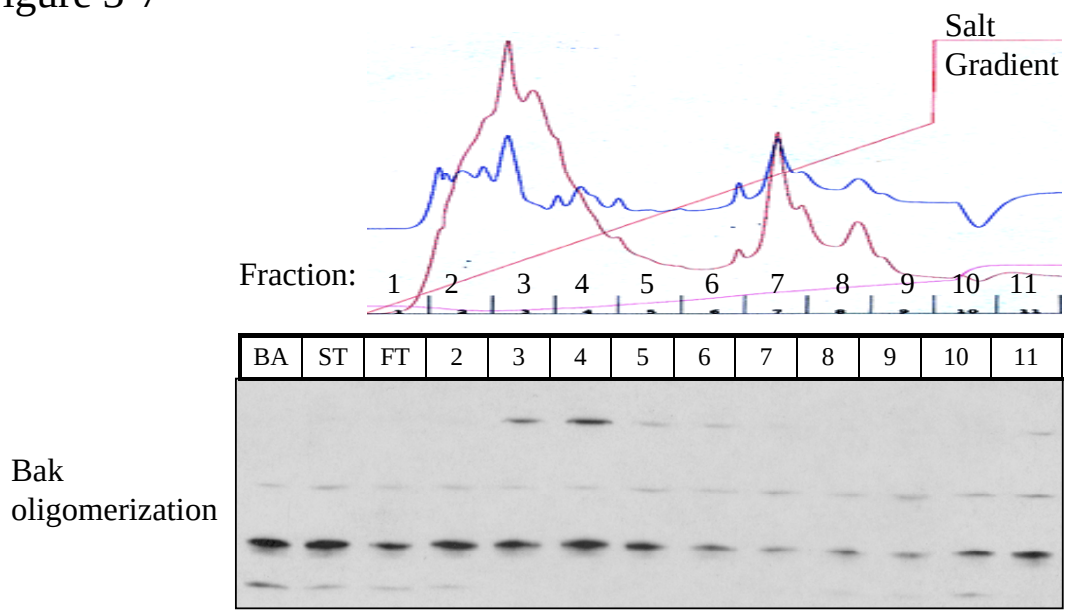

Figure 3-7 Q column behavior of activating factor. The gel filtration fractions 11, 12 and 13 were pooled, dialyzed and run on Mono Q using the SMART machine. Protein was eluted with a 0 to 500 mM gradient of NaCl and the flow through (FT) and eluted fractions were tested for activity using Bak oligomerization. As negative and positive controls, mitochondria were also incubated with Buffer A and sucrose alone (BA) and with UV cytosol starting material (ST).

A Bid Surprise

At this point, although the protein was far from purification, its biochemical characteristics were very reminiscent of another protein purified in the Wang lab, Bid. Bid is a BH3-only protein that is normally cleaved into its active form, tBid, by caspase-8 through activation of the extrinsic pathway (see chapter 1). In addition, uncleaved Bid is itself capable of releasing cytochrome c when present in sufficient quantity (Luo et al. 1998). Thus it made sense to check the active fractions for the presence of Bid (see figure 3-8). Bid is present in fractions 4 and 5, and there is priming activity in fractions 3, 4 and 5. So Bid does not correlate perfectly with activity, however its presence is cause for alarm because many

BH3-only proteins have similar biochemical profiles and it may be that this purification is merely concentrating BH3-only proteins.

Figure 3-8

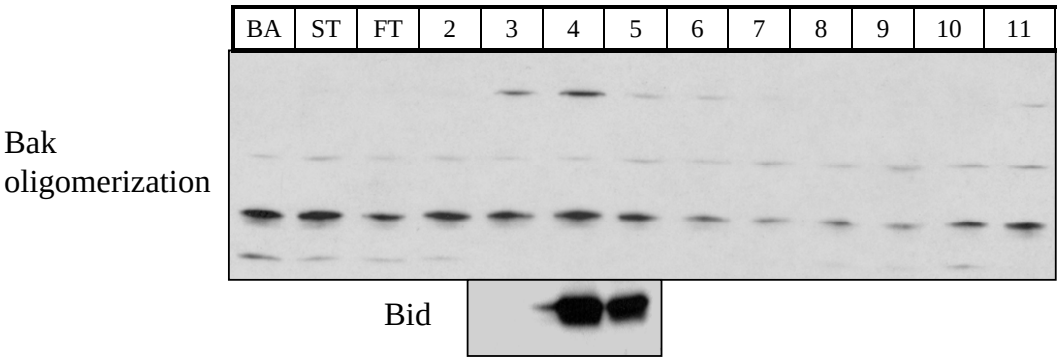

Figure 3-8 Bid partially correlates with activating factor. Q fractions as described in figure 3-7 were probed with antibody to Bid. Bid was present in significant quantities in fractions 4 and 5 as shown.

To confirm whether or not Bid plays a role in priming, Bid was immunodepleted from UV cytosol and the Bid-depleted cytosol tested for priming activity. As shown in figure 3-9A, beads alone had no effect on the ability of UV S100 to prime mitochondria whereas UV S100 depleted of Bid (-Bid lane, see lowest panel of figure 3-9A for Bid depletion) was incapable of priming mitochondria. Further confirmation of Bid's role came from incubating mitochondria with recombinant Bid protein. This also resulted in Bak oligomerization and cytochrome c release at levels of Bid that are comparable to the amount of Bid in UV S100 (see lower panel of figure 3-9). Although this does not prove that Bid is the only protein responsible for priming, it is at least a component of the activity being purified.

Figure 3-9

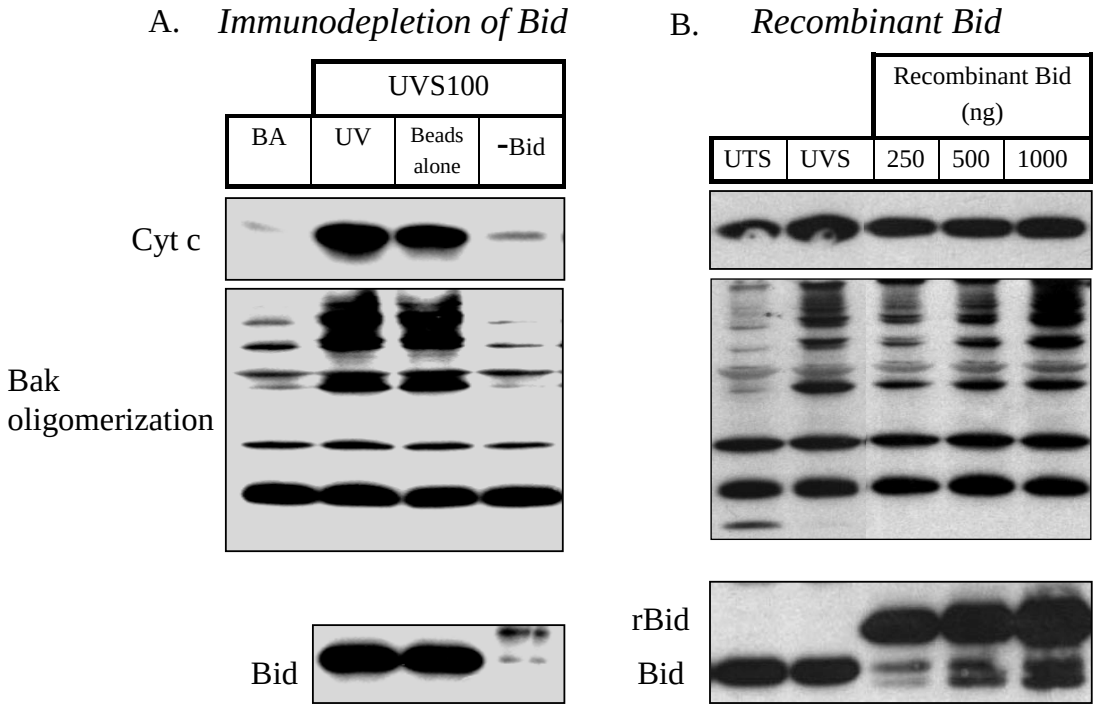

Figure 3-9 Immunodepletion of Bid removes priming activity and recombinant Bid can also prime mitochondria. A) UVS100 was incubated overnight with beads alone or Bid antibody-complexed beads and the resulting depleted S100 was used to prime mitochondria in vitro. The lower panel shows the Bid depletion. B) UTS100, UVS100 and recombinant Bid were used to prime mitochondria in vitro. The amount of Bid in UTS100, UVS100 and recombinant Bid is shown in the lower panel.

Cytosolic Inhibitors Revisited

Based upon observations that the amount of Bid does not seem to change after UV, there is no visible cleavage of Bid (data not shown), and recombinant Bid alone is capable of priming mitochondria, the question at this point was: how is Bid contributing to priming? It is helpful at this point to take a step back and review the Bcl-2 family briefly.

As discussed in Chapter 1, the Bcl-2 family is comprised of both anti- and pro-apoptotic molecules that antagonize one another. A healthy cell contains pro- and anti-

apoptotic molecules in a balance that prevents cell death -- but is not so tilted against death that the cell can never undergo apoptosis (as is the case with B cell lymphoma). Cytochrome c release and apoptosis can be induced by tilting this balance in two ways: 1) the amount or activity of pro-apoptotic molecules is increased; or 2) the amount or activity of anti-apoptotic molecules is decreased. Given that Min Fang demonstrated the presence of a cytosolic inhibitor that rapidly disappears after UV, it may be that priming is due to solely to the absence of this inhibitor rather than UV regulation of an activating molecule. In this scenario the absence of inhibitor allows constitutive BH3-only proteins, such as Bid, to prime mitochondria (see figure 3-3). Partial purification identified Bid as at least a component of priming. Identification of the cytosolic inhibitor had actually discovered two proteins, Bcl-x_L and Mcl-1.

Deepak Nijhawan had purified a cytosolic inhibitor of cytochrome c release and found it to be Bcl-x_L, an anti-apoptotic Bcl-2 member. That Bcl-x_L could block cytochrome c release was not surprising, however, finding Bcl-x_L in the cytosol was. Soon after the purification of Bcl-x_L, Min Fang investigated another Bcl-2 family member, Mcl-1, and found that Mcl-1 rapidly disappeared after UV. Further investigation of these molecules *in vivo* revealed that Mcl-1 rapidly disappears from both cytosol and mitochondria while Bcl-x_L translocates from cytosol to the mitochondria after UV (see figure 3-10). Both mechanisms serve to remove these inhibitors from the cytosol and their depletion alone may cause priming.

Figure 3-10

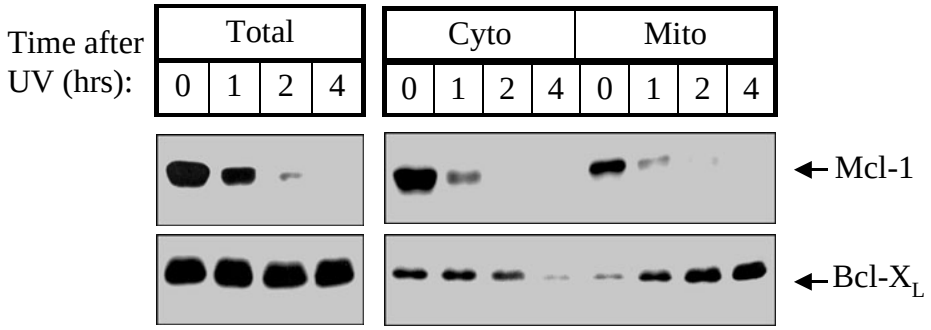

Figure 3-10 Mcl-1 rapidly decreases after UV and Bcl-X_L translocates from the mitochondria to the cytosol. HeLa cells were treated and harvested at the indicated times. The total cell contents, mitochondrial and cytosolic fractions were probed with antibodies to Mcl-1 and Bcl-X_L.

