BIOCHEMICAL PATHWAYS IN APOPTOSIS

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Dedication

To my parents who gave me infinite inspiration and support.

# BIOCHEMICAL PATHWAYS IN APOPTOSIS

by

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DISSERTATION

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**Acknowledgements**

This is by far the most important section of this document where I will have the chance to acknowledge all the people that made this work possible. The list is long, so long that I cannot reasonably expect it to be totally complete, but I will be sure not to omit the most important people. It takes many different types of contributions to make the work possible. One way to describe the effort is to make an analogy to a football organization that includes both players and coaches.

The players on our team included myself, Min Fang, Elie Traer, Qing Zhong, and Wenhua Gao. The game really began when Min first characterized an in vitro assay that reconstitutes UV dependent cytochrome c release. His initial characterization of the assay gave me the foothold to begin purifying proteins that regulate cytochrome c release. From there, Min Fang, Elie Traer and myself begin working towards all of the conclusions presented here. Our collaboration was very close; one person's experiment on Monday might alter another's on Tuesday. Each of us was necessary but neither

alone sufficient to finish the work. Like a football team, the offensive line has to block for the quarterback who has to make a perfect throw to a receiver who needs to run quickly and catch the ball. If any part the team fails, the play will not be executed. Outside of science, we all became close friends passionately searching for details and clues to answer the most intriguing questions. In the final stages of the project we got key contributions on recombinant protein technology and protein half-life studies from new additions to our lab, Wenhua Gao and Qing Zhong. They hastened our progress and we are grateful for their help. In addition, Hongjun Shu and Yingming Zhao gave us invaluable technical assistance in identifying our purified protein products. The pace and success of our work depended directly on their contribution. Renee Harold and especially Fenghe Du provided critical technical assistance throughout our project.

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BIOCHEMICAL PATHWAYS IN APOPTOSIS

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Caspases are a family of proteases that once activated execute apoptosis, a cellular suicide pathway. Activated caspases have a unique property to cleave and activate themselves. Once the first caspase is activated, it generates a chain reaction resulting in robust caspase activity and rapid death. The central question in apoptosis is to understand how the first caspase is activated. To address

this question, we present an assay that recapitulates de novo caspase activation in vitro. We describe how this assay was used to purify three proteins that were sufficient to reconstitute caspase activation in vitro: Apaf-1, cytochrome c, and caspase-9. The mechanism of caspase activation in vivo, however, is complicated by these proteins subcelluar localization. In the living cell, Apaf-1 and caspase-9 are cytoplasmic whereas cytochrome c is mitochondria, however, during apoptosis, cytochrome is released to the cytoplasm. In vitro, cytochrome c induces the formation of a stable Apaf-1/caspase-9 complex and caspase-9 autoactivation suggesting that cytochrome c release from the mitochondria to the cytosol is the rate-limiting event that leads to caspase activation. This conclusion shifted the focus of our studies upstream from what initiates caspase activation to what triggers cytochrome c release. The second part of the dissertation uses a biochemical approach to identify how cytochrome c release is regulated after exposure to ultraviolet light.

Ultraviolet light irradiation of HeLa cells triggers an apoptotic response mediated by mitochondria. Biochemical analysis of such a response revealed that the initial step leading to cytochrome c release is the complete disappearance of the mRNA of Mcl-1, an anti-apoptotic member of the Bcl-2

family. This event leads to the elimination of Mcl-1 protein from cells due to the short half-life of its protein. The block or delay of Mcl-1 disappearance by either proteasome inhibitors or Mcl-1 over-expression prevents subsequent steps of this apoptotic pathway including the translocation of Bax and Bcl-$x_L$ from cytosol to mitochondria and dephosphorylation of $Bim_{EL}$ on mitochondria. These sequential events lead to the oligomerization of Bax and Bak on the mitochondria, cytochrome c release and caspase activation.

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## Prior publications

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# Chapter 1

## *Programmed Cell Death or Apoptosis*

In the early twentieth century dead cells were most often identified in degenerative tissue but by the mid-twentieth century several different scientists surprisingly found cells dying during normal vertebrate development (Glucksmann, 1965). Cells dying during development exhibited morphological characteristics different from cells that suffered a toxic insult. During development, dying cells shrunk and condensed without compromising their membrane, but injured cells swelled and lost membrane integrity. These initial observations inspired a conceptual leap suggesting that during development, a subset of cells might activate an intrinsic cellular suicide program termed Programmed Cell Death (PCD). Further emphasizing the suicidal nature of PCD, during *Xenopus* development, cell death was inhibited by cycloheximide, suggesting that activation of the suicide pathway requires new protein synthesis (Tata, 1966). In 1972, Kerr and colleagues found that cells die during normal tissue turnover with the same morphology as those that die during development (Kerr et al., 1972). They called the process apoptosis, after the

Greek word meaning leaves falling from a tree. Today, the terms programmed cell death and apoptosis are used interchangeably.

Medical problems revolve around the life or death of the cell. The discovery of a regulated cellular suicide pathway completely changed the way biologists approached the question. Instead of simply understanding the nature of cellular defense against environmental toxins, modern biologists must also consider how and why cells *decide* to die.

***Apoptotic Pathways in C. elegans and Mammals***

The first molecular components of the apoptosis pathway were discovered through a series of genetic experiments in the worm, *C. elegans*. In *C. elegans,* 131 of 1090 somatic cells die predictably and precisely during development (Sulston, 1976). By selecting for mutant worms with defects in the apoptosis pathway, Horvitz and colleagues identified four genes important for apoptosis: *Ced-3*, *Ced-4*, *Ced-9* and *Egl-1* (Figure 1-1, upper panel) (Horvitz, 1999). This set of four genes can be divided into positive and negative regulators of apoptosis. *Egl-1*, *Ced-4* and *Ced-3* are required for apoptosis since loss-of-function mutations lead to the survival of essentially all cells that normally die (Conradt and Horvitz,

1998; Yuan and Horvitz, 1990). On the other hand, *Ced-9* negatively regulates apoptosis (Hengartner et al., 1992). The loss of *Ced-9* leads to inappropriate apoptosis whereas its over-expression completely blocks apoptosis.

The basic apoptosis pathway in *C. elegans* is evolutionarily conserved in vertebrates (Figure 1-1). *Ced-9* is homologous to the anti-apoptotic *Bcl-2* gene that was first identified as an over-expressed oncogene in a common human lymphoma (Hengartner and Horvitz, 1994). *Egl-1* is homologous to the BH3-only members of the Bcl-2 family, a set of proteins that induce apoptosis by antagonizing Bcl-2 activity. Ced-4 and its mammalian homologue Apaf-1 are adapter proteins that positively regulate the activation of Ced-3 and Caspase-9, respectively.

Ced-3 and Caspase-9 are specific cysteine proteases that cleave their substrates with signature specificity (Thornberry and Lazebnik, 1998). In living cells, they exist as inactive zymogens, procaspases, which are classified into two categories: initiators and effectors. Initiator caspases are the apoptotic trigger. Upon activation they cleave and activate effector caspases that execute the dismantling of the cell. Morphological characteristics of a dying cell including

chromatin condensation and DNA fragmentation are the direct result of caspase activity.

***Mitochondria and Caspase Activation***

Each cell possesses a "time bomb" of caspase activity; the activation of a small number of caspase molecules triggers a chain reaction resulting in the total dismantling of the cell. In order to avoid an unintended fatal "explosion", cells must precisely regulate caspase activation with many safeguards. Caspase activation is regulated by a set of pro-apoptotic proteins that are stored in the mitochondria. Early in apoptosis changes in the permeability of the outer mitochondrial outer membrane lead to the release of cytochrome c and Smac that trigger caspase activation (Figure 1-2).

Cytochrome c is a 13 kD soluble electron transfer protein located exclusively in the mitochondrial intermembrane space. During apoptosis, cytochrome c is released from the mitochondria to the cytosol where it binds Apaf-1 (Liu et al., 1996). Apaf-1 is a 130 kD cytosolic monomer consisting of three distinctive domains: a caspase recruitment domain (CARD), Ced-4 homologous domain, and a series of WD40 repeats (Zou et al., 1997). Cytochrome c binds to the WD40 domain of Apaf-1 and increases the affinity of Apaf-1 for either dATP or

ATP by approximately 10 fold (Jiang and Wang, 2000). The binding of nucleotide to Apaf-1 triggers formation of the "apoptosome", a multimeric Apaf-1/cytochrome c/dATP complex (Acehan et al., 2002). In the apoptosome, the exposed CARD domains of Apaf-1 recruit and catalyze the autoactivation of procaspase-9 (Li et al., 1997).

XIAP binds to the cleaved form of caspase-9 in the apoptosome and blocks it activity (Srinivasula et al., 2001). The IAP (Inhibitor of Apoptosis) family of proteins serves as a safety net for the cell against accidental cell death caused by transient releases of cytochrome c. During full-blown mitochondrial apoptosis, concurrent with cytochrome c, Smac (second mitochondrial activator of caspases) is released into the cytoplasm (Figure 1-3) (Du et al., 2000; Verhagen et al., 2000). Smac is a 55 kD that is targeted to the mitochondria by a 55 amino acid mitochondrial targeting sequence at its N-terminus. Once in the mitochondria, the targeting sequence is cleaved so that the N-terminus of the mature Smac molecule released into the cytoplasm begins with AVPI. These four residues are required and sufficient to bind to XIAP with high affinity and in so doing liberate cleaved caspase-9 from the shackles of XIAP (Chai et al., 2000). Activated caspase-9 in the apoptosome then is free to cleave and activate downstream

caspases such as caspase-3, caspase-6, and caspase-7, constituting the main caspase activity of apoptotic cells (Faleiro et al., 1997; Li et al., 1997).

The mitochondrial apoptosis pathway derived primarily from biochemistry and molecular biology has been genetically verified in mice by using gene knockout technology. Mice deficient in caspase-3, caspase-9, and Apaf-1 exhibit a similar phenotype, late embryonic to perinatal lethality due to a failure in nervous system development (Cecconi et al., 1998; Kuida et al., 1998; Kuida et al., 1996; Yoshida et al., 1998). The mice suffer from gross malformations in neuronal architecture as evidenced by severe neuronal hyperplasia that causes the forebrain to extrude from the skull (Figure 1-4). The strikingly similar phenotype between these different mice confirmed that these molecules are working in the same pathway.

In vitro experiments using embryonic fibroblasts from the knockout mice further validated the proposed linear caspase activation model: Apaf-1 → Caspase-9 → Caspase-3 → death morphology (Figure 1-3). Fibroblasts deficient in either Apaf-1 or caspase-9 release cytochrome c but do not activate Caspase-3 in response to multiple apoptotic stimuli (Hakem et al., 1998; Yoshida et al., 1998). Further downstream,

caspase-3 deficient cells treated with apoptotic stimuli do not exhibit downstream apoptotic markers such as DNA fragmentation and membrane blebbing (Janicke et al., 1998; Zheng et al., 1998). Just as Horvitz demonstrated in *C. elegans*, these experiments genetically defined a linear pathway for caspase activation in mammals.

Before its identification as an apoptosis activator, cytochrome c was originally known as a member of the electron transport chain involved in mitochondrial respiration and ATP synthesis. Because cytochrome c is important for something as vital as ATP generation, mice deficient in cytochrome c were not expected to survive. Surprisingly however, cytochrome c deficient mice do survive to E8.5 although the embryos are markedly reduced in size (Li et al., 2000). Cytochrome c deficient cells can be cultured in a special medium that accommodates their defect in mitochondrial respiration. As would be predicted, apoptotic stimuli do not activate caspase-3 in cytochrome c deficient cells. These cells are resistant to apoptosis induced by ultraviolet radiation, staurosporine and serum withdrawal. The defects in apoptosis are restricted to the mitochondrial death pathway; cytochrome c deficient cells are still susceptible to TNF induced apoptosis.

In contrast, mice deficient in XIAP or Smac do not have any gross phenotypic abnormalities (Harlin et al., 2001; Okada et al., 2002). In both cases, there are other known proteins with redundant function that may compensate. For example, N-IAP may compensate for a deficiency in XIAP. In the same way, Omi/HtrA2 may compensate for Smac; it like Smac is a protein released from the mitochondria that binds to and inactivates IAP (Hegde et al., 2002) (Suzuki et al., 2001).

Since the initial characterization of apoptosis, the "holy grail" of apoptosis has been to understand how and why a cell decides to execute itself. Biochemical and genetic experiments in mammalian systems pinpoint the release of mitochondrial proteins such as cytochrome c and Smac as the earliest known commitment steps to apoptosis. In the search for the life/death switch, the next question is to understand how and why the cell releases these proteins?

***Regulation of Cytochrome c Release by the Bcl-2 Family***

The founder of the Bcl-2 family, Bcl-2, was discovered at the translocation breakpoint t(14,18) of patients with Follicular B-cell lymphoma (Tsujimoto et al., 1985; Tsujimoto et al., 1984). In these patients, the heavy chain promoter drives the constitutive over-expression of Bcl-2 in B-cells

leading to a slow-growing lymphoma that is usually completely resistant to treatment. Two intriguing observations made the identification of Bcl-2 as an oncogene unique. First, in contrast to other known oncogenes such as Ras and Myc the exogenous expression of Bcl-2 did not increase the proliferation rate of cells. Secondly, immuno-localization and sub-cellular fractionation studies suggested that Bcl-2 is enriched in the outer mitochondrial membrane (Nguyen et al., 1993). Adams and Cory resolved the first mystery when they demonstrated that Bcl-2 might cause lymphoma by inhibiting apoptosis rather than inducing proliferation (Vaux et al., 1988). They showed that Bcl-2 over-expression blocked cell death in the absence of cell survival factors. Follicular B-cell lymphoma was the first example of a cancer that is caused by a failure to die rather than uncontrolled cell division. The importance of the mitochondrial localization of Bcl-2 remained a mystery until the discovery of cytochrome c. Once cytochrome c was identified as a caspase activator, the obvious prediction was that Bcl-2 regulates the release of cytochrome c from the mitochondria. Two different groups demonstrated that the over-expression of Bcl-2 inhibits the release of cytochrome c after a variety of different apoptotic stimuli (Kluck et al., 1997; Yang et al., 1997). The

description of Bcl-2's function solved many mysteries in apoptosis and in so doing also erased nagging skepticism about the importance of cytochrome c.

In the past decade, several new Bcl-2 family members have been identified most often by using screens for interacting proteins such as the yeast two hybrid (Gross et al., 1999). Interestingly, different members of the Bcl-2 family share significant sequence and structural homology but have opposing functions, pro- and anti-apoptotic (Figure 1-5)(Cory and Adams, 2002). In contrast to Bcl-2 which is anti-apoptotic, over-expression of pro-apoptotic Bcl-2 family members leads to cytochrome c release and inappropriate apoptosis (Kim et al., 1997; Oltvai et al., 1993; Vaux et al., 1988). The pro-apoptotic members of the family can be further subdivided into two groups based on sub-cellular localization and homology. A subset of the pro-apoptotic members such as Bid, Bim, and Bad share homology in a single domain, the BH3 domain, and localize to compartments other than the mitochondria. On the other hand, Bak and Bax contain four homologous domains (BH1-4). Bax is present in both the cytoplasm and mitochondria whereas Bak is exclusively localized to the mitochondrial membrane. However, after an apoptotic stimulus, Bax undergoes a conformational change and cytoplasmic Bax translocates to

the mitochondria where it integrates into the membrane (Hsu et al., 1997; Wolter et al., 1997).

Experiments using chemical cross-linkers suggest that during apoptosis mitochondrial Bax and Bak form higher order oligomeric complexes (Gross et al., 1998; Wei et al., 2000). Bak and Bax are required for the release of cytochrome c since Bax/Bak deficient fibroblasts are resistant to multiple apoptotic stimuli including staurosporine, ultraviolet radiation, etoposide, and serum withdrawal, but are still sensitive to TNF (Wei et al., 2001). Additionally, their respective oligomerizations are sufficient for cytochrome c release. Gross and colleagues use a FK1012 ligand and FK1012-Bax fusion proteins to artificially induce Bax oligomerization and cytochrome c release (Gross et al., 1998). BH3-only proteins regulate Bak and Bax oligomerization. During apoptosis, BH3 only proteins translocate from their outside compartments to the mitochondria where they activate the oligomerization of Bak and Bax.

### *Pro-Apoptotic Bcl-2 Family Members: BH3-Only Proteins*

Several different ligands such as TNF (tumor necrosis factor), Fas, or TRAIL can trigger the activation of the apoptotic pathway (Ashkenazi and Dixit, 1998; Ashkenazi and

Dixit, 1999). These ligands bind to their respective receptors, which are all homologous. Ligand binding activates the receptors to form a larger protein complex called the death inducing signaling complex (DISC) (Figure 1-2). The DISC contains an adaptor protein, FADD (Fas associated death domain containing), which recruits procaspase-8 and catalyzes its autoactivation. In some cell types, caspase-8 activation in the DISC is sufficient to trigger apoptosis (type 1) even in the absence of cytochrome c release (Scaffidi et al., 1998). However, in other systems (type 2), such as Fas induced hepatocellular apoptosis, cytochrome c release and caspase-9 activation are also required to complete the apoptosis pathway. Bid is a BH3-only family member that mediates cross talk between caspase-8 activation and cytochrome c release. Activated caspase-8 cleaves Bid, and the truncated Bid (tBid) translocates to the mitochondria and triggers the oligomerization of Bak and the release of cytochrome c (Li et al., 1998; Luo et al., 1998; Wei et al., 2000). The translocation of tBid requires the presence of cardiolipin in the mitochondria and is dramatically increased by tBid myristolation (Lutter et al., 2000; Zha et al., 2000).

The importance of cross talk between DISC and cytochrome c release is emphasized in Bid knockout mice. Wild type mice

injected with Anti-Fas die within hours due to massive hepatocellular apoptosis. Bid deficient mice, however, survive without any cytochrome c release or hepatocellular apoptosis presumably because Bid cleavage by caspase-8 is required for cytochrome c release and apoptosis (Yin et al., 1999). Similarly, Bax/Bak deficient mice are also resistant to Anti-Fas treatment. Following treatment, their hepatocytes show no cytochrome c release or effector caspase activation (Wei et al., 2001). By using mouse genetics, Korsmeyer and colleagues mapped a linear pathway for cytochrome c release consistent with biochemical data: Activated BH3 protein (tBid) → Bak/Bax oligomerization → Cytochrome c release (Figure 1-6).

Bim like Bid is another BH3-only protein that translocates to the mitochondria during apoptosis. In normal living cells, Bim is bound to the dynein motor complex (Puthalakath et al., 1999). After cells are induced to die by apoptosis, the Bim/LC8 complex dissociates from the cytoskeleton and translocates to the mitochondria where it induces cytochrome c release. Mice deficient in Bim have apoptotic abnormalities in lymphocytes (Bouillet et al., 1999). Unlike Bid, the regulation of Bim/LC8 translocation to the mitochondria is unknown.

Some BH3-only proteins such as Noxa and Puma are transcriptionally upregulated during apoptosis. In response to DNA damage, increased amounts of p53 induce the transcription of Puma and Noxa (Miyashita and Reed, 1995; Nakano and Vousden, 2001; Oda et al., 2000). An increase in the levels of these pro-apoptotic Bcl-2 family members is thought to tilt the delicate balance between opposing Bcl-2 family members in favor of apoptosis.

In contrast to death signals such as DNA damage and TNF treatment that activate BH3 only proteins, extracellular survival pathways such as IGF (insulin growth factor) inhibit these proteins. IGF activates the phosphotidylinositol-3 (PI3) kinase/Akt pathway, leading to phosphorylation of the Bad protein (Datta et al., 1997). Phosphorylated Bad is bound by the protein 14-3-3 and sequestered in the cytoplasm (Zha et al., 1996). During apoptosis, Bad is dephosphorylated, released by 14-3-3 and translocates to the mitochondria.

Different apoptotic stimuli activate BH3-only proteins in a variety of ways: post-translationally, proteolytic cleavage, and transcriptionally. Although BH3-only proteins are activated in different ways, once activated each BH3-only protein acts in a similar fashion. They translocate to the

mitochondria, induce a loss of mitochondrial membrane potential and release cytochrome c.

In 2001, Korsmeyer and colleagues summarized the current thinking on how the Bcl-2 family regulates mitochondrial apoptotic events (Figure 1-6) (Cheng et al., 2001). After an apoptotic stimulus, BH3-only proteins are post-translationally activated. Activated BH3-only proteins translocate to the mitochondria where they bind to and catalyze the oligomerization of Bax and Bak. Bax and Bak oligomerization induce cytochrome c release. Bcl-$x_L$ and Bcl-2 block apoptosis by binding to and inhibiting activated BH3-only proteins. The anti-apoptotic activity of Bcl-$x_L$ correlates with its ability to bind to BH3-only proteins but not Bax or Bak.

## Mechanism of ***Cytochrome*** c Release

There is no consensus as to the exact mechanism of cytochrome c release by the Bcl-2 family of proteins. One hypothesis is that changes in mitochondrial membrane permeability induce mitochondrial swelling causing outer membrane rupture (Vander Heiden et al., 1997). The permeability transition pore (PTP) is thought to regulate mitochondrial membrane permeability (Zamzami and Kroemer, 2001). The PTP is thought to be a large protein complex

containing both inner and outer mitochondrial membrane proteins including VDAC (Voltage dependent anion channel), ANT (Adenine nucleotide transporter), and CycD (Cyclophilin). In vitro, the PTP is permeable to molecules less than or equal to 1500 daltons. Opening of the PTP may lead to mitochondrial depolarization, loss of transmembrane potential, swelling and rupture. On the other hand, increases in outer membrane permeability may occur independent of swelling.

In some cases, cytochrome c release is independent of changes in mitochondrial transmembrane potential. In liposomes, VDAC forms a channel regulated by Bcl-$x_L$ that releases cytochrome c (Shimizu et al., 1999). Structural analyses of Bcl-$x_L$ and Bid suggest that Bcl-2 family members may have channel properties in the membrane (Chou et al., 1999; McDonnell et al., 1999; Minn et al., 1997; Muchmore et al., 1996). Bak or Bax oligomers may form a channel that increases cytochrome c permeability directly or alters ion balance leading to mitochondrial swelling and rupture (Kuwana et al., 2002).

***The Point of No Return***

Although apoptosis has been extensively studied in the context of vertebrate and invertebrate development, much of

the current enthusiasm in apoptosis research stems from its prevalence in common human diseases. Cells must walk a fine line; disease may be the result of too little or too much apoptosis. In cases such as cancer and chemotherapeutic resistance disease is the result of an apoptotic failure. On the other hand, during stroke, myocardial infarction, or Alzheimer's disease cells inappropriately die by apoptosis.

The quest to control cell fate, to eliminate unwanted cancer cells and preserve vital neurons, has driven researchers upstream to map a basic apoptosis pathway from the basic microscopic description of apoptosis to the activation of BH3-only proteins. Even though nearly 60,000 papers have been published on apoptosis, the most essential question remains. Where is the *point of no return*? When is it too late to save the cell? The cell may be committed to die even before cytochrome c release. Indeed, activated BH3-only proteins induce mitochondrial dysfunction and death even in the absence of cytochrome c release or caspase activation (Cheng et al., 2001). Hence, blocking cytochrome c release may not be enough to save a dying cell. If we hope to save an ischemic cardiac myocyte on the brink of death, then we must block the apoptosis pathway before the cell commits to die.

Our objective is to identify upstream events that regulate cytochrome c release in a model apoptosis system, ultraviolet radiation. Hopefully, we will charter far enough upstream to find out how and when the cell decides to pull the life-death switch.

***Ultraviolet Radiation Stress Response***

Of all the classical apoptosis inducers, ultraviolet (UV) radiation may be the most pertinent to health and disease. After all, in light of a rapidly diminishing ozone layer, increased exposure to harmful ultraviolet radiation is leading to more cases of malignant melanoma.

Damage inflicted by UV includes but is not necessarily limited to the formation of DNA pyrimidine dimers. Cells possess a complicated, two-tier response to UV induced damage. They activate a cell cycle checkpoint and in extreme cases when the damage is irreparable they commit apoptosis. These responses are mediated by members of the MAPK kinase family, JNK and p38, which induce apoptosis and G2-M arrest, respectively (Davis, 2000; Karin, 1998).

JNK is the earliest upstream factor that activates cytochrome c release in response to ultraviolet radiation. JNK over-expression induces apoptosis by exclusively

activating the mitochondrial death pathway. Bax/Bak deficient cells are resistant to JNK induced apoptosis (Lei et al., 2002).

Experiments testing the loss of JNK function confirm its requirement for ultraviolet induced cytochrome c release. After ultraviolet treatment, fibroblasts with all of the JNK genes deleted do not release cytochrome c and are resistant to apoptosis (Tournier et al., 2000). Although there are reports claiming that JNK phosphorylates Bcl-2 family members, there is no consensus as to how JNK activation leads to cytochrome c release (Yamamoto et al., 1999). Nonetheless, JNK is activated minutes after exposure to ultraviolet radiation placing it far upstream in the cytochrome c release pathway.

