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SMAC-Based Antagonists of the Inhibitors of Apoptosis

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SMAC-BASED ANTAGONISTS OF THE INHIBITORS OF APOPTOSIS

By

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A thesis submitted to the Faculty of Graduate and Post-doctoral Studies, University of Ottawa, in partial fulfillment of the requirements for the degree of

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For IAP

Abstract

Apoptosis signal pathways converge on the Caspases, a family of proteases that form a self-amplifying cascade, the activation of which generates the characteristic morphological and biochemical features of apoptotic cell death. The Inhibitors of Apoptosis (IAPs) are a family of proteins that bind and inactivate Caspases at both the initiation and terminal effector stages of this cascade. Over-expression of IAPs, as observed in cancer cell lines and tumour biopsy samples, results in a cellular phenotype of resistance to a variety of apoptotic stresses including chemotherapeutic drugs and irradiation. One negative regulator of IAP function, Smac (second mitochondrial activator of Caspases a.k.a.Diablo) has been identified as a mitochondrial protein that is processsed and released concomitantly with cytochrome c following an apoptotic stress. Proteolytic processing of the mitochondrial signal peptide sequence generates a novel amino terminus that interacts with XIAP and disrupts XIAP-meditated Caspase-9 inhibition. By disrupting the interaction of XIAP with Caspase-9, Smac is believed to promote apoptosis. Furthermore, the structure determinations of XIAP, in conjunction with either Caspases or Smac, have led to the identification of a critical pocket and groove on the surface of each BIR domain (Baculovirus IAP Repeat). Within XIAP BIR3, the binding pocket interacts with Caspase-9 and can be disrupted by the presence of Smac, which competes for the same site.

In this thesis work, the utility of Smac as a chemo-sensitizing agent for the treatment of cancer was enhanced by three different strategies. The first approach involved the development and characterization of an ubiquitin-Smac fusion system that circumvents the targeting of Smac to the mitochondria and results in the expression of a fully mature, biologically active Smac protein. The second approach entailed developing a strategy for increasing the affinity of Smac binding to the IAPs. A random peptide phage display screen

was used to identify novel, high affinity peptide ligands that bind to the IAP- BIR3 motifs. These peptide sequences were similar in structure to the amino terminus of Smac and Caspase-9. The data generated from these two approaches showed that both over-expressed, mature Smac and Smac-like peptide sequences bind to the IAPs *in vitro* and *in vivo* and sensitize cells to chemotherapeutic drugs. The third and final approach involved developing a Smac system that was a more potent apoptosis- inducer than wild-type Smac, which would be predicted to have greater therapeutic potential. It has been shown that Smac interactions with the IAP BIR domains may accelerate IAP auto-ubiquitination and destruction via the IAP carboxy terminal RING (Really Interesting New Gene) domain. Adding an IAP-RING domain to the carboxy terminus of Smac was predicted to enhance IAP degradation while maintaining the IAPs in a non-functional protein complex. Indeed, it was observed that generating a Smac protein with RING- conferred E3 ligase activity significantly decreased IAP levels, with a concomitant increase in the sensitivity to apoptosis in the presence of chemotherapeutic agents.

Acknowledgements

Reflecting on the past four years of my Ph.D. program the most notable recollection that I have of this time is how much I laughed. For that I thank Dr. Peter Liston and the members of his giddy lab, past and present, (Sandra Hurley, Tara Nevins, and Dan McManus) for making the lab a lively and positive environment. I also would like to thank Peter for his role as my supervisor. Peter's scientific talent and vast knowledge never ceases to amaze me and he is an example of what one should strive to be as a scientist.

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List of Abbreviations

AIF	Apoptosis Inducing Factor
AML	Acute Myelogenous Leukemia
PAF1	Apoptosis Protease Activating Factor 1
Apo2L/TRAIL	Apo2 Ligand/TNF-Related Apoptosis-Inducing Ligand
AS ODN	Anti-Sense ODN
BCL	B-Cell Lymphoma
BIR	Baculoviral IAP Repeat
BIRC	BIR Containing Protein
BRUCE	BIR-containing Ubiquitin Conjugating Enzyme
BSA	Bovine Serum Albumin
CARD	Caspase Recruitment Domain
CHK1	Checkpoint Kinase 1
c-IAP	Cellular IAP
CpIAP	*Cydia pomonella* IAP
CpVG	*Cydia pomonella* Granulosis Virus
DARK	*Drosophila* Apaf-1-Related Killer
dATP	Deoxyadenosine Triphosphate
DD	Death Domain
DED	Death Effector Domain
DIABLO	Direct IAP Binding Protein with Low PI
DIAP	*Drosophila melanogaster* IAP
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
EPR-1	Effector Cell Protease Receptor-1
ER	Endoplasmic Reticulum
FADD	Fas-Associated Protein with Death Domain
FCS	Fetal Calf Serum
GAPDH	Glyceraldehyde Phosphate Dehydrogenase
GST	Glutathione S-transferase
HEL299	Human Embryonic Lung 299 cells
HIAP	Human IAP
HIV/AIDS	Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome
HRP	Horseradish Peroxidase
IAP	Inhibitor of Apoptosis
IBM	IAP Binding Motif
IFNβ	Interferon β
IgG	Immunoglobulin
IRES	Internal Ribosome Entry Site
IκB	Inhibitor of κB
LB	Luria- Bertani Broth
MALT	Mucosa-Associated Lymphoid Tissue
MEF	Mouse Embryonic Fibroblasts
mRNA	messenger Ribonucleic Acid
MW	Molecular Weight

NAIP	Neuronal Apoptosis Inhibitory Protein
NF-κB	Nuclear Factor κB
NSCLC	Non-Small Cell Lung Carcinoma
ODN	Oligodeoxynucleotides
OpIAP	*Orgyia pseudosugata* IAP
OpNPV	*Orgyia pseudosugata* Nuclear Polyhedrosis Virus
PABP	Poly(A) Binding Protein
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCD	Programmed Cell Death
PCR	Polymerase Chain Reaction
PKB	Protein Kinase B
PMSF	Phenylmethylsulfonyl Fluoride
PTP	Permeability Transition Pores
RIAP1	Rat HIAP1
RIAP3	Rat XIAP
RING	Really Interesting New Gene
RNAi	RNA interference
SDS	Sodium Dodecyl Sulfate
siRNA	Small interfering RNA
SMA	Spinal Muscular Atrophy
SMAC	Second Mitochondrial Activator of Caspases
SMN	Survival Motor Neuron
TNFR1	Tumor Necrosis Factor Receptor 1
TNFα	Tumor Necrosis Factor α
TRADD	TNF Associated Death Domain
TRAF	TNF Receptor-Associated Factor
TsIAP	Testes Specific IAP
TUNEL	Terminal dUTP Nick End Labeling
Ub	Ubiquitin
UTR	Untranslated Region
UV	Ultraviolet
WST-1	Water Soluble Tetrazolium Salt
XAF1	XIAP-Associated Factor1
XIAP	X-linked Inhibitor of Apoptosis

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Chapter I

Literature Review

Programmed Cell Death

The development and maintenance of multi-cellular organisms is absolutely dependent on the processes which govern the balance between cell division and cell death. When these processes fail, there are pathological consequences which can result in failure of cell deletion during embryonic development, neurodegenerative diseases, or the development of cancer.

The established equilibrium between life and death is strictly controlled such that defective components are effectively eliminated by a process called "programmed cell death"(PCD). PCD has often been used interchangeably with the term apoptosis; however, there has been a recent recognition that apoptosis constitutes only one of three distinct mechanisms by which PCD can be executed.

Apoptosis, or type I PCD was originally described by Kerr *et al.* (1972) to distinguish between the type of cell death that occurs naturally during development and in the homeostatic regulation of the organism, versus the type of pathological cell death that is predominant in acute lesions following trauma. Apoptosis was defined by a group of morphological criteria, including chromatin condensation, margination and fragmentation of genomic DNA, membrane blebbing, and cellular fragmentation that can be targeted for phagocytosis by neighboring cells. The underlying biochemistry of these events has been extensively studied, and many, if not most of the key players have been identified (reviewed in Thorburn, 2004).

Type II PCD has been termed autophagy, which literally means "self-eating." Autophagy is a highly conserved cellular process found in organisms as diverse as yeast and mammals. It has been proposed that autophagy evolved initially as a response to starvation conditions, allowing unicellular organisms to recycle cellular contents in order to focus

remaining resources on critical metabolic pathways (Reggiori *et al.*, 2005). Autophagy differs from apoptosis both morphologically and biochemically. Autophagic cell death is characterized by the presence of autophagosomes, or vacuoles, and autophagolysosomes, which degrade cellular contents in a controlled manner through the action of lysosomal enzymes (reviewed in Baehrecke, 2005).

A third form of PCD (type III), called necrosis, exhibits many features of accidental, non-programmed cell death due to overwhelming cellular injury. Depending on the severity of the pathological insult, a continuum of apoptotic cell death features may be observed. In fact, some necrotic PCD models are often classified as "aborted apoptosis"; however, unlike apoptotic cell death, necrotic cell death is a more chaotic way of dying. Necrosis does not exhibit signs of chromatin condensation or plasma membrane blebbing and is often associated with organelle swelling and inevitably, cell lysis, followed by inflammatory tissue responses (reviewed in Leist and Jaattela, 2001).

Apoptosis is the most prevalent mechanism of PCD and is essential for the normal function of multi-cellular organisms. Various pathological conditions such as Alzheimer's disease, autoimmune diseases, HIV/AIDS and cancer have their genesis traced back to a dysregulation of apoptotic processes. The dysregulation of apoptosis, as it relates to cancer, is central to the work outlined in this thesis.

Apoptosis and Cancer

The development of cancer results when there is a failure in the control of the fundamental mechanisms that govern cell growth and cell death. The processes that regulate both cell proliferation and the destruction of potentially harmful cells are so closely linked

that the inappropriate activation of growth-promoting oncogenes often initiates cell death. In a healthy organism, cells typically die via apoptosis; however, the uninhibited cell proliferation characteristic of most cancer cells requires that apoptosis be suppressed. As solid tumors continue to grow, the resulting microenvironment often becomes deficient in growth factors, and lacks an adequate oxygen supply. Although these conditions would trigger apoptosis in normal cells, cancer cells continue to proliferate (reviewed in Hanahan and Weinberg, 2000; Evan and Vousden, 2001). Most approaches used in cancer treatment, such as chemotherapy and radiation therapy, kill cancer cells by inducing apoptosis; however, cancer cells often develop resistance to these types of therapies. Furthermore, many cancer therapies indirectly activate apoptosis by chemically or physically damaging DNA. This may have the unintended effect of generating a pool of heavily mutated cancer cells which increases the chances of developing resistance (reviewed in Lacasse *et al.*, 1999; Hanahan and Weinberg, 2000; Evan and Vousden, 2001). Insight into how apoptotic pathways are deregulated in cancer is therefore critical both to the understanding of how this disease develops and progresses, and for the development of reliable anti-cancer treatments. Thus, therapies that directly activate apoptosis would be predicted to be both safer and more effective than existing therapies.

At the molecular level, the evolving descriptions of mitochondrial dysfunction and Caspase activation as key elements of apoptosis have provided significant insight into the understanding of the molecular basis of cancer. In normal cells, limited activation of the Caspase cascade may be essential in cellular differentiation and/or replication (reviewed in Abraham and Shaham, 2004), thus making the preservation of Caspase control essential; however, in cancer cells it is often observed that Caspase activation pathways are impaired or absent, thereby preventing cells from dying when they should. Since the loss of Caspase

activation appears to be central to the prevention of most cell death events in cancer, finding strategies to overcome Caspase inhibition will be of value for the development of novel cancer treatments.

Control of Apoptotic Pathways

Caspases

Caspases are a class of cysteine-aspartyl proteases that are synthesized as precursor enzymes, or proenzymes. These proteases typically lie dormant in the healthy cell and in response to cell death stimuli are converted by proteolytic cleavage into active enzymes. Once activated, Caspases cleave their substrates after conserved aspartate residues and are responsible for most of the biochemical and morphological features of apoptotic cell death. The fundamental role of Caspases in the apoptotic process was first realized when CED-3, a cysteine protease required for apoptosis, was discovered in the nematode *Caenorhabditis elegans*. CED-3 was subsequently identified as a member of a highly conserved family of proteins present in not only *C. elegans*, but in a range of species from *D. melanogaster* to all vertebrates (reviewed in Hengartner, 2000). To date 14 distinct mammalian Caspases have been identified, 7 of which are central to the regulation of the apoptotic process.