Removal of Mcl-1 and Bcl-x_L Causes Priming

To test whether removal of Mcl-1 or Bcl-x_L causes priming, they were immunodepleted from untreated cytosol either alone, or in combination, and assayed for priming activity (see figure 3-11). As figure 3-11 demonstrates, depletion of either molecule resulted in the production of cytosol capable of priming mitochondria (see figure 3-11). In addition, depletion of both molecules also primed mitochondria. The depletion of both Mcl-1 and Bcl-x_L is demonstrated in the lower panels of figure 3-11.

Figure 3-11

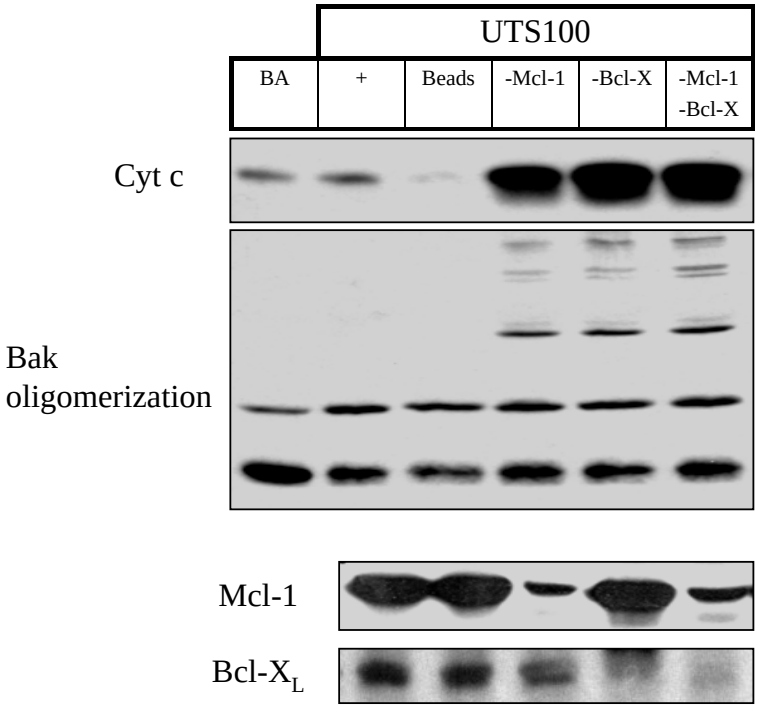

Figure 3-11 Untreated cytosol depleted of Mcl-1 and Bcl-X_L primes mitochondria. Untreated cytosol (UTS100) was incubated with beads alone or beads coupled to antibodies against Mcl-1 and Bcl-X_L and used to prime mitochondria in vitro. The depletion of Mcl-1 and Bcl-X_L is shown in the lower panels.

The above experiment provides insight into the mechanism of priming. Rather than activation of a protein, it is loss of inhibitors that causes priming *in vitro*. In the absence of cytosolic inhibitors, constitutive BH3-only proteins are able to cause Bak oligomerization and cytochrome c release. Since Bid is an abundant BH3-only protein, it is no surprise that purification partially isolated this protein.

Since the above experiments demonstrated that removal of inhibitor primes mitochondria *in vitro*, the next question was whether or not this was the mechanism of cytochrome c release *in vivo.*

Mcl-1

Comparing Mcl-1 and Bcl-x_L, the disappearance of Mcl-1 from cytosol is far more striking. As it turns out, Mcl-1 is a unique anti-apoptotic member of the Bcl-2 family (see figure 3-12). Mcl-1 contains a PEST domain (proline P, glutamic acid E, serine E and threonine T), a domain that results in protein with a short half-life (Rogers et al. 1986).

Figure 3-12

Mcl-1 is an unusual anti-apoptotic Bcl-2 family member

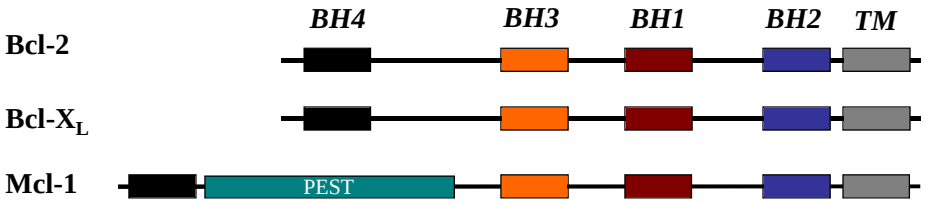

Figure 3-12 Comparison of Mcl-1 to other anti-apoptotic Bcl-2 family members reveals the unique presence of a PEST domain.

Removal of Mcl-1 is Necessary for Apoptosis

PEST domain proteins are degraded by the proteasome. Since there exist potent and specific proteasome inhibitors, it was easy to test the requirement of Mcl-1 degradation in UV-induced apoptosis. The proteasome itself is composed of a 20S catalytic core shaped like a cylinder. Caps on both ends increases the size of the proteasome to 26S, and the caps bind

polyubiquitin, unfold the protein and feed the amino acid chain through the 20S catalytic subunit. The proteasome inhibitor MG132 is a non-specific inhibitor of the proteasome. It irreversibly binds one of the proteases in the cylinder of the proteasome as well as other proteases. While MG132 is a very potent, efficient inhibitor it lacks specificity and affects many other proteases. Epoximicin is another proteasome inhibitor, but has a completely different mechanism. Isolated from fungus, it does not bind any of the proteases in the cylinder of the proteasome but reversibly inhibits the proteasome through a different binding site. Min Fang and Deepak Nijhawan used these inhibitors to pre-treat HeLa cells and the cells were subsequently exposed to UV. The results of the experiment are shown below in figure 3-13.

Figure 3-13

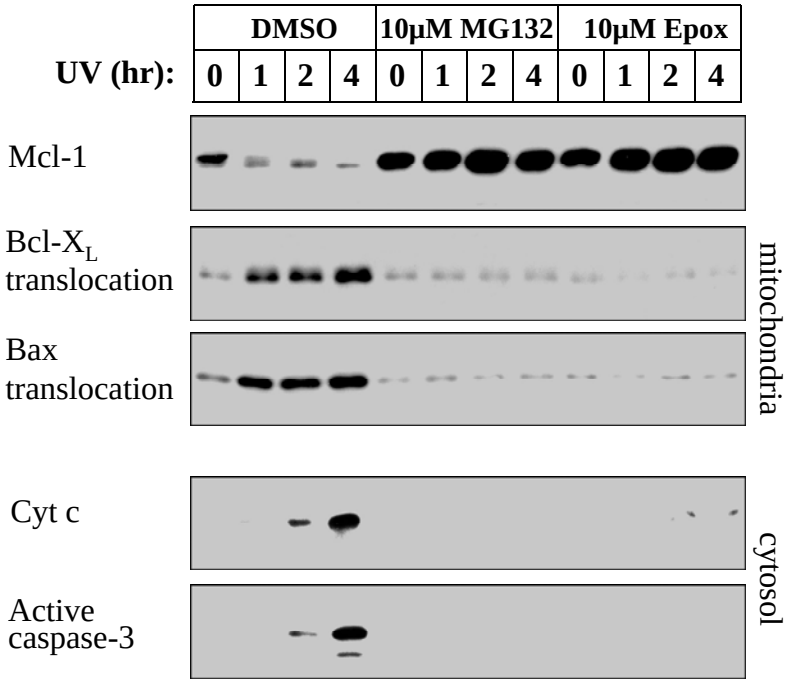

Figure 3-13 Proteasome inhibitors block cytochrome c release and caspase activation. MG132 and epoximicin were added to HeLa cells prior to UV treatment. The cells were then assay for total amount of Mcl-1, Bcl-XL/Bax translocation to mitochondria, cytochrome c release, and active caspase-3 by western blot of the appropriate fractions. Slide courtesy of Deepak Nijhawan.

MG132 and epoximicin both block the degradation of Mcl-1 by the proteasome as evidenced by the top panel of figure 3-13. Moreover, MG132 and epoximicin also block cytochrome c release and caspase-3 activation. Although Bax translocation was discussed in the introduction, but not specifically shown until now, Bax and Bcl-x_L both translocate to the mitochondria after UV (see the mitochondria blots in figure 3-13 and figure 3-10). Although this seems counterproductive, Min Fang has found that the total amount of translocated Bax exceeds that of Bcl-x_L, which may result in excess Bax on the surface of mitochondria resulting in Bax/Bak oligomerization and cytochrome c release. Regardless of the exact

mechanism, the ability of proteasome inhibitors to block these dowstream events suggested that the removal of Mcl-1 was an apical and required event in UV apoptosis.

In order to determine if the effect of proteasome inhibitors is indeed specific for Mcl-1, Deepak Nijhawan did the following experiment. Mcl-1 was specifically reduced in HeLa cells through RNAi (see figure 3-14). Then the cells were pretreated with MG132 (or DMSO as a control) and exposed to UV. Cells with Mcl-1 RNAi already have low levels of Mcl-1, and if MG132 is acting specifically through Mcl-1 then activation of caspase-3 in these cells should not be blocked by MG132. This was demonstrated to be the case (see figure 3-14). As a positive control, caspase activation in luciferase RNAi cells was still blocked by MG132. This elegant experiment delineated Mcl-1 as an apical checkpoint for apoptosis. When degradation of Mcl-1 is blocked, translocation of Bcl-x_L/Bax, Bak oligomerization and cytochrome c release are also blocked.

Figure 3-14

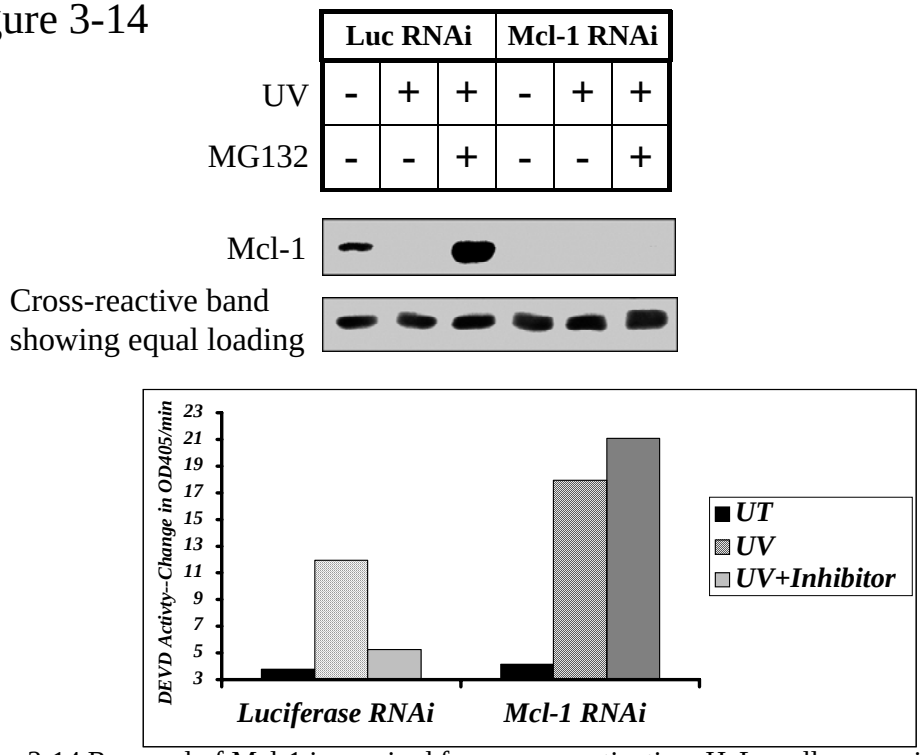

Figure 3-14 Removal of Mcl-1 is required for caspase activation. HeLa cells were either transfected with RNAi oligos to luciferase (as a control) or Mcl-1. Cells were then subjected to UV in the presence or absence of MG132. Cells were collected for western blot of Mcl-1 and measurement of caspase activity by cleavage of a fluorogenic substrate. Slide courtesy of Deepak Nijhawan.