Figure 1-1

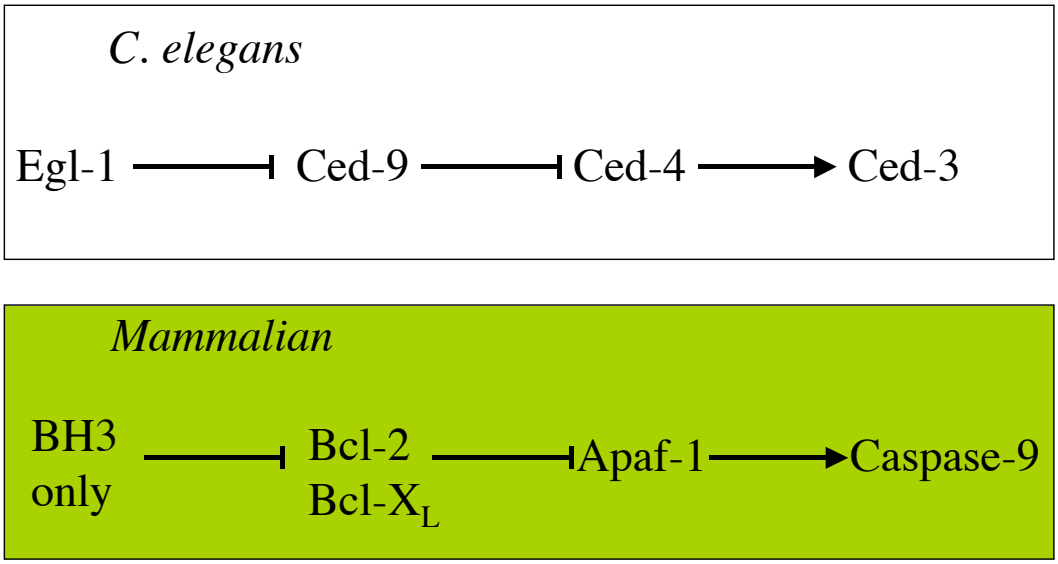



**Figure 1-1: Apoptosis is conserved between mammals and C. elegans.**

Figure 1-2

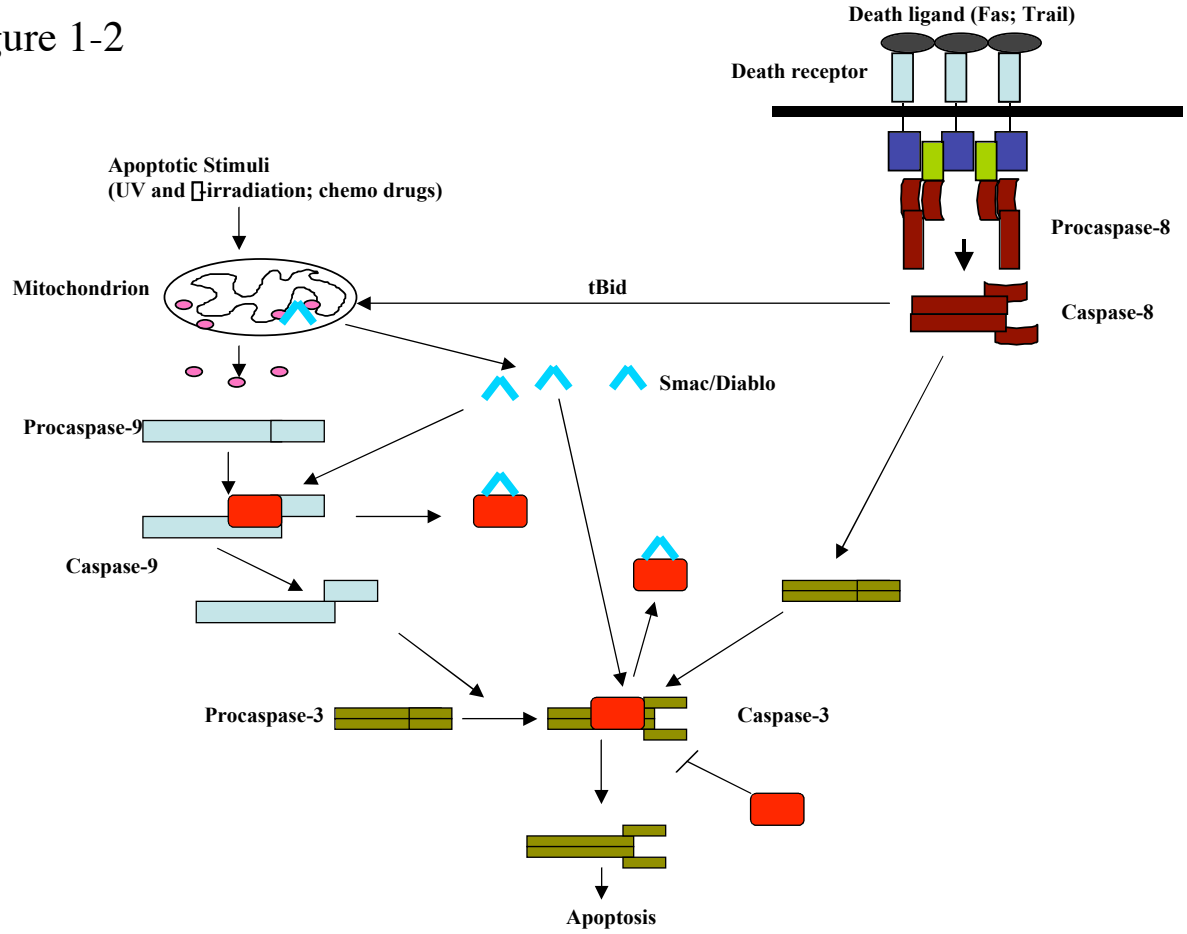



**Figure 1-2: Caspase activation pathways.**

Caspase-3 and caspase-8 are activated through the mitochondrial or death receptor pathway, respectively. Bid serves as a cross-talk protein. Caspase-8 can cleave Bid that then activates the mitochondrial pathway.

Figure 1-3

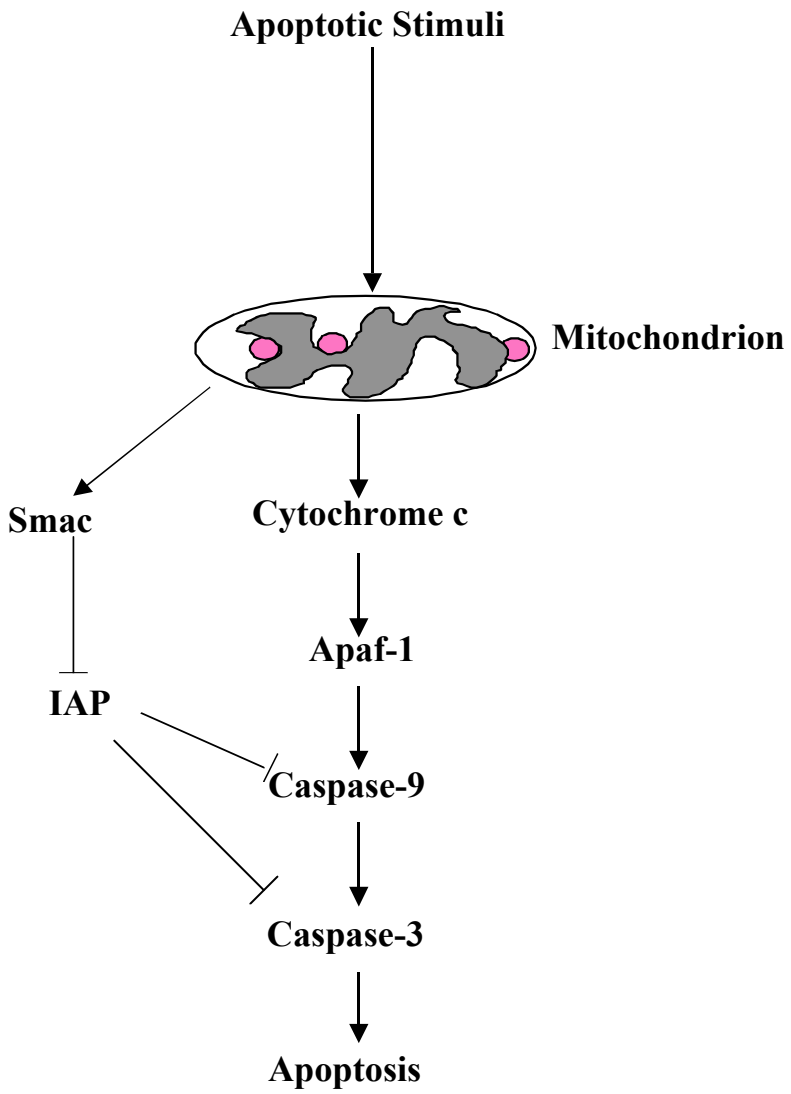



**Figure 1-3: The release of apoptogenic proteins from the mitochondria triggers apoptosis.**

IAP proteins negatively regulate cytochrome c mediated caspase activation. During apoptosis, Smac is concurrently released from the mitochondria with cytochrome c. Smac promotes

caspase activation by binding to IAP and relieving their inhibition.

Figure 1-4

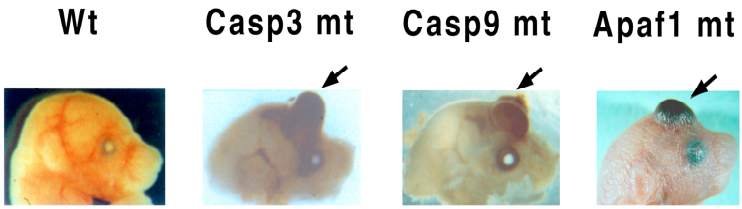
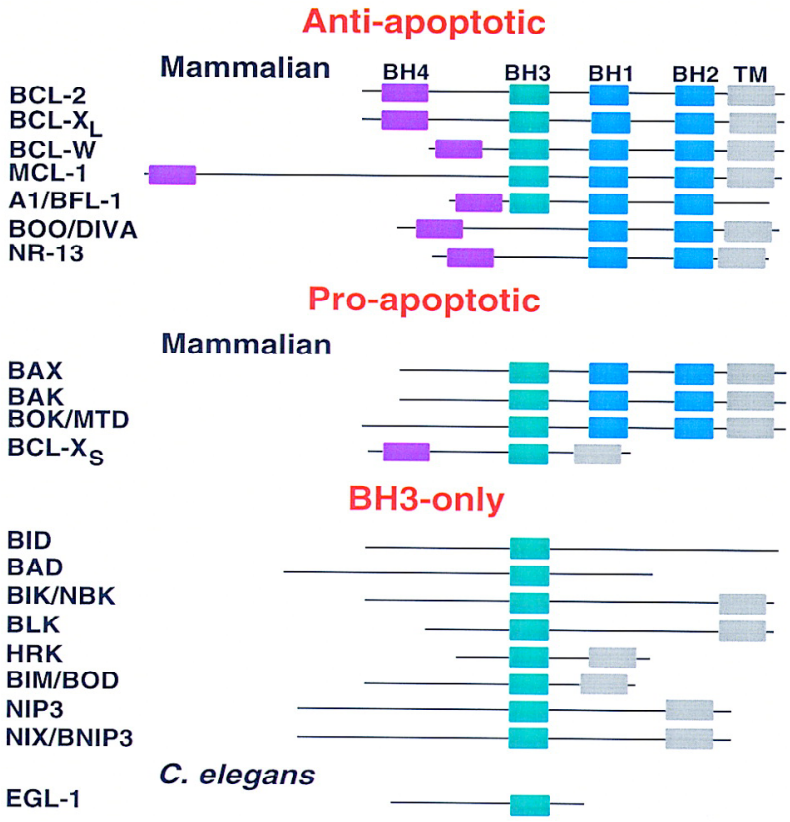



**Figure 1-4: Mice deficient in caspase activating proteins die at birth.**

Caspase-3, Caspase-9, and Apaf-1 knockout mice have a similar phenotype. Neurons fail to under apoptosis during development leading to severe neuronal expansion, forebrain extrusion, and perinatal death.

Figure 1-5




Gross, 1999

**Figure 1-5: Bcl-2 family of proteins.**

The Bcl-2 family is divided into pro and anti-apoptotic members. Family members are homologous in 4 different domains (BH1-4). Some members have a C-terminal transmembrane domain ™ that anchors them to the mitochondrial membrane. A subset of the pro-apoptotic members (BH3-only) is only homologous in the BH3 domain. These proteins are thought to communicate death signals to the mitochondria.

# Chapter 2

## *Introduction*

In the mid 1990's Alnemri and Dixit and their colleagues began to use degenerate PCR or hybridization techniques to clone proteins with DNA homology to the ced-3 protease (Fernandes-Alnemri et al., 1995). Caspase-3/CPP32 was one of the first mammalian ced-3 homologous proteins cloned in this way (Fernandes-Alnemri et al., 1994). Caspase-3 is a 32 kD protein that like other members of its family exists as an inactive zymogen (procaspase-3) (Tewari et al., 1995). During apoptosis, it is activated by proteolytic cleavage into a p20 and p11 fragment. The p20 fragment of caspase-3 is catalytically active. It will cleave many different intracellular substrates including PARP, DFF45, and procaspase-3 (Liu et al., 1997; Tewari et al., 1995). Since caspase-3 activation triggers a chain reaction by cleaving itself, it is not a straightforward problem to understand how caspase-3 activation initiates. For this reason, we attempted to develop an in vitro assay that recapitulates the initiation of caspase-3 activation by adding nucleotides to naïve (untreated) HeLa cell cytoplasmic extract (Liu et al., 1996). Using such an assay, we biochemically fractionated and

purified three proteins that reconstitute caspase-3 activation in vitro, cytochrome c, Apaf-1, and procaspase-9. The development of a caspase activation assay and its reconstitution was the result of many different experiments performed in our laboratory by colleagues (Liu et al., 1996; Zou et al., 1997). In this chapter, we will focus on only the following parts: caspase activation assay, reconstitution of purified components, necessity of caspase-9 and cytochrome c, and a proposed mechanism. We propose that cytochrome c is released from the mitochondria to the cytoplasm where it binds to Apaf-1 triggering the formation of a Apaf-1/caspase-9 complex. The formation of such a complex initiates caspase-9 auto-activation and subsequent caspase-3 cleavage.

***Nucleotides induces caspase activation in naïve extract***

Different nucleotides were added to naïve HeLa cell S100 in the presence of $S^{35}$ labeled procaspase-3. Incubation with 1 mM dATP or dADP leads to the proteolytic cleavage of the labeled procaspase-3 substrate (Figure 2-2). HeLa cytoplasm was divided into bound and flow through (FT) fractions on a SP-sepharose column. Both fractions were required to reconstitute dATP induced caspase activation. The SP- flow through was further fractionated on a hydroxylapatite column

into a flow through and bound fraction. The SP-bound (Apaf-2), SP-FT-HAP-Bound (Apaf-1), and SP-FT-HAP-FT (Apaf-3) fractions are all required to reconstitute dATP induced caspase activation (Figure 2-2, diagram). Purification of each fraction to homogeneity revealed that Apaf-1 was a novel protein with homology to ced-4, Apaf-2 was cytochrome c, and Apaf-3 was procaspase-9. Figure 2-2A shows the reconstitution of the caspase-3 activation reaction using three purified proteins: Apaf-1, cytochrome c, and Apaf-3. No single component or any combination of two was sufficient to activate caspase-3 (lanes 1–6). All three proteins plus dATP were required to cleave caspase-3 (lane 7). Using a polyclonal antibody generated against a recombinant caspase-9 fusion protein, we examined the cleavage of caspase-9 during the caspase-3 activation reaction by western blot analysis (Figure 2-2B). An anti-caspase-9 antibody recognized Apaf-3 confirming that Apaf-3 is caspase-9 (lanes 3 and 5–8). Caspase-9 was activated in the presence of Apaf-1, cytochrome c, and dATP regardless of whether caspase-3 was present (lanes 7 and 8), indicating that caspase-9 activation is independent of caspase-3 activity.

***Caspase-9 Is Required for Caspase-3 Activation***

To further demonstrate that caspase-9 is upstream of caspase-3, we immunodepleted caspase-9 from the HeLa cell S-100 fraction using the antibody against caspase-9. As is shown in Figure 2-3A, S100 depleted of either caspase-9 or cytochrome c failed to activate caspase-3 (lanes 3, 4, 7, and 8). Addition of purified Apaf-3 or cytochrome c to extracts depleted of the respective proteins restored the cleavage of caspase-3 (lanes 5, 6, 9, and 10). The endogenous caspase-3 precursor present in these extracts was cleaved in the same fashion as the $S^{35}$ labeled caspase-3 (Figure 2-3B).

***Apaf-1 and Caspase-9 Complex in the Presence of dATP and Cytochrome c***

To further explore the mechanism of caspase-9 activation, we studied the interaction of caspase-9 with Apaf-1 by co-immunoprecipitation using the antibody against caspase-9. The precipitates were analyzed by immunoblotting with an antibody against Apaf-1. As shown in Figure 2-4A, the anti-caspase-9 antibody precipitated Apaf-1 in the presence of cytochrome c, dATP and Apaf-3 (lane 10). Preimmune serum from the same animal did not precipitate Apaf-1 (lane 9). Omission of dATP

(lanes 7–8), cytochrome c (lanes 3–6), Apaf-3 (lanes 11–12), or Apaf-1 (lanes 1–2) obliterated co-immunoprecipitation of Apaf-1.

To further demonstrate that the interaction of caspase-9 with Apaf-1 requires cytochrome c and dATP, we performed the same co-immunoprecipitation experiment with HeLa S-100 immuno-depleted of cytochrome c (Figure 2-4B). This extract possessed no caspase-3 activating activity unless purified cytochrome c was added to the reaction (Figure 3, lanes 7–10). Apaf-1 was co-immunoprecipitated with caspase-9 in this extract only when both cytochrome c and dATP were added (lane 8). Preimmune serum did not precipitate Apaf-1 (lane 7).

***Discussion***

In these experiments, we present experimental data that suggest a mechanism for the activation of caspase-3/CPP32, a major caspase activity in cells undergoing apoptosis (Faleiro et al., 1997; Takahashi et al., 1997). The model, shown in Figure 2-5, indicates that caspase-3 activation begins when caspase-9 binds to Apaf-1 in a reaction triggered by cytochrome c and dATP. Active caspase-9 then cleaves and activates caspase-3, thereby setting in motion the events that lead to DNA fragmentation and cell death. This mechanism

pinpoints the release of cytochrome c as the key, initiating event in caspase activation. Our future efforts to understand upstream apoptosis regulation will focus on the release of cytochrome c.

# Figure 2-1

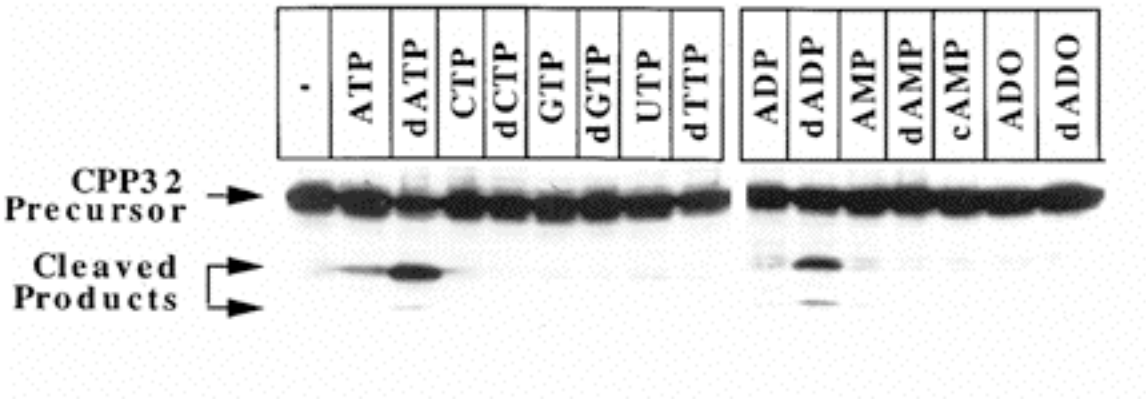



**Figure 2-1: dATP activates caspases de novo in HeLa extract.** The addition of nucleotides, dATP (1 mM) and to a lesser extent ATP, activate de novo caspase-3 cleavage in HeLa S100 (Liu et al., 1996).

## Figure 2-2

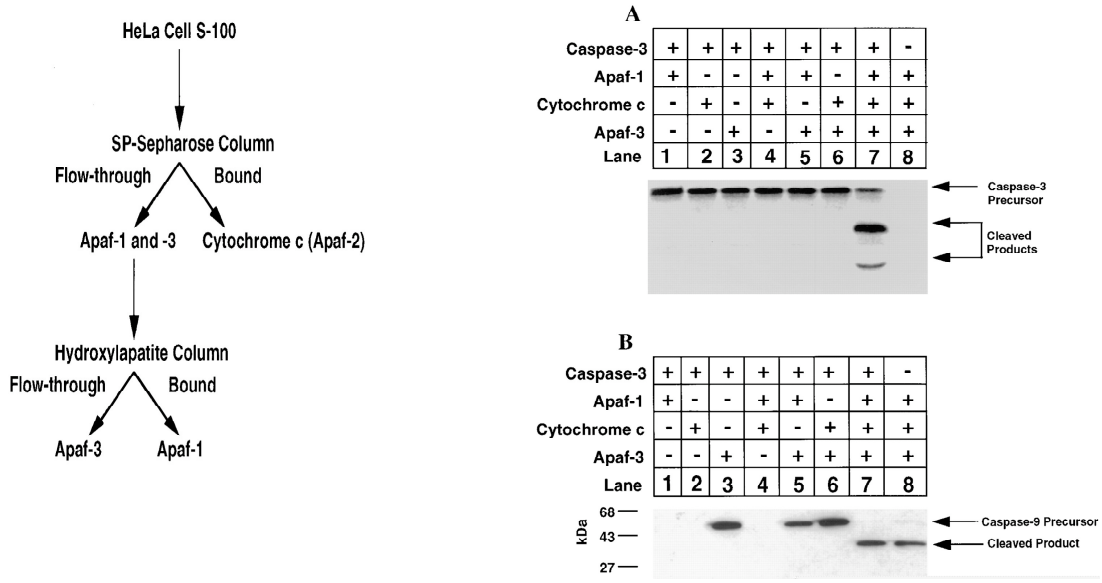



**Figure 2-2: Purification of Caspase Activation Activity.** Left panel a schematic that describes the biochemical separation of Apaf-1,2, and 3. (A) Reconstitution of Caspase-3 Activation with Purified Components. Aliquots (2 µl) of in vitro-translated, $^{35}$S-labeled, and affinity-purified caspase-3 were incubated at 30°C for 1 hr with aliquots of 2 µl Apaf-1 purified through the Mono Q column step as described in Zou et al., 1997 (lanes 1, 4, 5, and 7), or 1 µl (0.3 µg) cytochrome c (lanes 2, 4, 6, and 7), or 2 µl of purified Apaf-3 (12.5 ng) (lanes 3 and 5-7) in a final volume of 20 µl buffer A supplemented with 1 mM dATP and 1 mM MgCl2. In lane 8, Apaf-1, cytochrome c and Apaf-3 were incubated as described above in the absence of in vitro-translated 35S-labeled caspase-3.

After incubation, samples were subjected to 15% SDS-PAGE and transferred to a nitrocellulose filter. (B) The same filter as in (A) was probed with a polyclonal anti-caspase-9 antibody generated as described in the Experimental Procedures.

# Figure 2-3

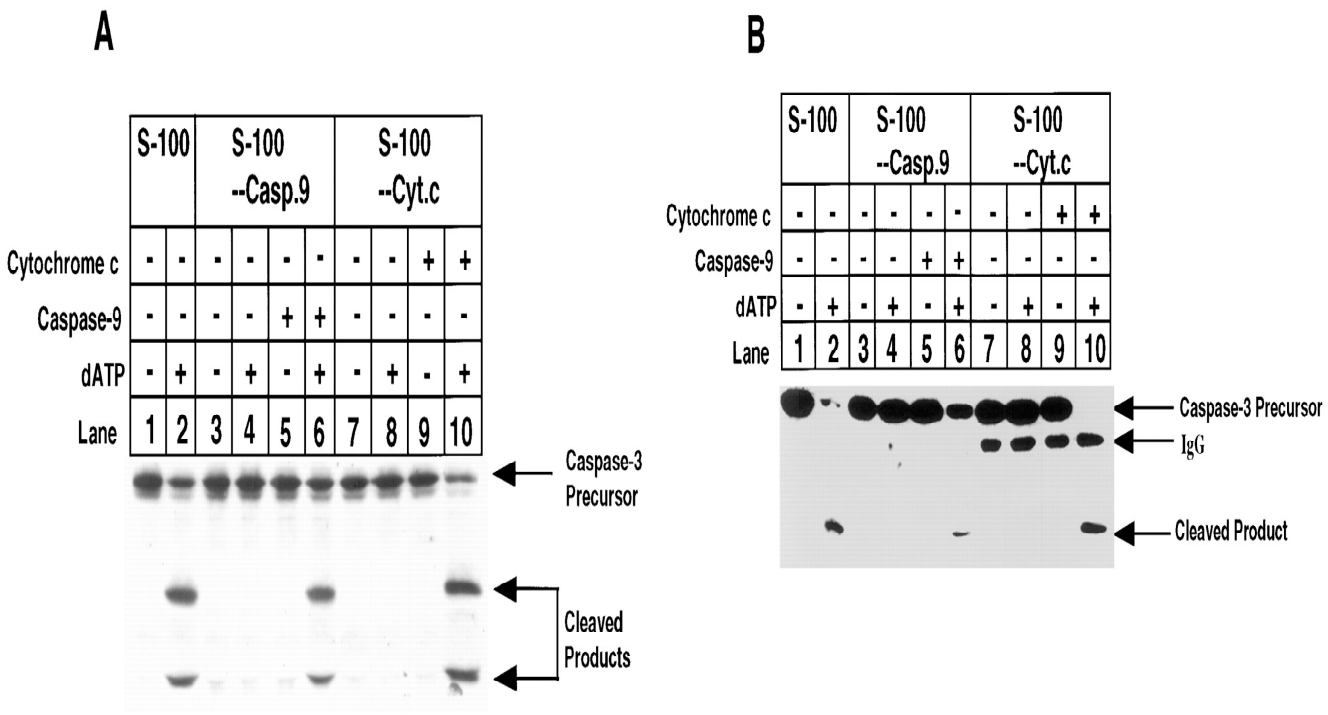



**Figure 2-3: Caspase-9 and Cytochrome c Are Required for Caspase-3 Activation.**

Depletion of caspase-9 from HeLa cell S-100 was performed as described in the Experimental Procedures. Depletion of cytochrome c was as described in Liu et al., 1996b . (A) Aliquots (50 µg) of HeLa cell S-100 (lanes 1 and 2), or HeLa S-100 immunodepleted of caspase-9 (lanes 3–6), or HeLa S-100 immunodepleted of cytochrome c (lanes 7–10) were incubated with aliquots (3 µl) of in vitro-translated, $^{35}$S-labeled

caspase-3 in the absence (lanes 1, 3, 5, 7, and 9) or presence (lanes 2, 4, 6, 8, and 10) of 1 mM dATP in a final volume of 20 µl of buffer A. Aliquots of 6 µl of purified Apaf-3 (37.5 ng) (lanes 5 and 6), or 0.2 µg purified cytochrome c (lanes 9 and 10) were supplemented to the indicated reactions. After incubating at 30°C for 1 hr, the samples were subjected to 15% SDS-PAGE, and the gel was subsequently transferred to a nitrocellulose filter. The filter was exposed to film for 3 days at -80°C. (B) Same reactions were performed as in (A) except the in vitro-translated, $^{35}$S-labeled caspase-3 was omitted. The samples were then subjected to 15% SDS-PAGE followed by electroblotting to a nitrocellulose filter. The filter was probed with 25 µg of a monoclonal antibody to caspase-3 (Transduction Laboratories).