Caspases in their proenzyme form contain three domains: an amino- terminal prodomain; a large subunit containing the active site cysteine within a conserved QACXG motif; and a carboxy- terminal small subunit. The prodomains are separated from the large subunit by an aspartate cleavage site and one or more aspartate cleavage sites are present in the linker region between the large and small subunits. Two cleavage events are required to activate Caspases into functional proteases. The first proteolytic cleavage divides the

proenzyme into large and small Caspase subunits. A second cleavage event removes the amino-terminal prodomain. The resultant, functional Caspase is a tetramer of two large p20 subunits and two small p10 subunits (reviewed in Wolf and Green, 1999) (Figure 1-1).

The presence of aspartate at the maturation cleavage sites is consistent with the ability of Caspases to auto-activate or be activated by other Caspases as part of an amplification cascade (Muzio *et al.*, 1998; Salvesen and Dixit, 1999). In this cascade, a multi-protein complex (the apoptosome) assembles in response to death stimuli that includes recruitment of the pro-Caspases. In this complex an adaptor molecule, Apaf-1 (apoptosis protease activating factor-1), binds to pro-Caspase-9. Pro-Caspase-9 is then autocatalytically activated via intrachain cleavage. This cleavage appears to only have a moderate effect on its catalytic activity in that fully processed Caspase-9 in isolation is only marginally active, similar to that of unprocessed Caspase-9 (Stennicke *et al.*, 1999; Srinivasula *et al.*, 2001). When Caspase-9 is in association with the apoptosome, there is a substantial increase in the catalytic activity of the processed, as well as the unprocessed Caspase-9 (Srinivasula *et al.*, 2001). Intrachain cleavage therefore appears to be relatively inconsequential to Caspase-9 activation, but rather its activation depends to a greater extent on conformational changes induced by association with the apoptosome (reviewed in Shi, 2004).

Activation of Caspases

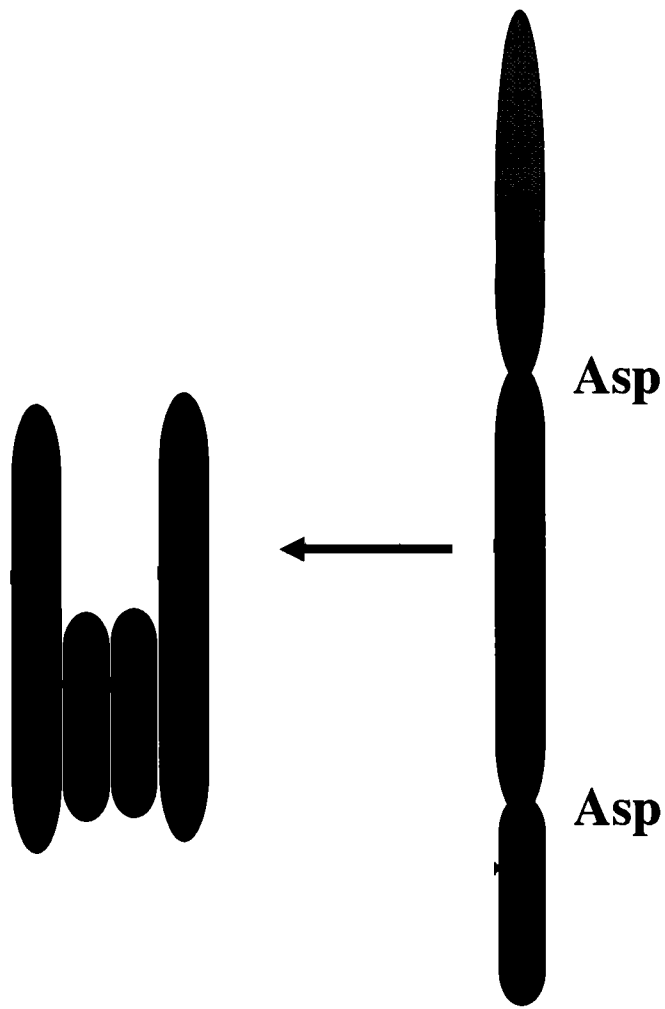
The mammalian Caspases have been classified as either initiator Caspases (Caspases -2, -8, -9, and -10) or effector Caspases (Caspases -3, -6, and -7) based on their structure and function. Initiator Caspases differ from the effector Caspases in that they have long N-terminal domains that allow them to interact with death effector domains (DED) or Caspase

recruitment domains (CARD) present in adaptor proteins such as FADD (Fas-associated protein with death domain) and Apaf-1, respectively. The activation of initiator Caspases results from cell death signaling through one of two pathways: 1) the extrinsic, death receptor pathway or 2) the intrinsic, mitochondrial pathway (Figure 1-2). An example of signaling through the extrinsic pathway occurs when there is binding of a so-called "death ligand" to a cell surface receptor, such as Fas/CD95/Apo-1, a member of the tumor necrosis factor (TNF) family of apoptosis- inducing receptors. Binding of trimeric Fas ligand to the Fas receptor triggers oligomerization and formation of a death-inducing signaling complex (DISC) comprised of Fas, the adaptor molecule FADD and pro-Caspase-8. The aggregation of pro-Caspase-8 in this complex results in its auto-activation and subsequently activation of downstream effector Caspases (reviewed in Nicholson, 2001; Goyal, 2001; Bratton *et al.*, 2001).

Apoptotic signals that trigger the activation of the intrinsic pathway result in the release of cytochrome c from the inter-mitochondrial membrane space into the cytosol. Binding of cytochrome c and dATP causes the adaptor molecule, Apaf-1, to form a large multimeric complex called the apoptosome. Apaf-1 in the apoptosome recruits pro-Caspase-9 through the interaction of their respective CARD domains. Pro-Caspase-9 is then processed by auto-catalysis (Zou *et al.*, 1999; Saleh *et al.*, 1999). Recent data suggests that following this proteolytic activation, there is a subsequent recruitment of Caspase-3 to the apoptosome (reviewed in Shi, 2004). Upon activation of the downstream effector Caspases, including Caspase-3 and -7, the disassembly of the cell occurs in what is known as the execution phase of apoptosis.

Figure 1-1. Schematic of Caspase structure.

Caspases are synthesized as inactive zymogens. The prodomain varies in length between initiator Caspases (long prodomains) (shaded) and effector Caspases (short prodomains) (solid). Cleavage at aspartate (Asp) sites results in an active tetramer consisting of two large (p20) and two small (p10) subunits.


Asp
Asp

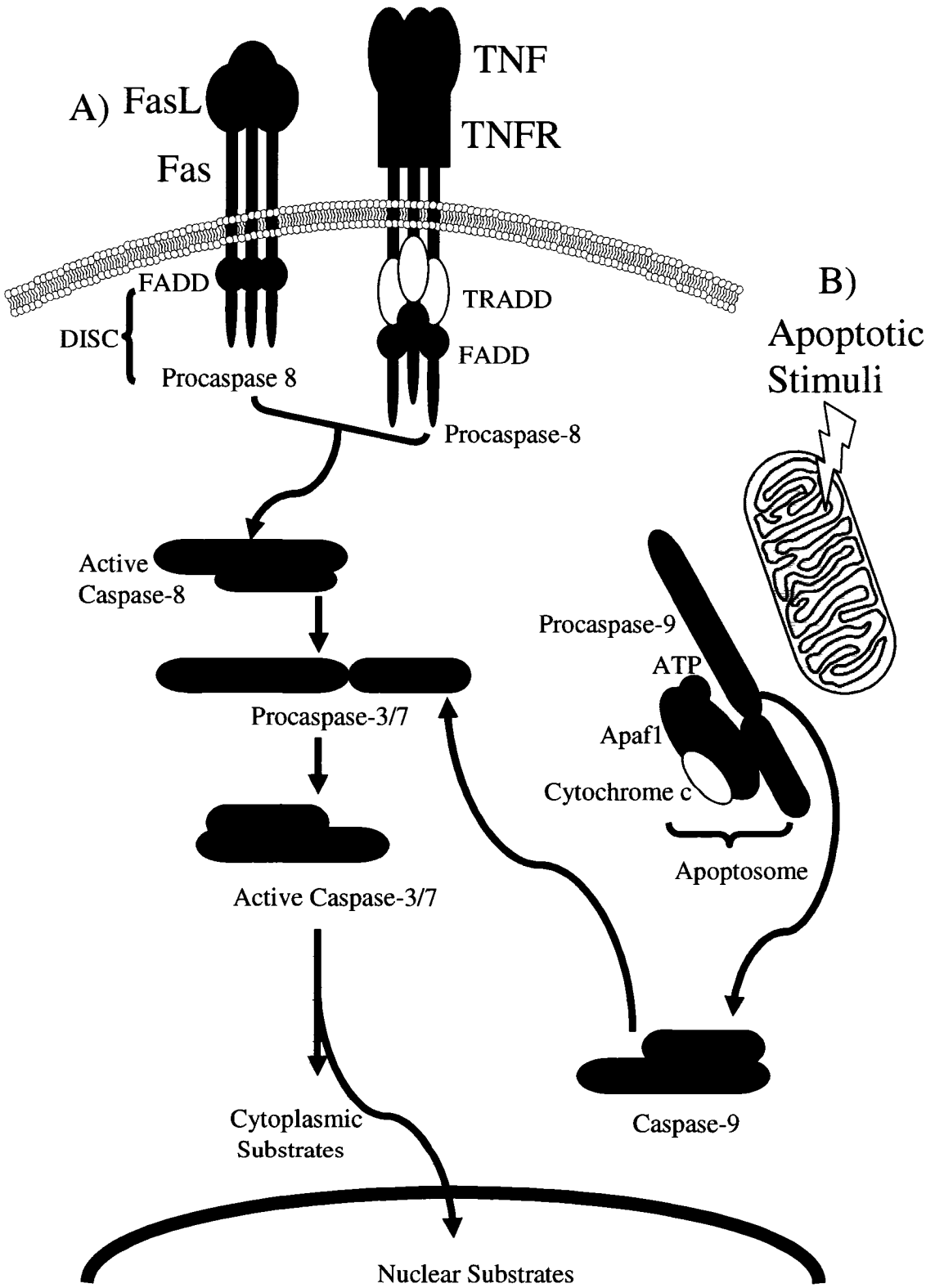
Bcl-2 Family of Proteins

The release of cytochrome c from the mitochondria into the cytosol is fundamental to apoptosome formation and subsequent downstream Caspase activation, in the intrinsic cell death pathway. The mechanism by which this release of cytochrome c comes about is controversial. Uncoupling of mitochondrial oxidative phophorylation is observed during apoptosis, resulting in the loss of mitochondrial transmembrane potential (Green and Reed, 1998). The opening of mitochondrial permeability transition pores (PTPs) is associated with this loss of potential and provides a mechanism by which cytochrome c might escape from the intermembrane space (Zamzami *et al.*, 1995). Other studies suggest that PTP opening is a secondary, Caspase-dependent event and that cytochrome c release precedes PTP opening (Bossy-Wetzel *et al.*, 1998). Therefore, the release of cytochrome c appears to be a consequence of a specific permeabilization event, which is independent of the generic membrane depolarization observed in a dying cell. In fact, there is evidence that members of the Bcl-2 protein family are key mediators of cytochrome c release in the context of apoptotic stimuli (Kluck *et al.*, 1997).

The *Bcl-2* gene was first identified as a proto-oncogene that is over-expressed by an immunoglobulin enhancer when chromosome translocations occur in follicular B-cell lymphoma. Unlike all other identified oncogenes, *Bcl-2* was found to inhibit cell death, rather than promote cell proliferation (Vaux *et al.*, 1988). Subsequently, CED-9, a functional homologue of Bcl-2, was identified as an apoptotic repressor in *C. elegans* (Vaux *et al.*, 1992). The Bcl-2 family determines whether or not the proCaspase/apoptosome complex can assemble. Evidence for this is based on the observation that anti-apoptotic members

Figure 1-2. Summary or intrinsic and extrinsic apoptosis signaling pathways.