Removal of Mcl-1 is not Sufficient for Apoptosis

In addition to demonstrating that Mcl-1 is responsible for the ability of proteasome inhibitors to block caspase activation, the RNAi experiment also implied that removal of Mcl-1 is not sufficient for apoptosis because RNAi of Mcl-1 did not induce cell death (see figure 3-14). Further demonstrating that removal of Mcl-1 is not sufficient for apoptosis is the inability of the transcriptional inhibitor Actinomycin D and the translational inhibitor cycloheximide to induce cell death (see figure 3-15). Actinomycin D and cycloheximide do

not cause cytochrome c release and caspase activation anywhere near the level of activity of UV, yet all three treatments dramatically reduce the amount of Mcl-1.

Figure 3-15

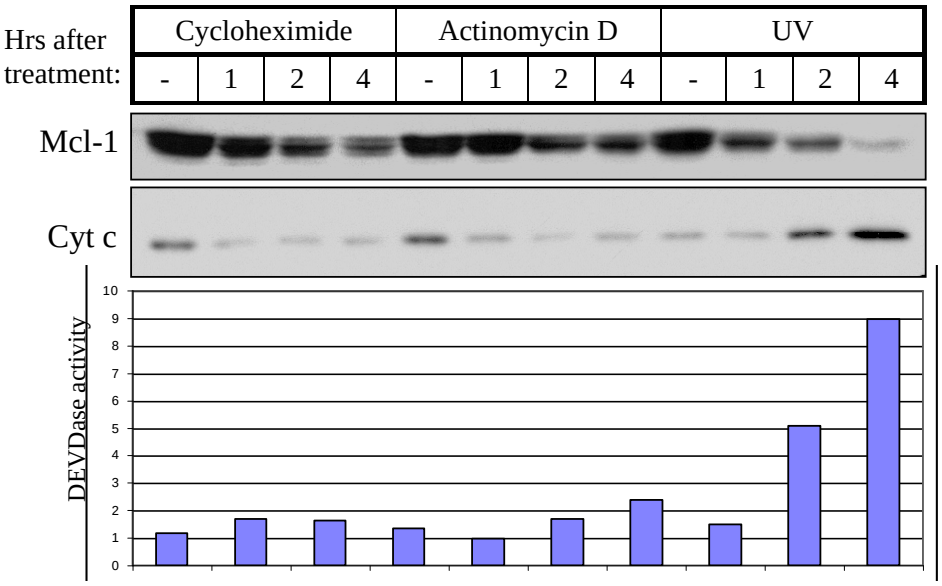

Figure 3-15 Removal of Mcl-1 is not sufficient for apoptosis. HeLa S3 cells were treated with cycloheximide, actinomycin D or UV for the hours indicated. The cells were then harvested with the cytosol collected for cytochrome c release and the cell pellet used to determine Mcl-1 levels and caspase activity using a fluorogenic assay.

Since it was possible to prime mitochondria with untreated S100 that was deprived of either Mcl-1 or Bcl-x_L, it seemed reasonable that cycloheximide could also prime mitochondria due to its ability to drastically reduce the amount of Mcl-1. As demonstrated in figure 3-16, cycloheximide is able to prime mitochondria *in vitro* as efficiently as UV (see lanes 3 and 4).

Figure 3-16

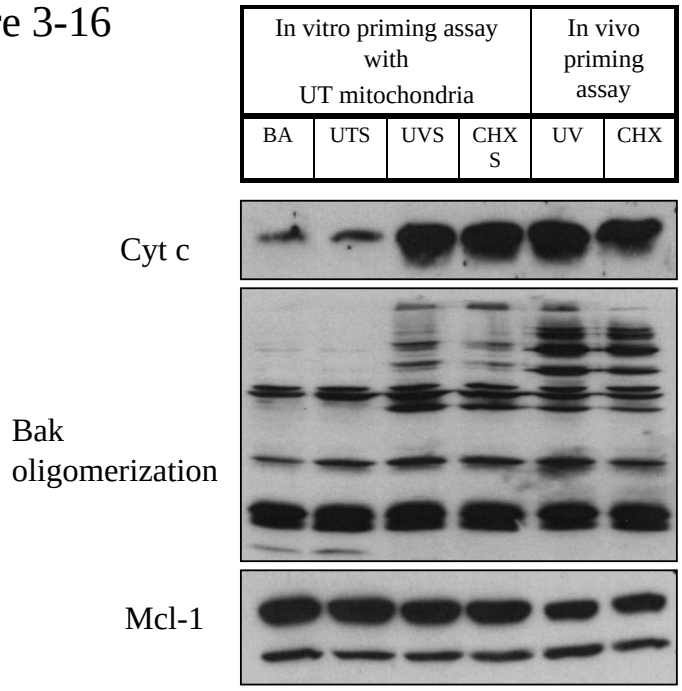

Figure 3-16 Cycloheximide is able to prime mitochondria as efficiently as UV. Untreated mitochondria were primed in vitro using cytosol from untreated cells (UTS), 1 hour UV treated (UVS) or 1 hour cycloheximide treated (CHXS) and assayed for cytochrome c release and Bak oligomerization. Untreated (first lane - BA), UV-treated and cycloheximide-treated mitochondria were isolated and tested for in vivo priming by cytochrome c release and Bak oligomerization. The bottom panel is the amount of Mcl-1 on the mitochondria.

Additionally, mitochondria isolated from cells treated for 1 hour with either UV or cycloheximide are primed *in vivo*. Comparing lanes 1, 5 and 6 in figure 3-16 demonstrates clearly that both UV and cycloheximide can prime mitochondria efficiently, and thus the priming assay is not so much a measure of a specific activator but rather the loss of inhibitor, specifically Mcl-1 (the amount of Mcl-1 is decreased on the mitchondria as well, but the exposure in figure 3-16 makes it difficult to appreciate). As shown in figure 3-15 however, UV is able to induce caspase activation in four hours whereas cycloheximide and Actinomycin D are not. Priming is therefore reflective of an initial loss of Mcl-1 required for

in vivo cytochrome c release, but it is not reflective of how UV specifically induces cytochrome c release and cell death *in vivo*.

A Second Hit is Necessary

If removal of Mcl-1 is not sufficient to induce cell death then there must be another event required to induce cytochrome c release and caspase activation, a second hit. This second hit is downstream of Mcl-1 since MG132 blocks the removal of Mcl-1 and prevents Bcl-x_L/Bax translocation, Bak oligomerization and cytochrome c release. However, the second hit is not reflected in the priming assay, it is only a reflection of the amount of Mcl-1.

An Indicator of Cell Health?

As mentioned earlier, Mcl-1 is an unusual member of the Bcl-2 family. It was originally discovered as a gene upregulated during in B cell maturation (Mcl is short for myeloblast cell lineage) and is thought to prevent apoptosis during that process. Mcl-1 has an intrinsically short half-life due to its PEST domain and therefore its levels can be rapidly modulated through either transcription or translation. One method of Mcl-1 regulation is through the JAK/STAT pathway in response to IL-6 (Puthier et al. 1999). This transcriptionally upregulates Mcl-1, resulting in increased amounts of Mcl-1 protein. However, the effect is transitory due to Mcl-1's intrinsically short half-life. Mcl-1 is like an immediate-early response gene in this regard and thus it makes sense that it is the apical

molecule in UV-induced apoptosis. Many forms of cellular stress, such as UV and viral responses, cause a decrease in translation and as a result, Mcl-1 protein levels will decrease in accordance with its intrinsically short half-life. This has the effect of lowering the threshold for apoptosis because Mcl-1 removal is not sufficient to induce apoptosis. In some cell lines, such as multiple myeloma cell lines there is a drastic upregulation of Mcl-1 and these cells can be killed by cycloheximide or Mcl-1 antisense alone (Zhang et al. 2002). However in most cells it is likely that Mcl-1 removal is just the first of at least two, and perhaps more, events that need to happen in order for the cell to undergo apoptosis. The next chapter investigates what this second hit might be.

CHAPTER FOUR
Role of Bim_{EL} Phosphorylation in UV-induced Apoptosis

SECOND HIT

What is downstream of Mcl-1 disappearance?

As discussed in the last chapter, removal of Mcl-1 is necessary but not sufficient for cytochrome c release and caspase activation. Cycloheximide, an inhibitor of translation, and Actinomycin D, an inhibitor of transcription, can effectively reduce the amount of Mcl-1 but do not cause cytochrome c release and caspase activation in four hours (see figure 3-15). This is in stark comparison to UV, which causes robust caspase activation at four hours. Since removal of Mcl-1 is not enough to induce apoptosis alone, there must be another event that is also required for cytochrome c release and caspase activation. We refer to this event as the second hit, although it may be comprised of many separate events. Since proteasome inhibitors prevent degradation of Mcl-1 and block caspase activation despite the second hit of UV (figure 3-13), we reasoned that a component of the second hit may be specifically bound and antagonized by Mcl-1.

Mcl-1 preferentially binds Bim_{EL}

A stable cell line expressing FLAG-tagged Mcl-1 was used to immunoprecipitate Mcl-1, either alone or in the presence of UV. The immunoprecipitated Mcl-1 and bound proteins were run on an SDS-PAGE gel and stained using colloidal Coomassie blue. As

expected (figure 4-1), UV decreases the total amount of Mcl-1. However, the only significantly bound protein -- either before or after UV -- that we identified was Bim_{EL} (Bim_{EL} is also in the UV lane by western blot, but there was not enough to see by Coomassie Blue staining).

Figure 4-1

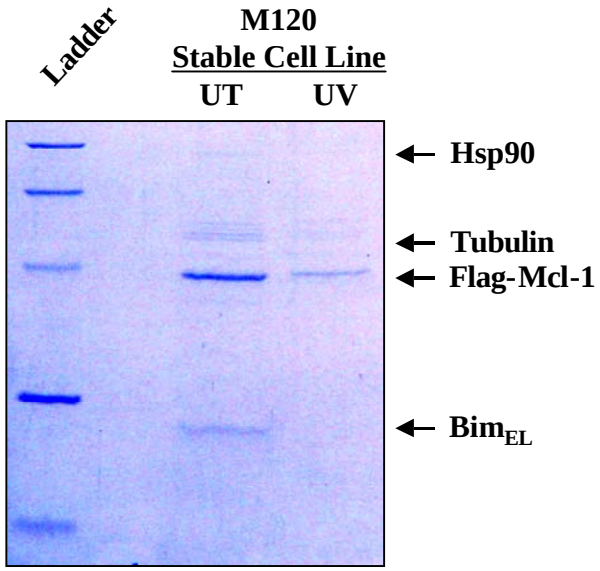

Figure 4-1 Mcl-1 preferentially binds Bim_{EL}. A FLAG-tagged Mcl-1 stable cell line was used to immunoprecipitate FLAG-Mcl-1 and bound proteins both before and after UV. The identified bands are indicated.

In HeLa cells, we found that Bim_{EL} is predominantly located on the mitochondrial membrane (data not shown), consistent with its localization in T cells (Zhu et al. 2004). Endogenous mitochondrial Bim_{EL} is completely immunoprecipitated by Mcl-1 antibodies, indicating that Mcl-1 indeed binds Bim_{EL} very specifically (figure 4-2). This is in agreement

with the immunoprecipitation from the Mcl-1 stable cell line and has also been demonstrated in lymphocytes (Opferman et al. 2003).