# Figure 2-4

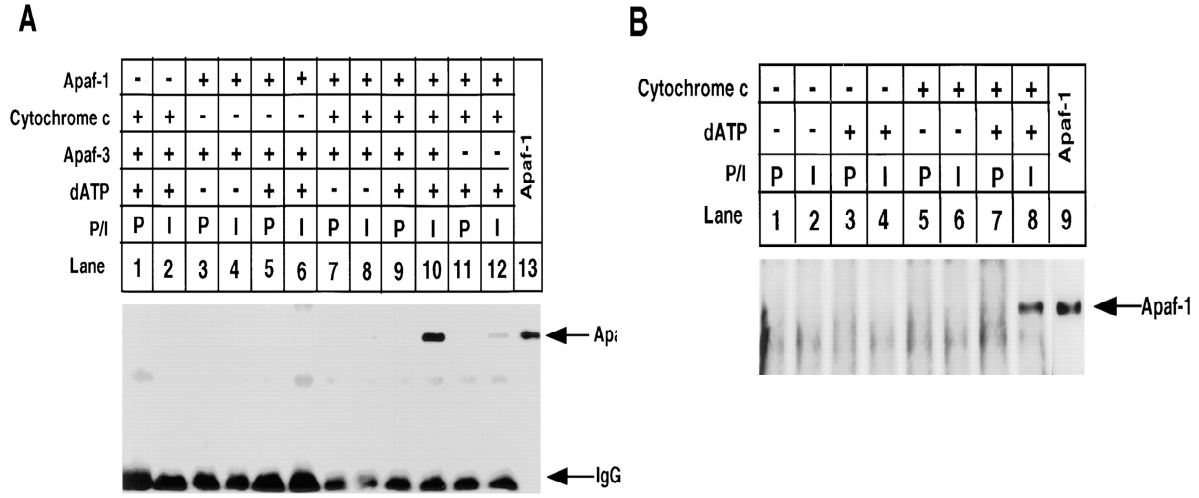



**Figure 2-4: Formation of an Apaf-1/Caspase-9 complex.**

Apaf-1 and Apaf-3 were separated by a hydroxylapatite column as described in Zou et al., 1997 and used for the immunoprecipitation experiment. Aliquots of 50 µl of the Apaf-1 fraction, 100 µl of the Apaf-3 fraction, 0.5 µl (10 mg/ml) of purified cytochrome c, and dATP to a final concentration of 1 mM were added as indicated to a final volume of 500 µl of buffer A. 500 µg of BSA was also added to all reactions to reduce nonspecific binding to the beads. After incubating at 30°C for 20 min, aliquots (25 µl) of anti-caspase-9 antibody/protein A beads prepared as in Experimental Procedures were added to the reaction. After incubation with

rotation overnight at 4°C, the beads were centrifuged and washed with 1 ml of buffer A three times. The beads were then resuspended in 60 µl of 1% SDS loading buffer. After boiling for 5 min, the beads were pelleted by centrifugation and the supernatants were collected. Aliquots of 15 µl of resulting supernatants were subjected to 8% SDS-PAGE followed by electroblotting to a nitrocellulose filter. Purified Apaf-1 (5 ng) was loaded on lane 13. P: preimmune serum, I: immune serum. (B) Aliquots of 120 µl (900 µg protein) of the HeLa cell S-100 extract immunodepleted of cytochrome c (see Figure 2-3) were incubated in the absence (lanes 1, 2, 5, and 6) or presence (lanes 3, 4, 7, and 8) of 1 mM dATP at 30°C for 20 min in a final volume of 500 µl of buffer A. Purified cytochrome c (5 µg) was added to lanes 3, 4, 7, and 8. Purified Apaf-1 (5 ng) was loaded on lane 9. After incubation, the samples were subjected to immunoprecipitation and Western blot analysis as described in (A).

# Figure 2-5

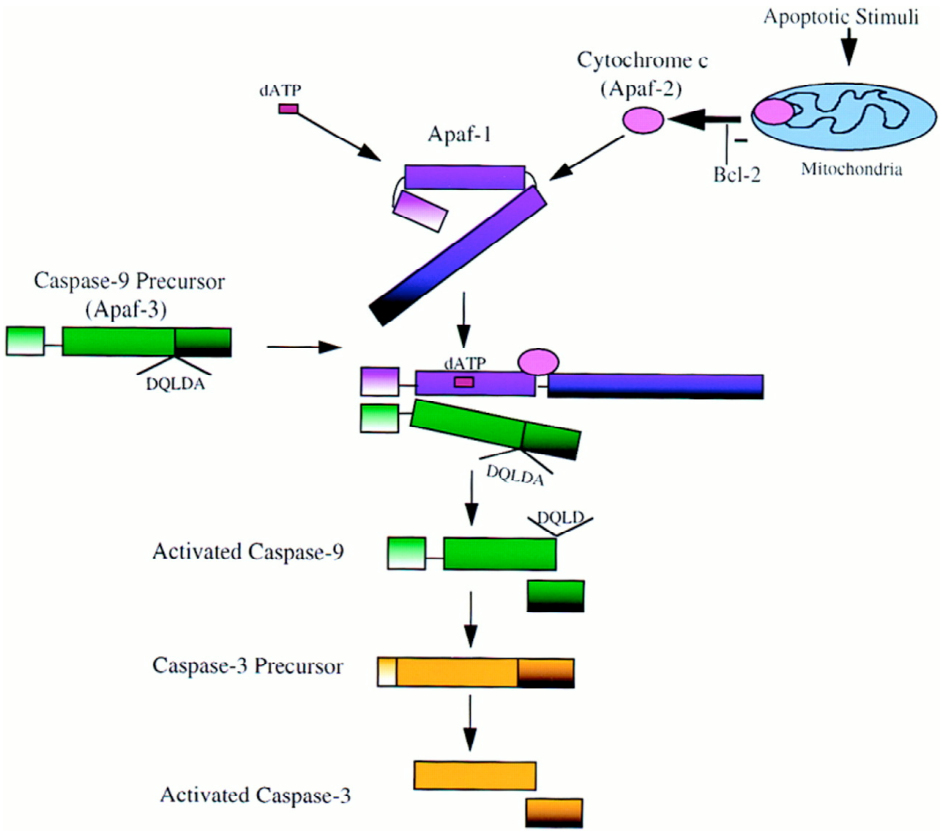



**Figure 2-5: Model for caspase activation.**

Our results predict that cytochrome c release is the rate-limiting step in the activation of caspase-3. Cytochrome c released from the mitochondria binds to Apaf-1. The cytochrome c/Apaf-1 complex then recruits procaspase-9 and catalyzes its autoactivation. Caspase-9 is an upstream caspase that once activated will cleave caspase-3.

# Chapter 3

## *Introduction*

In order to eventually setup biochemical systems for studying cytochrome c release, we established a model of apoptosis in HeLa cells treated with ultraviolet light. HeLa cells treated with UV exhibit mitochondrial changes consistent with apoptosis including cytochrome c release, bak oligomerization, and caspase activation. By using pan-caspase inhibitors, we showed that both cytochrome c release and bak oligomerization are upstream, caspase independent events. Then using HeLa cells treated with UV for only a short while, we recapitulated UV dependent cytochrome c release in vitro. One of the biochemical activities we derived was a cytoplasmic inhibitor of cytochrome c release in naïve (untreated) HeLa cells. The specific inhibitory activity was higher in cells that express the oncogene Bcr-Abl than HeLa cells. Using cytoplasmic extract from Bcr-Abl expressing cells, we fractionated and purified a protein, Bcl-$x_L$, which is both necessary and sufficient for the inhibitory activity. The

HeLa cells systems were more complex; in HeLa cells, Mcl-1 in addition to Bcl-$x_L$ was required for inhibition.

***Activation of mitochondrial apoptotic pathway in response to UV-irradiation***

After receiving a strong dose of UV irradiation, cultured HeLa cells exhibit synchronized characteristic apoptotic changes beginning at 2 hours post irradiation. After 4 hours, most of cells die by apoptosis. As shown in Figure 3-1, these changes include the oligomerization of Bak, appearance of cytochrome c in the cytosol, activation of caspase-9 and caspase-2, cleavage of DFF45/ICAD (not shown) and fragmentation of chromatin DNA (not shown).

When cells were irradiated in the presence of a pan-caspase inhibitor, z-VAD-fmk, caspases-9 activation was completely blocked (right panel). However, the oligomerization of Bak and release of cytochrome c remained intact, indicating that these two events are upstream of caspase activation. Caspase-2 activation was also blocked by this concentration of pan-caspase inhibitor, suggesting that caspase-2 activation is not required for UV-induced cytochrome c release in HeLa cells as it is in oncogene transformed human fibroblasts (Lassus et al., 2002).

### *Homogenized UV-treated HeLa Cell Mixtures Release Cytochrome c*

In an attempt to reproduce UV induced cytochrome c release in vitro, we incubated homogenized cell mixtures from HeLa cells harvested at early time points after UV (Figure 3-2A). Although cytochrome c release does not occur until 2-4 hours after UV (Figure 3-1, lane 3-4), cell mixtures prepared from cells harvested only one hour after UV treatment release cytochrome c after an incubation at 37°C (Figure 3-2B, lane 2) but not 4°C (not shown).

### *Mitochondrial Priming*

Since the whole cell mixture made from UV treated cells releases cytochrome c, we reasoned that at this early time point, the mitochondria alone might exhibit an apoptotic phenotype including cytochrome c release and bak oligomerization. Mitochondria isolated from cells just 30-60 minutes after UV-irradiation readily formed oligomerized Bak and released cytochrome c when incubated in vitro (Fig. 3-2C, lanes 2-3). In contrast, mitochondria from untreated cells did not form oligomerized Bak or release cytochrome c under the same conditions (lane 1). Mitochondria from 30-60 minutes post-UV

irradiated cells are therefore primed to release cytochrome c in vitro even though it takes at least another hour for them to release their cytochrome c in vivo.

***Inhibitors in normal cytosol prevent cytochrome c release from primed mitochondria***

The delay of cytochrome c release in vivo compared to in vitro suggested the presence of an inhibitor in the cytosol. To test this hypothesis, mitochondria from 60 minutes post UV-irradiation cells were isolated and incubated with cytosol (S100) from cells that either never saw UV (naïve), or cells 30-120 minutes post UV irradiation (Figure 3-3A). As shown in Figure 3-3B, cytosol from naïve cells efficiently inhibited cytochrome c release from primed mitochondria isolated from cells 60-minutes post-UV irradiation (lane 2). The inhibition was gradually lost from cytosol isolated from cells that were cultured with increasing time after UV irradiation (lanes 3-5).

***Cells that Express Bcr-Abl have Increased Inhibitory Activity***

Expression of the Bcr-Abl fusion protein is the direct cause of the majority of chronic myelogenous leukemia (CML). Since cells that express Bcr-Abl do not release cytochrome c after receiving an apoptotic stimulus, we suspected that Bcr-

Abl might inhibit cytochrome c release by increasing the activity of our cytoplasmic inhibitor(s). We compared the inhibitory activity between cytosol harvested from HL60 cells that stably express Bcr-Abl or vector alone (Amarante-Mendes et al., 1998). Cytosol from cells that express Bcr-Abl has higher specific inhibitory activity than cytosol from cells transfected with the vector (Neo)(Figure 3-4B). K562 cells express Bcr-Abl and are derived from a CML patient (Klein et al., 1976). After UV treatment, K562 cells fail to release cytochrome c and do not commit apoptosis. In accordance, they also have higher specific inhibitory activity (Figure 3-4C). STI571 is a small molecule that specifically inhibits the tyrosine kinase activity of Bcr-Abl (Carroll et al., 1997; Druker et al., 1996). Cytosol harvested from K562 cells that were cultured in the presence of STI571, have reduced inhibitory activity (Figure 3-4D, lane 2 and 7) suggesting that the increased inhibitory activity in K562 cells is the result of Bcr-Abl. The inhibitory activity from HeLa cell cytoplasm (Figure 3-3B, lane 3-5) but not K562 cytosol disappears in cytosol from UV treated cells (Figure 3-4D, lanes 3-6).

***Biochemical Fractionation of a Cytoplasmic Inhibitor of Cytochrome c Release***

We fractionated K562 S100 using ammonium sulfate, mono Q, size-exclusion chromatography and hydroxylapatite (not shown). Inhibitory activity remains in the supernatant after the addition of 20% ammonium sulfate to S100 (Figure 3-5A, lanes 6 and 7) but pellets with 30% and 40% (Figure 3-5A, lanes 8-11). Inhibitory activity elutes from mono Q column as a single peak at approximately 200 mM NaCl (Figure 3-5B). Inhibitory activity fractionates into two peaks on a size-exclusion column: fractions 1 and 2 (Figure 3-5C upper panel, lanes 2 and 3) are very large complexes that are not resolved by the column, and fractions 8 and 9 (Figure 3-5C upper panel, lanes 9 and 10) that migrate at a predicted size of 30 kD. We predicted that the inhibitory factor(s) in fractions 1 and 2 might aggregate into very large protein complexes or might be non-specifically associated with aggregates. We used a buffer containing 6M Urea to disrupt the formation of aggregates or prevent non-specific interactions. The inhibitory factor(s) renatures after the urea is dialyzed out (not shown), so we fractionated the activity on a size-exclusion column in a buffer that contains 6M Urea. After dialysis, the activity now fractionates in a single peak (Figure 3-5C lower panel, lanes 7-9). The predicted size is

larger likely because the denatured protein has an increased surface area.

***Purification of an Cytoplasmic Inhibitor of Cytochrome c Release***

Since inhibitory activity renatures without any loss in activity, we used a purification scheme that combines size-exclusion and mono Q steps with and without urea (Table 3-1). The final step was performed on a 100 μl Mono Q column in order to concentrate the activity. Different dilutions of the final fractions were assayed so that we could identify the precise activity peak (Figure 3-6A). The entire peak fraction was run on an SDS-PAGE and then stained with coomassie (Figure 3-6B). We assayed dilutions of fractions from each purification step in order to determine the activity per volume, total activity, activity yield and enrichment (Table 3-2). Three individual bands (A-C) in the peak fraction were excised and digested with trypsin. Individual peptides were separated by HPLC and their exact mass determined by mass spectrometry. Peptide fingerprinting results revealed that band B consisted of two different protein products: Bcl-$x_L$ and an unknown protein (Figure 3-7A). The identity of two peptides, ELVVDFLSYK and EAGDEFELR, derived from Bcl-$x_L$ were confirmed by fragmentation

followed by mass spectrometry (Figure 3-7B, C).

***Bcl-$x_L$ is a cytoplasmic inhibitor of cytochrome c release***

We measured the inhibitory activity and the levels of Bcl-$x_L$ in S100, 30% ammonium sulfate pellet, and size-exclusion fractionation (Figure 3-8A) and mono Q (Figure 3-8B). The inhibitory activity in all of the fractions correlated with the levels of Bcl-$x_L$.

We used antibodies to Bcl-$x_L$ to immuno-deplete it from K562 cytosol (Figure 3-9A, lanes 2 and 4). Cytosol immuno-depleted of Bcl-$x_L$ did not have any inhibitory activity suggesting that Bcl-$x_L$ is necessary for inhibition (Figure 3-9B, lane 4).

***Mcl-1 and Bcl-$x_L$ are cytosolic inhibitors of cytochrome c release in HeLa cytoplasm***

We were most interested in understanding the regulation of inhibitors of apoptosis after UV treatment. However, since UV treatment eliminates inhibitory activity in HeLa cytoplasm (Figure 3-3B, lanes 3-5) but not K562 (Figure 3-4D, lanes 3-6), we repeated Bcl-$x_L$ immuno-depletion experiments on HeLa cytosol. In contrast to K562, HeLa cell cytoplasm depleted of Bcl-$x_L$ still retained inhibitory activity (Figure 3-11, lane 4). We hypothesized that there may be other anti-apoptotic Bcl-2

family members in addition to Bcl-$x_L$ in HeLa cytoplasm that inhibit cytochrome c release. The cytosol from naive HeLa cells was fractionated by ammonium sulfate precipitation, ion exchange Mono Q column chromatography, gel-filtration column chromatography (not shown), hydroxylapatite chromatography (not shown) and assayed for inhibition of cytochrome c release from UV primed mitochondria. The column behavior of inhibitory activity from HeLa cytoplasm was the same as that from K562. As shown in the upper panel of Figure 3-10A, 30% ammonium sulfate precipitated the activity (lanes 4, 6). The precipitated activity was subsequently loaded onto a Mono Q column and activity peak was eluted from the column by approximately 200 mM of NaCl (Figure 3-10B, upper panel, lane 9).

We suspected that another homologous anti-apoptotic Bcl-2 family member might be required in HeLa cells so we probed the column fractions using antibodies against other Bcl-2 family members in addition to Bcl-$x_L$. As shown in the middle and lower panels of Figure 3-10A and 3-10B, we found that both Bcl-$x_L$ and Mcl-1 co-purified with the inhibitory activity perfectly.

We tested whether the inhibitory activity in naïve cytosol was due to Mcl-1 and Bcl-$x_L$ by immuno-depleting these two

proteins individually and in combination from naive S100. As shown in the middle and lower panels of Figure 3-11, antibodies against Mcl-1 and Bcl-$x_L$ successfully depleted these proteins from naïve cytosol (lanes 3-5). Cytosol individually depleted of Mcl-1 (lane 3) or Bcl-$x_L$ (lane 4) lost some activity while cytosol depleted of both Mcl-1 and Bcl-$x_L$ (lane 5) completely lost its ability to inhibit cytochrome c release. In HeLa cytoplasm both Mcl-1 and Bcl-$x_L$ are necessary for inhibitory activity whereas Bcl-$x_L$ is primarily responsible for inhibition in K562 cytoplasm.

***Bcl-$x_L$ and Mcl-1 are sufficient to block cytochrome c release***

To confirm that Bcl-$x_L$ and Mcl-1 are sufficient to inhibit cytochrome c release from UV treated mitochondria, purified recombinant Bcl-$x_L$ and Mcl-1 were incubated with UV primed mitochondria. As shown in the upper panel of Figure 3-12, 3 nM of Mcl-1 or 10 nM of Bcl-$x_L$ was able to completely block cytochrome c when added directly to UV treated mitochondria (lanes 6, 11). The amounts of recombinant Bcl-$x_L$ and Mcl-1 in the reaction were comparable with that of the Bcl-$x_L$ and Mcl-1 present on the mitochondria and in naïve cytosol (middle and lower panels, lanes 1-4). Since we diluted the cell pellet fivefold when making S100, the estimated actual concentration of

Bcl-$x_L$ and Mcl-1 in HeLa cytoplasm is about 150 nM and 10 nM respectively.

The inhibitory activity in HeLa cells is lost after UV treatment. Since the inhibitory activity is provided by both Bcl-xL and Mcl-1, these two proteins activity in the cytosol change after UV. Our subsequent experiments will evaluate how the properties of Bcl-$x_L$ and Mcl-1 in vivo change after UV treatment.

***Discussion***

Biochemical studies revealed that two anti-apoptotic members of the Bcl-2 family, Bcl-$x_L$ and Mcl-1 are in vitro inhibitors of cytochrome c release that are regulated by UV light. It is known that over-expression of either Bcl-$x_L$ or Mcl-1 blocks cytochrome c release and apoptosis, but the mechanism by which they are regulated during apoptosis is still unclear. In the next chapters, we will evaluate how UV regulates endogenous Mcl-1 and Bcl-$x_L$.

# Figure 3-1

| Time after UV (hr): | DMSO | | | | 120$\mu$M z-VAD | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 4 | 0 | 1 | 2 | 4 |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |

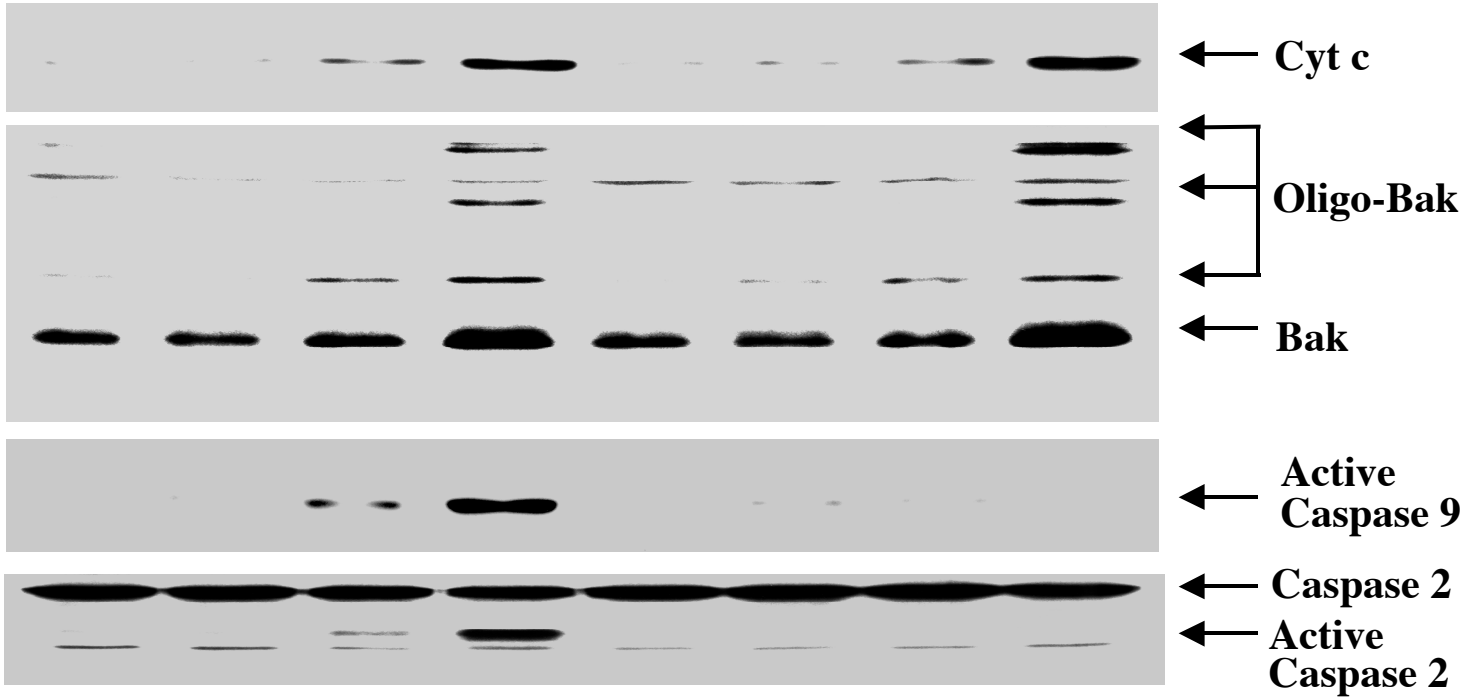
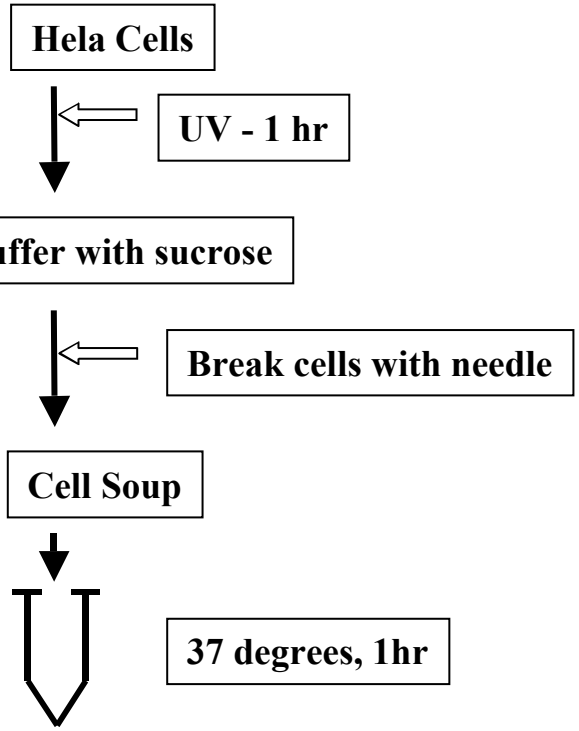


**Figure 3-1: UV induced cytochrome c release from HeLa cells is caspase independent.**

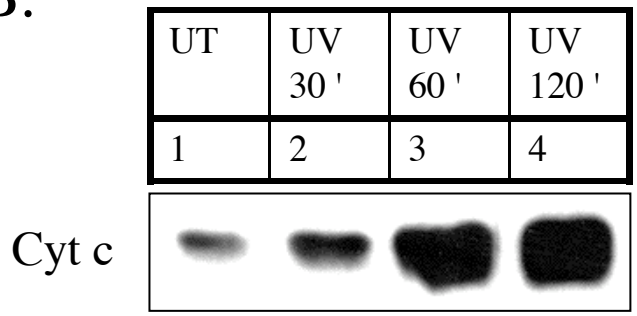
Mitochondria and S100 were harvested from HeLa cells treated with UV for different amounts of time as described in Experimental Procedures. In lanes 5-8, we pre-incubated the cells in the presence of Z-VAD.fmk, a broad-spectrum caspase inhibitor. Cytochrome c, Caspase-9 and Caspase-2 were analyzed in S100. Bak oligomerization was analyzed in mitochondrial fractions as described in Experimental Procedures.