Death receptor- mediated signaling of apoptosis results from Fas ligand binding to the Fas/CD95/Apo-1 receptor or from TNFα binding to TNFR1 (**A**). Recruitment of TRADD and/or FADD results in pro-Caspase-8 activation and subsequent downstream Caspase-3 activation. Mitochondrial-mediated apoptotic signaling results in cytochrome c release and in formation of the apoptosome complex consisting of Apaf1 and Caspase-9 (**B**). Following subsequent capase-3 activation, disassembly of the cell occurs.


A) FasL
Fas
TNF
TNFR
FADD
DISC
Procaspase 8
TRADD
FADD
Procaspase-8
Active
Caspase-8
Procaspase-3/7
Active Caspase-3/7
Cytoplasmic
Substrates
Nuclear Substrates
B)
Apoptotic
Stimuli
Procaspase-9
ATP
Apaf1
Cytochrome c
Apoptosome
Caspase-9

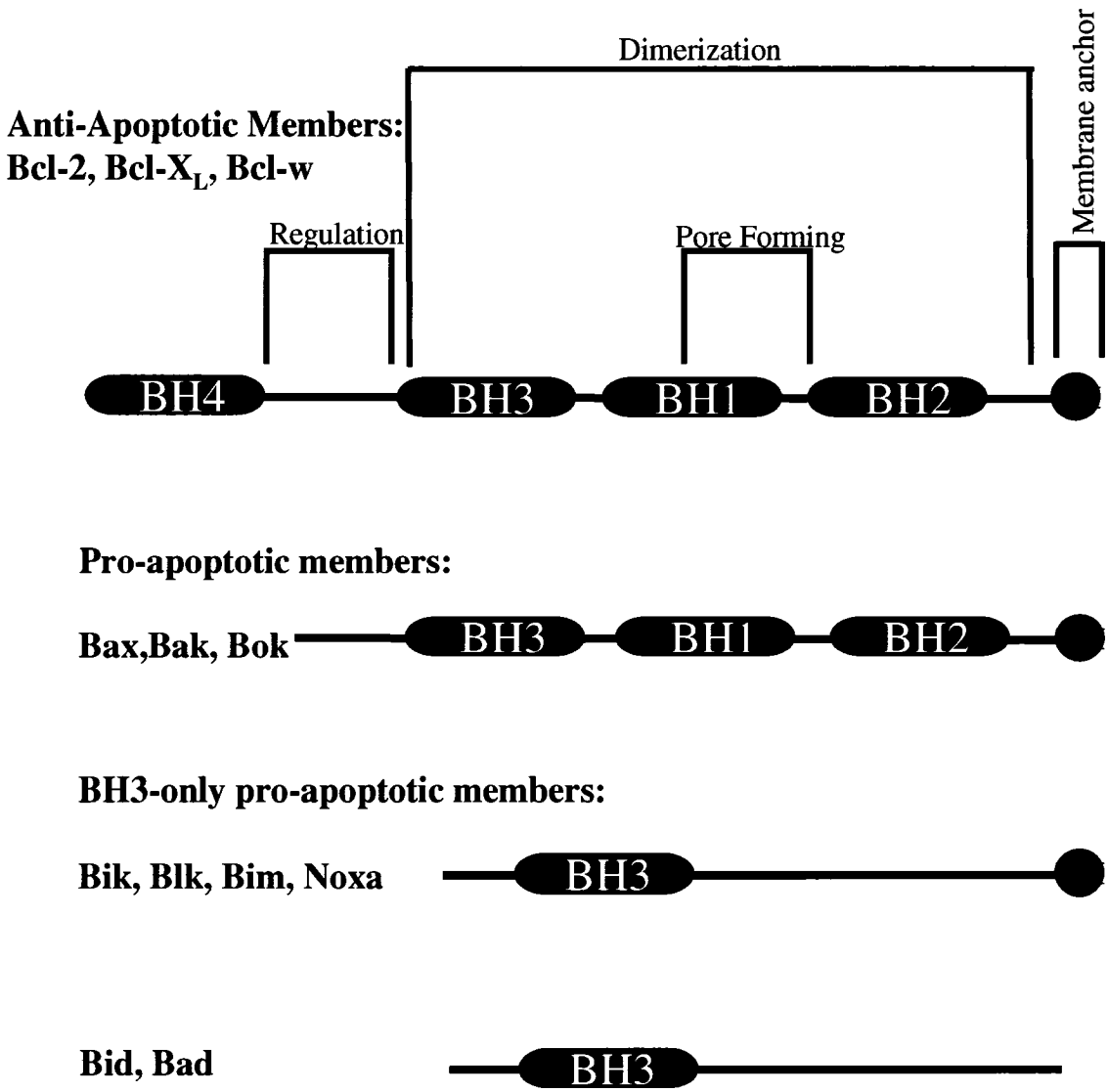
such as Bcl-2, and its nematode counterpart CED-9, prevent apoptosome formation, but their pro-death family members can over-ride this anti-apoptotic activity (reviewed in Adams and Cory, 2001). At least 20 other Bcl-2 family members have been identified in mammals. The key determinant of a Bcl-2 related protein is the presence of at least one of four conserved Bcl-2 homology (BH1-BH4) domains. The Bcl-2 family members are divided into three categories of proteins according to their structure and function and are defined as: anti-apoptotic members; pro-apoptotic members; and BH3-only pro-apoptotic members (Figure 1-3) (reviewed in Zimmerman *et al.*, 2001).

The anti-apoptotic members of the Bcl-2 family usually contain all four BH domains and include: Bcl-2; Bcl-X_L; Bcl-W; A1; and Mcl-1. These proteins potently inhibit apoptosis in response to many cytotoxic insults. They possess a carboxy-terminal transmembrane domain (TM) that targets them to the outer mitochondrial membrane, endoplasmic reticulum, and the nuclear envelope. In healthy cells Bcl-2 resides as an integral membrane protein, whereas Bcl-X_L and Bcl-W require an apoptotic signal to become tightly associated with the membrane (reviewed in Cory and Adams 2002).

The pro-apoptotic members of the Bcl-2 family, Bax and Bak, are essential for apoptosis in many cell types and this is supported by the fact that mice deficient in both of these genes show a severe developmental phenotype (Lindsten *et al.*, 2000). In healthy cells Bax exists as a cytosolic monomer, or is loosely attached to membranes. Following a death stimulus, Bax undergoes a conformational change and integrates into the outer mitochondrial membrane where it oligomerizes (reviewed in Gross *et al.*, 1999; Cory and Adams, 2002). The oligomerization of Bax and Bak are thought to permeabilize the outer mitochondrial membrane, thus permitting the efflux of apoptotic proteins, such as cytochrome c, into the cytosol. The mechanism by which they become activated and over-ride Bcl-2 is still a topic

Figure 1-3. Bcl-2 family members.

The Bcl-2 family members can be divided into 3 groups of proteins according to their structure and function. The anti-apoptotic family members usually contain all four BH domains and include Bcl-2, Bcl-X_L, and Bcl-w. The pro-apoptotic members are lacking the BH4 domain and include Bax, Bak, and Bok. Finally, the BH3-only pro-apoptotic family members only contains the BH3 homology domain and include Bid, Bad, Bim, Bik, Blk, and Noxa.


Anti-Apoptotic Members:
Bcl-2, Bcl-X$_L$, Bcl-w
Dimerization
Membrane anchor
Regulation
Pore Forming
BH4
BH3
BH1
BH2
Pro-apoptotic members:
Bax,Bak, Bok
BH3
BH1
BH2
BH3-only pro-apoptotic members:
Bik, Blk, Bim, Noxa
BH3
Bid, Bad
BH3

of debate (reviewed in Cory and Adams, 2002). There is however, one theory which suggests that Bax and Bak directly form channels in the mitochondrial membrane. Consistent with this idea is the fact that Bax oligomers can form pores in liposomes that allow passage of cytochrome c (Saito *et al.*, 2000).

The BH3-only proteins appear to be the initiators of apoptosis in response to developmental cues or intracellular damage. In healthy cells BH3-only proteins are held in check by a variety of strategies. As examples: Bim and Bmf are sequestered to the microtubule or actin components of the cytoskeleton by interactions with a dynein light chain; Bad is maintained via phosphorylation and subsequent interaction with a scaffold protein, 14-3-3; Bid is synthesized as a precursor protein and remains inactive until proteolytically cleaved; and Noxa is controlled at the transcriptional level where expression is induced by p53 in response to DNA damage (Zha *et al.*, 1996; Puthalakath *et al.*, 1999; Oda *et al.*,2000). In response to a cell death signal these pro-apoptotic family members are activated and translocate to intracellular membranes where they neutralize the anti-apoptotic Bcl-2 homologues. The BH3-only proteins, however, cannot kill in the absence of Bax and Bak, and consequently function upstream in the same pathway (Wei *et al.*, 2001; Zong *et al.*, 2001).

Inhibition of Caspase Function

All apoptotic signaling pathways converge on the Caspases making these proteases the ultimate effectors of apoptotic cell death. It is absolutely critical to the development and survival of an organism that the Caspases are tightly regulated, as the inappropriate activation of these enzymes can have severe consequences with respect to the development of various disease conditions. Over-activation of Caspases has been implicated in certain

neurodegenerative disorders, whereas inappropriate suppression is implicated in the development of many cancers. The first level of Caspase regulation is seen in their structure and activation. As described, Caspases are synthesized as inactive zymogens and are only activated via signaling through strictly controlled pathways. A second level of regulation involves the specific inhibition of active Caspases by naturally occurring inhibitors.

Cellular proteins have been identified that inhibit specific initiator Caspases. There is one family in particular, the Inhibitors of Apoptosis (IAP) proteins that function as intrinsic regulators of the Caspase cascade. The IAPs are the only known endogenous proteins that regulate the activity of both initiator and effector Caspases. Controlled expression of the IAPs has been shown to influence cell death in a variety of contexts and is believed to have important consequences with respect to human cancer.

IAPs

The IAP family

The IAPs effectively suppress apoptosis induced by a variety of stimuli, including death receptor activation, growth factor withdrawal, ionizing radiation, viral infection, and genotoxic damage. The first IAP genes identified were the baculoviral genes *CpIAP* (from *Cydia pomonella* granulosis virus CPGV) and *OpIAP* (from *Orgyia pseudosugata* nuclear polyhedrosis virus OpNPV), where they were found to compensate for the loss of function of p35, a baculoviral Caspase inhibitor protein (reviewed in Salvesen and Duckett, 2002). The IAPs are believed to be used by baculoviruses to allow for viral propagation by preventing a defensive apoptotic response in host insect cells (reviewed by Uren *et al.*, 1998). Since the discovery of the baculoviral IAPs, numerous cellular homologues have been identified in a

range of species from *Drosophila* to vertebrates. The first mammalian IAP, NAIP (Neuronal Apoptosis Inhibitory Protein), was identified during a positional cloning effort to identify the causative gene for Spinal Muscular Atrophy (SMA) (Roy *et al.*, 1995). Although deletions within the *naip* gene were initially believed to be the genetic defect in SMA, it was later shown that deletions of the neighboring *survival motor neuron (SMN)* gene caused the disease (Lefebvre *et al.*, 1995). The loss of functional NAIP has however, been suggested to be a factor in determining the severity of the disease (reviewed in Gendron and MacKenzie, 1999). Since the discovery of NAIP (BIR containing protein 1/BIRC1), the IAP family has expanded to include: X-linked inhibitor of apoptosis (XIAP / MIHA / hILP / BIRC4); cellular IAP1/ Human IAP2 (c-IAP1 / HIAP2 / MIHB / BIRC2); cellular IAP2/ Human IAP1 (c-IAP2 / HIAP1 / MIHC / BIRC3) (Duckett *et al.*, 1996; Liston *et al.*, 1996; Rothe *et al.*, 1995; Uren *et al.*, 1996); Testis –specific IAP (Ts-IAP / hILP2 / BIRC8) (Lagace *et al.*, 2001; Richter *et al.*, 2001); BIR-containing ubiquitin conjugating enzyme (BRUCE / Apollon/ BIRC6) (Chen *et al*., 1999; Hauser *et al.*, 1998); Survivin (BIRC5) (Ambrossini *et al.*, 1997); and Livin (Livin / KIAP / ML-IAP / BIRC7) (Kasof and Gomes, 2001; Lin *et al.*, 2000; Vucic *et al.*, 2000) (Figure 1-4).