Figure 4-2

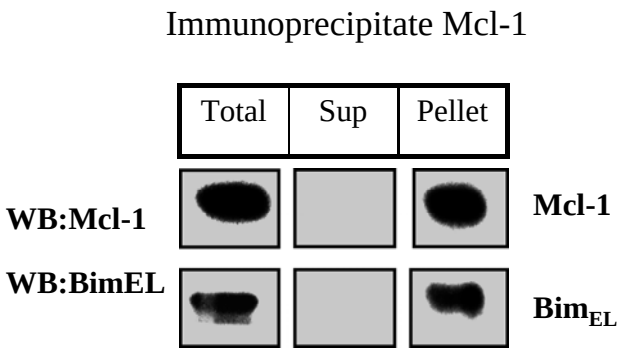

Figure 4-2 eEndogenous Mcl-1 can immuno-precipitate Bim_{EL} from mitochondrial membrane. HeLa S3 cells were fractionated into cytosol and mitochondrial heavy membrane fractions. Mitochondrial membrane was solubilized with 1% Triton and anti-Mcl-1 antibodies were used to immunoprecipitate Mcl-1, the total, supernatant, and pellet fractions were probed with antibodies to Mcl-1 and Bim_{EL}.

A Bim Refresher

Some background on Bim was given in Chapter 1 but at this point more detail and up to date information is needed.). There are three major transcriptional variants of Bim: Bim_{EL}, Bim_L and Bim_S; shorthand for extra long, long and short, respectively (figure 4-5). Bim_{EL} and Bim_L are more abundant species and have identical amino acid composition with the exception of an alternatively spliced intron present in Bim_{EL} (O'Connor et al. 1998; O'Reilly et al. 1998). Bim_S is the least abundant but the most potent of the three and contains a unique

N-terminus. Bim_L, and the related protein Bmf, were originally proposed to induce apoptosis by translocation. In healthy cells Bim and Bmf are sequestered to the cytoskeleton by dynein light chain LC8. Dynein light chain is complexed with intermediate and heavy chains to form the dynein motor complex. The dynein motor complex is involved in retrograde organelle transport and moves along microtubules in an ATP-dependent manner. After a DNA damage stimulus, such as UV light, Bim translocates from the microtubule cytoskeleton to the mitochondria where it antagonizes Bcl-2 and leads to cytochrome c release (Puthalakath et al. 1999; Puthalakath et al. 2001).

Bim is also regulated by transcription, but this mechanism seems to function primarily in neuronal cells. Two separate groups found that Bim levels dramatically increase in neuronal cell lines that were deprived of nerve growth factor (NGF) (Harris and Johnson 2001; Putcha et al. 2001; Whitfield et al. 2001). The removal of NGF induces apoptosis in a c-jun N-terminal kinase (JNK) dependent manner, and the induction of Bim_{EL} could be blocked by expression of dominant negative c-jun.

Bim_L and Bim_{EL} have identical amino acid composition with the exception of an alternatively spliced intron present in Bim_{EL} (figure 4-5). Bim_{EL} and Bim_L both contain a consensus dynein light chain binding motif (figure 4-5) but detailed studies of binding to dynein motor complexes (Puthalakath et al. 1999) has been primarily with Bim_L. Other studies have also recently found that Bim_{EL} is mostly mitochondrial (Zhu et al. 2004), implying that these molecules may be regulated in different manners.

During our studies, many reports of Bim_{EL} phosphorylation were published (Ley et al. 2003; Luciano et al. 2003; Ley et al. 2004). These papers identified serines contained in the

unique region of Bim_{EL} (serines 55, 65 and 73 -- see figure 4-3) as ERK1/2 phosphorylation sites. They further reported that the phosphorylation of Bim_{EL} is involved regulating its degradation with the phosphorylated Bim_{EL} having a shorter half-life than the dephosphorylated form. This is consistent with the role of the MEK1/ERK1/2 signaling cascade in response to growth factors (Vaudry et al. 2002) because the phosphorylation of Bim_{EL} would lead to increased Bim_{EL} turnover and thus tip the balance away from apoptosis.

Figure 4-3

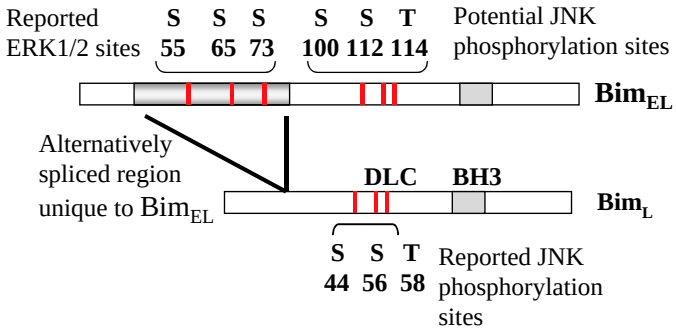

Figure 4-3 Diagram of Bim_{EL} and BimL. BimL is missing an alternatively spliced intron present in Bim_{EL}. The reported ERK sites of Bim_{EL} are indicated as are the reported JNK phosphorylation sites of BimL. The dynein light chain binding domain (DLC) contains serine 56 of Bim_L and serine 112 of Bim_{EL}.

But other reports have different conclusions. Another paper has identified serine 65 in particular as a site for phosphorylation by JNK (Putcha et al. 2003). These studies were done in neuronal cells where JNK is known to transcriptionally increase Bim_{EL}, so in their new model JNK is proposed to have a two-fold effect in increasing Bim_{EL}, first through transcription and then by making it more active through direct phosphorylation.

JNK has also been reported to phosphorylate the smaller isoform Bim_L and regulate its binding to dynein light chain (Lei and Davis 2003). This model would account for the

regulation of translocation since JNK activation is needed to disrupt the binding to DLC. In particular phosphorylation of threonine 56 prevented DLC from binding to Bim_L (Lei and Davis 2003). In most cells though, Bim_L is the less abundant isoform. In HeLa cells it is very difficult to detect (if present at all) with Bim_{EL} being the major isoform.

And yet another system using IL-2 withdrawal from lymphocytes found that Bim_{EL} is dephosphorylated (Seward et al. 2003). This dephosphorylation is associated with cell death and in this model dephosphorylation of Bim_{EL} was reported to make Bim_{EL} more potent in inducing apoptosis.

In short, there are many conflicts in the field. Even though others published phosphorylation data during our study, the meaning of this phosphorylation remained unclear -- especially as it relates to apoptosis.

Bim_{EL} is dephosphorylated after UV

After UV, mitochondrial Bim_{EL} is dephosphorylated within four hours (figure 4-3), a time course that is consistent with caspase activation. The shifted band on western blot was determined to be phosphorylation by treating mitochondria with phosphatase to reproduce the shift (data not shown) and based upon similar data of Bim_{EL} phosphorylation by others (see above discussion). Although phosphorylated Bim_{EL} is a specific target of caspases (Chen and Zhou 2004) (cleavage of phosphorylated Bim_{EL} would also account for the observed shift), zVAD only partially attenuates the shift of Bim_{EL} indicating that the observed shift with UV is not solely due to caspase cleavage of phosporylated Bim_{EL} (figure 4-3). Furthermore, HA-tagged Bim_{EL} transfected into Bcl-x_L overexpressing cells reproduces the

dephosphorylation and western blot shift of endogenous Bim_{EL}, even though these cells have no caspase activation (figure 4-6 and 4-7).

Figure 4-4

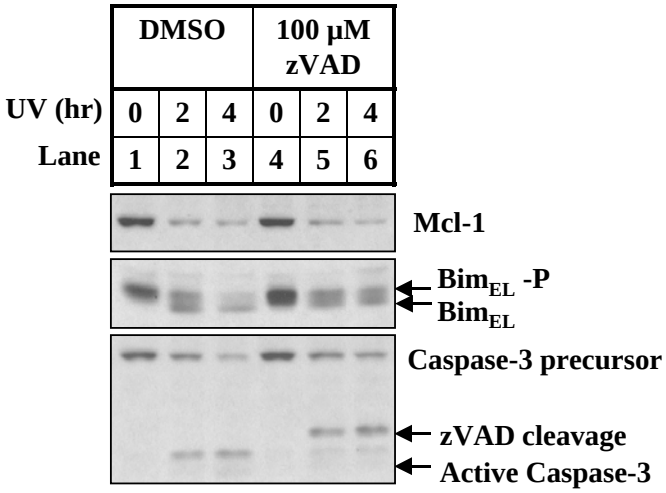

Figure 4-4 Bim_{EL} shifts after UV independently of caspase activation. HeLa S3 cells were treated with UV either in the presence or absence of zVAD and the mitochondria were isolated and probed by western for Mcl-1 and Bim. The cytosol was used to the caspase western blot. The zVAD cleavage refers to the band produced by caspase-3 in the presence of zVAD -- this cleavage is not an active caspase however.

It is possible that Bim_{EL} is dephosphorylated due solely to disappearance of Mcl-1. However, Bim_{EL} is not dephosphorylated when Mcl-1 is decreased with cycloheximide, nor does Mcl-1 RNAi have any effect on the Bim_{EL} dephosphorylation, either untreated or in response to UV (figure 4-5). Therefore, dephosphorylation of Bim_{EL} is downstream of UV treatment, which causes caspase activation, and not downstream of Mcl-1 disappearance caused by RNAi or cycloheximide treatment, which does not cause caspase activation. Additionally, since Bim_{EL} dephosphorylation occurs in a time course consistent with caspase activation, it appeared to be a good candidate for the second hit.

Figure 4-5

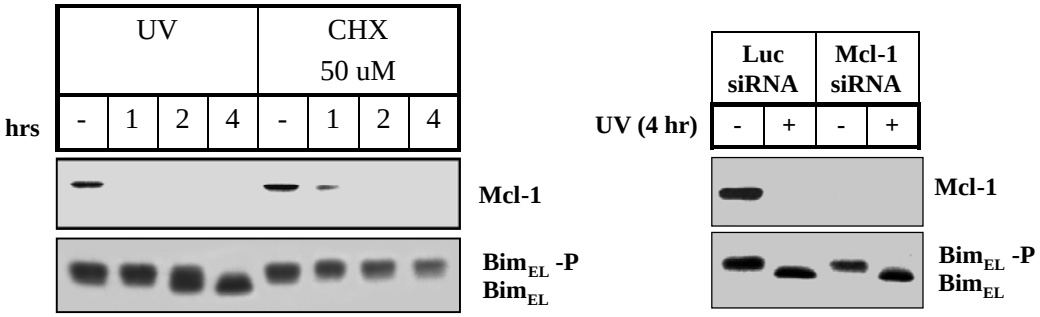

Figure 4-5 Bim dephosphorylation is not due to Mcl-1 disappearance. HeLa S3 cells were treated with UV or cycloheximide (CHX) for the times indicated. The mitochondria were then isolated and amounts of Mcl-1 and Bim determined by western. HeLa attached cells were transfected with oligofectamine using either a control siRNA against luciferase or an Mcl-1 specific oligo. Lysates from untreated and UV-treated samples were used for westerns with Mcl-1 and Bim.

Identification of Bim$_{EL}$ phosphorylation sites

As discussed earlier, many reports of Bim$_{EL}$ phosphorylation were published as we were working on Bim$_{EL}$ phosphorylation. A diagram of Bim$_{EL}$ and Bim$_{L}$ are shown in figure 4-3 as a reference for further discussion of phosphorylation sites.

Although Bim$_{EL}$ was clearly dephosphorylated after UV, not all phosphorylations are detectable by western blot. Therefore an unbiased approach was used to determine all possible phosphorylations that occur both before and after UV treatment. HA-tagged Bim$_{EL}$ was transfected into a Bcl-x$_{L}$ stable cell line to prevent caspase cleavage of HA-Bim$_{EL}$. The HA-Bim$_{EL}$ was immunoprecipitated, run on an SDS-PAGE gel and stained with Coomassie blue stain. The transfected HA-Bim$_{EL}$ shifts with UV treatment, reproducing the shift of endogenous Bim$_{EL}$ after UV (figure 4-6). Three bands were cut from the gel: 1) the

phosphorylated untreated Bim_{EL} band, 2) the unshifted Bim_{EL} band, and 3) the UV treated HA-Bim_{EL}. The bands were then trypsinized and analyzed by mass spectrometry. Two peptides corresponding to the same phosphorylation site were identified from Band 1. As represented pictorially in figure 4-6, these peptides are phosphorylated at serine 73. Serine 73 and the corresponding trypsin peptide fragment are in the region of Bim_{EL} distinct from Bim_L, which contains three consensus ERK phosphorylation sites of a serine or threonine followed by a proline: serine 55, serine 65 and serine 73.