# Figure 3-2

A.




B.




C.

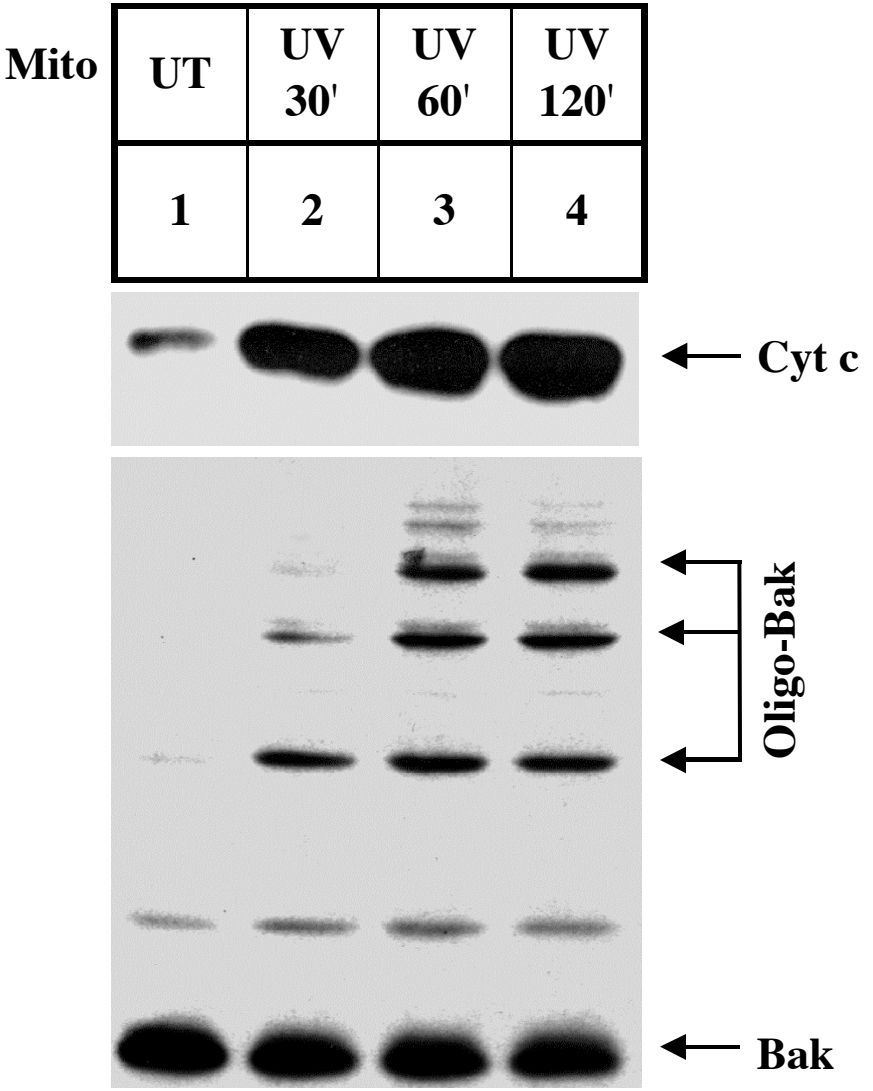


**Figure 3-2: UV dependent cytochrome c release reconstituted in vitro.**

(A) Schematic describing how UV dependent cytochrome c release was reproduced in vitro using HeLa cell mixtures. (B) Cell mixtures (1 mg/ml) made from HeLa cells left untreated (lane 1), or treated with UV 30 min (lane 2), 60 min (lane 3), or 120 min (lane 4) prior were incubated in vitro for 15 min at 37°C. Following incubation, the mixtures were pelleted and the supernatants were analyzed for cytochrome c. (C) Mitochondria from UV treated cells release cytochrome c in vitro. Mitochondria (.67 mg/ml) from cells untreated (lane 1), UV treated for 30 min (lane 2), 60 minutes (lane 3), and 120 minutes (lane 4) were incubated in vitro for 30 min at 37°C. Following incubation, the mitochondria were pelleted and analyzed as described in Experimental Procedures.

# Figure 3-3

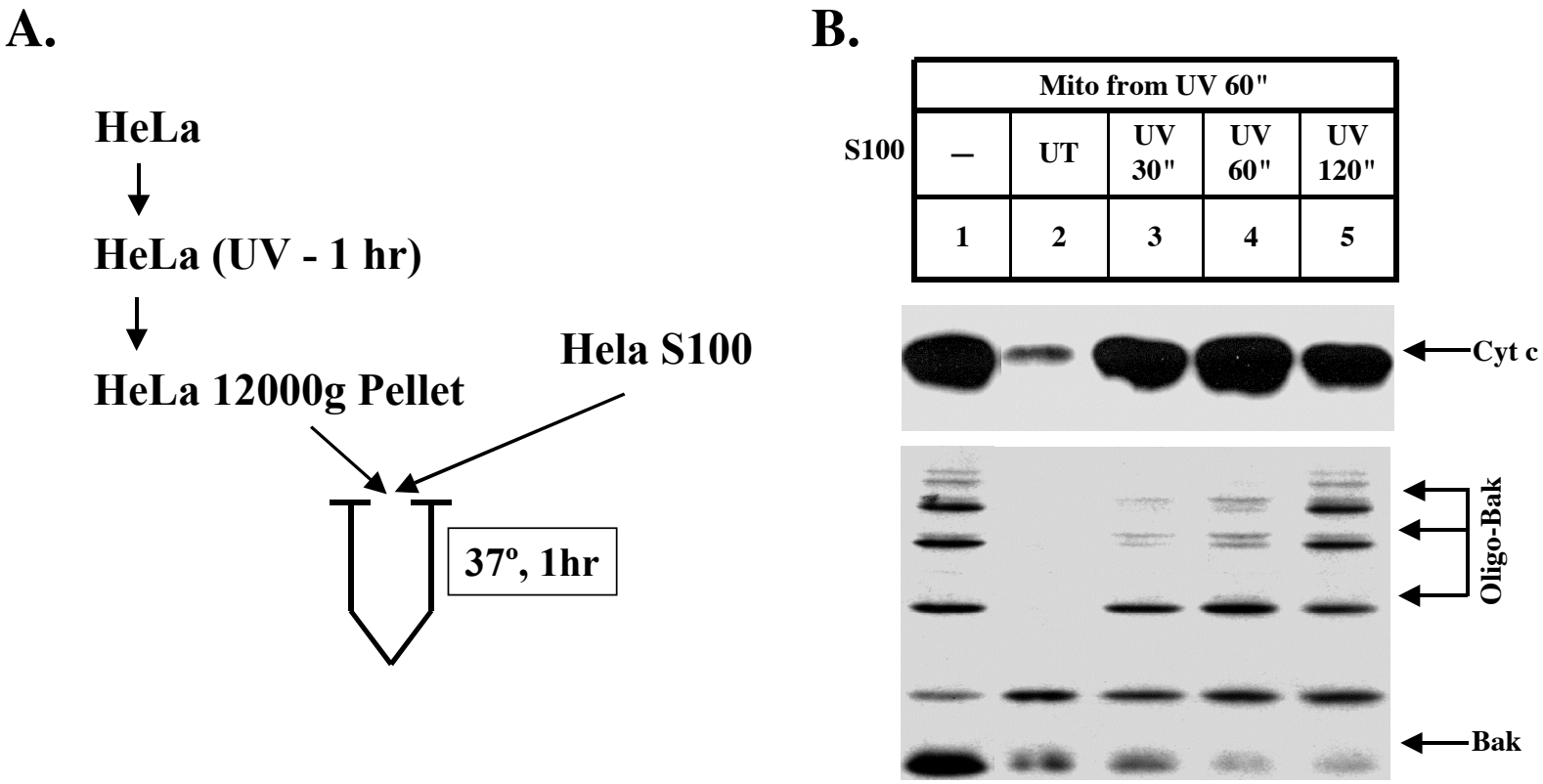

A.
HeLa
HeLa (UV - 1 hr)
HeLa 12000g Pellet
Hela S100
37°, 1hr
B.
Mito from UV 60"
S100
–
UT
UV 30"
UV 60"
UV 120"
1
2
3
4
5
Cyt c
Oligo-Bak
Bak

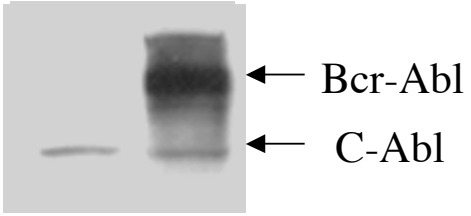
**Figure 3-3: Untreated cytoplasmic extract inhibits release of cytochrome c from primed mitochondria.**

Schematic of assay combining "primed" mitochondria with different S100 samples. S100 inhibits cytochrome c release from primed mitochondria. Mitochondria (0.67 mg/ml) from cells treated with UV for 1 hour were co-incubated (lanes 1-5) at 37°C for 30 min with only buffer (lane 1) or HeLa S100 (4 mg/ml) from untreated (lane 2), UV treated for 30 min (lane 3), 60 min (lane 4), or 120 min (lane 5). Mitochondria and supernatants were analyzed for Bak oligomerization and cytochrome c release, respectively.

# Figure 3-4

**A.**

| Neo | Bcr-Abl |
|---|---|
| 1 | 2 |




**B.**

| | HL60 Neo | | | | | HL60 Bcr-Abl | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| UV Mit | + | - | + | + | + | + | - | + | + | + | + |
| Protein (□g) | | 100 | 10 | 30 | 60 | 100 | 100 | 10 | 30 | 60 | 100 |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |

**C.**

| | Hela S100 | | | | | | | K562 S100 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| UV | + | - | + | + | + | + | + | - | + | + | + | + | + |
| Protein mg/ml | | 4 | 4 | 2 | 1 | 0.5 | .25 | 4 | 4 | 2 | 1 | 0.5 | .25 |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |

**D.**

| S100 | | UT | UV .5 hr | UV 1 hr | UV 2 hr | UV 4 hr | UT | UV .5 hr | UV 1 hr | UV 2 hr | UV 4 hr |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |

**Figure 3-4: Inhibition of cytochrome c release is higher in cytoplasm from cells that express Bcr-Abl.**

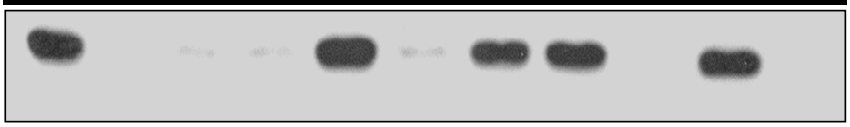
(A) HL60 cells stably express Bcr-Abl (Amarante-Mendes et al., 1998). (B) Inhibitory activity is higher in cells that express Bcr-Abl. S100 prepared from HL60 cells that express Bcr-Abl (lanes 7-11) or vector alone (lanes 2-6) were tested for inhibitory activity. (C) K562 cells have higher inhibitory activity than HeLa cells. S100 prepared from HeLa cells (lanes 2-7) or K562 cells (lanes 8-13) was tested for inhibitory activity. (D) Inhibitory activity was measured from S100 that was prepared from K562 cells that were incubated with STI571 (25 □M) for 24 hrs (lanes 7-11) or vehicle alone (lanes 2-6) that had been left untreated or UV treated for the indicated amount of time.

# Figure 3-5

**A.**

| | S100 | | 10% $(NH_4)_2SO_4$ | | 20% $(NH_4)_2SO_4$ | | 30% $(NH_4)_2SO_4$ | | 40% $(NH_4)_2SO_4$ | |
|---|---|---|---|---|---|---|---|---|---|---|
| Fraction | | | Sup | Pel | Sup | Pel | Sup | Pel | Sup | Pel |
| UV12GP | + | - | + | + | + | + | + | + | + | + |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |

Cyt C

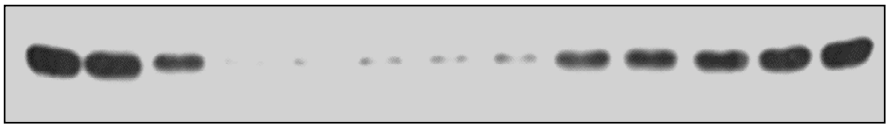


**B.**

150 mM NaCl — 250 mM NaCl

| Fraction | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |

Cyt C



**C.**

| Fraction | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |

Cyt C — Buffer A

Cyt C — 6M Urea/Buffer A

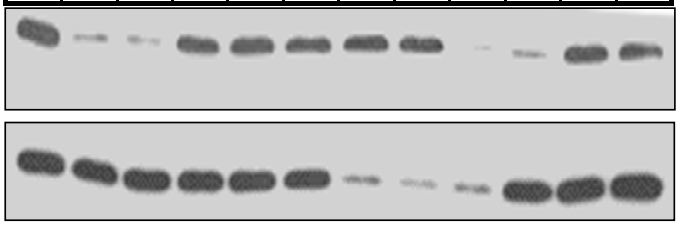
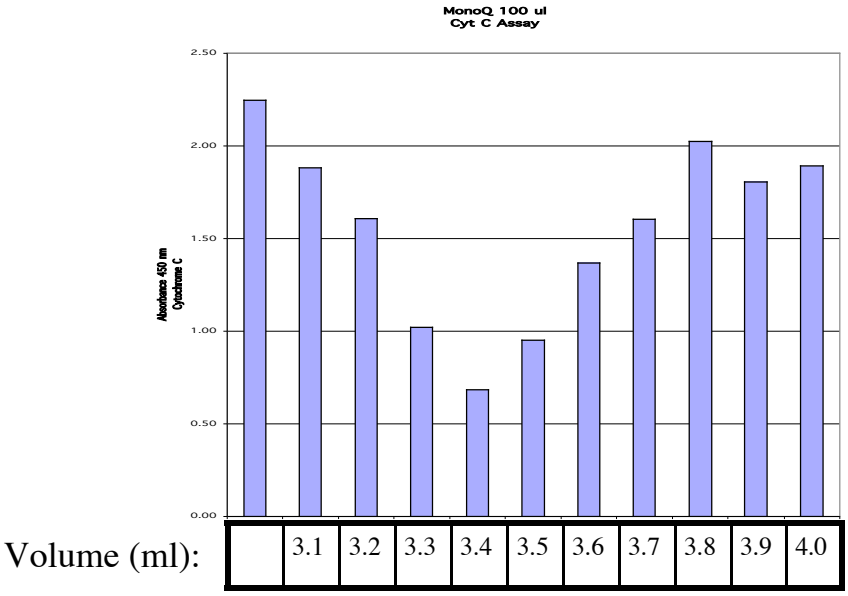


**Figure 3-5: Biochemical Fractionation of Inhibitory Activity**

(A) Inhibitory activity precipitates with 30% ammonium sulfate. Ammonium Sulfate was added to 1 ml of K562 S100 (6 mg/ml; pre-incubated with SP-sepharose beads to remove any contaminating cytochrome c) up to the indicated percentage. After rotation at 4°C for 1 hour, the precipitates were pelleted. The supernatants and pellets resuspended in 1 ml of buffer A were dialyzed overnight in buffer A. 10 μl of the dialyzed samples were assayed for inhibitory activity in a 50 μl reaction volume. (B) Inhibitory activity elutes as a single peak on a Mono Q column. Partially purified fractions from K562 cells were loaded onto a mono Q column and eluted between 150 mM and 250 mM NaCl. The fractions were dialyzed and assayed for inhibitory activity. (C) Inhibitory activity migrates as a single peak on a size exclusion column run in the presence of 6M Urea. 30% ammonium sulfate pellets from K562 S100 were resuspended in 1 ml of buffer A (upper panel) or 1 ml of buffer A/6M Urea (lower panel). Each fraction was fractionated on a size exclusion column in either buffer A (with 100 mM NaCl (upper panel) or buffer A/6M Urea with 100 mM NaCl (lower panel). After each run, all of the 1 ml fractions were dialyzed in buffer A overnight and assayed for inhibitory activity.

# Figure 3-6

## A.


MonoQ 100 ul
Cyt C Assay
Absorbance 450 nm
Cytochrome C
2.50
2.00
1.50
1.00
0.50
0.00
Volume (ml):
3.1
3.2
3.3
3.4
3.5
3.6
3.7
3.8
3.9
4.0

## B.

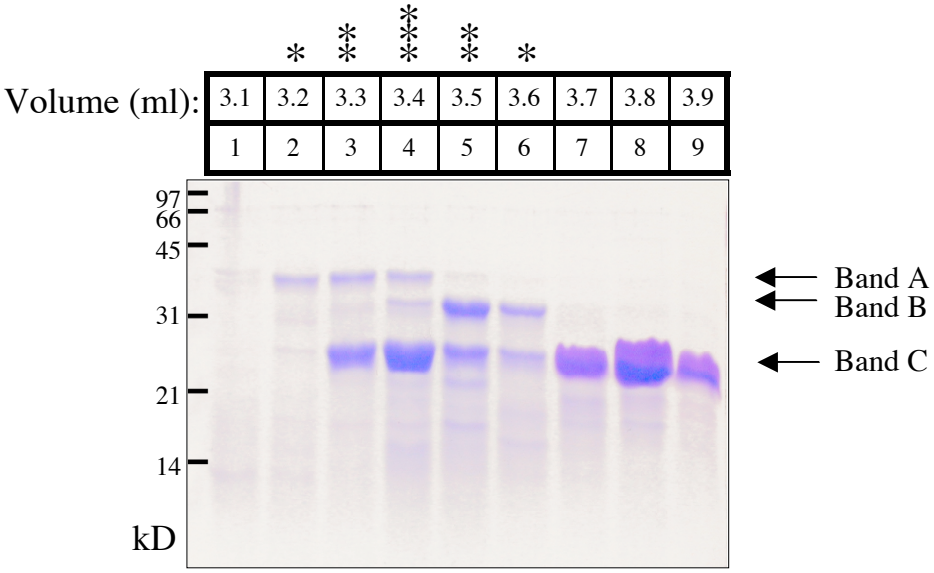

*
**
***
**
*
Volume (ml):
3.1
3.2
3.3
3.4
3.5
3.6
3.7
3.8
3.9
1
2
3
4
5
6
7
8
9
97
66
45
31
21
14
kD
Band A
Band B
Band C

**Figure 3-6: Final Step of purification of inhibitory activity.** (A) Activity profile from Mono Q fractions of purification. 0.1 μl of final Mono Q fractions between 150 mM to 250 mM NaCl following step 6 of the purification (see experimental procedures) were assayed in 50 μl final volume for inhibitory activity. (B) 80 μl of the indicated fractions were run on a 15% SDS-PAGE gel that was subsequently stained with coomassie.

# Table 3-1

## Purification Protocol

| | Step | Buffer |
|---|---|---|
| **0** | **K562 S100** | **Buffer A** |
| **1** | **Ammonium Sulfate 30% Pellet** | **Buffer A** |
| **2** | **Superdex 200 26/60 Gel Filtration** | **Buffer A/ 6M Urea** |
| **3** | **Hi trap Q (5 ml)** | **Buffer A/ 6M Urea** |
| **4** | **CHT-II HAP** | **Buffer A** |
| **5** | **Superdex 75** | **Buffer A** |
| **6** | **Mono Q (100 ul)** | **Buffer A** |

Table 3-1: Purification Scheme

The purification step uses the size exclusion and Q sepharose steps in the presence of 6M Urea and then again in only buffer A. The CHT-II HAP column was preceded with a Hi-trap Heparin SP column.

# Table 3-2

## Purification Table

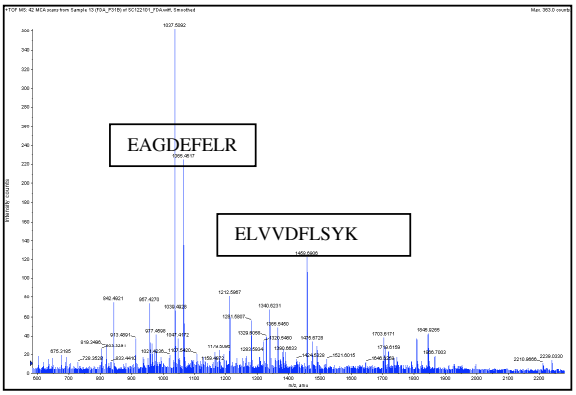
| Step | Total Protein (mg) | Protein Yield (%) | Total Activity Units | Activity Yield (%) | Fold Purified |
|---|---|---|---|---|---|
| K562 S100 | 4533 | 100 | 66667 | 100 | 1 |
| 30% $(NH_4)_2SO_4$ Pellet | 730.2 | 16.1 | 60000 | 90.0 | 5.6 |
| Superdex 26/60 | 6.800 | 0.15 | 25000 | 37.5 | 250 |
| Hi Trap Q (5 ml) | 1.200 | 0.026 | 5333 | 8.00 | 303.4 |
| CHT II (1 ml) | .4710 | 0.010 | 2500 | 3.75 | 361 |
| Superdex 75 | .1970 | 0.0044 | 2000 | 3.00 | 685 |
| Mono Q (100 ul) | 0.006 | 0.0001 | 400 | 0.60 | 4533 |

Table 3-2: Purification Table

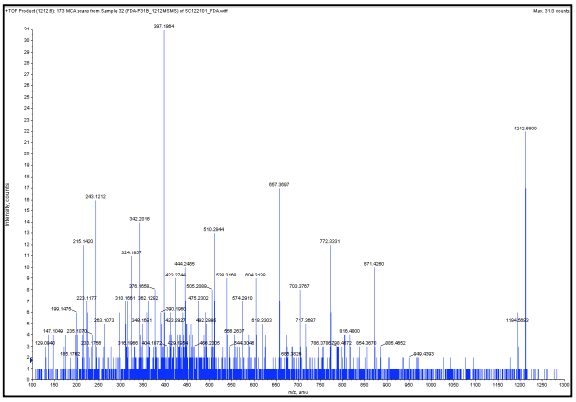
Aliquots from each step of the purification were dialyzed in buffer A and 3 different dilutions were assayed. Cytochrome c inhibition was measured by ELISA immunoassay (R & D Systems). We calculated the total activity units for each fraction by arbitrarily designating a level of cytochrome c release inhibition as a single activity unit. Protein concentrations were determined by Bradford analysis.

# Figure 3-7

**A.**




**B.** ELVVDFLSYK



**C.** EAGDEFELR

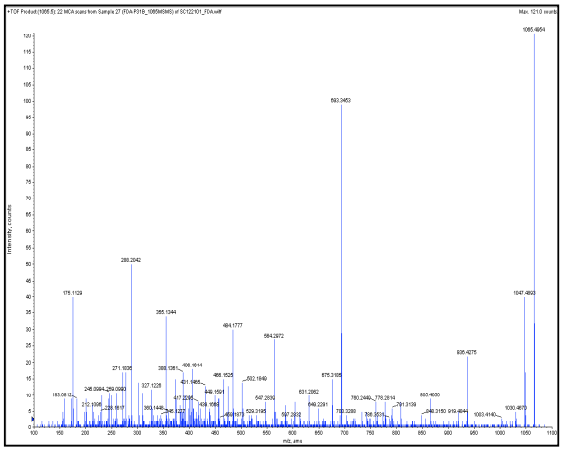


**Figure 3-7: Protein fingerprinting analysis identifies Bcl-$x_L$ in active fractions.**

(A) Band B from Figure 3-6 was digested with trypsin and the resulting fragments were purified by HPLC as described in experimental procedures. Peptides were identified from two different proteins. Two peptides of mass 1065.4517 and 1458.6906 corresponded to peptides from Bcl-$x_L$. The remaining peptides identified an unknown protein. (B and C) Two peptides that identified Bcl-$x_L$ were confirmed by individual peptide fragmentation. After HPLC purification, the mass of the resulting fragments was determined by mass spectrometry.