IAP Expression

XIAP

Human *XIAP* mRNA appears to be ubiquitously expressed in all adult and fetal tissues. XIAP has been reported to inhibit apoptosis in response to a large variety of apoptotic stimuli including ultraviolet radiation, TNFα, Fas Ligand, menadione (a free radical inducer), and etoposide (a topoisomerase II inhibitor) (reviewed in LaCasse *et al.*,

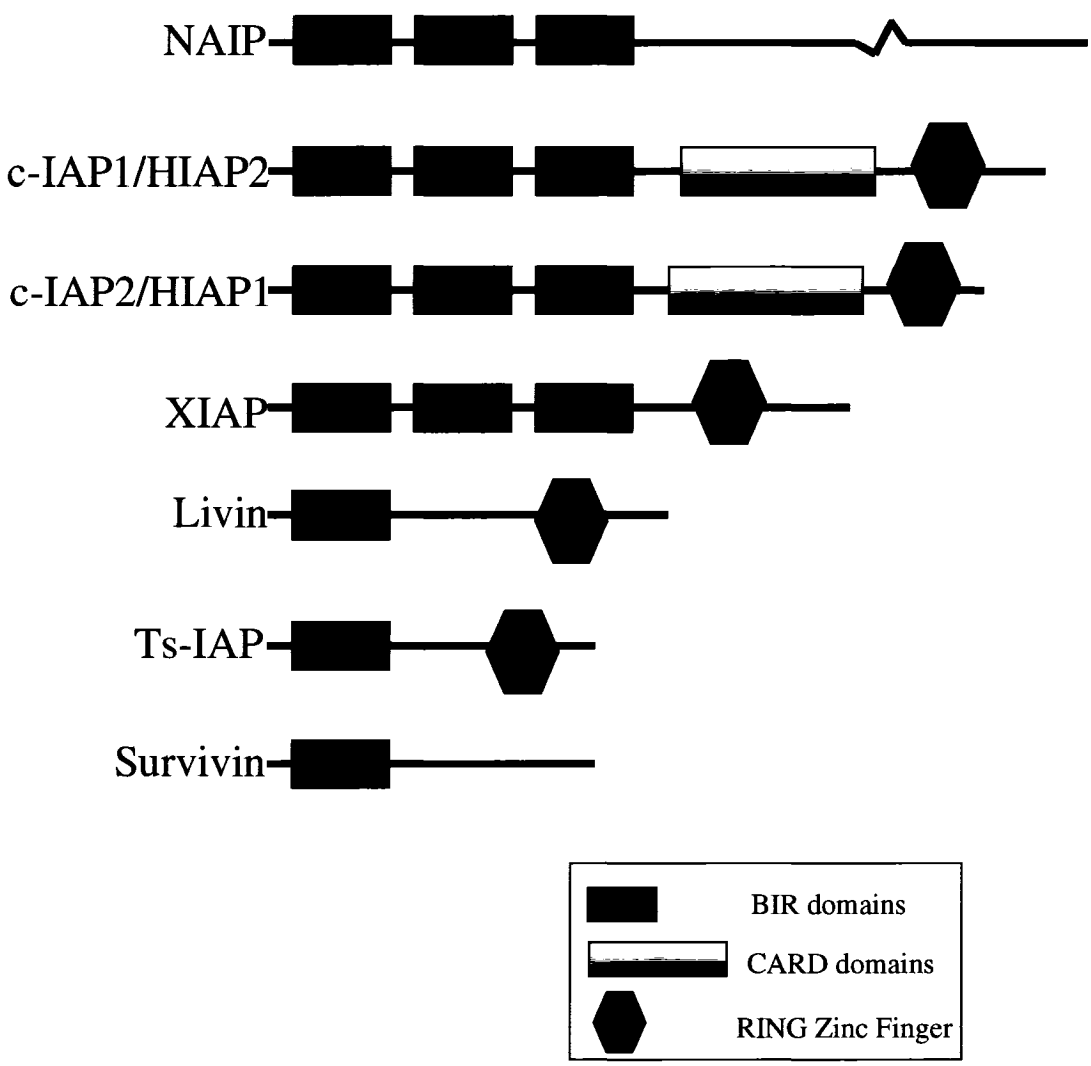
1998; Liston *et al.*, 1996). Central to the ability of XIAP to block apoptosis are the BIR domains which have been shown to directly inhibit both initiator and effector Caspases. The extensiveness of *XIAP* mRNA expression has led to the suggestion that XIAP may be the IAP family member that provides a "housekeeping" function in the prevention of apoptosis in healthy cells. Expression of *XIAP* appears elevated in many cancer cell lines (Liston *et al.*, 1996), reinforcing the idea that *XIAP* expression level may control the apoptotic threshold of cells.

Interestingly, the XIAP transcript has long 5' and 3' UTRs which could provide a means for regulating the levels of XIAP protein independent of transcriptional control of the mRNA. Long 5' UTRs are rarely found in eukaryotic cells and interfere with the normal ribosome scanning method of translation (reviewed in Kozak, 1991). To overcome this, eukaryotic genes with long 5' UTRs have often been shown to translate their proteins through a cap-independent internal ribosome entry site (IRES). IRES elements permit ribosome binding near the translational start site of the message. An IRES element has been identified in the human XIAP 5' UTR and permits XIAP expression in cells under stress conditions that typically block cap-dependent translation (Holcik *et al.*, 1999; Holcik and Korneluk, 2000; Holcik *et al.*, 2000). Thus, XIAP protein may be preferentially up-regulated relative to other cellular proteins under the same stress conditions. In fact, it has been observed that under conditions of low dose γ-irradiation, XIAP translation is up-regulated in an IRES-dependent manner (Holcik *et al.*, 2000).

Although less is known about c-IAP1 and c-IAP2, they have also been shown to inhibit the initiator and effector Caspases. C-IAP1 and c-IAP2 were first identified in a complex with TNFα receptor 2, where they interact with TNFα-receptor-associated factors

Figure 1-4. Domain structure of the IAP family.

The presence of at least one BIR domain is the defining characteristic of the IAP family. The human IAPs possess either one (Survivin, Livin, TsIAP) or three tandem BIR domains (XIAP, HIAP1, HIAP2, NAIP) (indicated by blue boxes). Along with the BIR domains, several IAPs contain a RING-zinc finger domain at the carboxy terminus (indicated by green hexagon). HIAP1 and HIAP2 both possess a Caspase recruitment domain (indicated by yellow box) in the linker region between the BIR domains and the RING domain.


NAIP
c-IAP1/HIAP2
c-IAP2/HIAP1
XIAP
Livin
Ts-IAP
Survivin
BIR domains
CARD domains
RING Zinc Finger

1 and -2 (TRAFs). The recruitment of these IAPs to the TNFR2 complex requires the presence of both TRAF1 and TRAF2 and is mediated by the interaction between the BIR domains of the IAPs and the TRAF domains (Rothe *et al.*, 1995; Roy *et al.*, 1997). The TNFR1 complex is also able to recruit c-IAP1 through its interaction with TRAF2 via the adaptor molecule TNFα-receptor associated death domain protein (TRADD). The TNFα receptor (TNFR) can mediate both survival and death signals. For example, pro-survival signals result from an observed increase in the expression of c-IAP1 and c-IAP2 following activation of the NF-κB transcription factor in response to TNFR signaling. In this context, c-IAP1 and c-IAP2 have been proposed to play a specialized role in protecting cells from TNFα-induced apoptosis by reducing the level of Caspase-8 activation (Chu *et al.*, 1997; Wang *et al.*, 1998). Whereas the survival signals are transduced by TRAF2/c-IAP1 interactions, pro-death signals are mediated through TRADD/FADD binding to Caspase-8 (Fotin-Mleczek *et al.*, 2002)

The other IAP members, Livin and Survivin, have a distinctly different pattern of expression and function compared to XIAP, c-IAP1 and c-IAP2. Livin, also called ML-IAP (melanoma-IAP) or KIAP (kidney IAP), is expressed at high levels in embryonic tissues and in transformed cells (Vucic e*t al.*, 2000). There are two splice variants of Livin, Livin α and Livin β. The two splice variants display different tissue distribution and anti-apoptotic characteristics; however, high levels of both splice variants are observed, specifically, in melanoma and colon cancer. Over-expression of either Livin isoform blocks apoptosis induced via the extrinsic, death receptor pathway. Interestingly, the two isoforms behave differently in the presence of the apoptosis-inducing drugs staurosporine and etoposide; Livin α protects against staurosporine and Livin β protects against etoposide, but not vice

versa (Ashhab *et al.*, 2001). The anti-apoptotic properties of Livin cannot be solely attributed to its BIR domain due to the fact that there is no difference between the BIR domains of the two Livin isoforms, yet they still respond differently to apoptotic triggers (Ashhab *et al.*, 2001).

Survivin is most highly expressed during fetal development, and is transcriptionally restricted to expression during the G2/M phase of the cell cycle where it is proposed to function as a mitotic spindle checkpoint protein (Li *et al.*, 1998; Jiang, *et al.*, 2001). Survivin appears to associate with Caspase-3 during mitosis and seems to suppress Caspase-mediated cleavage of centrosome-asssociated p21waf1 (reviewed in Janse and Zangemeister-Wittke, 2002). The distinct localization of Survivin to the mitotic spindles and its critical function in cytokinesis suggests that this protein functions in cell cycle rather than apoptosis regulation (Li *et al.*, 1998). Interestingly, the BIR domain in Survivin is most similar to the three dimensional structure of XIAP BIR3 and it is predicted that the anti-apoptotic action of this IAP involves the inhibition of Caspase-9 (Shi *et al.*, 2000).

IAP Structure and Function

The IAPs are structurally similar in that they all contain one or more 70-80 amino acid Baculoviral IAP Repeat (BIR) domains in which the core of the domain consists of the variable sequence $C(X)_2C(X)_6W(X)_3D(X)_5H(X)_6C$, where X is any amino acid. The cysteine- and histidine- rich BIR domain chelates zinc and forms a globular structure that consists of four or five alpha helices and a variable number of anti-parallel β-pleated sheets (reviewed in Clem, 2001). As summarized in Figure 1-4, the prototypical mammalian IAP family members contain a carboxy-terminal RING (Really Interesting New Gene) zinc finger. In XIAP, c-IAP1 and c-IAP2, this RING domain has been shown to possess E3