Figure 4-6

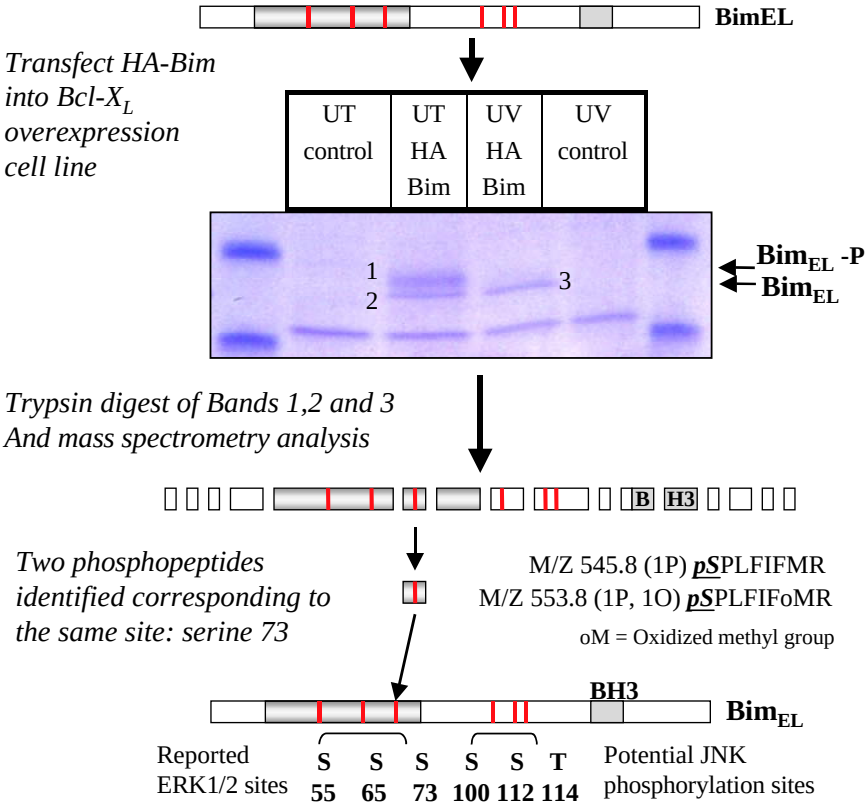

Figure 4-6 Identification of Bim $_{EL}$ phosphorylation sites.

To confirm whether or not these sites were involved in the shift of Bim_{EL} after UV, aspartate and alanine mutations were constructed, transfected into a Bcl-x_L stable cell line and treated with UV. Mutation of serines 55, 65 and 73 (the site we identified) to alanine or aspartate were made individually and in combination. Mutation of just serine 65 largely reproduced the shift and mutation of all three sites (S55, S65 and S73) completely abolished any shift with UV. This triple mutation also reproduced western bands the same size as phosphorylated and dephosphorylated wild type HA-Bim. These results are consistent with previous reports of phosphorylation at this site (Ley et al. 2003; Luciano et al. 2003; Ley et al. 2004).

Figure 4-7

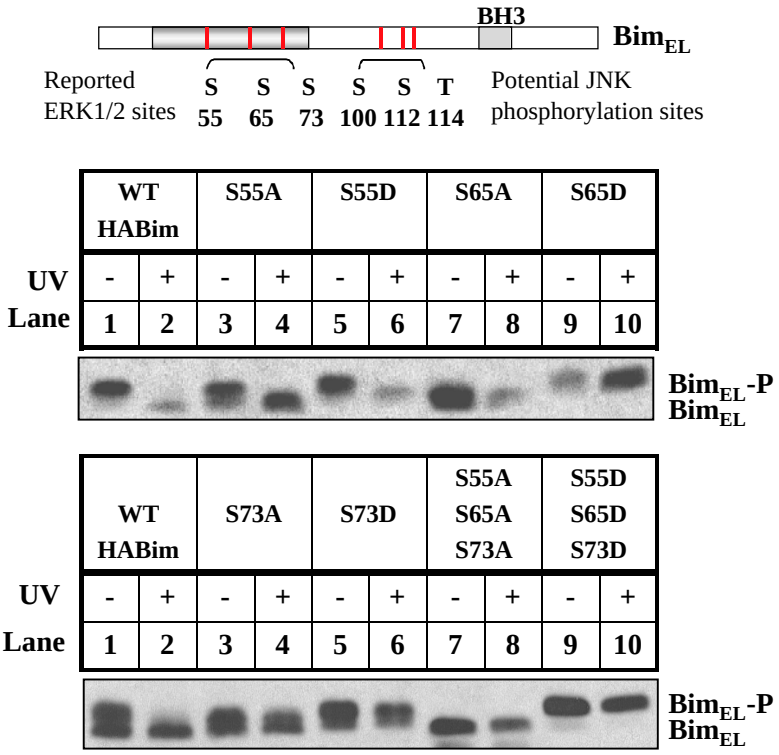

Figure 4-7 Serine 65 is the site most responsible for the Bim $_{EL}$ shift after UV. Alanine and aspartate mutations corresponding to the reported ERK1/2 sites were made both individually and in combination to an HA-tagged Bim $_{EL}$ construct. The constructs were then transfected into a Bcl-XL overexpression cell line and were left untreated, or treated with UV.

The likely reason that phosphorylation at serine 65 was not identified with mass spectrometry is because of the size of the trypsinized peptide that contains that site. Figure 4-6 schematically shows the approximate size of Bim$_{EL}$ peptides after trypsinization. Serines 55 and 65 are contained in a peptide fragment 47 amino acids long (figure 4-6). This peptide is too large for mass spectrometry to identify phosphorylation sites. By comparison, serine 73 is contained on a peptide of 8 amino acids. The majority of the remaining peptides were in the range of 5 to 25 amino acids in length and therefore phosphorylations in sufficient quantity

could theoretically be identified by mass spectrometry. However, negative results cannot be inter*pr*eted as definitive proof that a site is not phosphorylated.

Since serines 55, 65 and 73 correspond to consensus MEK/ERK sites, U0126, a specific inhibitor of the MEK1 pathway, was used to inspect whether or not Bim_{EL} could be dephosphorylated by U0126 (figure 4-8). The effectiveness of U0126 was determined by western blot for phosphorylated ERK1/2, a major substrate of MEK1. U0126 caused complete Bim_{EL} dephosphorylation, even more profoundly than UV, implying that Bim_{EL} is downstream of the MEK1/ERK1/2 pathway as reported by others (Ley et al. 2003; Ley et al. 2004). It also became clear from this experiment that UV attenuates ERK1/2 phosphorylation in a manner similar to U0126, which would seem to account for the dephosphorylation of Bim_{EL} after UV. However, we first wanted to test whether or not dephosphorylated Bim_{EL} was more potent than phosphorylated Bim_{EL}.

Figure 4-8

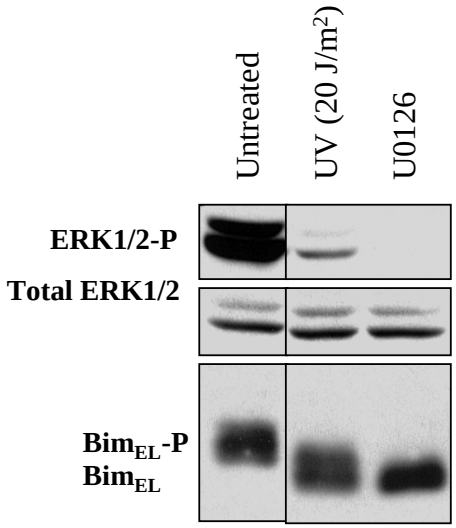

Figure 4-8 The MEK1 inhibitor U0126 also causes Bim_{EL} dephosphorylation. A Bcl-X_L stable cell line was treated with UV and U0126 for four hours. The cells were then collected and assayed for phospho-ERK1/2 as a control for U0126 and Bim.

Bim_{EL} dephosphorylation is not the second hit

To test whether or not this phosphorylation plays any role in UV-induced apoptosis. Serines 55, 65 and 73 were mutated to alanine or aspartate and transfected into HeLa cells. A day later the cells were harvested and assayed for caspase activity. As shown in figure 4-9, there was no significant difference in caspase activation between the mutated constructs. Western blot indicated that all constructs expressed similar amounts of protein (figure 4-9). This result implies that Bim_{EL} dephosphorylation at serines 55, 65 and 73 does not significantly change Bim_{EL}'s potency.

Figure 4-9

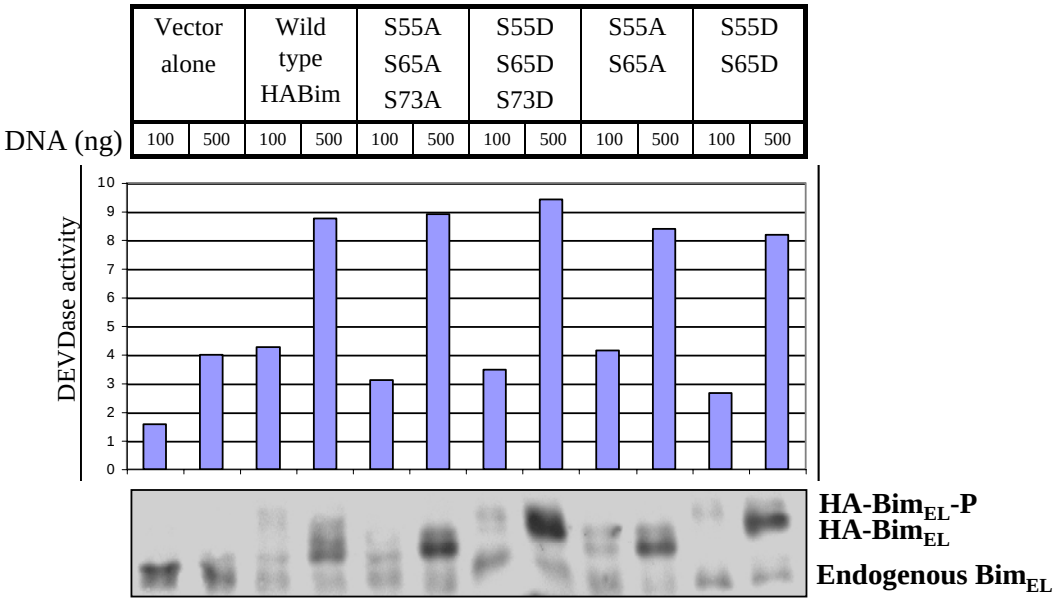

Figure 4-9 Mutation of phosphorylation sites does not alter BimEL's ability to induce apoptosis. HA-tagged BimEL constructs were mutated to alanine or aspartate to mimic phosphorylation and transfected into HeLa attached cells. The next day the cells were harvested and assayed for caspase activity and run on SDS-PAGE gel to confirm similar expression levels.

Another possibility is that phosphorylation of Bim_{EL} affects binding to Mcl-1. To test this, wild type and mutated constructs were transfected into a FLAG-tagged Mcl-1 stable cell line. The FLAG-Mcl-1 was immunoprecipitated with anti-FLAG beads and the total (T), supernatant (S) and pellet (P) were run on an SDS-PAGE gel and probed with antibodies to Mcl-1 and Bim. Mcl-1 binds both alanine and aspartate mutations very efficiently (figure 4-10). This corroborates our previous immunoprecipitation experiments with endogenous Mcl-1. In that case as well, both phosphorylated and dephosphorylated Bim_{EL} are immunoprecipitated by Mcl-1 (figure 4-2).