# Figure 3-8

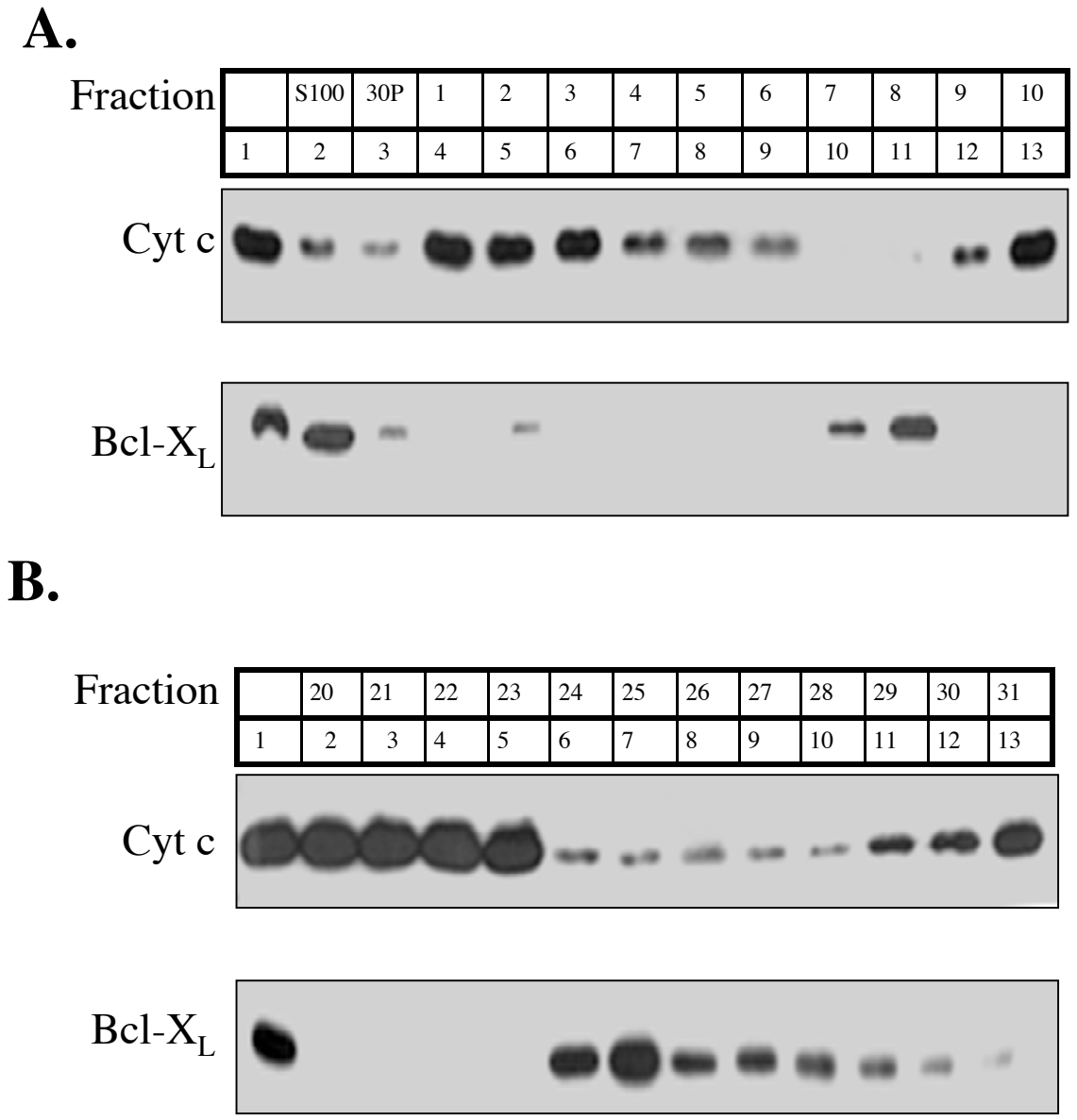



**Figure 3-8 Bcl-$x_L$ correlates with inhibitory activity**

(A) Bcl-$x_L$ correlates with inhibitory activity on a size exclusion column. 30 ml of K562 S100 (6 mg/ml)

was run through the first two steps of purification (see Experimental Procedures). In the upper panel, 20 μl of S100 (lane 2), 2.5 μl of the resuspended 30% ammonium sulfate pellet after overnight dialysis in buffer A (lane 3), and 20 μl of each dialyzed gel filtration fraction (lanes 4-12) were assayed for inhibitory activity in a 50 μl reaction volume. 15 μl of each fraction was also analyzed by western blot for the levels of Bcl-$x_L$ (lower panel). (B) Bcl-$x_L$ correlates with inhibitory activity on a mono Q column. 25 ml of size exclusion fractions from (A) (lanes 10-11) were collected, dialyzed, and loaded onto a 1 ml mono Q column. Fractions were eluted on a 40 ml gradient from 100mM to 250mM NaCl in buffer A, dialyzed and assayed (5 μl of 50 μl total volume) for inhibitory activity. 20 μl of each fraction was also analyzed by western blot for the levels of Bcl-$x_L$ (lower panel).

# Figure 3-9

**A.**

| | Beads | | Anti-Bcl-$x_L$ | |
|---|---|---|---|---|
| S100 | Sup | Pel | Sup | Pel |
| 1 | 2 | 3 | 4 | 5 |

**B.**

| | | Beads | Anti-Bcl-$x_L$ |
|---|---|---|---|
| | S100 | Sup | Sup |
| 1 | 2 | 3 | 4 |

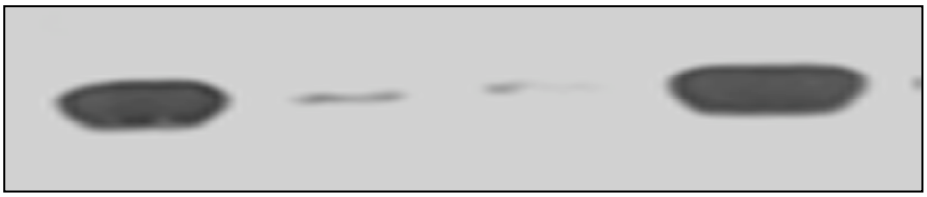


**Figure 3-9: Bcl-$x_L$ is necessary for inhibitory activity**

Bcl-$x_L$ is required for inhibitory activity in K562 S100. 350 µl of K562 S100 (3 mg/ml) were incubated overnight with 5 µl of buffer (lane 2-3) or Anti-Bcl-$x_L$ (Cell Signaling) (lane 4-

5). The following day, protein A agarose beads pre-incubated with BSA were added with rotation for several hours at 4°C. The beads were removed by centrifugation and the pellets were eluted with 1x SDS loading buffer. K562 S100, pellets and supernatants were analyzed for Bcl-$x_L$ (A). K562 S100 and supernatants from mock and Bcl-$x_L$ immuno-depleted S100 were evaluated for inhibitory activity.

# Figure 3-10

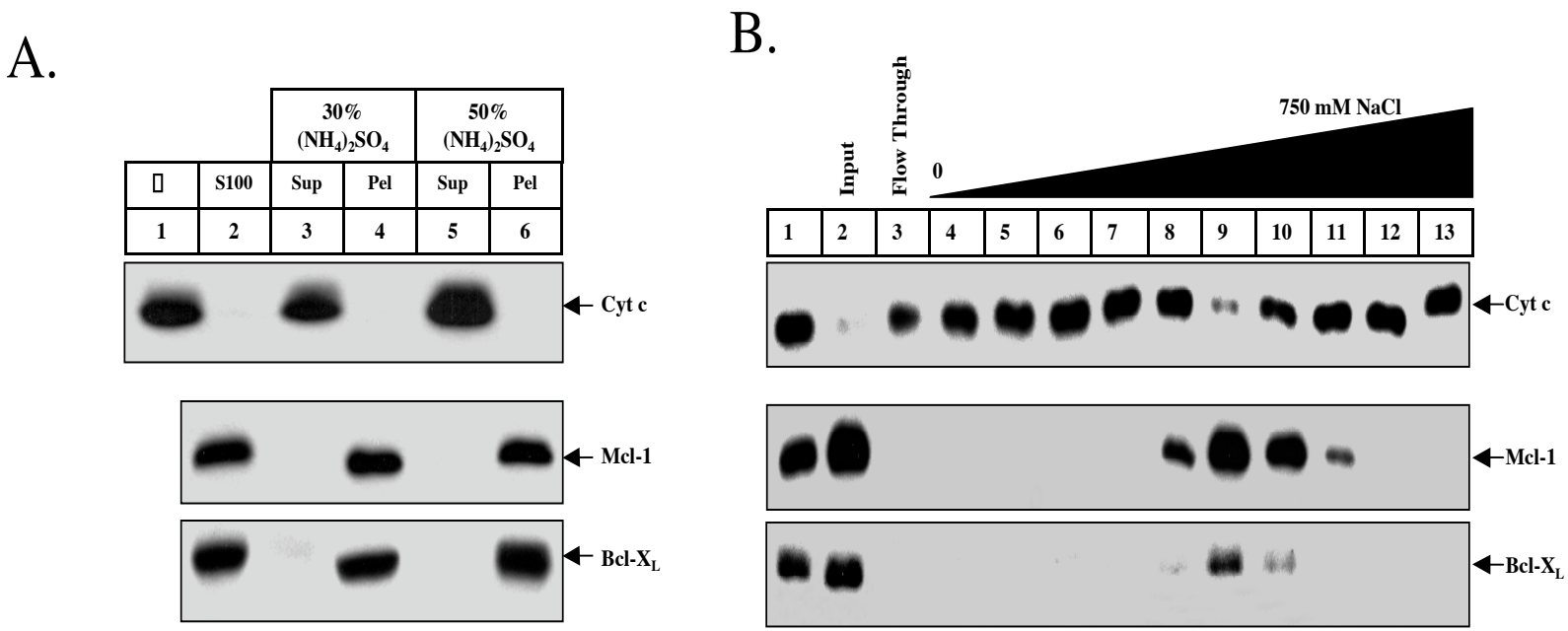

A.
30% (NH4)2SO4
50% (NH4)2SO4
□
S100
Sup
Pel
Sup
Pel
1
2
3
4
5
6
Cyt c
Mcl-1
Bcl-XL
B.
Input
Flow Through
0
750 mM NaCl
1
2
3
4
5
6
7
8
9
10
11
12
13
Cyt c
Mcl-1
Bcl-XL

**Figure 3-10: Inhibitors in HeLa S100**

Biochemical Fractionation and Identification of Inhibitors. (A) Mcl-1 and Bcl-$x_L$ correlate with inhibitory activity after ammonium sulfate fractionation. Ammonium sulfate was added to 1 ml of S100 (5 mg/ml) up to 30% (lanes 3,4) or 50% (lanes 5,6). Following incubation at 4°C for 1 hr, the supernatant (Sup) and pellet (Pel) were separated by centrifugation (20000g). The pellet was resuspended in 1 ml of Buffer A. Both the supernatants and pellets were dialyzed in Buffer A overnight. All fractions were assayed for inhibitory activity (as described in Experimental Procedures) and evaluated for Mcl-1 and Bcl-$x_L$. (B) Mcl-1 and Bcl-$x_L$ correlate with inhibitory activity after a two-step purification. HeLa S100 (36 mg) was precipitated with 30% ammonium sulfate. The resulting pellet (7.5 mg) was resuspended and dialyzed in Buffer A and then loaded onto a 1 ml Hi-trap Q Sepharose column (Amersham) equilibrated in Buffer A. The protein was eluted with a gradient from 0 – 750mM NaCl (in Buffer A) over 14 ml. Inhibitory activity was assayed for buffer alone (Lane 1), input (Lane 2), Q- flow through (Lane 3), and fractions eluting from Q sepharose (Lane 4-13). The amount of Mcl-1 and Bcl-$x_L$ in each sample and the mitochondria (lane 1) was measured by western blot.

# Figure 3-11

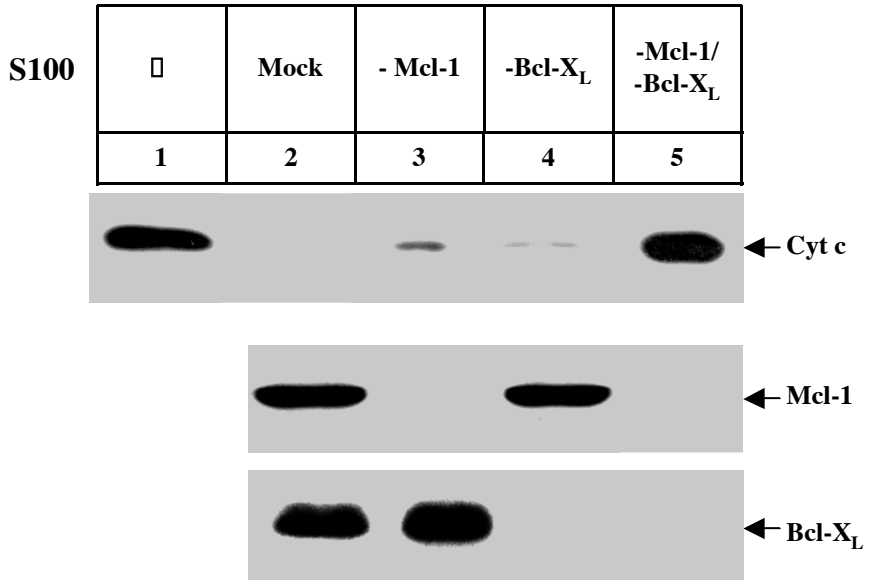


**Figure 3-11: Mcl-1 and Bcl-$x_L$ are both necessary**

Inhibitory activity is lost in S100 immunodepleted with Mcl-1 and Bcl-$x_L$. HeLa S100 was immunodepleted as described in Experimental Procedures. Inhibitory activity was assayed in buffer alone (lane 1), S100 (lane 2), S100 mock-immunodepleted (lane 3), depleted of Mcl-1 (lane 4), Bcl-$x_L$ (lane 5), or both Mcl-1 and Bcl-$x_L$ (lane 6). The amount of Mcl-1 and Bcl-$x_L$ was determined by western blot for each S100 sample.

# Figure 3-12

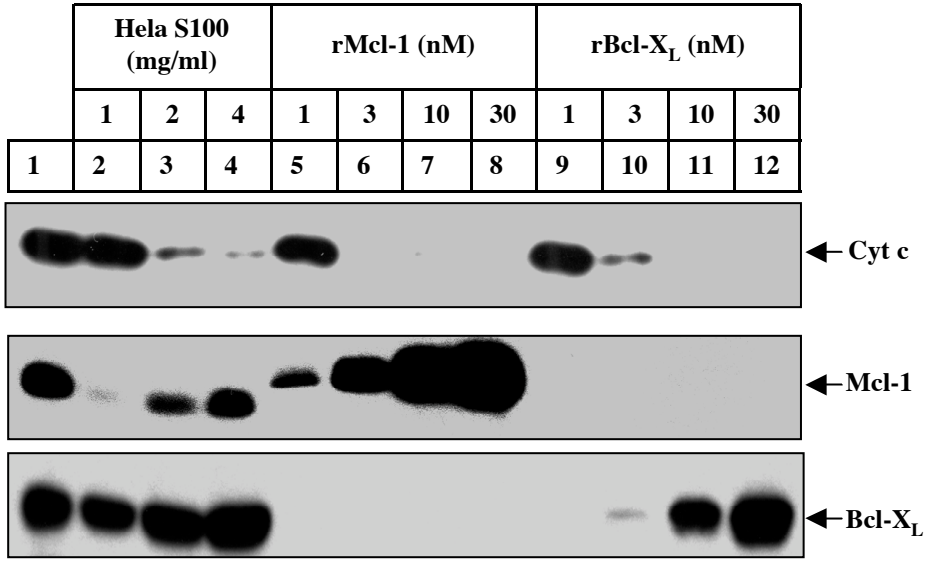

Hela S100 (mg/ml)
rMcl-1 (nM)
rBcl-X$_L$ (nM)
1 2 4 1 3 10 30 1 3 10 30
1 2 3 4 5 6 7 8 9 10 11 12
Cyt c
Mcl-1
Bcl-X$_L$

**Figure 3-12: Mcl-1 or Bcl-$x_L$ is sufficient for inhibition.** Recombinant Mcl-1 (rMcl-1) and Bcl-$x_L$ (rBcl-$x_L$) both have inhibitory activity. rMcl-1 and rBcl-$x_L$ were prepared as described in Experimental Procedures. S100, rMcl-1 and rBcl-$x_L$ were analyzed for inhibitory activity. The levels of Mcl-1 and Bcl-$x_L$ in recombinant fractions were compared to those in S100. Solubilized mitochondria were analyzed for Mcl-1 and Bcl-$x_L$ in order to compare the levels of these proteins in mitochondria to those in the fractions.

# Chapter 4

## *Introduction*

Mcl-1 and Bcl-$x_L$ are localized to the cytoplasmic and mitochondrial extract. After UV treatment Mcl-1 disappears in both compartments and Bcl-$x_L$ translocates from the cytoplasm to the mitochondria. Proteasome inhibitors block the disappearance of Mcl-1. By inhibiting Mcl-1 degradation after UV, proteasome inhibitors inhibit cytochrome c release and related events. The disappearance of Mcl-1 is upstream of and required for the translocation of Bcl-$x_L$.

## *The levels and distribution of Mcl-1 and Bcl-$x_L$ after UV irradiation*

The inhibition of cytochrome c release in naïve cytosol is relieved after UV-irradiation (Figure 3-3B, lanes 3-5). We therefore examined the levels of Mcl-1 and Bcl-$x_L$ in cytosol and mitochondria isolated from naïve and UV treated cells. As shown in Figure 4-1, most of the Mcl-1 was gone from cytosol and mitochondria 1 hour after UV-irradiation and it completely disappeared from cells after 2 hours (lanes 2-4). On the other hand, the total amount of Bcl-$x_L$ in cells did not change even 4

hours after UV-irradiation but the cytosolic Bcl-$x_L$ started to decrease 1 hour after UV irradiation and disappeared around 4 hours when full-blown apoptosis was observed. Unlike Mcl-1, there was a corresponding increase of Bcl-$x_L$ on mitochondria, indicating that Bcl-$x_L$ translocates from cytosol to mitochondria after UV-irradiation (lanes 10-12, middle panel). As a loading control, cytochrome c oxidase, a mitochondrial inner membrane protein, and a cross-reactive cytosolic protein remained unchanged (lower panel).

The elimination of Mcl-1 is not limited to UV irradiation or to HeLa cells. These events are also associated with UV induced apoptosis in a fibroblast cell line. Mcl-1 disappears, Bcl-$x_L$ translocates from the cytoplasm to the mitochondria, and cytochrome c is released in human skin fibroblasts treated with UV-irradiation (Figure 4-2). Other reagents that induce apoptosis through genotoxic stress such as □-irradiation (Figure 4-3) or etoposide (Figure 4-4) also efficiently induce Mcl-1 elimination (upper and lower panels) and subsequent apoptotic changes in HeLa cells including cytochrome c release and caspase-3 activation (middle panels).

***The Proteasome is required for the elimination of Mcl-1***

The complete disappearance of Mcl-1 in response to UV irradiation suggested that the proteasome might be involved in degrading Mcl-1. To test this hypothesis, two different proteasome inhibitors, MG132 and epoxomicin, were used to pre-treat HeLa cells before UV-irradiation (Meng et al., 1999; Palombella et al., 1994). As shown in Figure 4-5, both proteasome inhibitors efficiently block the degradation of Mcl-1 (lanes 5-12). Concomitantly, they also prevent cytochrome c release and caspase activation in vivo.

***Elimination of Mcl-1 is required for Bcl-$x_L$ and Bax translocation to mitochondria***

Interestingly, treatment with proteasome inhibitors also prevented Bcl-$x_L$ translocation from cytosol to mitochondria (Figure 4-5). Bax is another member of the Bcl-2 family that also translocates from cytosol to mitochondria during apoptosis (Wolter et al., 1997). Both proteasome inhibitors also blocked Bax translocation to mitochondria (Figure 4-5).

To test the hypothesis that the anti-apoptotic proteasome inhibitor effect was specifically due to the accumulation of Mcl-1, we first eliminated Mcl-1 by transfecting siRNA molecules complementary to a 21 nucleotide region of Mcl-1 (Elbashir et

al., 2001) and then tested the capacity of epoxomicin to block UV induced cytochrome c release. We added epoxomicin 45 minutes or 90 minutes after UV-irradiation and examined cytochrome c release and the level of Mcl-1 protein in these cells. As shown in Figure 4-6, in luciferase siRNA transfected cells, adding epoxomicin 45 or 90 minutes after UV-irradiation still blocked cytochrome c release and Mcl-1 disappearance (lanes 2-4). However, epoxomicin failed to block cytochrome c release when cells were transfected with Mcl-1 siRNA (lanes 6-8). This data suggested that proteasome inhibitors block downstream events including Bcl-$x_L$ and Bax translocation, cytochrome c release and caspase activation by preventing the degradation of Mcl-1.

The specific upstream effect of Mcl-1 was also studied in two stable HeLa cell lines that over-express Mcl-1 under a CMV promoter. In response to UV irradiation, Mcl-1 expressed from the transgene decreased at the same rate as that of the endogenous gene (Fig. 4-7, lanes 5-12). Nevertheless, 4-hours post UV irradiation, there was still some transgenic Mcl-1 protein left in the cytosol and mitochondria. Consistently, cytochrome c, Bcl-$x_L$ and Bax did not translocate in the Mcl-1 over-expressing cells 4 hours post UV irradiation in comparison with a control HeLa cell line that only expressed the Neo vector (lanes 1-4), indicating that elimination of Mcl-1 is an

upstream event for Bcl-$x_L$ and Bax translocation.

To further verify that Mcl-1 degradation is upstream of Bcl-$x_L$ translocation, we examined the levels of Mcl-1 in a Bcl-$x_L$ over-expressing HeLa cell line (Fig 4-8A, 4-8B). The cytochrome c release in response to UV irradiation was blocked in this cell line (not shown) but Mcl-1 disappears and bax translocates (not shown) at the same rate as Mcl-1 in control cells (Figure 4-8B).

***Discussion***

Our findings suggest that the disappearance of Mcl-1 is a general response to genotoxic stress. Gamma irradiation, etoposide, UV, and mitomycin (not shown) treatment all cause a decrease in the levels of Mcl-1 early in the apoptotic program. Previous reports suggested that the levels of Mcl-1 are regulated by an ubiquitin dependent proteasomal degradation (Zhang et al., 2002). In accordance, we were able to block the disappearance of Mcl-1 with two different proteasome inhibitors. In addition, the proteasome inhibitors also blocked other apoptotic events including cytochrome c release, bax translocation, bcl-$x_L$ translocation, and caspase-3 activation. Unlike Mcl-1, there was no a priori reason to believe that the proteasome independently regulates these other events. More likely, the Mcl-1 is regulated upstream of

these other events. Consistent with this theory, when Mcl-1 was specifically removed, the proteasome no longer protected cells from UV induced apoptosis. Furthermore, if the levels of Mcl-1 are increased by over-expression, all other events are blocked. The focus of the next chapter is to address the next upstream step, how Mcl-1 levels are regulated by UV.

# Figure 4-1

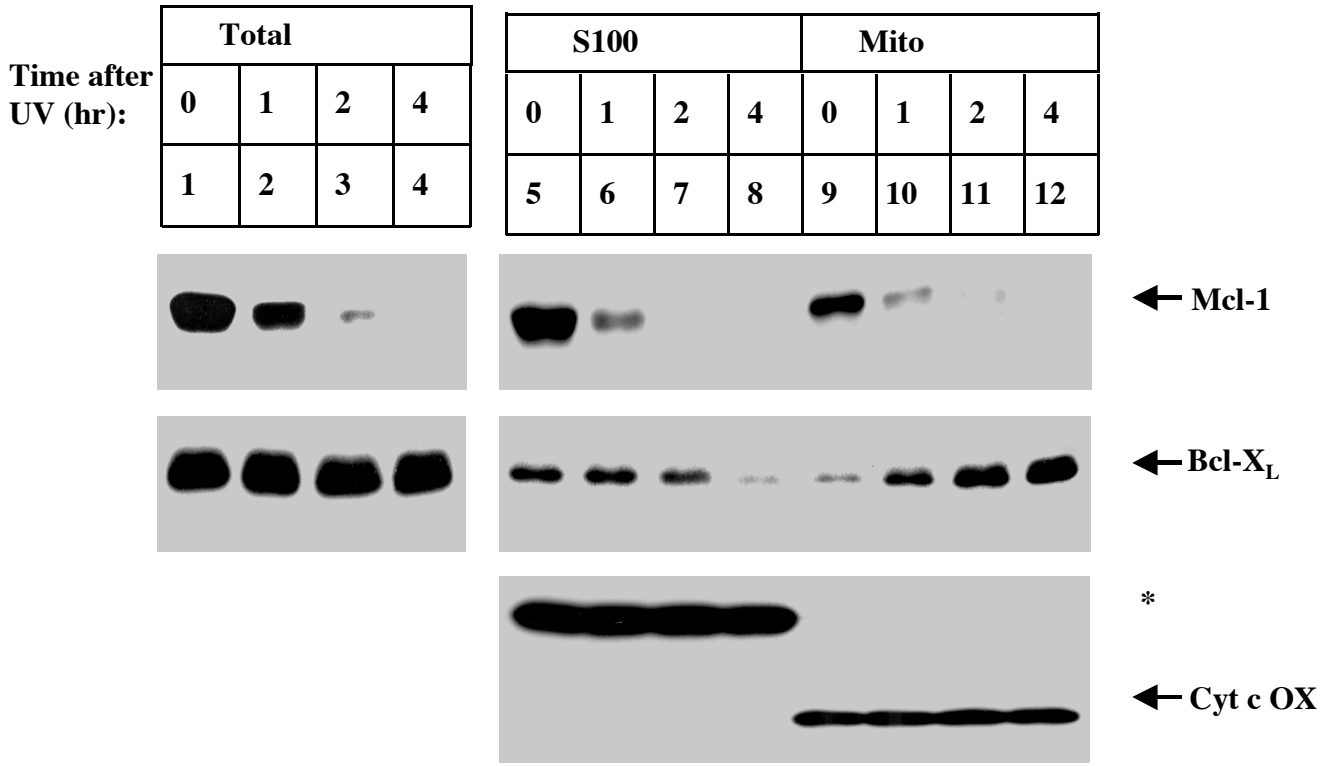

Time after UV (hr):
Total
0
1
2
4
1
2
3
4
S100
Mito
0
1
2
4
0
1
2
4
5
6
7
8
9
10
11
12
Mcl-1
Bcl-X$_L$
*
Cyt c OX

**Figure 4-1: UV treatment induces the disappearance of Mcl-1 and the translocation of Bcl-$x_L$.**

HeLa cells left untreated or treated with UV for different amounts of time were either harvested for total cell lysate or fractionated into mitochondria (Mito) and S100. The levels of Mcl-1, Bcl-$x_L$, and cytochrome c oxidase were determined in total cell lysate (lanes 1-4), S100 (lanes 5-8), and Mito (lanes 9-12).