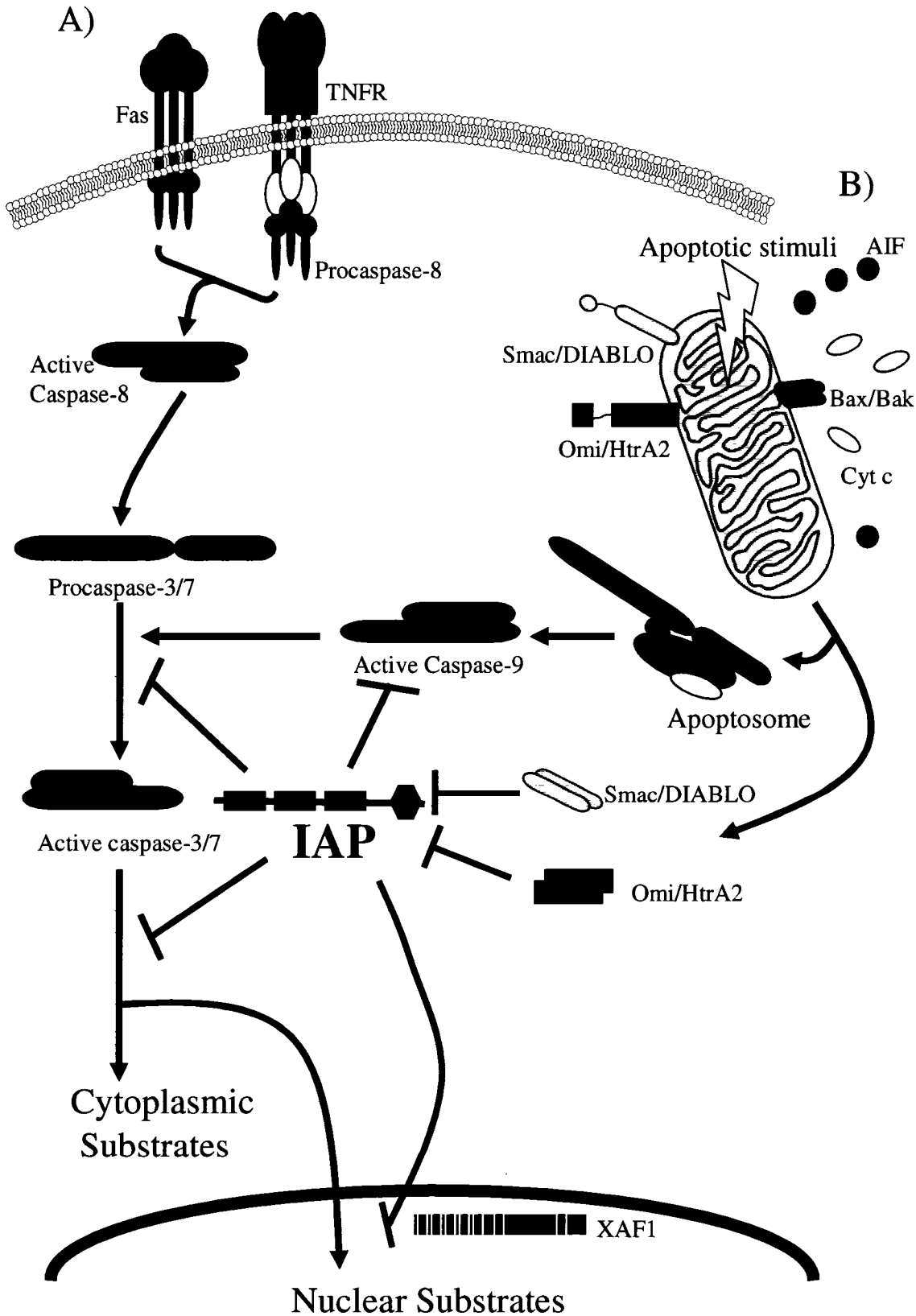
ubiquitin ligase activity, directly regulating self-ubiquitination and degradation (Yang *et al.*, 2000).

Unique to c-IAP1 and c-IAP2 is the presence of a CARD domain (Figure 1-4). CARD domains are a subfamily of a larger group of related protein folds found in cell death proteins. CARDs typically mediate oligomerization with other CARD containing proteins. This is similar to other proteins with related fold structures called the death domains (DD) and the death effector domains (DED) (reviewed in Martin, 2001). The function of CARDs in the IAPs is not yet known but they undoubtedly form protein-protein interactions, possibly with Apaf-1, and with some of the DD or DED-containing proteins (reviewed in Martin, 2001).

Following the discovery of the IAPs was the immediate observation that the expression of these proteins, in a variety of cell lines, effectively inhibits the apoptotic process when mediated by either the intrinsic or extrinsic pathways (Figure 1-5). The mechanism by which the IAPs inhibit apoptosis was first identified by Deveraux *et al.* (1997). In these studies, XIAP was shown to be ineffective at inhibiting the initial cleavage of Caspase-3 by Caspase-8 signaling, but was able to prevent the subsequent processing of Caspase-3 into its mature subunits. Therefore XIAP inhibits extrinsic apoptotic signaling, not by interfering directly with Caspase-8 activation, but rather via inhibition of the downstream effector Caspases (Deveraux *et al.*, 1997; Deveraux *et al.*, 1998). Furthermore, XIAP was shown by protein-protein interaction studies to bind specifically to Caspase-3 and -7, but not to capases-1,-6, or -8 (Deveraux *et al.*, 1997). *In vitro* assays later confirmed by blocking cytochrome c –induced activation of pro-Caspase-9 that XIAP, c-IAP1 and c-IAP2 could prevent downstream proteolytic processing of pro-Caspases-3, -6, and -7 (Figure 1-3) (Deveraux *et al.*, 1998).

Figure 1-5. IAPs effectively inhibit the apoptotic process when mediated via the intrinsic or extrinsic pathways.

Following the initial downstream Caspase-3 cleavage, induced by Caspase-8 signaling, the IAPs effectively block any subsequent processing of capase-3 into its mature, active form **(A)**. The IAPs are not only able to effectively block Caspase-3 processing, but are also able to effectively inhibit cytochrome c-induced activation of Caspase-9 as occurs in the intrinsic death pathways **(B).**


A)
Fas
TNFR
Procaspase-8
Active
Caspase-8
Procaspase-3/7
B)
Apoptotic stimuli
AIF
Smac/DIABLO
Bax/Bak
Omi/HtrA2
Cyt c
Active Caspase-9
Apoptosome
Smac/DIABLO
Active caspase-3/7
IAP
Omi/HtrA2
Cytoplasmic
Substrates
XAF1
Nuclear Substrates

To further dissect the exact mechanism of IAP-mediated Caspase inhibition, the ability of various fragments of XIAP to suppress both *in vitro* Caspase activation and apoptosis in intact cells was examined. Utilizing recombinant proteins composed of the BIR1+BIR2 domains and the BIR3+RING domains, more precise interactions were observed in which BIR2 specifically inhibited Caspases-3 and -7 and BIR3 inhibited Caspase-9 activity (Takahashi *et al.*, 1998; Deveraux *et al.*, 1999). In favor of the idea that the IAPs may potentially regulate the Caspases through distinct domains was the observation that following Fas-induced apoptosis XIAP itself becomes cleaved into two distinct fragments; one consisting of BIR1 and BIR2 and the other consisting of BIR3 and the RING domain (Deveraux *et al.*, 1999). The cleavage of XIAP though, has so far only been observed in cells that are in the late stages of apoptosis and may merely be a function of general protein degradation during the apoptotic process, rather than the consequence of an anti-apoptotic mechanism (Deveraux *et al.*, 1999; Johnson *et al.*, 2000).

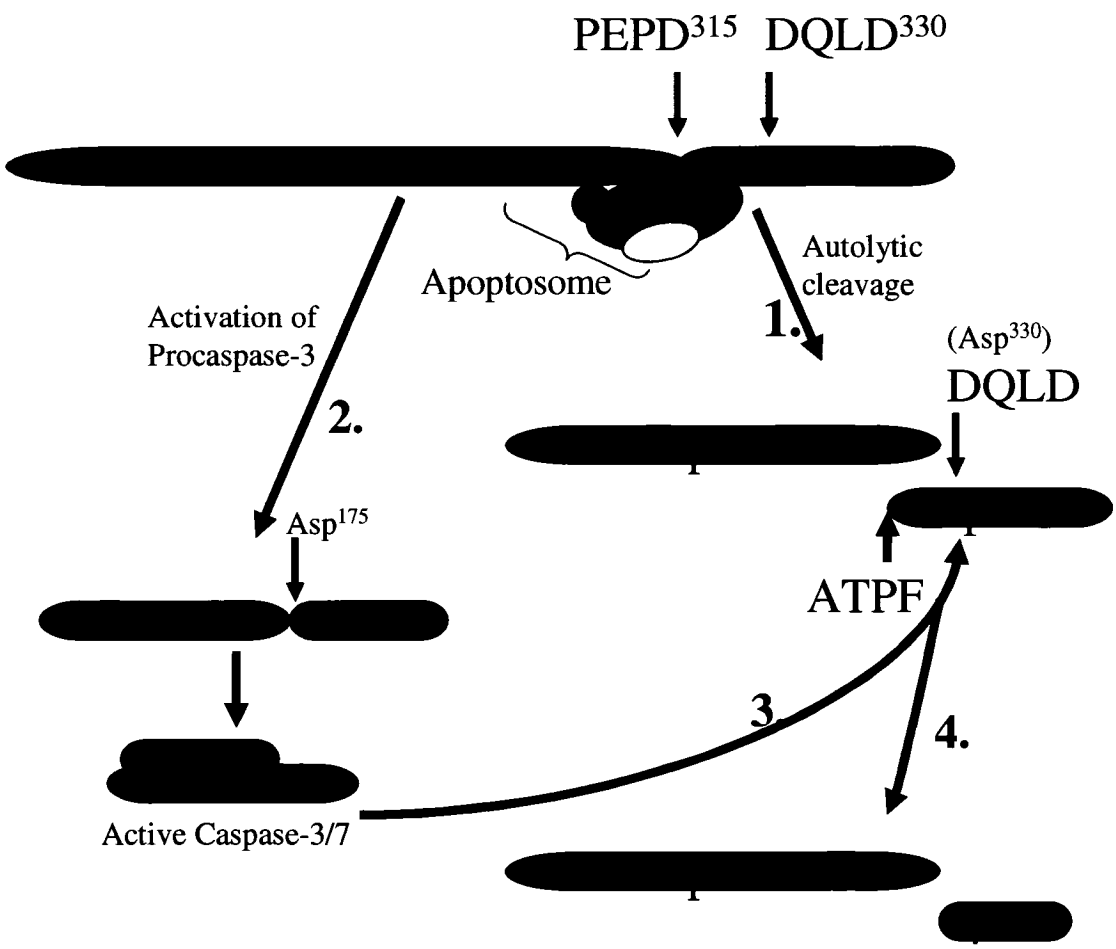
Crystallography and mutagenesis studies established that individual BIR domains of the IAPs do indeed have different mechanisms of Caspase inhibition. Complexes of XIAP BIR2 with Caspases-3 and -7 indicate that the linker region between BIR1 and BIR2 is the only IAP element which has contact with the Caspases, whereas the BIR2 domain is hidden in the crystal structure (Huang *et al.*, 2001). Using GST-fusions with the XIAP linker region alone, it was observed that Caspase-3 is inhibited and the BIR2 domain is most likely irrelevant in this interaction (Huang *et al.*, 2001); however, the interaction with Caspase-7 is slightly different in that the BIR2 domain is required to stabilize the linker interaction with this Caspase (Suzuki *et al.*, 2001). In essence, the effector capases appear to be inhibited simply by the steric hindrance due to BIR2 and/or its linker blocking the substrate entry site.

IAP Inhibition of Caspases

The BIR3 domain of XIAP, c-IAP1 and c-IAP2 (Deveraux *et al.*, 1998; Bratton *et al.*, 2001) and the single BIR domain in Livin (Vucic *et* al., 2000) and Ts-IAP (Lagace *et al.*, 2001) have been shown to directly bind to and inhibit Caspase-9. Furthermore, the mechanism of XIAP interaction with Caspase-9 differs from the interactions with Caspases -3 and -7. Catalytic cleavage of Caspase-9 occurs at Asp^{315} to yield large (p35) and small (p12) Caspase-9 subunits. The newly generated amino-terminus of the p12 subunit starts with amino acid sequence ATPF. This peptide sequence binds to a pocket on the surface of XIAP BIR3. Fifteen amino acids downstream is a Caspase-3 cleavage site at Asp^{330} (Figure 1-6). The co-crystal structure of Caspase-9 complexed with XIAP BIR3 reveals that the BIR3 domain forms a heterodimer with a Caspase-9 monomer (Shiozaki *et al.*, 2003). In effect, XIAP BIR3 stabilizes Caspase-9 in an inactive state, by preventing Caspase-9 homodimerization, which is essential for its autocatalytic activity. Interestingly, further cleavage by Caspase-3 at Asp^{330} in the p12 subunit of Caspase-9 generates a p10 subunit that lacks the XIAP binding motif. Therefore, a feedback proteolytic mechansism was proposed as follows: Caspase-9 activation in the apoptosome results in pro-Caspase-3 cleavage, thereby activating Caspase-3. Caspase-3 feedback cleavage at the Asp^{330} in the p12 Caspase-9 subunit removes the XIAP BIR3 recognition motif of Caspase-9, thereby preventing IAP inhibition and increasing the enzymatic activity of Caspase-9 (Figure 1-5) (Srinivasula *et al.*, 2001; Zou *et al.*, 2003). Although this is an attractive model, there is evidence to suggest that removal of the IAP-binding motif may not prevent XIAP association, and that surface contacts between XIAP and the Caspase-3-generated p10 fragment of caspase 9 can still maintain this interaction (Zou *et al.*, 2003).