Figure 4-10

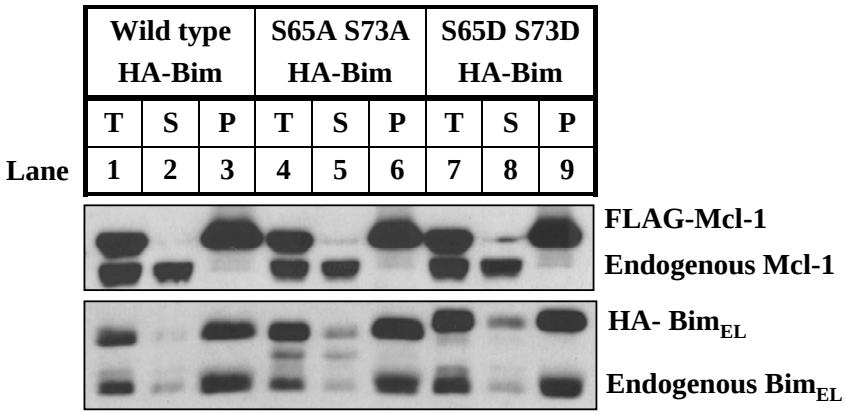

Figure 4-10 Mcl-1 binds both phosphorylated and unphosphorylated Bim_{EL} mutants. A FLAG-tagged Mcl-1 stable cell line was transfected with wild type, S65A/S73A or S65D/S73D HA-tagged Bim_{EL}. The FLAG-Mcl-1 was immunoprecipitated with anti-FLAG beads and the total (T), supernatant (S) and pellet(P) were probed with antibodies to Mcl-1 and Bim. The FLAG-Mcl-1, HA- Bim_{EL} and endogenous proteins are indicated.

UV inactivates the MEK/ERK pathway in a dose dependent manner

Figure 4-8 demonstrates that UV causes an inactivation of the MEK1/ERK1/2 pathway in HeLa cells. As shown in figure 4-11, ERK1/2 is dephosphorylated (and therefore inactivated) in a dose-dependent manner after UV. This dephosphorylation of ERK1/2 corresponds to dephosphorylation of Bim_{EL}. UV treatments as low as one and three J/m2 still cause Mcl-1 disappearance but only mildly decrease ERK1/2 phosphorylation and Bim dephosphorylation (figure 4-11). However at our original dose of 20 J/m2, UV causes both an inactivation of the MEK/ERK pathway and Bim dephosphorylation, although not as much as U0126.

Figure 4-11

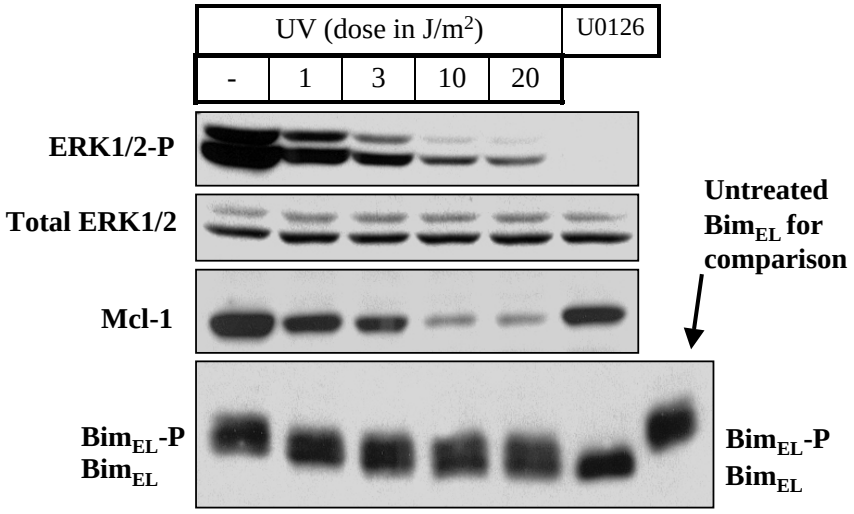

Figure 4-11 UV inactivates MEK/ERK in a dose-dependent manner. A Bcl-X_L stable cell line was treated with increasing amounts of UV and with U0126, a MEK1 inhibitor. After four hours, cells were harvested and assayed for phospho-ERK1/2, total ERK1/2, Mcl-1 and Bim.

Inactivation of the MEK/ERK pathway and Bim_{EL} dephosphorylation are unlikely to be major determinants of caspase activation because even at lower doses of UV there is still significant caspase activity in HeLa S3 cells (figure 4-12). Although UV-dependent attenuation in phosphorylated ERK1/2 does correlate with caspase activation (figure 4-12), U0126 causes no caspase activation itself. But this may be due to the presence of Mcl-1, which we know is necessary to remove first.

Figure 4-12

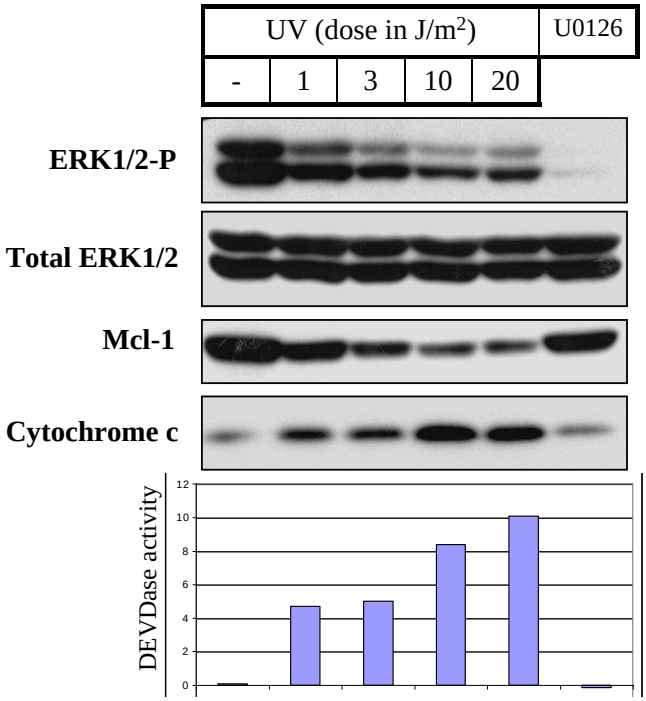

Figure 4-12 Inactivation of the MEK1/ERK1/2 cascade does not correlate with cytochrome c release and caspase activation. HeLa S3 cells were treated with increasing amounts of UV. After four hours, cytosol was collected for cytochrome c and the cell pellet used to determine phospho-ERK1/2, total ERK1/2 and Mcl-1 levels.

Inhibition of the MEK1/ERK1/2 pathway is not the second hit

In order to test if inhibition of the MEK1/ERK1/2 pathway is the second hit itself, rather than dephosphorylation of Bim_{EL}, HeLa cells were treated with cycloheximide to remove Mcl-1 and U0126 to inhibit MEK1. Although cycloheximide decreases the level of Mcl-1 and U0126 drastically inhibits phosphorylated ERK1/2, there is very little caspase activity in these cells and no more than cycloheximide alone. Further, U0126 does not increase caspase activation in combination with UV. Therefore, inhibition of the MEK1/ERK1/2 pathway cannot be the second hit either.

Figure 4-13

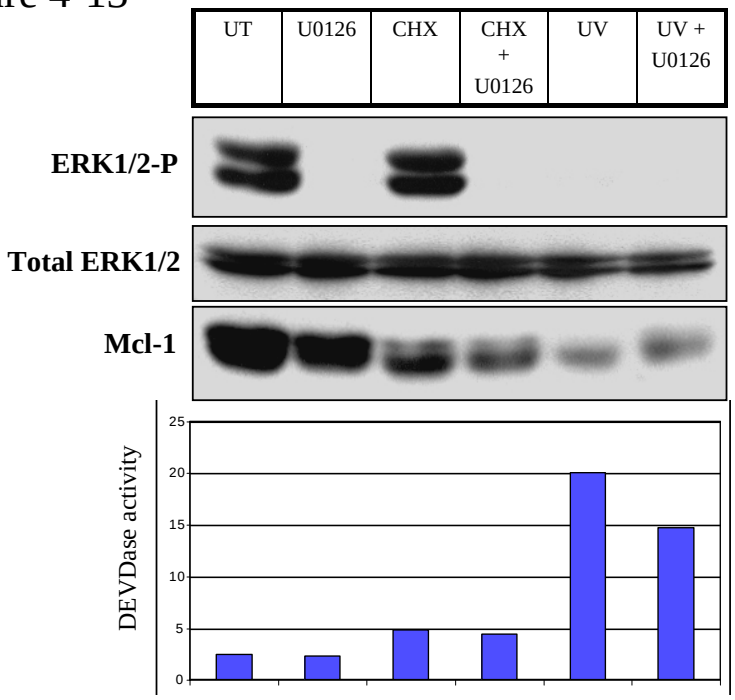

Figure 4-13 Mcl-1 removal and Bim_{EL} dephosphorylation do not induce cytochrome c release. HeLa S3 cells were treated with cycloheximide (CHX), the MEK1 inhibitor U0126 or UV or in combination. After four hours, the cells were collected and assayed for caspase activity. Cell lysate was used for western blot of phospho-ERK to confirm U0126 activity and Mcl-1 to confirm cycloheximide activity.

Is JNK the second hit?

JNK does not phosphorylate Bim_{EL} in vitro

Although we did not sequence any sites that were phosphorylated after UV, JNK has been previously shown to phosphorylate Bim_L (Lei and Davis 2003). We considered that JNK may phosphorylated Bim_{EL} as well and that perhaps a phosphorylation and not a dephosphorylation may be the second hit. The reported JNK phosphorylation sites in Bim_L,

and corresponding Bim_{EL} sites, are diagrammed in figure 4-3. In order to test whether or not JNK phosphorylates Bim_{EL}, we made recombinant wild type Bim_{EL} protein and incubated the protein with immunoprecipitated JNK from both untreated and UV-treated HeLa cells (figure 4-14). The GST-c-jun substrate was phosphorylated specifically and robustly by immunoprecipitated JNK from UV-treated cells, however the GST control and Bim_{EL} proteins were not (figure 4-14). In addition, other recombinant Bcl-2 family members were tested to see if any of these proteins were phosphorylated by JNK. There is background phosphorylation of many of these proteins, but there is no induction with UV and the amount of phosphorylation is much less than the GST-c-jun positive control. HA-tagged Bim_{EL} constructs were also transfected into the FLAG-Mcl-1 stable cell line and immunoprecipitated with anti-FLAG antibodies to further test whether Mcl-1 binding might be necessary for phosphorylation of Bim_{EL}. As with recombinant protein, immunoprecipitated Bim_{EL} was not phosphorylated by JNK (data not shown). These results suggest that Bim_{EL} is not phosphorylated directly by JNK in response to UV.

Figure 4-14

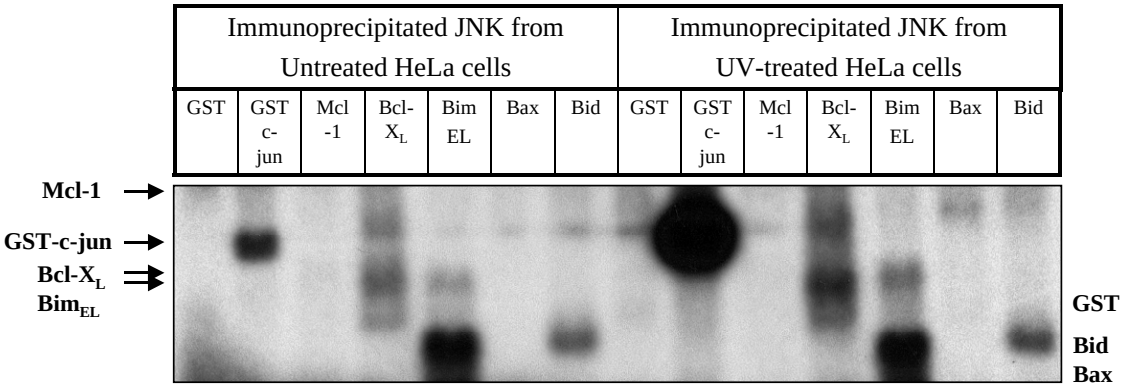

Figure 4-14 JNK does not phosphorylate Bim_{EL} in vitro or other Bcl-2 family members tested. JNK was immunoprecipitated from untreated and UV-treated HeLa cell lysate to get inactive and active JNK ,respectively. The immunoprecipitated JNK was then incubated with approximately 1 ug of recombinant protein and γ-^{32}P-ATP for 30 minutes. The reactions were then run on an SDS-PAGE gel, transferred to nitrocellulose membrane and exposed to film overnight. Similar quantity of protein was verified by Pounceau S staining (not shown).