# Figure 4-2

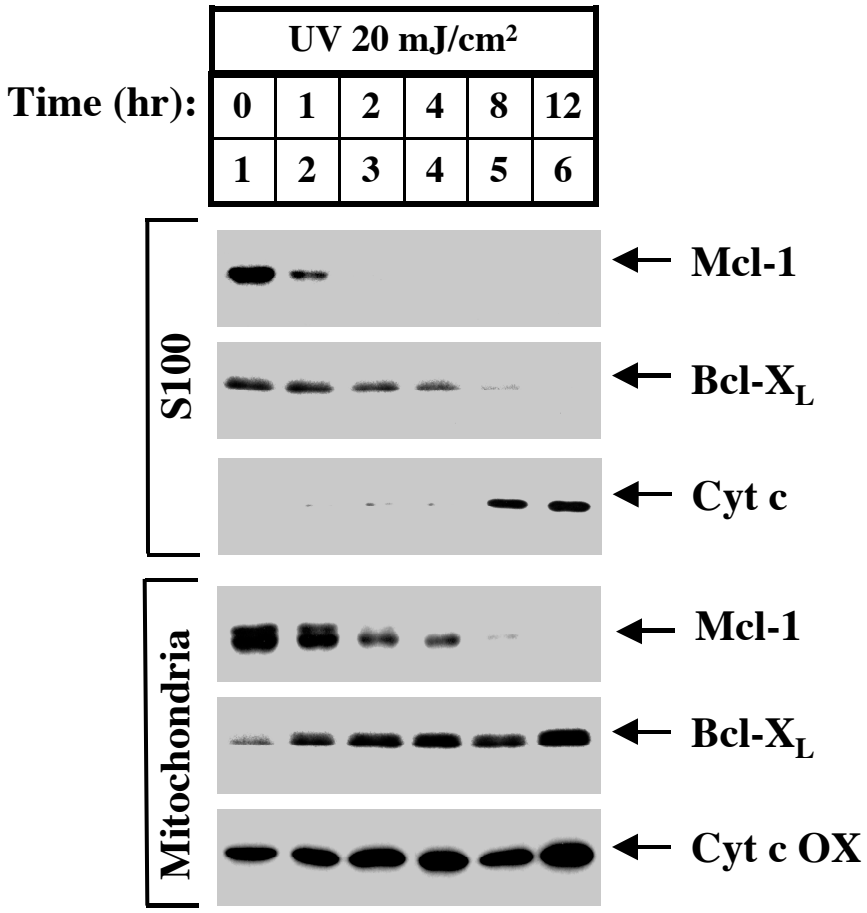



**Figure 4-2: Mcl-1 disappears in UV induced apoptosis of a human fibroblast cell line.**

5 x $10^6$ human fibroblast cells were treated (as described in Experimental Procedures) with UV at different time points

before harvest. Mcl-1, Bcl-$x_L$ and cytochrome c levels were compared in the S100. Mcl-1, Bcl-$x_L$ and cytochrome c oxidase were compared in the mitochondria.

# Figure 4-3

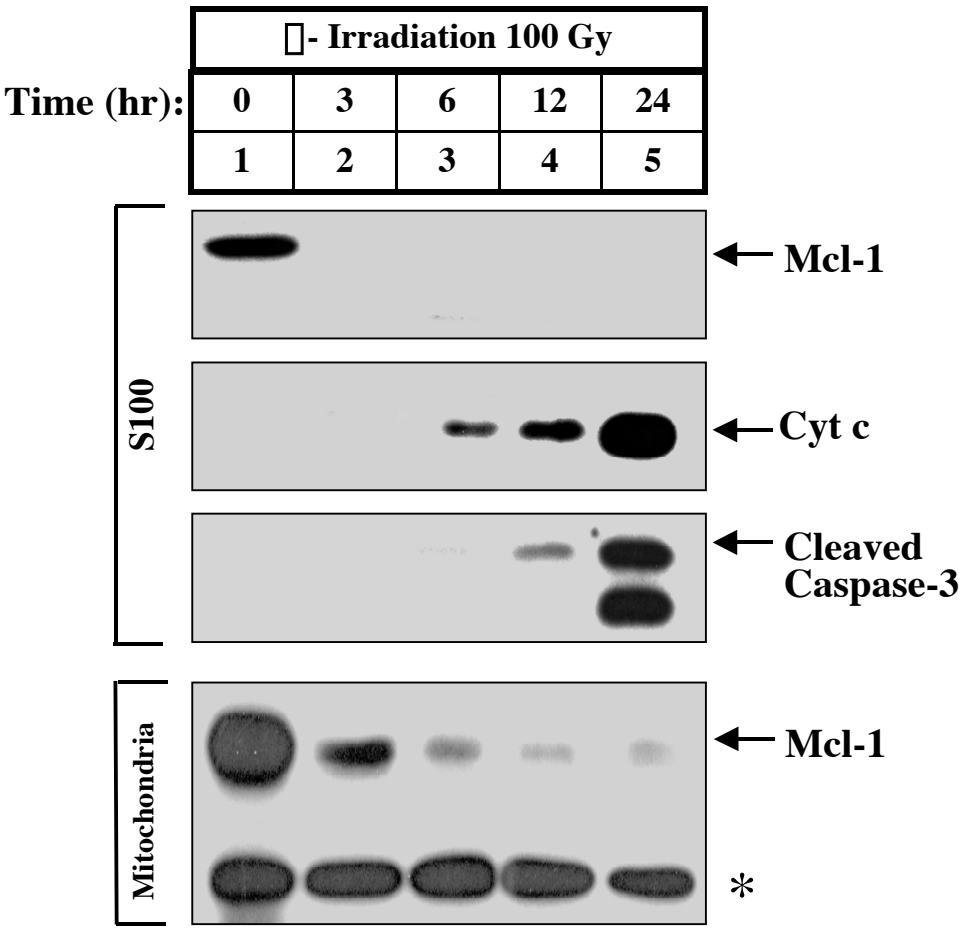



**Figure 4-3: Mcl-1 disappears during apoptosis induced by 100 Gy γ - Irradiation treatment.**

Mitochondria and S100 were isolated from HeLa cells left untreated (lane 1) or 3 hr (lane 2), 6 hr (lane 3), 12 hr

(lane 4), 24 hr (lane 5) after treatment with 100 Gy □ -Irradiation treatment. The levels of cleaved caspase-3, cytochrome c and Mcl-1 were measured in S100. Mcl-1 mitochondrial levels were compared between the samples. The (*) denotes a cross-reactive band that indicates equal loading.

# Figure 4-4

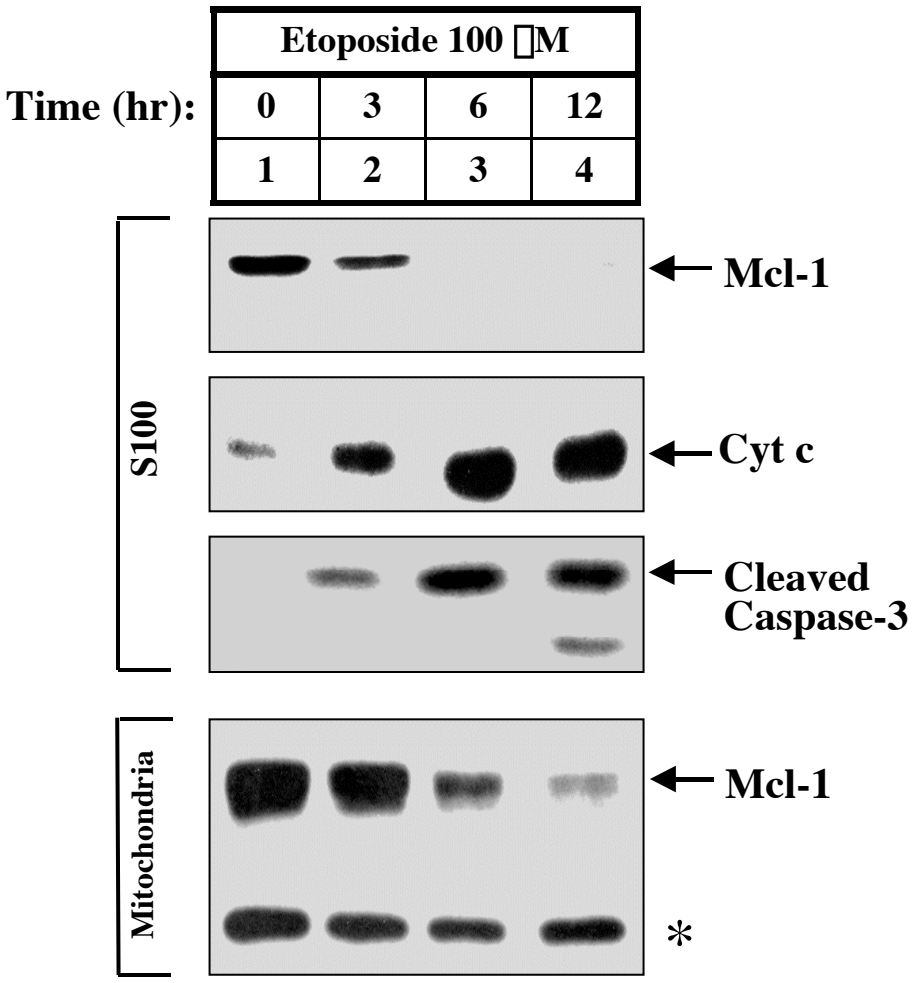



**Figure 4-4: Mcl-1 disappears during apoptosis induced by etoposide treatment.**

Mitochondria and S100 were isolated from HeLa cells that were

left untreated (lane 1) or treated with 100 μM Etoposide for 3 hr (lane 2), 6 hr (lane 3), or 12 hr (lane 4). The levels of cleaved caspase-3, cytochrome c and Mcl-1 were measured in S100. Mcl-1 mitochondrial levels were compared between the samples. The (*) denotes a cross-reactive band that indicates equal loading.

# Figure 4-5

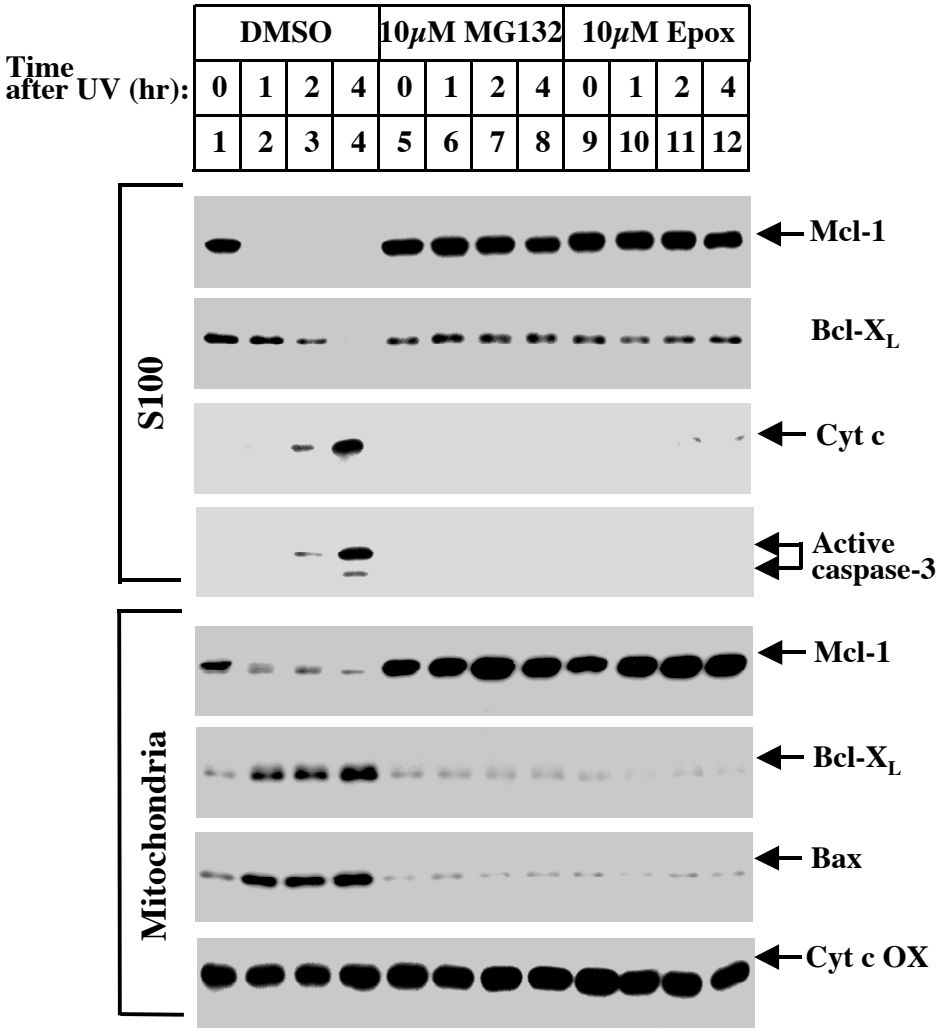



**Figure 4-5: Proteasome inhibitors block Mcl-1 disappearance and apoptosis.**

Proteasome inhibitors block the decrease of Mcl-1, the translocation of Bcl-$x_L$, and other apoptotic events after UV

treatment. Mitochondria and S100 were fractionated from HeLa cells treated with UV for different amounts of time and pretreated with DMSO (lanes 1-4), 10 μM MG132 (lanes 5-8), or 10 μM Epoxomicin (lanes 9-13). The levels of Mcl-1, Bcl-$x_L$, Bax, and cytochrome c oxidase were measured in the mitochondria. The levels of Mcl-1, Bcl-$x_L$, cytochrome c, and cleaved Caspase-3 were measured in the cytosol.

# Figure 4-6

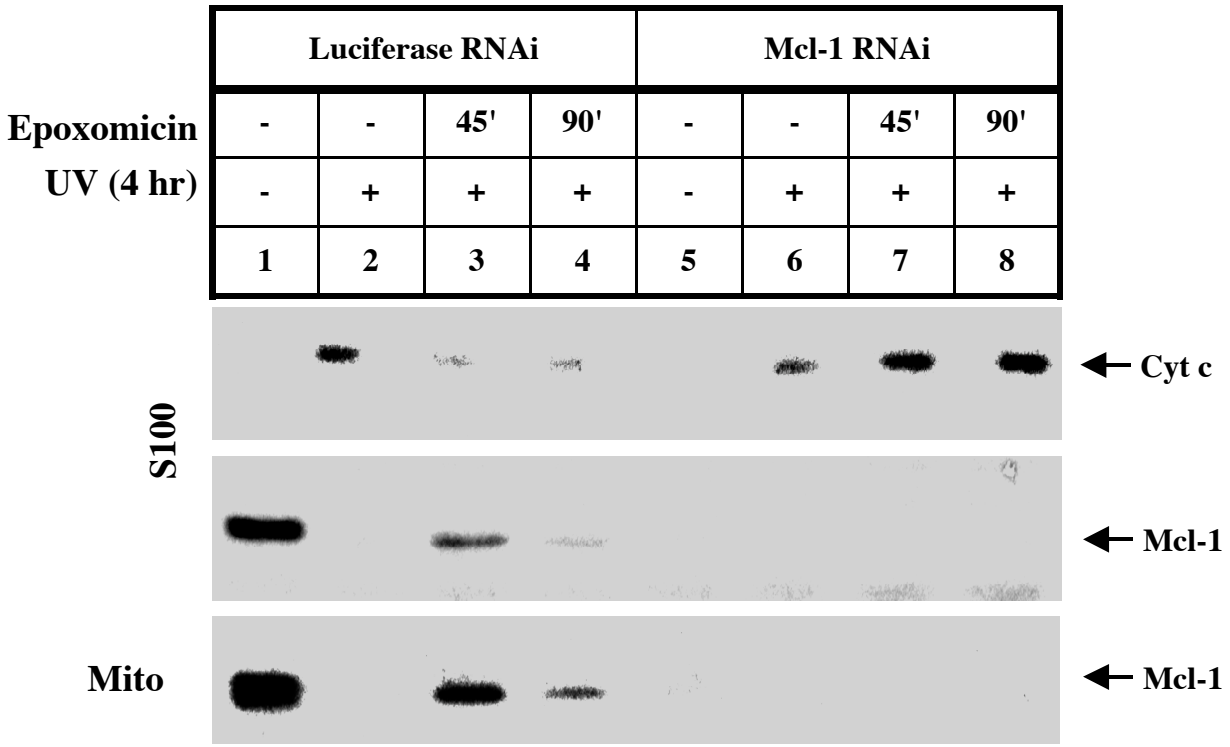

Luciferase RNAi
Mcl-1 RNAi
Epoxomicin
UV (4 hr)
- - 45' 90' - - 45' 90'
- + + + - + + +
1 2 3 4 5 6 7 8
S100
Mito
Cyt c
Mcl-1
Mcl-1

**Figure 4-6. Mcl-1 is required for proteasome inhibitor attenuation of apoptosis.**

Epoxomicin fails to rescue UV treated HeLa cells pretreated with Mcl-1 RNAi. HeLa cells were transfected with Luciferase (1-4) or Mcl-1 siRNA using Oligofectamine (Invitrogen) as described in Experimental Procedures. 24 hours later, the cells were left untreated (lanes 1 and 5), or treated with UV (lanes 2-4, 6-8). In the treated samples, epoxomicin (10 M) was added 45 (lane 3,7) or 90 minutes (lane 4,8) after UV treatment. Mitochondria and S100 were isolated from cells that were collected and harvested 4 hours after the UV treatment. Mitochondria were analyzed for Mcl-1 and the levels of Mcl-1 and cytochrome c were measured in S100.

# Figure 4-7

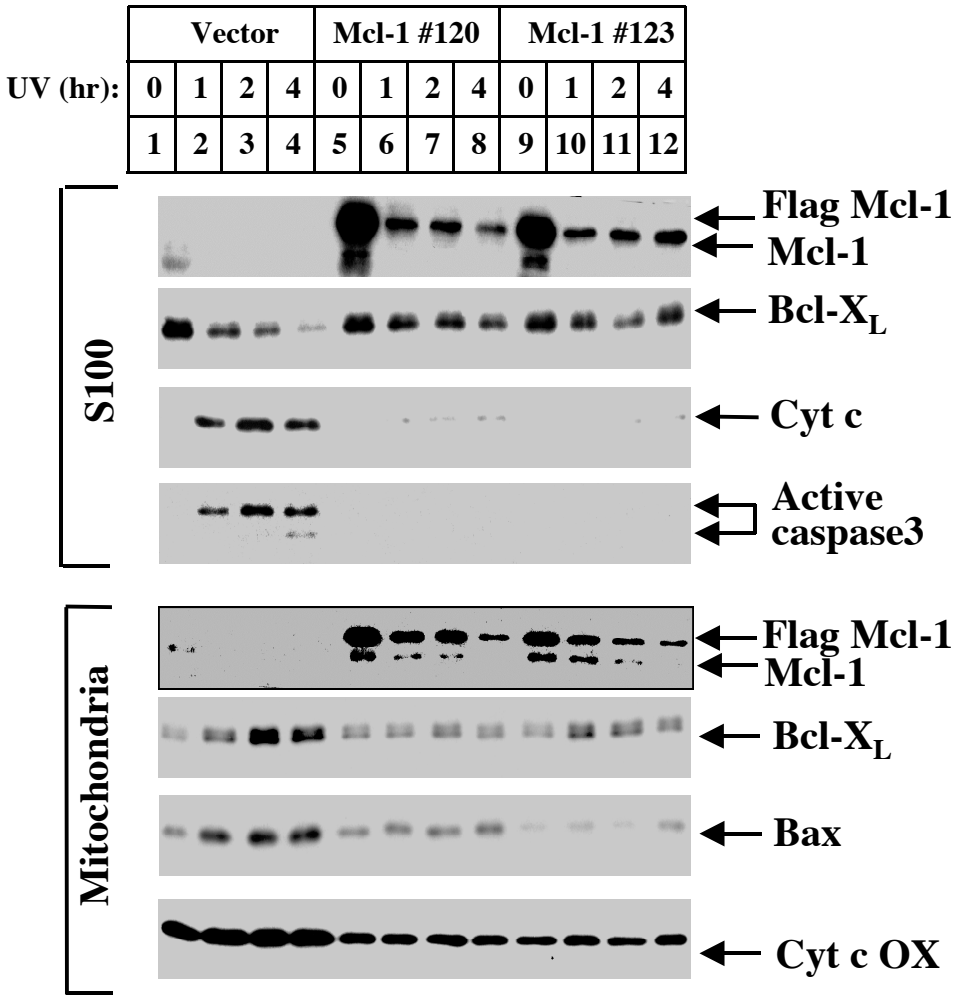



**Figure 4-7: The removal of Mcl-1 is required for apoptosis.** Mcl-1 overexpression delays downstream apoptotic events. After UV treatment, mitochondria and S100 were isolated from vector control (lanes 1-4) and two independent Flag-Mcl-1 overexpressing lines: line 120 (lanes 5-8) and line 123

(lanes 9-12). The levels of Mcl-1, Bcl-$x_L$, Bax, and cytochrome c oxidase were measured in the mitochondria. The levels of Mcl-1, Bcl-$x_L$, cytochrome c, and cleaved Caspase-3 were measured in the cytosol.

# Figure 4-8

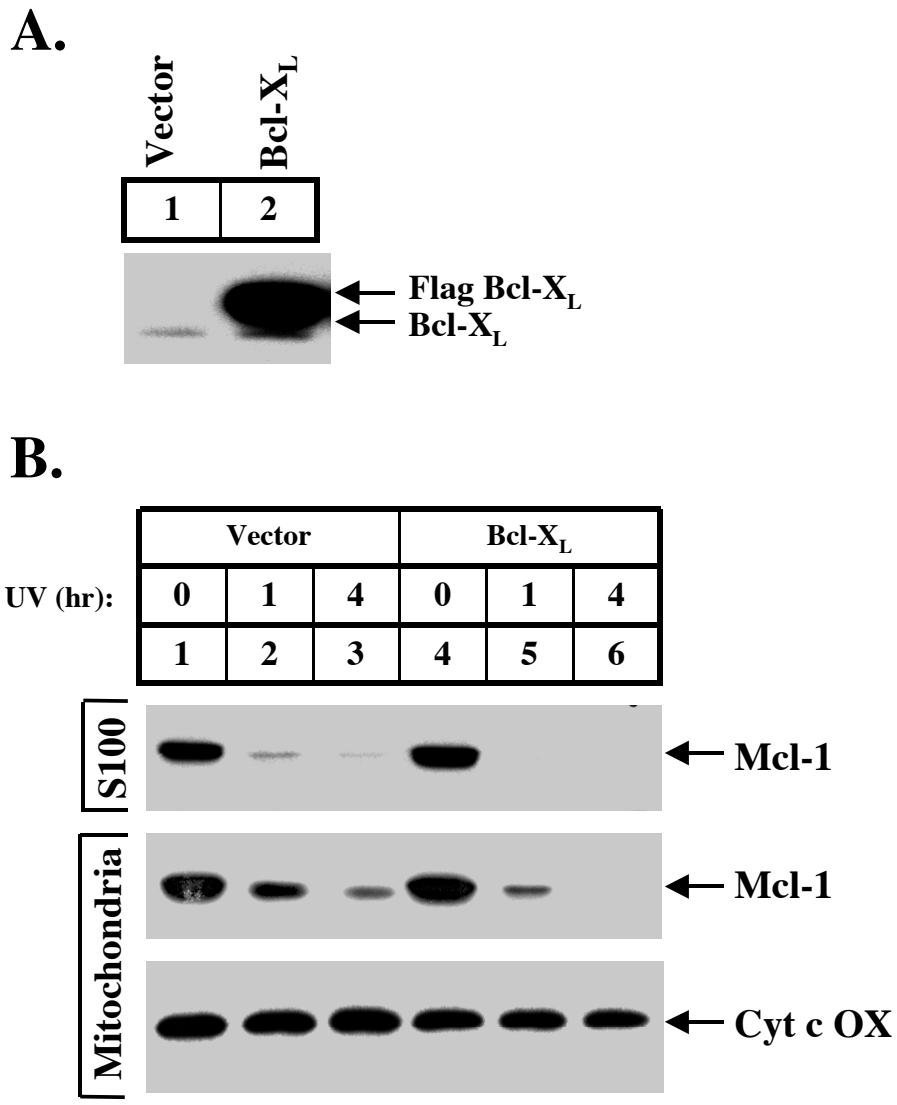



**Figure 4-8: The disappearance of Mcl-1 is upstream of Bcl-$x_L$.** (A) The levels of Bcl-xL were measured in total cell lysate prepared from vector and Flag-Bcl-xL overexpressing cells. (B) Mcl-1 disappearance is upstream of Bcl-xL. Mcl-1 levels

were analyzed in mitochondria and S100 fractionated from Vector (lanes 1-3) or Flag-Bcl-xL (lanes 4-6) overexpressing cells treated with UV for one (lane 2 and 5) or four hours (lanes 3 and 6).