Figure 1-6. Catalytic cleavage of Caspase-9.

Cleavage at Asp^{315} results in large (p35) and small (p12) Caspase-9 subunits. At the amino terminus of p12 is a XIAP BIR3 recognition site, with the sequence ATPF **(1)**. Cleavage of Caspase-9 results in activation of proCaspase-3 **(2)**. In a feedback mechanism active Caspase-3 is then able to further cleave Caspase-9 at the Asp^{330} site in the p12 subunit **(3),** generating a p10 subunit that lacks the XIAP binding motif **(4)**. The enzymatic activity of capase-9 is effectively increased.


PEPD315 DQLD330
Apoptosome
Autolytic cleavage
1.
(Asp330)
DQLD
Activation of Procaspase-3
2.
Asp175
ATPF
3.
4.
Active Caspase-3/7

One recent study describes a "two-site interaction mechanism" to illustrate how BIR domains are able to achieve specificity and potent inhibition of their target Caspases. This model describes a conserved IAP Binding Motif (IBM) interacting groove that participates in inhibition by binding neoepitopes that are revealed once Caspase activation occurs. These IBM interacting grooves are the most conserved surface feature of BIR domains and are found on many BIR domains including BIR2 and BIR3 of XIAP and BIR1 and BIR2 of DIAP1, an orthologue in *Drosophila melanogaster* (reviewed in Tenev *et al.*, 2005; Scott *et al.*, 2005). In spite of their sequence conservation, there is convincing evidence for the differential roles of the BIR domains in the regulation of Caspase activity. To summarize, Caspase-3 is inhibited exclusively by the linker region between BIR1 and BIR2, whereas Caspase-7 inhibition requires both the linker region and the BIR2 domain of XIAP. It is evident that Caspase-9 is inhibited in direct response to BIR3 binding, thereby preventing further Caspase-9 activation as well as suppressing downstream effector Caspase activity. Thus far, none of the IAP BIR1 domains have been shown to have any Caspase inhibiting activity; however, Akt/PKB (protein kinase B)-mediated phosphorylation at serine 87 within BIR1 reduces auto-ubiquitination and stabilizes XIAP (Dan *et al.*, 2004). BIR1 therefore appears to play a regulatory role rather than directly participating in apoptosis suppression.

IAP Protein Regulation

Ubiquitination and Degradation

The ubiquitination and subsequent proteasomal degradation of the IAPs may be a key regulatory event in the apoptotic program. Furthermore, the carboxy-terminal RING domain of the IAPs is believed to be the component which mediates both the ubiquitination and

degradation of the IAPs, as well as that of their substrates. (Yang *et al.*, 2000; Suzuki *et al.*, 2001). RING domain-containing proteins possess E3 ubiquitin ligase activity and function as specific adapters by recruiting target proteins to a multi-component complex containing an E2 enzyme. Earlier studies on the RING domains of XIAP and c-IAP1 showed that this domain is involved in the ubiquitination and degradation of the IAPs in response to apoptotic triggers. Following treatment with glucocorticoids or etoposide, XIAP and c-IAP1 were rapidly degraded in a proteasome-dependent manner that could be blocked in the presence of proteasome inhibitors (Yang *et al.*, 2000). Other studies showed that XIAP and c-IAP1 were able to promote the ubiquitination and proteasomal degradation of Caspases-3 and -7, thereby enhancing the anti-apoptotic effect of the IAPs (Suzuki *et al.*, 2001). Although the RING domain has been clearly shown to be involved in the ubiquitination and degradation of the IAPs, it remains unclear whether this activity enhances the anti-apoptotic activity of the IAPs or actually antagonizes the activity. Studies using RING-deletion mutants have provided evidence for both scenarios. In one study, over-expression of RING-deleted XIAP and c-IAP1 mutants in cell culture resulted in the loss of auto-ubiquitination and proteasomal degradation, and conferred better protection against apoptotic stressors than wild-type XIAP and c-IAP1 (Yang *et al.*, 2000); however, in a separate study, RING-deletion mutants of XIAP were found to be less effective than wild-type XIAP at preventing apoptosis induced by the over-expression of Caspase-3 or Fas (Suzuki *et al.*, 2001).

XIAP ubiquitination sites were identified and the role of IAP ubiquitination was examined by site directed mutagenesis of key lysine residues. Overexpression of wild-type or XIAP mutant protein in cultured cells did not reveal any differences in their ability to protect against Bax or Fas-induced apoptosis (Shin *et al.*, 2003). These findings suggest that ubiquitin mediated destruction of the IAPs may not be as significant as believed, at least in

the context of over-expressed proteins (Shin *et al.*, 2003). Alternatively, there is a possibility that RING domains may function to suppress apoptosis in conditions of low apoptotic stimulus by the ubiquitination of Caspases, whereas high levels of apoptotic stress trigger self degradation of the IAPs leading to apoptosis.

While c-IAP1 and c-IAP2 are typically present in low abundance in the cell, it has been proposed that c-IAP2 levels are maintained at low levels via constitutive ubiquitination and subsequent degradation by c-IAP1 (Conze *et al.*, 2005). Insight into to c-IAP1 E3 ligase function was determined with the generation of c-IAP1 knockout mice. Although these mice appeared to be both viable and fertile, they had significantly elevated c-IAP2 protein levels with normal mRNA levels, suggesting that in a normal setting, c-IAP1 ubiquitination of c-IAP2 leads to low protein levels of c-IAP2 (Conze *et al.*, 2005). Further co-expression and *in vitro* binding studies of c-IAP1 and c-IAP2 along with TRAF1/2, demonstrated that the c-IAPs and TRAF proteins form a multimeric complex. In this complex TRAF1 and TRAF2 appear to function as adaptors, bringing c-IAP1/2 together, allowing c-IAP1 to ubiquitinate c-IAP2 (Conze *et al.*, 2005). In a previous study c-IAP1 was observed to have a pro-apoptotic function by causing the ubiquitination and proteasome-mediated degradation of TRAF2 in response to TNFR2 signaling and thus sensitize cells to TNFα–induced cell death (Li *et al.*, 2002). Thus it appears that c-IAP family members function as ubiquitin protein ligases and are capable of regulating one another as well as components of the TNFR2 signaling complex.

Recent studies demonstrate that the ubiquitination and degradation of XIAP depends on phosphorylation status. More specifically, XIAP has been identified as a substrate for Akt/PKB. Akt/PKB is a serine/threonine kinase, known to promote cell survival and

suppress apoptosis in a number of cell lines when induced by a variety of cell death stimuli. Protein sequence analysis shows that the Akt/PKB phosporylation sequence RXRXXS/T is found within the BIR1 domain of XIAP (Dan *et al.*, 2004). Phosphorylation of XIAP by Akt/PKB inhibits cisplatin-induced auto-ubiquitination, thereby reducing XIAP degradation. Furthermore, increased levels of XIAP are associated with decreases in cisplatin-stimulated Caspase-3 activity and apoptosis (Dan *et al.*, 2004).

Negative Regulators of the IAPs

Several proteins play critical roles in maintaining an adequate balance between too much and too little apoptosis. To help maintain this balance are classes of proteins which act as negative regulators of the IAPs, and specifically interact with and relieve their Caspase-inhibitory effects.

XAF1

XAF1 (XIAP-Associated Factor 1) is a novel, zinc finger-rich protein that was first identified by a yeast two-hybrid screen with XIAP. The ability of XAF1 to bind with XIAP was confirmed by *in vitro* experiments. Using purified, recombinant proteins it was shown that XAF1 directly associates with XIAP to antagonize XIAP-mediated Caspase-3 inhibition (Liston *et al.*, 2001). Using recombinant adenoviruses to over-express XAF1 in cell cultures, XAF1 was shown to reverse XIAP-mediated protection against chemotherapeutic drugs such as cisplation and etoposide. In contrast to the cytosolic localization of XIAP, XAF1 protein appears to be localized in the nucleus. Following an apoptotic stress XAF1 is able to elicit XIAP re-localization from the cytosol to the nucleus, perhaps as a means of sequestering XIAP (Liston *et al.*, 2001).

XAF1 is ubiquitously expressed in normal tissues, but is found at extremely low levels in the majority of the NCI 60 cell-line panel of cancer cells (Fong *et al.*, 2000). Studies show that restoring XAF1 expression increases the sensitivity of tumor cell types to apoptotic triggers. Gene array analysis revealed that xaf1 mRNA can be up-regulated in response to interferon-β (IFN-β) treatment (Leaman *et al.*, 2002). Although the melanoma cell lines used in this study were found to be resistant to Apo2L/TRAIL (Apo2 Ligand/Tumor Necrosis Factor-related Apoptosis-inducing Ligand), pre-treatment with IFN-β sensitized the cells to Apo2L/TRAIL-induced apoptosis, ostensibly due to the up-regulation of XAF1 protein. XAF1 over-expression via transient transfection indicated that the constitutive expression of XAF1 was able to sensitize the cells to Apo2L/TRAIL to the same degree as IFN pre-treatment (Leaman *et al.*, 2002). When other studies examined the expression levels of XAF1 in human colon cancer tissues, low levels of xaf1 mRNA, compared to adjacent healthy tissues of the same individuals, were found. Furthermore, XAF1 over-expression in cultured colon carcinoma cell lines displayed an increase in Apo-2L/TRAIL and drug induced apoptosis (Tu *et al.* submitted). Therefore, these studies show that the over-expression of XAF1 strongly influences cellular sensitivity to the pro-apoptotic effects of Apo2L/TRAIL. XAF1 has also been shown to selectively kill other human tumor cells including pancreatic and breast cancer cell lines. When these human tumor cell lines and tissue matched, normal cell lines were transduced with adenoviral *XAF1*, approximately 80% of the tumor cells underwent apoptosis compared to 5-10% in the normal cell lines (Yang *et al.*, 2003). The down-regulation or loss of *XAF1* expression in cancer cells is believed to contribute to apoptosis suppression through unrestricted XIAP activity. Thus, *XAF1* is a promising candidate for cancer gene therapy in that it effectively and specifically induces apoptosis and sensitizes tumors to cytotoxic therapies.

Smac/DIABLO

Smac/DIABLO (Second mitochondrial activator of Caspases, a.k.a. Direct IAP Binding protein with Low PI) is a 25 kDa mitochondrial protein that promotes apoptosis via its ability to eliminate IAP-mediated Caspase inhibition. The ability of Smac to prevent IAP inhibition of Caspases makes this protein a functional human equivalent to the *Drosophila* proteins Reaper, Grim and Hid (Du *et al.*, 2000; Verhagen *et al.*, 2000).

Initial mitochondrial targeting of Smac is dependent upon a 55 amino acid sequence found at its amino-terminus. This sequence is proteolytically cleaved upon the induction of apoptosis, enabling the eventual release of mature Smac into the cytosol via Bax/Bak channels. Upon receiving an apoptotic signal via the intrinsic mitochondial stress pathway, both Smac and cytochrome c are released with similar kinetics. Although the exact mechanism of Smac release from the mitochondria is not entirely known, one study suggests that, despite their significant size difference, the Smac dimer (100kDa) and cytochrome c (12 kDa) are released through the same Bax- or Bak-formed membrane pores. This mechanism is supported by the fact that Bcl-2 over-expression can inhibit both cytochrome c and Smac release (Adrain *et al.*, 2001); whereas Bid-induced permeabilization of the outer-mitochondrial membrane induces the rapid and complete release of cytochrome c and Smac from the intermembrane space (Madesh *et al.*, 2002). A recent study showed that Smac release is inhibited by m-calpain, indicative of Bax involvement (Gorka *et al.*, 2004). Furthermore, it was observed using a combination of confocal microscopy and laser scanning cytometry that following camptothecin treatment there is a Bax-dependent, simultaneous release of Smac and cytochrome c from the mitochondria (Gorka *et al.*, 2004).