However, JNK may be involved in caspase activation. The JNK inhibitor SP600125 was used to pre-treat HeLa cells that were then exposed to UV. SP600125 decreased the amount of caspase activation in response to UV (figure 4-15). This is in accordance with experiments from fibroblasts deficient for JNK1 and JNK2 (Tournier et al. 2000), which have reduced cytochrome c release and caspase activation in response to UV. This would be consistent with JNK being the second hit or a component of the second hit. However, our results suggest that although JNK may be involved in cytochrome c release and caspase activation, it is not acting directly on Bim_{EL}.

Figure 4-15

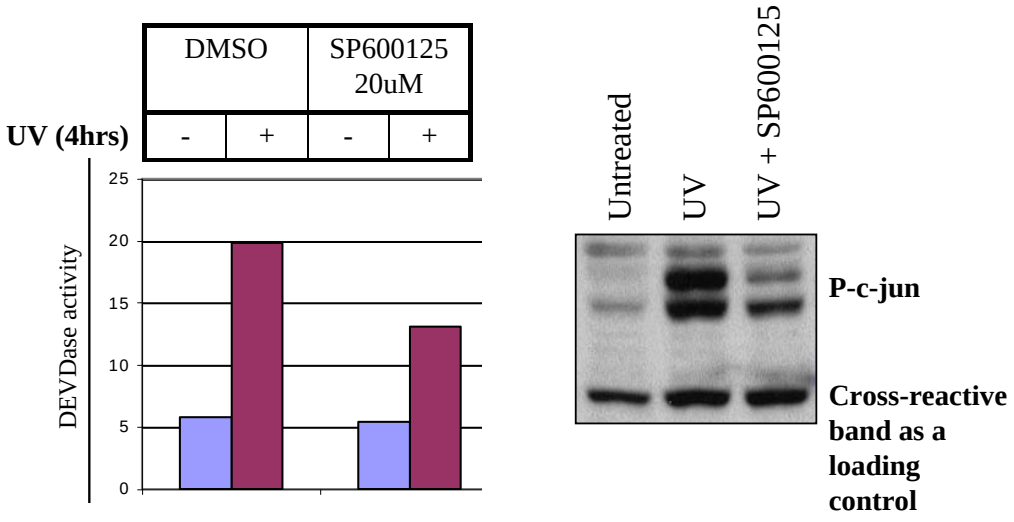

Figure 4-15 Inhibition of JNK inhibits caspase activation. HeLa S3 cells were treated with DMSO or the JNK inhibitor SP600125 for 1 hour and then treated with UV. Four hours after UV the cells were harvested, lysed and the lysate assayed for caspase activity using a fluorogenic substrate. The western blot indicates the inhibition of phospho-c-jun by active JNK after UV.

Further tests of JNK as the second hit were inconclusive. Addition of active JNK to sucrose-buffered cell soup extracts did not cause any release of cytochrome c (data not shown). Moreover, activation of JNK by transfection did not cause an appreciable increase in caspase activity, even in combination with Mcl-1 RNAi (data not shown). So despite the evidence that JNK may indeed be the second hit, we do not have any evidence of this beyond the inhibitor data, which is far from conclusive. However, our results indicate that JNK does not phosphorylate Bim_{EL}, which means that the relevant target of JNK in UV-induced cell death remains a mystery. *In vitro* assays and standard molecular biology techniques so far have not yet been able to dissect how JNK contributes to cytochrome c release and apoptosis.

Discussion

In response to DNA-damaging events such as UV or etoposide, Mcl-1 levels dramatically decrease. Although this decrease in Mcl-1 is required, it is not sufficient to induce cytochrome c release and caspase activation. Accordingly, removal of Mcl-1 acts as a sensitizing event that reduces the threshold for cytochrome c release and caspase activation (Fig. 2D).

In addition to Mcl-1 depletion, a second hit is required for cytochrome c release and caspase activation. Bim_{EL} is both selectively bound by Mcl-1 and dephosphorylated after UV. Although Bim_{EL} dephosphorylation appeared to be a good candidate for the second hit, our results demonstrate that Bim_{EL} dephosphorylation is due to UV inhibition of the ERK1/2 pathway. Inhibition of ERK1/2 dephosphorylates Bim_{EL} but does not accelerate caspase activation. Dephosphorylation of Bim_{EL} is therefore a secondary event unrelated to regulation of cytochrome c release.

Even though there have been many reports of phosphorylation affecting Bim's apoptotic ability we have found no evidence that this is the case in UV-induced apoptosis. RNAi experiments against Mcl-1 accelerate the rate of caspase activation, which is consistent with its role as an apical signal in apoptosis. RNAi against Bim_{EL} is not as easy to determine. Cells one day after RNAi treatment have only a slight reduction in caspase activity even though the level of Bim is clearly reduced. On the other hand, two days after RNAi treatment, there is a further reduction of Bim_{EL} and a much more significant decrease in

caspase activation after UV (data not shown). This may be due to Bim_{EL} RNAi lowering the threshold for cytochrome c release in a manner similar to Mcl-1 RNAi. However, the specific requirement of Bim_{EL} is difficult to determine from RNAi. All in all, Bim_{EL} RNAi is very difficult to interpret in this system.

Although UV attenuates ERK1/2, one kinase that is strongly activated by UV and required for UV-induced apoptosis in mouse embryonic fibroblasts (Tournier et al. 2000) is c-Jun NH2-terminal kinase (JNK). Despite the evidence of JNK in UV-induced cell death, the apoptosis-specific substrates of JNK have remained elusive. Recently, JNK was reported to phosphorylate Bim_L after UV (Lei and Davis 2003). The phosphorylation sites of Bim_L are also conserved in Bim_{EL}. Although mass spectrometry did not identify phosphorylated Bim_{EL} peptides after UV, we tested if JNK phosphorylates Bim_{EL} *in vitro*. Recombinant GST-c-jun substrate was phosphorylated robustly by immunoprecipitated JNK from UV-treated cells whereas recombinant Bim_{EL} was not (data not shown). This suggests that Bim_{EL}, unlike Bim_L, is not phosphorylated by JNK.

This discrepancy between Bim_{EL} and Bim_L may be explained by differential regulation of Bim_{EL} and Bim_L. Bim_L, and the related protein Bmf, have been demonstrated to induce apoptosis by translocation from the cytoskeleton to the mitochondria (Puthalakath et al. 1999; Puthalakath et al. 2001). In healthy cells Bim_L and Bmf are sequestered to the cytoskeleton by dynein light chain (DLC). DLC is complexed with intermediate and heavy chains to form the dynein motor complex, which is involved in retrograde organelle transport along microtubules. After a DNA damage stimulus, such as UV light, Bim_L translocates from the microtubule cytoskeleton to the mitochondria where it antagonizes BCL-2 and leads to

cytochrome c release (Puthalakath et al. 1999; Puthalakath et al. 2001). JNK phosphorylation of Bim_L disrupts binding to DLC, which is proposed to cause Bim_L translocation to the mitochondria (Lei and Davis 2003). On the other hand, we found that Bim_{EL} is already located on the mitochondrial membrane. Mitochondrial localization of Bim_{EL} has also been observed in T cells (Zhu et al. 2004), implying that Bim_{EL} is regulated differently than Bim_L.

The only clear phosphorylation of Bim_{EL} in HeLa cells that we could find was due to ERK1/2. Phosphorylation by ERK1/2 has been reported to regulate Bim_{EL} half-life in response to serum. Signaling through the EGF pathway causes MEK1 and ERK1/2 activation, leading to phosphorylation of Bim_{EL} and a shorter half-life, thereby decreasing the amount of the pro-apoptotic molecule Bim_{EL} (Ley et al. 2003; Luciano et al. 2003; Ley et al. 2004; Marani et al. 2004). Conversely, withdrawal of serum leads to dephosphorylation and accumulation of Bim_{EL} through extension of its half-life. Although dephosphorylation might feasibly result in accumulation of Bim_{EL} in UV treated cells, U0126 does not cause any increase in caspase activity even though it completely dephosphorylates Bim_{EL}. Furthermore, we found that UV does not change the amount of Bim_{EL} over a four-hour time course (data not shown). These results suggest that Bim_{EL} dephosphorylation is likely to play a more important role over longer time periods, such as serum withdrawal or IL-2 withdrawal from lymphocytes (Seward et al. 2003) and is less important for acutely induced apoptosis by UV.

Regulation of Bim_{EL} in response to serum withdrawal also dovetails nicely with regulation of Mcl-1. In the presence of serum, Mcl-1 levels rapidly increase (data not shown) and Bim_{EL} is phosphorylated and decreases over a longer time course. The end result is that cells are more resistant to apoptosis. On the other hand, in the absence of serum Mcl-1 levels

decline (data not shown) and Bim_{EL} is dephosphorylated. The end result is a longer Bim_{EL} half-life causing it to accumulate and tip the balance towards cell death.

Despite the special relationship between Bim_{EL} and Mcl-1, the required removal of Mcl-1 after UV appears to sensitize mitochondria after UV rather than specifically release a modified protein. Sensitization of mitochondria is consistent with Mcl-1 RNAi reducing the threshold for caspase activation and Bim_{EL} RNAi increasing the threshold. Although Mcl-1 may specifically antagonize some other molecule, we were not able to identify it. Our results indicate that the second hit is likely to come from another source.

Our study contributes mostly negative data to the field, but it is important to share the approaches we tried, and the results obtained to further push the study of upstream regulation of cytochrome c release. It was, and remains, the most important question in apoptosis and disease.

APPENDIX A
Experimental Procedures

Reagents

The following antibodies were used for western blots: Monoclonal Bak Ab-1 (Oncogene), polyclonal Bid ((Luo et al. 1998), polyclonal caspase-3 (cell signaling), monoclonal caspase-9 (Cell Signaling), monoclonal cytochrome c (Pharmingen), polyclonal Bcl-x_L (Cell signaling), monoclonal Mcl-1 (Pharmingen), polyclonal Mcl-1 S-19 (Santa Cruz), monoclonal phospho-c-jun KM-1(Santa Cruz) , polyclonal phospho-ERK1/2 (Cell Signaling), polyclonal ERK1/2 (Cell Signaling), and polyclonal Bax N-20 (Santa Cruz). Polyclonal Bim (Stressgen) was used for all western blots with one exception; monoclonal Anti-Bim (Chemicon) was used to test Mcl-1 immunoprecipitates. Immunoprecipiations or immunodepletions for Mcl-1 and Bcl-x_L were performed with polyclonal Mcl-1 (Santa Cruz). Z-VAD.fmk, actinomycin D, cycloheximide, MG132, U0126, SP600125, and epoxomicin were obtained from Calbiochem. All other chemicals were obtained from Sigma and of the highest grade unless otherwise noted.