# Chapter 5

## *Introduction*

Our hypotheses to explain the disappearance of Mcl-1 protein after UV are the following: 1) the half-life of the protein is reduced after UV; 2) the synthesis of the protein is reduced after UV. Pulse-chain analysis demonstrated the latter to be true. The levels of Mcl-1 mRNA drop soon after UV leading to reduced synthesis. The existing level of Mcl-1 protein is degraded rapidly due to its intrinsically short half-life.

## *UV-induced Mcl-1 elimination is due to disappearance of its mRNA*

UV induced Mcl-1 elimination could be due to accelerated protein degradation or inhibited synthesis. To sort through these possibilities, we did a pulse chase experiment using an anti-Mcl-1 antibody to immunoprecipitate the newly synthesized $S^{35}$-labeled Mcl-1 and the protein was chased with or without UV-irradiation. As shown in Figure 5-1A-B, Mcl-1 is a short-lived protein with a half-life of about 40 minutes. Interestingly, the half-life of Mcl-1 was the same with or without UV

irradiation, even though the total amount of Mcl-1 protein was dramatically decreased after UV-irradiation.

The above experiment indicates that Mcl-1 protein synthesis must be blocked after UV irradiation. To demonstrate that directly, we labeled the cells with $S^{35}$ methionine at the same time as UV irradiation. After 60 minutes of pulsing, we analyzed newly synthesized Mcl-1 by immunoprecipitation. Indeed, newly synthesized Mcl-1 was dramatically decreased after cells were exposed to UV irradiation and the decrease was not inhibited by MG132 (Fig. 5-2).

The stop in Mcl-1 protein synthesis could be due to direct translation inhibition or elimination of its mRNA. To distinguish these two possibilities, we analyzed the level of Mcl-1 mRNA isolated at different time points post UV irradiation. As shown in Figure 5-3, Mcl-1 mRNA showed a dramatic decrease after UV irradiation and completely disappeared within one hour of UV irradiation. In contrast, the level of GAPDH mRNA remained the same even four hours after UV irradiation.

***Discussion***

Two hypotheses to explain the disappearance of Mcl-1 mRNA mimics those proposed for the protein: 1) the half-life of the mRNA is reduced by UV or 2) the synthesis of new mRNA is

reduced by UV. We believe the second hypothesis will be true since the half-life of Mcl-1 mRNA is short, so that any cessation in synthesis would lead to its rapid disappearance (Yang et al., 1996).

# Figure 5-1

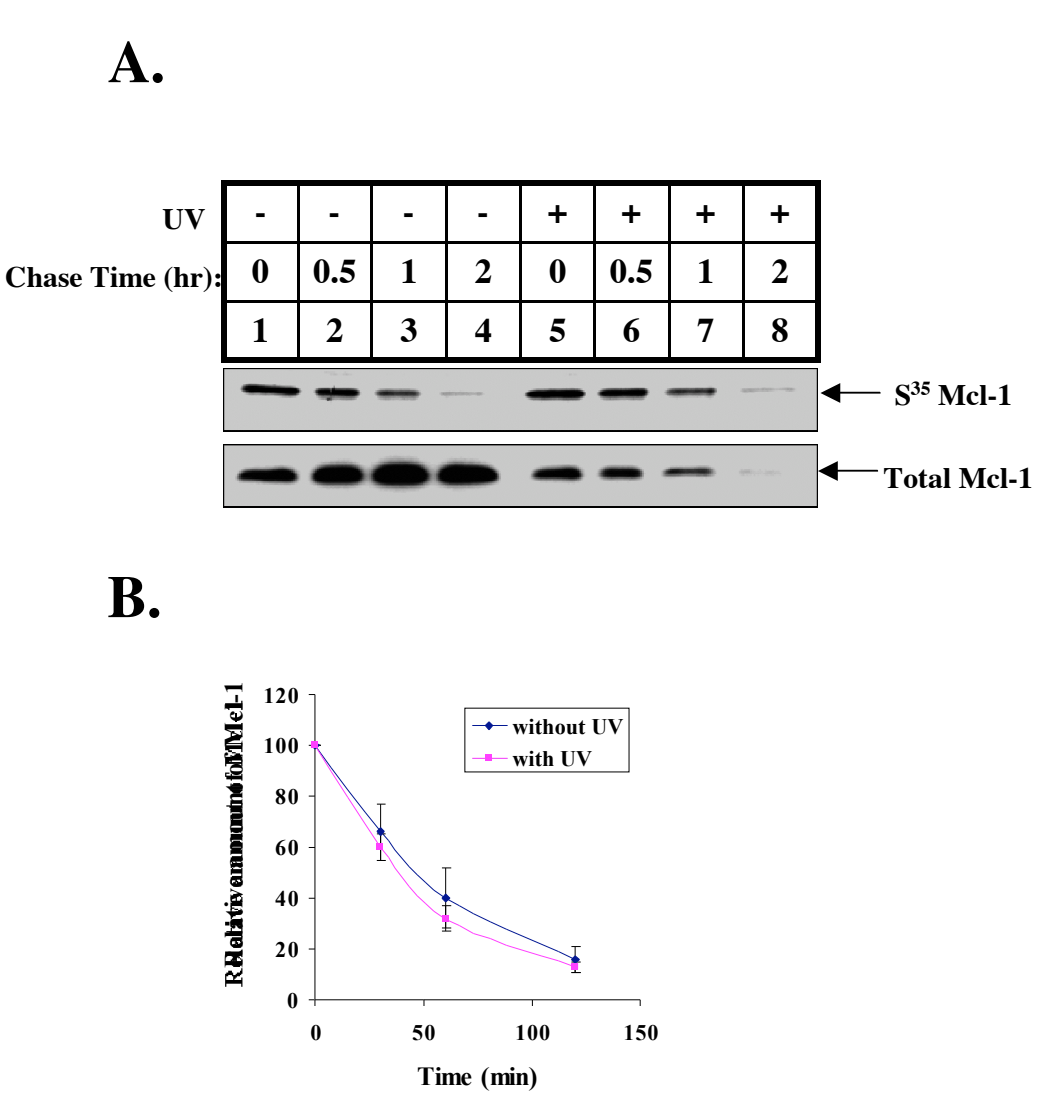



**Figure 5-1: Half-life of Mcl-1 with or without UV treatment.** HeLa cells were incubated in methionine starvation medium for 30 minutes before adding [$^{35}$S] methionine to pulse for one hour. Following the pulse, cells were either UV treated (lanes 5-8) or untreated (lanes 1-4), and then immediately

chased by complete medium for 0 (lane 1 and 5), 30 min (lane 2 and 6), 60 min (lane 3 and 7), or 120 min (lane 4 and 8). The levels of remaining Mcl-1 were analyzed by SDS-PAGE of Mcl-1 immunoprecipitates as described in Experimental Procedures. The amount of $^{35}S$ labeled Mcl-1 was quantified by phosphoimager analysis and plotted with respect to time. Values are representative of three independent experiments.

# Figure 5-2

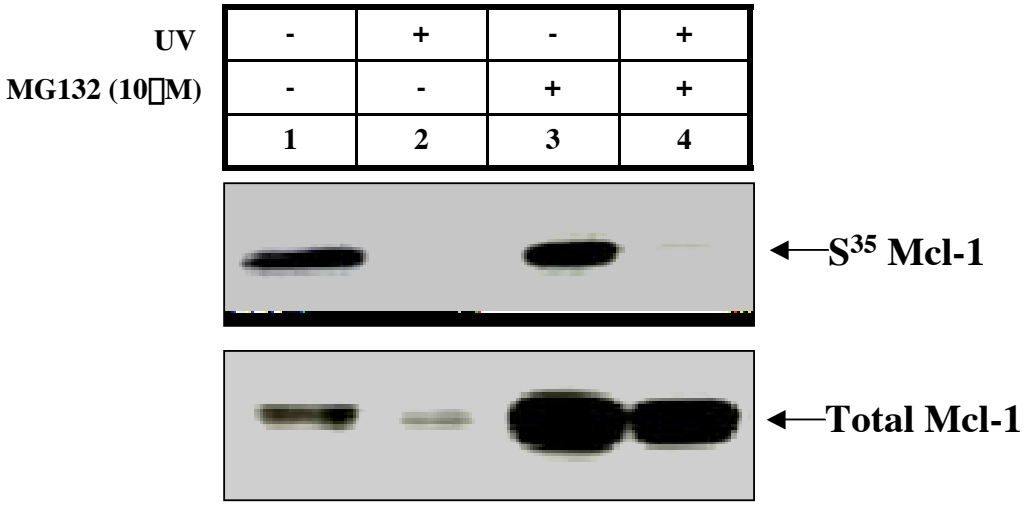



**Figure 5-2: Synthesis of Mcl-1 was blocked by UV irradiation.** HeLa cells were either left untreated (lane 1), treated with UV (lanes 2), pretreated with MG132 alone (lanes 3), pretreated with MG132 for 1 hr before UV (lane 4), and then methionine starved for 30 min followed by 1 hr of labeling with [$^{35}$S] methionine. After labeling, the synthesis of new

Mcl-1 was measured by SDS-PAGE of Mcl-1 immunoprecipitates and total Mcl-1 levels were determined by western blot.

# Figure 5-3

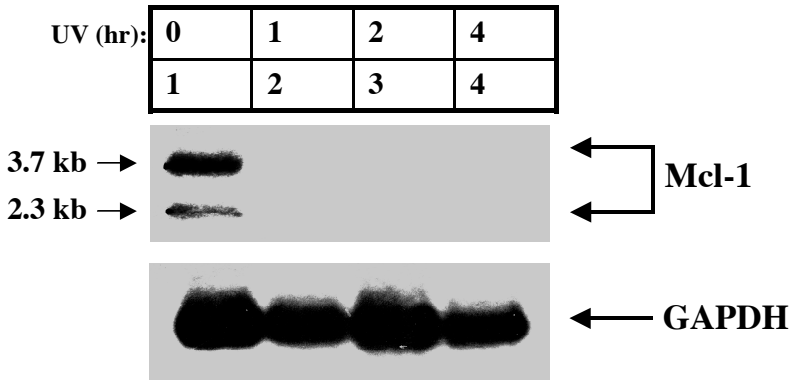



**Figure 5-3: Mcl-1 mRNA levels decrease after UV treatment.** mRNA was extracted with QuickPrep Micro mRNA Purification kit (Amersham) from HeLa cells untreated (lane 1) or treated with UV for different amounts of time (lanes 2-4). 400 ng of mRNA from each sample was subjected to electrophoresis and blotted as described (Dunn and Sambrook, 1980). Northern blot was hybridized separately with probes for Mcl-1 and GAPDH in ULTRAhyb (Ambion). The probe for Mcl-1 was derived from a 1 kb PCR fragment containing the entire coding sequence that was labeled by random-primed (DECAprime II, Ambion/ $^{32}$P dCTP, Amersham).

# Chapter 6

## *Introduction*

In the previous chapters, we have demonstrated that the disappearance of Mcl-1 protein is an essential apical step in the apoptotic pathway that leads to cytochrome c release. In the next chapter, we show that its elimination albeit necessary is not sufficient. In order to activate cytochrome c release, the cell must endure another "hit". We propose that the dephosphorylation of $Bim_{EL}$ is a candidate "second hit".

## *Elimination of Mcl-1 is necessary but not sufficient to cause apoptosis*

To verify that the disappearance of Mcl-1 protein is due to the inhibition of its synthesis, we treated HeLa cells with a transcriptional inhibitor actinomycin D or a translational inhibitor cycloheximide (CHX) and checked the fate of Mcl-1 protein in the same time course as UV irradiation. As shown in Figure 6-1, Mcl-1 protein was also eliminated at a similar rate with UV, actinomycin D, or CHX.

In contrast to UV-irradiation, treatment of HeLa cells with actinomycin D or cycloheximide up to 4 hours did not cause

apoptosis even though Mcl-1 protein was gone after 1 hour, suggesting that the elimination of Mcl-1 protein is not sufficient to cause apoptosis. Consistently, there was no noticeable Bax and Bcl-$x_L$ translocation or cytochrome c release after these treatments (lanes 5-12). We therefore hypothesized that additional signals (hits) are needed to trigger Bcl-$x_L$ translocation and cytochrome c release in UV treated cells.

***Dephosphorylation of pro-apoptotic protein $Bim_{EL}$ is a candidate additional apoptotic signal***

We reasoned that if an additional hit is needed to further advance the apoptotic process induced by UV-irradiation, such a hit has to somehow be connected to Mcl-1 since the second hit cannot happen if Mcl-1 levels are kept artificially high either by proteasome inhibitors or Mcl-1 over-expression (Fig 4-5,4-7). The site of a second hit is also most likely the mitochondria since cytosolic Mcl-1 is in its free form and no interacting proteins could be found (not shown). We therefore searched for Mcl-1 interacting proteins on the mitochondria by immuno-precipitating Mcl-1 from mitochondria lysate made in 1% NP40. Since Mcl-1 is known to interact with pro-death members of the Bcl-2 family of proteins, we analyzed the possible Mcl-1 interacting proteins by western blotting Mcl-1 precipitates. As

shown in Figure 6-2, we found that mitochondrial Mcl-1 bound $Bim_{EL}$, a BH3-only pro-apoptotic protein. Remarkably, all the solubilized $Bim_{EL}$ co-precipitated with anti-Mcl-1 antibody (lane 5). In contrast, other pro-apoptotic proteins including Bax, Bak, Bad, Bid, and Bik were not precipitated with this antibody (not shown).

$Bim_{EL}$ precipitated by Anti-Mcl-1 was not the result of a cross-reaction because $Bim_{EL}$ did not precipitate if the cells were treated with UV-irradiation to eliminate Mcl-1 (lane 6). Under these conditions, all of the $Bim_{EL}$ stayed in the supernatant (lane 4) suggesting that $Bim_{EL}$ bound to Mcl-1 is liberated when Mcl-1 is degraded. Interestingly, $Bim_{EL}$ liberated in response to UV irradiation migrated more quickly on the SDS-gel compared to $Bim_{EL}$ from untreated (lanes 1-2). The faster migration of $Bim_{EL}$ is likely due to dephosphorylation since such a change in SDS-gel movement could be reproduced by treatment with a phosphatase (not shown). The dephosphorylation is downstream since actinomycin D or CHX do not cause $Bim_{EL}$ dephosphorylation even though Mcl-1 was completely gone under these conditions (Figure 6-5).

If $Bim_{EL}$ dephosphorylation is the second hit required for UV-induced cytochrome c release, the prediction would be that such an event should be downstream of Mcl-1 degradation. To

test that, we examined $Bim_{EL}$ dephosphorylation in response to UV irradiation in cells where Mcl-1 degradation was blocked by proteasome inhibitors, in Mcl-1 over-expressing cell lines and in a Bcl-$x_L$ over-expressing cell line. As shown in Figure 6-3, proteasome inhibitors completely blocked $Bim_{EL}$ dephosphorylation (lanes 5-12). $Bim_{EL}$ dephosphorylation was also significantly delayed in Mcl-1 over-expressing cell lines (Figure 6-4A); however, $Bim_{EL}$ dephosphorylation was not affected in the Bcl-$x_L$ over-expressing cells (Figure 6-4B).

# Figure 6-1

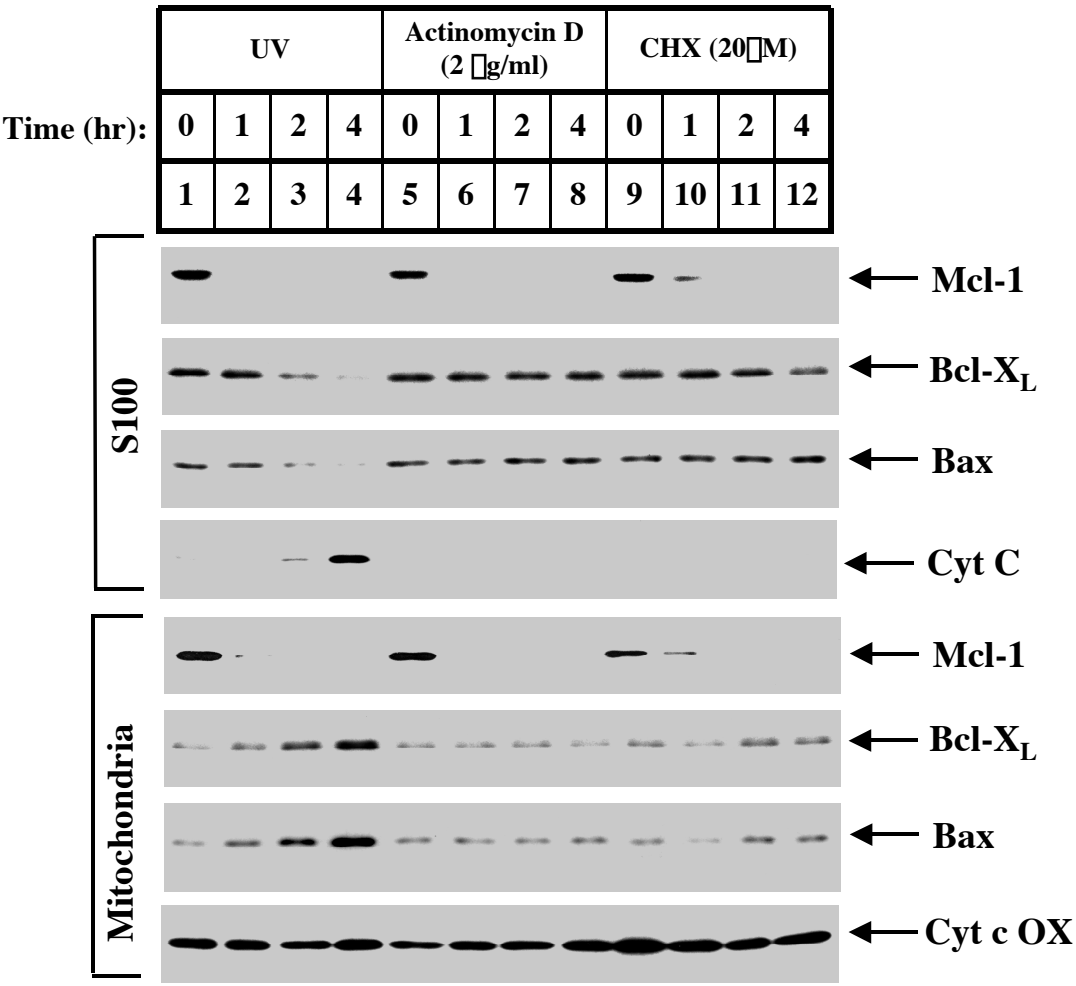



**Figure 6-1: Reduced Mcl-1 levels are not sufficient for UV induced cytochrome c release.**

(A) Protein synthesis inhibitors induce the disappearance of Mcl-1 but do not trigger apoptosis. HeLa cells were either left untreated (lanes 1,5, and 9), treated with UV (lanes 2-

4), Actinomycin D (2 μg/ml) (lanes 6-8), or CHX (20 μM) (lanes 10-12) and harvested at different time points after treatment.

# Figure 6-2

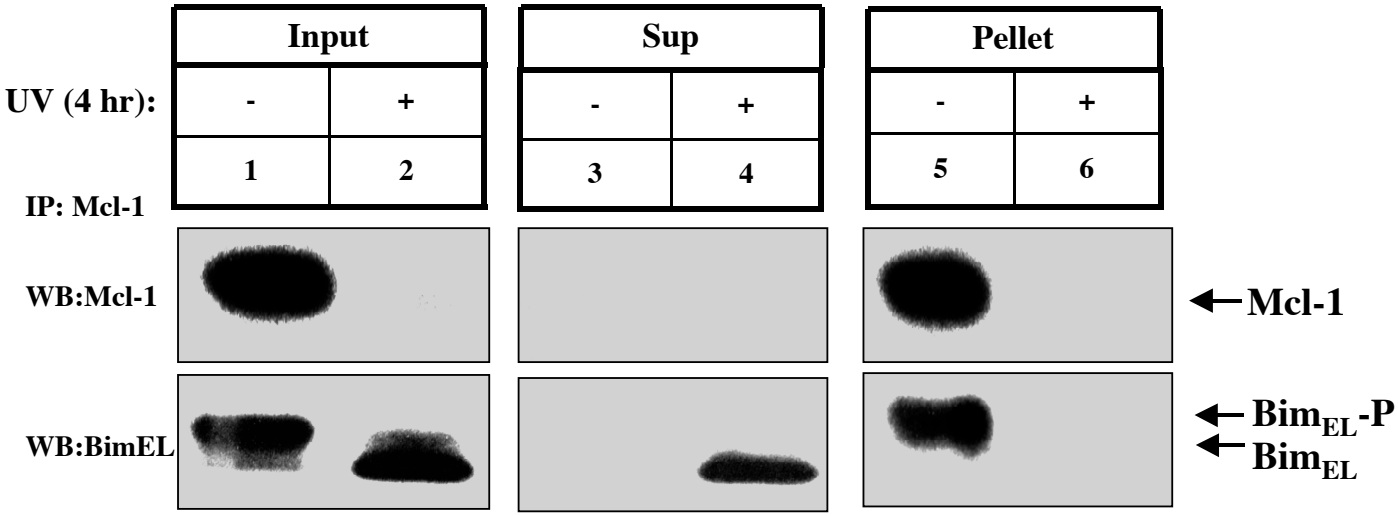

Input
Sup
Pellet
UV (4 hr):
-
+
-
+
-
+
1
2
3
4
5
6
IP: Mcl-1
WB:Mcl-1
WB:BimEL
Mcl-1
Bim$_{EL}$-P
Bim$_{EL}$

**Figure 6-2: Mitochondrial Mcl-1 quantitatively binds $Bim_{EL}$.** Mitochondria were harvested from HeLa cells that were either left untreated or UV treated four hours prior. Mcl-1 was immunoprecipitated as described in Experimental Procedures. The levels of Mcl-1 and $Bim_{EL}$ were measured in the input, supernatant, and immunoprecipitates of untreated and UV treated mitochondrial samples.

# Figure 6-3

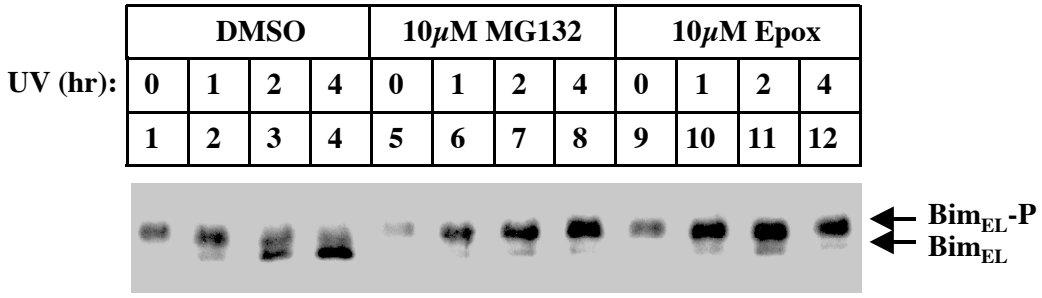



**Figure 6-3: Proteasome inhibitors block $Bim_{EL}$ dephosphorylation after UV treatment.**

The phosphorylation state of $Bim_{EL}$ was determined by western blotting mitochondrial samples from Fig 4-5.