Other studies, however, suggest that Smac and cytochrome c are released through different mechanisms. In these studies, mouse embryonic fibroblasts (MEFs) from Bax null

mice failed to release both Smac and cytochrome c following TRAIL-induced apoptosis. This indicates that in the receptor-mediated pathways of apoptosis, Bax is critical for the release of mitochondrial apoptotic proteins (Kandasamy *et al.*, 2003). Furthermore, in a single knock-out of either Bid, Bax, or Bak, mitochondrial Smac release, but not cytochome c release was prevented. Cytochrome c release was only prevented in MEFs that were generated from a double knockout for Bax and Bak (Kandasamy *et al.*, 2003). Therefore, it was concluded from these experiments that release of Smac and cytochrome c from mitochondria is, in fact, differentially regulated in receptor-mediated pathways of apoptosis (Kandasamy *et al.*, 2003). Differences in the mechanism of Smac and cytochrome c release were also observed in the presence of Caspase inhibitors. In these conditions Smac release is prevented whereas cytochrome c is permitted, suggesting that Smac efflux from the mitochondria is a Caspase-catalysed event (Adrain *et al.*, 2001). It may be that Smac release simply requires further membrane permeability due to increased or persistent mitochondrial damage.

Smac Isoforms

Several Smac isoforms have recently been identified and studies using these proteins have suggested that the pro-apoptotic function of Smac may be mediated by additional, non-IAP mechanisms. Smacβ, an alternatively spliced isoform of Smac, lacks the mitochondrial targeting sequence found in full length Smac. *In vitro* experiments show that Smacβ interacts with purified XIAP protein; however, in intact cells, XIAP binding is not observed. Smacβ is still considered to be pro-apoptotic due to its ability to potentiate apoptosis following death receptor and chemical stimuli (Roberts *et al.*, 2001). Unlike Smacβ, Smac3, a more recently identified isoform of Smac, contains both an NH_2-terminal mitochondrial

targeting sequence and an IAP binding motif. Following an apoptotic stress, Smac3, like Smac, is released from the mitochondria into the cytosol where it interacts with the BIR2 and BIR3 domains of XIAP. There are some reports that the Smac3 isoform is unique in that it is able to induce the acceleration of XIAP auto-ubiquitination and destruction, whereas Smac only seems to have this effect on c-IAP1 and c-IAP2 (Fu *et al.*, 2003; Yang and Du, 2004); however, another report describes the ability of Smac to antagonize both XIAP auto-ubiquitination as well as XIAP-dependent ubiquitination of Caspase-7. By disrupting IAP-Caspase interactions and repressing the ubiquitin ligase activities of the IAPs, Smac may effectively prevent Caspase demise via ubiquitination (Creagh *et al.*, 2004). Although Smac and its isoforms may differ in how they potentiate apoptosis, the involvement of all Smac proteins with the IAPs, at multiple levels, is an effective measure to ensure that the apoptotic program can proceed.

Omi/HtrA2

Following the identification of Smac was the discovery of another mitochondrial IAP binding protein called Omi (a.k.a.HtrA2) was discovered (Hegde *et al.*, 2002; Verhagen *et al.*, 2002; van Loo *et al.*, 2002; Martins *et al.*, 2002). Omi/HtrA2 is a mammalian homologue of the *Escherichia coli* bacterium heat-inducible serine protease, known as HtrA. Omi was predicted to have a function similar to that found in bacteria where HtrA behaves as a chaperone or protease that determines whether misfolded proteins are refolded or targeted for destruction (Verhagen *et al.*, 2002). Similar to Smac, Omi contains an amino-terminal mitochondrial targeting sequence which is cleaved upon import into the mitochondria. Proteolytic cleavage of the targeting sequences exposes a conserved IAP-binding motif at the amino-terminus with the tetrapeptide sequence AVPS. Following an apoptotic stress, mature

Omi/HtrA2 is released together with mature Smac from the mitochondria into the cytosol where it interacts with the IAPs and promotes apoptotic cell death (Hegde *et al.*, 2002; Verhagen *et al.*, 2002). Omi, however, exerts its pro-apoptotic activity both by its ability to disrupt Caspase-IAP interaction, as well as its serine protease activity. Furthermore, Omi has been shown to potentiate apoptosis through the direct proteolytic processing of its IAP protein targets (Srinivasula *et al.*, 2003; Yang *et al.*, 2003). Studies have demonstrated that when Omi is over-expressed in cells, there is an observed increase in degradation products of XIAP and cleavage products of c-IAP1. Additionally, when endogenous Omi levels are suppressed by RNA interference, IAP degradation and/or cleavage is not observed and cells become desensitized to TRAIL and etoposide treatment (Srinivasula *et al.*, 2003; Yang *et al.*, 2003). A recent study indicates that as well as potentiating apoptosis via proteolysis of the IAPs, Omi may also enhance Caspase activation (Suzuki *et al.*, 2004). In this study, when Omi was over-expressed in the cytosol as an ubiquitin fusion protein, there was an indirect induction of outer mitochondrial membrane permeabilization, followed by cytochrome c-dependent Caspase activation (Suzuki *et al.*, 2004).

The serine protease activity of Omi may also induce apoptosis in a Caspase-independent manner through the proteolysis of unidentified cellular targets (Hegde *et al.*, 2002; Verhagen *et al.*, 2002). Although cytosolic targets for Omi have not been identified, the proteolytic removal of its 133 amino acid mitochondrial targeting sequence is a self catalyzed event (Savopoulos *et al.*, 2000). This raises the possibility that other mitochondrial targeting sequences, including that of Smac, may be processed by Omi.

GSTP1/eRF3

The discovery of proteins that function as IAP antagonists via a tailor-made IAP binding motif (IBM), such as those found in Smac and Omi, has made way for the identification of other IBM containing proteins that are found not only in the mitochondria but in other organelles. One recently identified IBM-containing protein is an isoform of the polypeptide chain-releasing factor GSPT1/eRF3 protein that is localized to the endoplasmic reticulum (ER). GSPT1 typically functions via the interaction with poly(A)-binding protein (PABP) and acts as a polypeptide chain release factor in translation. As with other IAP-binding proteins, GSPT1 is proteolytically processed following an apoptotic stress to reveal a conserved NH_2-terminal IBM with the amino acid sequence AKPF (Hegde *et al.*, 2003). Processed GSPT1 has been shown to interact with the IAPs, promote Caspase activation, ubiquitinate IAPs, and induce apoptosis (Hegde *et al.*, 2003). The fact that GSPT1 is in the ER stresses the importance of cross-talk between organelles during both the initiation and execution phases of apoptosis.

Chk1

Checkpoint kinase 1 (Chk1) is a dual function kinase that: 1) is active during the S-M phase transition of the cell cycle, regulating Cdc25A function and 2) prevents mitotic progression in the presence of DNA damage. From a domain-based databank search for IBM-containing protein homologues, Chk1 was identified as a candidate that contains an N-terminal motif, MAVPF, identical to that present in the *Drosophila* Hid protein. This Chk1 motif is also homologous to the N-terminus of processed Caspase-9, Smac, and the *Drosophila* homologues Grim and Reaper (Galvan *et al.*, 2004). Chk1 and XIAP reportedly form complexes in association with condensed chromosomes aligned at the metaphase plate

during mitosis. To date Chk1 is the only identified IAP-interacting protein that is not implicated directly in apoptotic processes; rather, the interaction between Chk1 and XIAP may involve cross-talk between the mechanisms that control cell cycle and apoptosis (Galvan *et al.*, 2004).

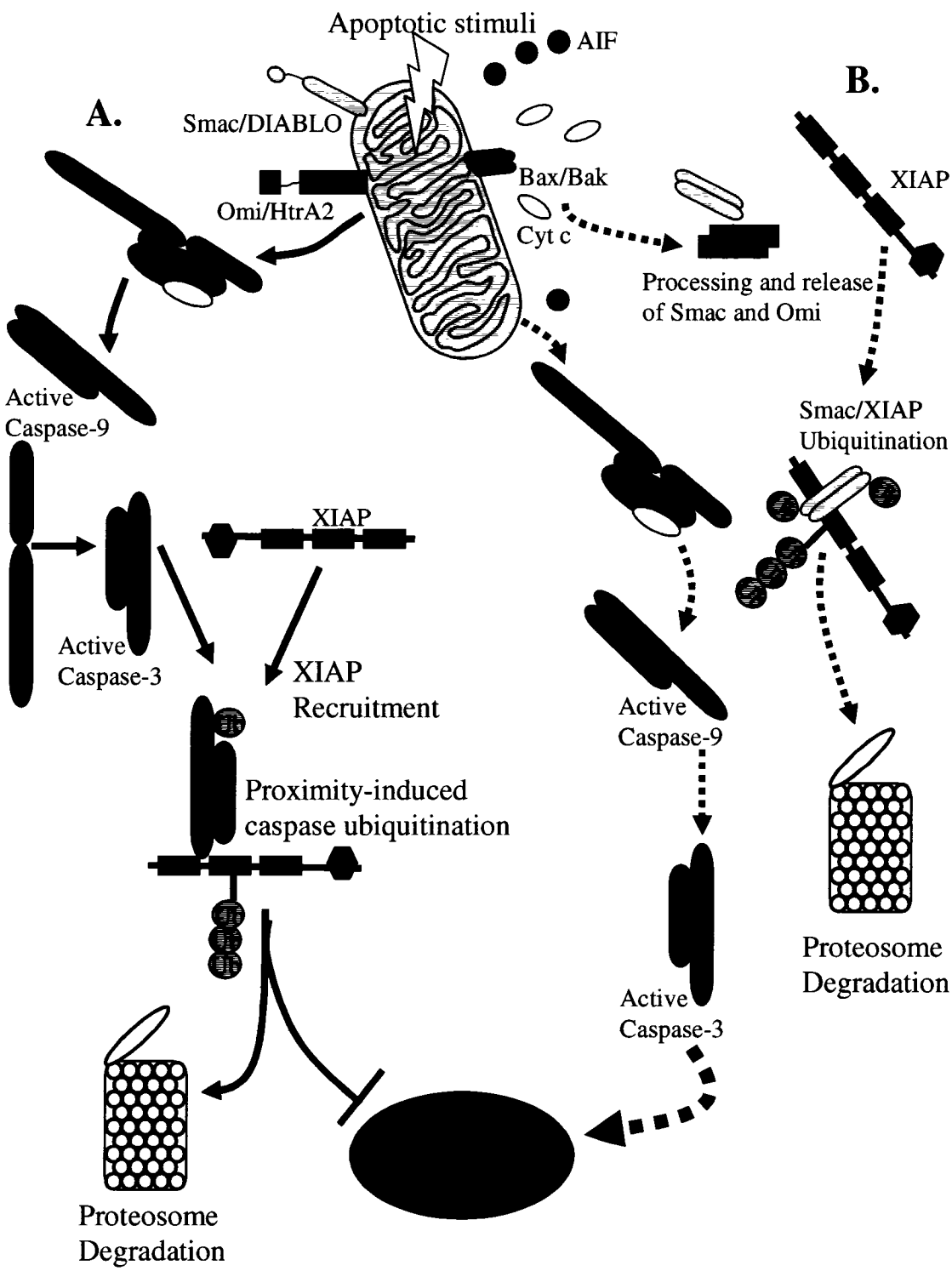
The IAPs and Apoptosis

In this review, the significance of capase-IAP interactions in mediating apoptosis has been established. The complexity of IAP regulation that is required to keep apoptotic pathways in check, either by ubiquitination and degradation pathways, or via protein-protein interactions with negative regulators such as XAF1, Smac and/or Omi, has also been presented. I will now discuss how these various regulatory mechanisms function together in a general model of apoptotic cell death (Figure 1-7).