Cell Culture, UV treatment, and cellular fractionation

HeLa cells were plated in a 15 cm dish at a density of 2 x 10^7 cells (or equivalent amount in 6-well dish) in DMEM medium supplemented with 10% FBS. Several hours before treatment, the media was replaced with 13 ml of fresh medium (1 ml for 6-well plates). The cover of each dish was removed and the cells were treated in a Stratagene

stratalinker with 20 J/m^2 of ultraviolet irradiation (254 nm). At the indicated times after treatment, cells were scraped, collected, and washed once in PBS. The cell pellet was resuspended in 5 times the volume of Buffer A (20 mM Hepes, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF and Complete Protease Inhibitor, Roche) supplemented with 250 mM sucrose, 1 mM $NaVO_4$ and 0.1 μM okadaic acid. The resuspended cell pellet was incubated on ice for 15 minutes before the cells were broken by passing them through a 22 gauge needle 25 times. The resulting broken cell mixture was centrifuged in three sequential steps: 1,000g; 10,000g; and 100,000g. The 10,000g pellet was considered the "mitochondrial" fraction and the 100,000g supernatant (S100) the cytosol. Mitochondrial pellets were lysed in 0.1%NP-40 Buffer A with 1 mM $NaVO_4$ and 0.1 μM okadaic acid, normalized for protein and analyzed by SDS-PAGE. Protein concentrations were normalized using the Bio-Rad Bradford reagent.

2D Gel Electrophoresis

Mitochondria were fractionated as described above and lysed in 4%CHAPS, 7 M urea, 2 M thiourea, 1% IPG Buffer (Amersham), 20 mM Tris pH 9.0, 1 mM $NaV0_4$, 0.1 μM okadaic acid (Alexis Biochemicals), and Complete protease inhibitor (Roche). 50 μg of sample was labeled with Cy3 or Cy5 dyes (Amersham RPK 0273 and RPK 0275) in the dark on ice for 30 min. The reaction was quenched by adding 1 μl of 10 mM free lysine and incubated for another 10 minutes on ice. The Cy3 and Cy5 labeled samples were then mixed together and the total volume increased to 350 μL using rehydration buffer: 4%CHAPS, 7 M urea, 2 M thiourea and 1% IPG Buffer (Amersham). The labeled sample was then focused on

24 cm Immobiline Drystrip (Amersham) using an IPGPhor (Amersham). The following parameters were used to focus protein: 50 μA per IPG strip; all steps at 20 degrees Celsius; S1, Step-n-hold Rehydration 0 hrs; S2, Step-n-hold 30 V for 12 hrs; S3, Step-n-hold 1000 V for 1000 Vhrs; S4 Step-n-hold 8,000 V for 60,000 Vhrs. The Immobiline strips were then equilibrated and reduced with 50mM Tris pH 8.8, 6 M urea, 35% glycerol, 2%SDS and 5 mg/ml DTT for 10 minutes at room temperature with gentle rocking. A second equilibration to prevent oxidation was performed in 50mM Tris pH 8.8, 6 M urea, 35% glycerol, 2%SDS and 45 mg/ml iodoacetamide. Then the strip was placed on top of a 12% SDS-PAGE gel, held in place with a small amount of agarose in SDS running buffer, and run according to standard protocol. The Cy dyes were then scanned using a Typhoon gel scanner (Amersham) and analyzed with DeCoder software (Amersham).

Bak Oligomerization

Mitochondria (1 mg/ml) were incubated in buffer A with 250 mM sucrose in the presence of 1 mM Bismaleimidohexane (Calbiochem) for 30 min at room temperature. (Wei et al. 2000) Following incubation the mitochondria were pelleted and resuspended in 1 x SDS loading buffer. Samples were subjected to SDS-PAGE and blotted with monoclonal Anti-Bak.

Priming

Mitochondria were isolated from HeLa S3 cells as described above. 200 μg of mitochondria was incubated with 600 μg of S20 or S100 and incubated at 37 degrees Celsius

for 30 minutes. The mitochondria were pelleted with a 10,000G spin, the supernatant removed, and the mitochondria pellet resuspended in 100 μL of Buffer A containing 250 mM sucrose and 150 mM NaCl. This was incubated again for 15 minutes at 37 degrees. The sample was then divided into two 50 μL aliquots and one was oligomerized with BMH (see above) while the other was pelleted and the supernatant run on SDS-PAGE and probed for cytochrome c.

RNA interference

RNAi knockdown experiments were done according to previous reports (Elbashir, 2001). Annealed, purified, and desalted double stranded siRNA, Luciferase (AACGUACGCGGAAUACUUCGA), Mcl-1 (AAGAAACGCGGUAAUCGGACU) Bim2 (siGENOME D-004383-02), and Bim3 (siGENOME D-004383-03) were ordered from Dharmacon Research, Colorado. 1.5×10^5 HeLa cells were plated in a 6-well plate and the next day the cells were transfected with 200 nM of siRNA in Opti-MEM medium (Invitrogen) without FBS using Oligofectamine reagent (Invitrogen) according to the manufacturer's transfection protocol. 2 days later, the medium over the cells was adjusted to 1 ml and they were treated with UV light as described above.

Immunoprecipitation

For immunodepletion and Mcl-1 immunoprecipitation experiments, samples were incubated with beads precoupled to antibody. Protein A agarose beads (Santa Cruz) (0.5 ml bed volume) were incubated in 1 ml of PBS with 1 mg/ml of BSA overnight at 4°C alone,

with 100 μg polyclonal Anti-Mcl-1, Bcl-x_L or Bid. After incubation, the beads were pelleted by centrifugation and washed at least 3 times. For immunoprecipitation experiments, harvested mitochondria were lysed in 1% NP40 in Buffer A with 100 mM NaCl. 10 μl of precoupled beads were incubated in 100 μl of mitochondrial (2 mg/ml) lysate in lysis buffer overnight at 4 degrees. The beads were pelleted, washed three times with lysis buffer and eluted in 100 μl of a SDS gel loading buffer.

FLAG-Mcl-1 Immunoprecipitation

8 15 cm dishes were seeded with M120 stable cell line (Nijhawan et al. 2003) and 4 treated with UV. The cells were then harvested and lysed in 4ml of 0.1%NP-40 Buffer A supplemented with Complete protease inhibitors (Roche). 11ml of 0.1%NP-40 Buffer A with 750mM NaCL and 15 μl of Anti-FLAG M2 beads (Sigma) were added to bring the volume to 15ml and incubated at 4°C overnight. The pellet was then washed 7 times with 0.1%NP-40 Buffer A with 750mM NaCl and FLAG-Mcl-1 eluted with 0.5 mg/ml 3X FLAG peptide (Sigma) in 0.1%NP-40 Buffer A. The eluted sample was then analyzed by SDS-PAGE and Colloidal Coomassie Stain (Invitrogen). Immunoprecipitation with transfected HA-Bim_{EL} constructs was similar but preceded by transfection 1 day earlier.

HA-Bim_{EL} Immunoprecipitation

2 μg of HA-Bim in pCMV was transfected with 60 μl PLUS reagent and 90 μl Lipofectamine (Invitrogen) into 15 cm dishes seeded with 10 x 10^7 B5 Bcl-x_L stable cells

(Nijhawan et al. 2003) the day before. 4 dishes were UV-treated and incubated for 5 hours. The cells were then lysed, along with control transfected and untransfected cells, in 8 ml of 1%Triton X-100 Buffer A containing 150mM NaCl, 1 mM $NaVO_4$, 0.1 μM okadaic acid, and 200 nM Staurosporine. 80 μl of anti-HA matrix (Roche) was added to the lysate, the salt adjusted to 650 mM NaCl, and incubated at 4°C overnight. The next day the pellet was washed 2 times in 8 M urea and 15 times in 1%Triton Buffer A with 150 mM NaCl to remove all traces of urea. HA-Bim_{EL} was eluted by boiling in SDS dissociation buffer (20 mM Tris pH 7.5 50 mM NaCl 5 mM DTT and 1%SDS). The eluted lysate was diluted with 300 ul of 1%Triton X-100 Buffer A containing 150mM NaCl, 1 mM $NaVO_4$, 0.1 μM okadaic acid, and 200 nM Staurosporine. 30 μl of anti-HA matrix was added and incubated at 4°C overnight. The next day the pellet was washed 3 times with 1%Triton X-100 Buffer A with 1 M NaCL and 3 times in 1%Triton X-100 150mM NaCl and HABim_{EL} eluted by boiling in SDS sample buffer. The eluted sample was analyzed using SDS-PAGE and stained with Coomassie (Bio-Rad).

Transfection

Transfections were performed using Lipofectamine PLUS (Invitrogen) according to manufacturer's recommendations. For 6-well transfections, 2×10^5 cells were plated the day before and transfected using 4 μl PLUS and 4 μl Lipofectamine.

Mcl-1 and Bcl-x_L overexpressing stable cell lines

Full length Mcl-1 cDNA was cloned into HindIII and BamHI of p3XFLAG-CMV-10 (Sigma). The resulting fusion protein was full length Mcl-1 with three flag epitopes at the N-terminus followed by a two amino acid linker. The full length Bcl-x_L was cloned into XhoI and NdeI of pCDNA 3.1 (-) (Invitrogen) with a single Flag tag at the N-terminus followed by a short linker sequence. Empty vector or subcloned plasmids were transfected into 5 x 10^5 attached HeLa cells grown in DMEM with 10% FBS using Lipofectamine Plus transfection reagent (Invitrogen) according to the manufacturers protocol. Two days later, the cells were transferred to 20 100 mm dishes in DMEM with 10% FBS and 0.5 mg/ml of G418. After several weeks, individual clones were lifted and tested for expression of the transgene.

Recombinant Mcl-1 and Bcl-x_L

The cDNA of full-length human Mcl-1 was subcloned into NdeI and SapI sites of pTYB1 vector (New England Biolabs). Full length Bcl-x_L cDNA was cloned into NdeI and XhoI of the pet15B vector (Novagen). BL21 (DE3) cells transformed with pTYB1-Mcl-1 or pet 15b-Bcl-x_L were grown at 30°C in LB-Amp until the OD (600 nm) was 0.4 - 0.8. For Bcl-x_L, IPTG was added to 1 mM for 4 hours at 30 ° and for Mcl-1 0.3 mM for 6 hr at 20°C to induce expression of the fusion protein. After induction, the bacteria were harvested and sonicated. For pTYB-Mcl-1, the supernatant was loaded onto chitin resin and the fusion protein was cleaved to elute full-length recombinant Mcl-1 without modification according to the protocol from the manufacturer (New England Biolabs). The eluted recombinant Mcl-1 protein was purified in two additional consecutive steps: Hitrap Q sepharose and Superdex

200 size exclusion chromatography. For His-Bcl-x_L, the supernatant was run over Ni-NTA resin (Qiagen), equilibrated in buffer A. The column was washed with buffer A with 1M NaCl and the recombinant protein was eluted with 250 mM imidazole in buffer A. The eluted recombinant Bcl-x_L was then further purified and concentrated on a Hitrap Q sepharose column.

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VITAE

Elie Abraham Traer was born in Mariemont, Ohio, on January 12, 1973, the son of Nancy Louise Traer and Robert Allen Traer. After completing his work at Albany High School, Albany, California in 1991, he entered the University of Chicago, Chicago, Illinois. He received the degree of Bachelor of Arts in Chemistry and also fulfilled the requirements for a Bachelor of Science in Mathematics in June of 1995. During the following three years he was employed as a laboratory technician at the University of Utah, Salt Lake City, Utah working for Stephen M. Prescott, MD. In June, 1998 he entered the Medical Scientist Training Program at the University of Texas Southwestern Medical School at Dallas, Texas. He did his graduate work with Xiaodong Wang, PhD and defended his thesis in September, 2004. He received his Doctorate of Medicine and Doctorate of Philosophy degrees in June, 2006. He is married toJennifer Beth Dunlap, whom he met at UT Southwestern. Their son, Noah Cade Traer, was born in August, 2002.

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