# Figure 6-4

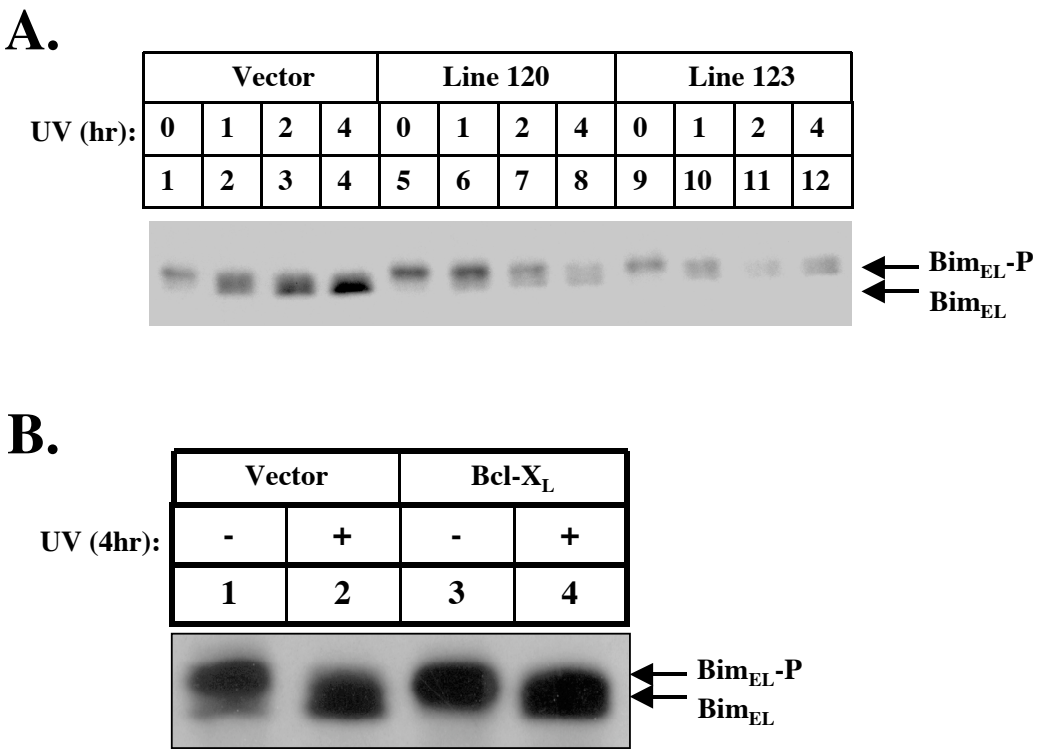



**Figure 6-4: Bim dephosphorylation is downstream of Mcl-1 disappearance and upstream of Bcl-$x_L$.**

(A) Mcl-1 overexpression delays $Bim_{EL}$ dephosphorylation after UV treatment. The phosphorylation state of $Bim_{EL}$ was determined by western blotting mitochondrial samples from Fig

5A. (B) $Bim_{EL}$ dephosphorylation is upstream of Bcl-$x_L$. $Bim_{EL}$ phosphorylation was determined by a western blot of mitochondrial lysate from vector (lanes 1-2) or Bcl-$x_L$ overexpressing cells (lanes 3-4) (See Figure 4-8) that were left untreated (lanes 1 and 3) or treated with UV for four hours (lanes 2 and 4).

# Figure 6-5

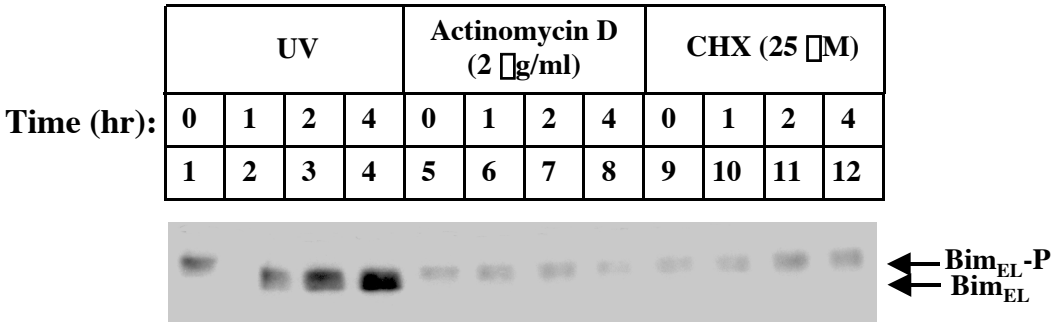



**Figure 6-5: Bim dephosphorylation is specific to UV.**

Protein synthesis inhibitors induce the disappearance of Mcl-1 but not the dephosphorylation of $Bim_{EL}$. The phosphorylation state of $Bim_{EL}$ was determined by western blotting mitochondrial samples from Fig 6-1.

## Experimental Procedures

### *Reagents*

The following antibodies were used for western blots: Monoclonal Bak Ab-1 (Oncogene), monoclonal cytochrome c (Pharmingen), polyclonal Bcl-$x_L$ (Cell signaling), monoclonal Mcl-1 (Pharmingen), monoclonal caspase-2 (Pharmingen), polyclonal caspase-3 (cell signaling), monoclonal caspase-9 (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 immuno-depletions for Mcl-1 and Bcl-$x_L$ were performed with polyclonal Mcl-1 (Pharmingen) and polyclonal Anti-Bcl-$x_L$ (Cell Signaling). Z-VAD.fmk, actinomycin D, cycloheximide, MG132, and epoxomicin were obtained from Calbiochem.

### *UV Treatment and Cellular Fractionation.*

HeLa or K562 cells were plated at a density of 2 x $10^7$ cells in a 15 cm dish with DMEM or RPMI medium supplemented with 10% FBS respectively. Several hours before treatment,

each dish was replaced with 13 ml of fresh medium. The cover of each dish was removed before the cells were treated in a Stratagene stratalinker with 200 $mJ/cm^2$ of ultraviolet irradiation (254 nm). HeLa cells were also treated with gamma irradiation from cesium source or etoposide (Sigma). At the indicated times after treatment, cells were scraped, collected, and washed once in PBS. The cell pellet was resuspended in 5 x 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) supplemented with 250 mM sucrose. 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 soup was centrifuged in three sequential steps in order to separate mitochondria from cytosol: 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 solubilized in 1% Chaps in Buffer A unless otherwise indicated. Protein concentration was normalized using the Bio-Rad Bradford reagent.

***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.

***In vitro Assays for Mitochondrial Priming and Inhibitory Activity.***

Mitochondria (.67 mg/ml) alone or with S100 (4 mg/ml) were incubated as indicated in buffer A with 250 mM sucrose for 15 minutes at 37°C in buffer A with 250 mM sucrose and 150 mM NaCl. Following incubation, the mitochondria were pelleted and the supernatant was tested for cytochrome c release. The mitochondria pellet was tested for Bak oligomerization as described above. During biochemical fractionation, in order to increase our yield we made S100 from HeLa cells using a buffer without sucrose. Hypotonic buffers cause mitochondrial rupture and cytochrome c contamination in the S100. (Liu et al., 1996) Contaminating cytochrome c was removed from the S100 by incubating with SP-sepharose XL resin (Amersham) for 1

hour at 4 degrees. Cytochrome c binds SP resin, but our activity does not (data not shown).

***Purification of Bcl-$x_L$ from K562 cells.***

K562 cells were grown in 200 liters quantities at a density of 7 x $10^5$ cells/ml in RPMI medium supplemented with 10% fetal bovine serum at the National Cell Culture Center (Minnesota). Cells were collected by centrifugation at 3000 rpm and washed twice with PBS. The cell culture center shipped the fresh cell pellets to our laboratory overnight. Upon the receiving the shipment, we resuspended the cells in five times the volume of Buffer A supplemented with leupeptin, aprotinin, and pepstatin A. After fifteen minutes incubation on ice, the resuspended cells were sheared in a 100 ml dounce homogenizer with 25 strokes. The S100 fraction was recovered and frozen after sequential centrifugations as described above in "UV Treatment and Cellular Fractionation." 400 ml of thawed K562 S100 (6 mg/ml) was spun at 20,000g to remove any insoluble fractions. Ammonium sulfate was added to the supernatant up to 30% and incubated with stirring at 4°C. After 90 minutes, the mixture was spun at 20,000g for 15 min at 4°C to pellet the precipitate. The supernatant was removed and the pellet resuspended in 120 ml of Buffer A supplemented

with 6M Urea. The resuspended solution was cleared by centrifugation and filtration (0.45 micron) in that order. 6 ml of the cleared solution was loaded onto a superdex 26/60 size exclusion column (Amersham) equilibrated in Buffer A with 6M Urea twenty consecutive times. All of the fractions (12.5 ml) were dialyzed and assayed. Two active fractions from each run (500 ml total) were loaded onto a 5 ml Hi Trap Q sepharose column (Amersham). The column was eluted in a single step from 100mM to 300mM NaCl in Buffer A with 6M Urea. The dialyzed active fraction was then run through a 1 ml heparin hi trap (Amersham) followed by a 1 ml CHT-II (Type II hydroxylapatite – Biorad). The CHT-II column was eluted in a single 50 mM potassium phosphate step. The eluted fraction (1.5 ml) was loaded three separate times (0.5 ml/run) onto a superdex 75 (25 ml) (Amersham). Active gel filtration fractions were loaded onto a 100 μl Mono Q column and eluted from a 100 mM to 300 mM NaCl gradient. 6,3,1, 0.5, 0.3 and 0.1 μl fractions were assayed from each 100 μl fractions. 80 μl of the remaining fractions were run on SDS-PAGE (15%) and stained with coomassie. Three bands in the most active fraction were excised and individually digested with trypsin (Promega) and the mass of the resulting peptides was

determined after separation on a capillary reverse-phase FPLC column and mass spectrometry.

***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.

***Mcl-1 RNA interference.***

RNAi knockdown experiments were done according to previous reports (Elbashir, 2001). Annealed, purified, and desalted double stranded siRNA, Luciferase (AACGUACGCGGAAUACUUCGA) and Mcl-1 (AAGAAACGCGGUAAUCGGACU) were ordered from Dharmacon Research, Colorado. 7 x $10^5$ HeLa cells were plated on day 0. On day 1, 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. After 4 hours, FBS was added to a final concentration of 10%. On Day 2, the medium over the cells was adjusted to 6 ml and they were treated with UV light as described above.

***Immunoprecipitation***

For immuno-depletion 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, or 100 μg polyclonal Anti-Bcl-$x_L$. After incubation, the beads were pelleted by centrifugation and washed at least 5 times. For immuno-depletion reactions, 50 μl of beads were incubated with 500 μl of S100 (5 mg/ml) overnight with rotation at 4°C. After incubation, the beads were pelleted and the supernatant was collected as immunodepleted S100. 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.

For pulse chase experiments, radiolabeled cells were lysed in cold RIPA lysis buffer (10 mM Tris-Hcl pH 7.4, 1% NP-40, 1mM EDTA, 0.1% SDS, 150 mM NaCl) with fresh proteinase inhibitors followed by breaking through a 25-gauge syringe 20 times. Equivalent amounts of cell extract were adjusted to equal volumes with RIPA buffer, precleared with protein A agarose beads, and clarified by centrifugation. Lysates were then incubated with polyclonal Anti-Mcl-1 overnight at 4°C, and the immune complexes were precipitated with protein A agarose beads for 4-6 hours. The immunoprecipitates were

washed five times RIPA buffer and incubated at 95°C for 5 min in SDS sample buffer. Samples were subjected to (SDS-PAGE) followed by autoradiography.

***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.

***Mcl-1 Pulse Chase Experiments.***

HeLa suspension cells were plated at a density of 5 x $10^6$ cells per 10-cm-diameter dish. For the pulse-chase, the cells were washed with phosphate-buffered saline (PBS), starved in methionine/ cysteine-free DMEM (GibcoBRL) at 37°C for 30 min, then labeled with 200 to 500 µCi of [$^{35}$S] cysteine/methionine (ICN) per plate in at 37°C for 60 min. After labeling, the cells were chased with complete DMEM containing 10% FBS and 2M cold methionine at 37°C for the indicated amount of time. A total of $10^7$ cells were collected at each time point. Cells were subsequently subjected to immunoprecipitation analysis as described above.

## Discussion

***Cytochrome c release: caspase dependent or independent.***

It is not surprising that the discovery of cytochrome c as a caspase activator was met with skepticism. Nobody ever imagined that a protein required for life sustaining activities such as mitochondrial respiration also served as a suicide trigger. Following the initial discovery a series of biochemical, genetic and cell biology experiments confirmed that cytochrome c is released during apoptosis and triggers caspase activation. However, cytochrome c mediated caspase activation is not conserved in lower species such as *C. elegans*. The lack of evolutionary conservation in the cytochrome c pathway inspired some controversy as to its role as an apoptotic initiator.

The caspase activation pathway in *C. elegans* is significantly different from that in the mammalian system (Figure 1-1). In *C. elegans*, the activation of ced-3, a caspase homolog, is regulated by its interaction with the Apaf-1 homolog ced-4. The Bcl-2 homolog, ced-9, binds to ced-4 and inhibits its activation of ced-3. In the mammalian system, Bcl-2 blocks caspase activation indirectly, it

prevents the release of cytochrome c which induces caspase activation through Apaf-1. Some researchers doubt that Bcl-2 has the same anti-apoptotic effect in both systems through an entirely different biochemical mechanism. Intuitively, these scientists believe that it is more likely that Bcl-2 like ced-9 directly inhibits caspase activation by interacting with Apaf-1. Accordingly, an interaction between anti-apoptotic Bcl-2 family members has been reported but remains controversial.

In this model, cytochrome c mediated caspase activation amplifies rather than initiates the apoptotic program. Consistent with this theory, several recent reports suggest that caspase-2 is activated early during apoptosis and leads to cytochrome c release. Transformed human fibroblasts in which the levels of caspase-2 have been reduced by RNA interference do not release cytochrome c during apoptosis (Lassus et al., 2002). After an apoptotic stimulus, caspase-2 immuno-localization changes from predominantly nuclear to cytoplasmic (Paroni et al., 2002). Once released, activated caspase-2 can directly release cytochrome c from mitochondria or indirectly release cytochrome c by cleaving Bid into tBid (Guo et al., 2002). In a separate but supporting study, Strasser and colleagues used lymphocytes harvested from mice

deficient in Apaf-1 or caspase-9 to study cytochrome c independent caspase activation (Marsden et al., 2002). Even though the elimination of these proteins abolishes the cytochrome c mediated caspase activation pathway, they found that lymphocytes still possess caspase activity after an apoptotic stimulus.

Although several reports suggest that caspase activation is upstream of the mitochondria, studies in other systems reveal a caspase independent cytochrome c release pathway. For example, mouse embryonic fibroblasts deficient in two pro-apoptotic Bcl-2 family members, Bak and Bax, do not release cytochrome c or activate caspases after various apoptotic stimuli (Wei et al., 2001). Furthermore, mice deficient in caspase-2 have no gross phenotypic abnormality and caspase-2 null fibroblasts still die by apoptosis (Bergeron et al., 1998). In our model system, UV treatment of HeLa cells, caspase-2 activation is not necessary for cytochrome c release. Following UV, the pan caspase inhibitor Z-VAD, blocks the activation of caspase-2, 3, and 9 but does not affect mitochondrial changes such as bak oligomerization and cytochrome c release (Figure 3-1). As is the case with the TNF/Fas pathways, caspase-2 activation pathways may be cell type specific.

Our objective is to understand and define the caspase independent cytochrome c release pathway in HeLa cells treated with UV. The first clue toward understanding cytochrome c release came from an answer to a long-standing mystery in the field: Why was the anti-apoptotic Bcl-2 protein localized to the mitochondria? Two different groups demonstrated that over-expression of Bcl-2 blocked the release of cytochrome c from the mitochondria. Presently, Bcl-2 and other family members are thought to work at the outer mitochondrial membrane to regulate the release of cytochrome c.

***The balancing act of pro- and anti-apoptotic Bcl-2 family of proteins***

The Bcl-2 family is composed of both pro- and anti-apoptotic proteins that share sequence and structural homology with each other (Cory and Adams, 2002). Most of the initial studies of the Bcl-2 family members utilized over-expression as a means of characterizing the apoptotic nature of the proteins (Oltvai et al., 1993; Vaux et al., 1988). While effective at separating pro- and anti-apoptotic characteristics, over-expression has not elucidated how these molecules are regulated in the cell.

The pro-apoptotic members have recently been separated

into two functional classes using genetics: Bax/Bak and BH3-only proteins. The Bax/Bak double knockout mouse demonstrated that these two proteins are required for cytochrome c release (Lindsten et al., 2000; Wei et al., 2001; Zong et al., 2001). BH3-only proteins such as Bad, Bim, or Bid remove the inhibition of anti-apoptotic Bcl-2 family members on Bax/Bak oligomerization and/or cause such an oligomerization directly through a possible "hit-and-run" mechanism (Cheng et al., 2001; Wei et al., 2001; Zong et al., 2001). On the other hand, mouse knockout models have proven less effective at sorting out the functions of the anti-apoptotic Bcl-2 family members. Mcl-1 null embryos have a pre-implantation lethal phenotype revealing only that Mcl-1 has an important and early role in development (Rinkenberger et al., 2000). The knock-out of Bcl-$x_L$ was also early embryonic lethal and the embryo displayed widespread apoptosis indicating that this gene is also important early in development (Motoyama et al., 1995).

***Mcl-1 is the apical sensor for apoptotic stimuli***

We used in vitro fractionation and reconstitution to distinguish early modifications of Bcl-2 family members in response to UV irradiation and then characterized whether there was any dependence between these molecular steps. We found

that a drop in Mcl-1 levels is the apical step in a signal transduction pathway composed of Bcl-2 family members that leads to cytochrome c release. When Mcl-1 degradation is blocked or its levels are artificially increased, downstream apoptotic events such as Bcl-$x_L$ and Bax translocation, Bim dephosphorylation, cytochrome c release, and caspase activation are all blocked or delayed. In contrast, upstream events such as the disappearance of Mcl-1, Bim dephosphorylation, and Bax translocation were not eliminated in cells that over-express Bcl-$x_L$. These results indicate that Mcl-1 functions upstream of Bcl-$x_L$, Bax and $Bim_{EL}$ in HeLa cells.

Since Mcl-1 regulation is an upstream event, it is important to understand how this molecule is regulated. We found that the estimated half-life of Mcl-1 (40 min) is unchanged after UV treatment, however UV induces a rapid disappearance of Mcl-1 mRNA that leads to a stop in Mcl-1 synthesis. Since Mcl-1 protein turns over quickly, the UV induced stop in synthesis leads to a rapid disappearance in Mcl-1. The elimination of Mcl-1 is not limited to UV irradiation or to HeLa cells. Genotoxic stress induced by etoposide or □-irradiation also efficiently induces Mcl-1 elimination and subsequent apoptotic changes in HeLa cells (Figure 4-3,4-4). Mcl-1 disappearance is also upstream of Bcl-$x_L$/ Bax

translocation and cytochrome c release in human skin fibroblasts treated with UV-irradiation (Figure 4-2).

In response to oxidative stress, Mcl-1 is regulated by phosphorylation. After oxidative stress, activated JNK kinases phosphorylate and inactivate Mcl-1. JNK kinases are also important upstream mediators in UV induced apoptosis. Cells deficient in JNK1 and JNK2 fail to release cytochrome c or go through apoptosis after UV treatment (Tournier et al., 2000). In some systems, or maybe with a less severe dose of UV, Mcl-1 activity might be reduced by JNK mediated deactivation rather than total disappearance. Another possibility is that UV induced JNK activation triggers a different arm of the apoptotic pathway other than Mcl-1 deactivation such as the dephosphorylation of Bim. Future experiments using specific inhibitors of JNK or JNK deficient cells will help define its role in other apoptotic events after UV such as the disappearance of Mcl-1 and/or the dephosphorylation of $Bim_{EL}$.

***Sequential events to cytochrome c release***

In HeLa cells, Mcl-1 degradation and the subsequent liberation of $Bim_{EL}$ are required for cytochrome c release, however it is not sufficient. Treatment with transcriptional or protein synthesis inhibitors predictably cause a rapid decrease

in Mcl-1 but do not trigger Bcl-$x_L$ and Bax translocation or cytochrome c release. In addition to the removal of Mcl-1, UV induced cytochrome c release requires at least one more "hit" in HeLa cells. In other systems it appears that Mcl-1 disappearance is both necessary and sufficient for apoptosis. For example, it is possible to induce apoptosis in a multiple myeloma cell line merely by treating it with inhibitors of protein synthesis or by specifically removing Mcl-1 with anti-sense oligonucleotides (Zhang et al., 2002). In this system, depletion of Mcl-1 is the only hit needed for apoptosis.

$Bim_{EL}$ dephosphorylation is a candidate "second hit" since it is a UV-induced event. Inhibition of protein synthesis liberates $Bim_{EL}$ by triggering the disappearance of Mcl-1 but does not lead to $Bim_{EL}$ dephosphorylation (Fig. 7-5). How dephosphorylated $Bim_{EL}$ triggers further downstream apoptotic events such as Bax and Bak oligomerization is not clear since no stable interaction between these proteins can be detected (not shown). It may work in a "hit-and run" fashion as has been proposed for tBid (Korsmeyer et al., 2000). Like Mcl-1, Bcl-$x_L$ and Bax are also present in both cytosolic and mitochondrial compartments. Unlike Mcl-1, their total amount does not change during apoptosis but rather cytosolic Bax and Bcl-$x_L$ simultaneously translocate to the mitochondria, an event that is

concurrent with the decreased activity of Bcl-$x_L$ in cytosol (Hsu et al., 1997). On mitochondria, Bcl-$x_L$ is bound to Bax suggesting that their translocation is a coordinated event that also marks the inactivation of Bcl-$x_L$ as a cytochrome c release inhibitor (Chao et al., 1995; Sedlak et al., 1995). Our experiments reveal a biochemical pathway through which ultraviolet light induces mitochondrial damage leading to apoptosis. An upstream biochemical pathway that begins with Mcl-1 may provide new insights into the pharmacological manipulation of apoptosis in human disease.

***Cancers: Too little Apoptosis***

The rapidly expanding interest to delineate the apoptotic pathway is mostly motivated by a desire to control cell fate in human disease. In some cases, drugs are needed to specifically induce apoptosis in malignant cancer cells that do not have a functional apoptotic program. For example, Chronic myelogenous leukemia (CML) is a rare cancer that is caused by a chromosomal translocation between chromosomes 9 and 22 that results in the expression of a novel fusion protein, Bcr-Abl. The Bcr-Abl protein has constitutive tyrosine kinase activity that leads to aberrant cell proliferation and a failure to die by apoptosis. CML cell fail to die because of Bcr-Abl induced over-expression

of Bcl-$x_L$ (Amarante-Mendes et al., 1998). Inhibition of Bcr-Abl by a new drug, STI571, reduces the levels of Bcl-$x_L$ and restores a normal apoptotic response after UV (not shown). This is one successful example in which the restoration of the apoptotic program in cancer cells might resensitize them to treatment.

***Cellular Stress: Too Much Apoptosis***

On the flip side, therapy is also needed to block death in disease cells that die by apoptosis. These situations include acute crises such as ischemic injury in stroke and myocardial infarction as well as chronic disease such as Alzheimer's and Parkinson's disease. The problem in preventing death is more complex because there is no way to know when the cell has passed the "point of no return". For example, Bax/Bak deficient cells which fail to release cytochrome c still die presumably because of abnormal mitochondrial function even in the absence of cytochrome c release and caspase activation (Xiang et al., 1996). For that reason, we pursued the delineation of apoptosis pathways upstream of cytochrome c release hoping that future anti-apoptosis therapies that inhibit apoptosis upstream of mitochondria may be more effective than those that inhibit caspases directly. The sequential nature of the apoptotic signal transduction pathway revealed by this study offers several new

points for potential intervention. For instance, specific inhibition of Mcl-1 mRNA or protein degradation may effectively prevent apoptosis and death from a toxic insult. The effectiveness of drugs that block the degradation of Mcl-1 will depend on why Mcl-1 is no longer synthesized. If the changes in Mcl-1 mRNA levels are specific either by reduced transcription or increased synthesis, then drugs that block these events will be effective. However, if the disappearance of Mcl-1 is the result of a non-specific, global reduction in transcription and protein synthesis, then drugs that block Mcl-1 protein degradation may prevent apoptosis but will not rescue the cells from death. In this case, mitochondrial events during apoptosis are already past the "point of no return".

***Stress, Synthesis and Apoptosis***

Apoptotic stimuli such as UV have been shown to globally reduce the rate of transcription and translation. UV light leads to the hyper-phosphorylation and ubiquitination of RNA polymerase II reducing the initiation of new transcription (Luo et al., 2001; Rockx et al., 2000). UV also blocks protein translation by stimulating the GCN kinase to phosphorylate EIF2 (Elongation Initiation Factor) that inhibits new protein translation (Deng et al., 2002). Global reductions in synthesis

will lead to the disappearance of Mcl-1 whose mRNA and protein have a short half-life. In this case, the existing levels of Mcl-1 mRNA and protein might determine how much time the cell has to repair its damage and restart synthesis before cytochrome c is released and apoptosis begins. In accordance, growth factor cell survival pathways such as the AKT/PI3 kinase pathway protect the cell from stress by upregulating Mcl-1 mRNA levels (Huang et al., 2000; Liu et al., 2001; Wang et al., 1999). The inhibition of protein synthesis is not a response restricted to UV, but is a common response to a variety of stresses including nutrient deprivation (Clemens, 2001). If apoptosis were a response to a long-term shutdown in synthesis, then it would explain how thousands of different compounds all trigger the same biochemical apoptotic pathway. It is unlikely that each compound triggers a specific pathway for cytochrome c release but more likely that a synthesis shutdown serves as a universal stress response. Consistent with this theory, the over-expression of Bcl-2 blocks cytochrome c release, caspase activity and other mitochondrial changes but did not increase long term cell survival after growth factor withdrawal (Nunez et al., 1990). In this case, damage may be irreparable and the cell will still die. Cells may have "died" due to a permanent cessation in synthesis long before they exhibit signs of

apoptosis. Cytochrome c release, caspase activation and the subsequent dismantling of the cell may not be “cellular suicide” but rather a form of “cellular cremation”.

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**Vitae**

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