Apoptotic cell death can be triggered either via the intrinsic or the extrinsic pathway where eventually all signaling converges on the effector Caspases-3 or -7. Apoptotic stresses acting through the intrinsic pathway result in the release of cytochrome c (most likely via Bax-or Bak- channel formation in the outer mitochondrial membrane) resulting in Caspase-9 activation. The consequences of cytochrome c release are determined by the activity of the IAPs. Pre-exposure to apoptotic stresses or the presence of high levels of IAPs may inhibit activated Caspase-9 and any subsequent effector Caspase activation (Figure 1-5). The ubiquitin ligase activity of the IAPs may target the bound Caspases for proteasome degradation, thereby aborting the apoptotic process. This type of regulation could be considered a safety mechanism to prevent transient or incidental cytochrome c leakage from eliciting an unnecessary, full-blown apoptotic response. In the case where sufficient

Figure 1-7. Mechanisms that regulate apoptotic cell death.

The presence of high levels of the IAPs results in IAP-induced Caspase inhibition. Caspase-IAP interaction triggers autoubiquitination of both the IAP and of the Caspase itself. The entire complex is degraded and the apoptotic process is aborted **(A)** (solid line). Alternatively, if a cell receives a sublethal apoptotic stess, endogenous IAPs are saturated by interactions with Smac/DIABLO and/or Omi/HtrA2. Proteosome-mediated degradation of IAP-Smac and or IAP-Omi complexes results in IAP depletion, allowing for unrestricted Caspase activation **(B)** (dotted line).


Apoptotic stimuli
AIF
A.
B.
Smac/DIABLO
Bax/Bak
Omi/HtrA2
Cyt c
XIAP
Processing and release
of Smac and Omi
Active
Caspase-9
Smac/XIAP
Ubiquitination
XIAP
Active
Caspase-3
XIAP
Recruitment
Active
Caspase-9
Proximity-induced
caspase ubiquitination
Proteosome
Degradation
Active
Caspase-3
Proteosome
Degradation

Caspase-9 activation occurs, the effector Caspases-3 and-7 will become activated, triggering more significant or persistent changes in mitochondrial membrane permeability. This in turn releases more cytochrome c, as well as Apoptosis Inducing Factor (AIF), Smac, and Omi. AIF translocates to the nucleus where it is involved in chromatin condensation and degradation. Meanwhile, Smac and Omi are able to bind to and sequester IAPs so that unrestricted Caspase activation can proceed (Figure 1-7).

With Smac and Omi positioned in the mitochondria, their inhibition of IAP-Caspase interaction should predictably occur only when there is apoptotic signaling via the intrinsic pathway; however, Smac has also been shown to exert its pro-apoptotic effects on apoptotic processing through the extrinsic or death receptor pathway by functioning at the level of the effector Caspases. Death receptors and their ligands such as Fas/FasL and TRAIL/trail receptor are able to initiate Caspase activation independently of the mitochondria. Since both the intrinsic and extrinsic pathways converge on the effector Caspases-3 and -7, high levels of IAPs are able to prevent Caspase activation at this distal point, thereby terminating the receptor pathway via the inhibition of Caspase-3. Given that the mitochondria can be by-passed in the death receptor pathway, the mechanism of Smac release may not be intuitively obvious; however, it seems that in some cell types, stimulation of death receptors results in Caspase-8 cleavage and the subsequent activation of the pro-apoptotic Bcl-2 family member Bid (Gross *et al.*, 1999; Li *et al*,. 2002). When Bid becomes cleaved its truncated form is able to stimulate the formation of Bax- or Bak- pores in the outer mitochondrial membrane. This in turn induces the release of cytochrome c and Smac, and formation of the Apaf-1/ Caspase-9/apoptosome. Since Caspase-8 is not sensitive to direct IAP inhibition, persistent activation of the death receptor pathway should result in the relief of inhibition on downstream effector Caspases by the release of Smac via the secondary activation of the

mitochondrial pathway (reviewed in Wang, 2001). Thus, there is cross-talk between several signaling pathways to ensure that once an appropriate cell death signal is given, the apoptotic pathway is fully engaged.

IAPs and Cancer

Upon examining IAP expression in a variety of cancer cell lines and primary tumor biopsy samples, elevated levels were observed to be present in nearly all cancer types (Ambrosini *et al.*, 1997; LaCasse *et al.*, 1999; Vucic *et al.*, 2000; Tamm *et al.*, 2000; Fong *et al.*, 2001; Li *et al.*, 2001). The most dramatic example of IAP over-expression in tumors was observed with Survivin. As discussed earlier, Survivin expression is limited to embryonic tissues and many different tumor types, but absent in most healthy, adult tissues (Ambrosini *et al.*, 1997; Vucic *et al.*, 2000). Furthermore, the presence of Survivin in patient tumor biopsy samples correlates with poor prognosis, increased rates of treatment failure, and relapse (reviewed in Mesri *et al.*, 2001). The prognostic significance of IAP over-expression is less clear for some of the other IAPs. For example, XIAP protein levels correlate with disease severity and prognosis in acute myelogenous leukemia (AML) (Tamm *et al.*, 2000), but not in non-small cell lung carcinoma (NSCLC) (Ferreira *et al.*, 2001a; Ferreira *et al.*, 2001b; Liu *et al.*, 2001). These results suggest that the expression levels of the IAPs could be expected to have a significant impact in the development and maintenance of cancer due to their central role in the regulation of apoptosis.

Genetics of IAPs in cancer

There is direct genetic evidence that IAPs are able to function as oncogenes. This is seen in the case of the c-IAPs where chromosome amplification of the 11q21-q23 region,

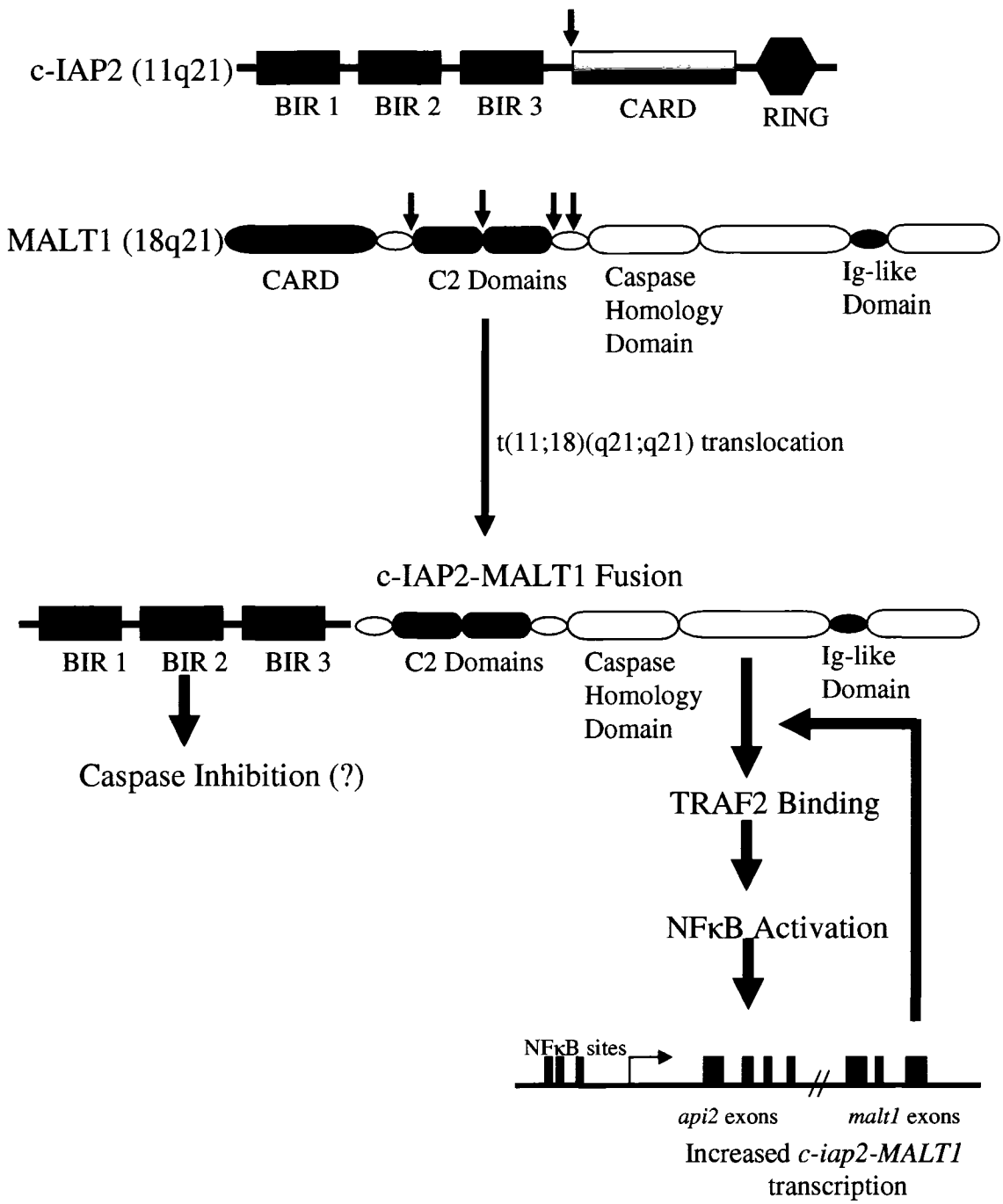
encompassing both c-IAP1 and c-IAP2, has been observed in a variety of malignancies, including medulloblastomas, renal-cell carcinomas, glioblastomas, gastric carcinomas and non-small cell lung carcinomas. Esophageal squamous-cell carcinomas frequently display this amplification, and transcriptional profiling has identified c-IAP1 as the sole target that is consistently over-expressed in these tumors (Imoto *et al.*, 2001). More recent findings have also shown amplification of 11q22 in primary tumors of non-small cell lung and small cell lung cancers. Analysis indicated that both c-IAP1 and c-IAP2 were over-expressed in the primary lung carcinomas (Dai *et al.*, 2003).

Further genetic evidence implicating the IAPs as potential oncogenes was found in extranodal marginal zone mucosa-associated lymphoid tissue (MALT) B-cell lymphomas. The translocation events t(1;14)(p22;q32) and t(11; 18)(q21;q21) have been well documented in MALT lymphomas and involves both NF-κB activation and c-IAP2. The less frequently observed translocation t(1;14)(p22;q32) juxtaposes the Bcl-10 gene on chromosome 1p22 adjacent to the immunoglobulin heavy chain (IgH) gene on chromosome 14 where the IgH enhancer causes Bcl-10 over-expression (Vega and Medeiros, 2001).

The more frequent translocation event, present in approximately 50% of extranodal MALT lymphomas, involves a gene rearrangement of the *MALT1* locus 18q21 with the c-IAP2- gene (also known as *API2*), located at 11q21 resulting in the c-IAP2-MALT fusion t(11;18)(q21;q21) (Dierlamm *et al.*, 1999; Remstein *et al.*, 2000; Baens *et al.*, 2000; Hosokawa, 2005). This gene fusion encodes a chimeric protein consisting of the c-IAP2 BIR domains fused in-frame to the carboxy-terminus of MALT1 (Uren *et al.*, 2000) (Figure1-8). Typically, gastric MALT lymphomas are associated with chronic inflammation due to *Helicobacter pylori* infection, and can be treated with antibiotic therapy; however, the majority of these lymphomas which do not respond to antibiotics exhibit the c-IAP2-MALT1

Figure 1-8. c-IAP2 is involved in MALT lymphoma.

The more frequently occurring t(11;18)(q21;q21) translocation disrupts the c-IAP2 gene (indicated by arrow), *api2*, on chromosome 11q21and the MALT1 gene, *mlt*, on chromosome 18q21. The resulting novel protein, c-IAP2-MALT1, possesses the HIAP1 BIR domain (arrow) fused to the carboxy terminus of MALT1 at one of four different points (indicated by four arrows on MALT 1 protein diagram). This schematic represents a resulting fusion protein. These proteins are both anti-apoptotic and NFκB-activating through TRAF2 binding. A positive feedback amplification loop is established in which the fused gene is expressed under the control of the *c-iap2* promoter, which contains several NFκB sites.


c-IAP2 (11q21)
BIR 1
BIR 2
BIR 3
CARD
RING
MALT1 (18q21)
CARD
C2 Domains
Caspase Homology Domain
Ig-like Domain
t(11;18)(q21;q21) translocation
c-IAP2-MALT1 Fusion
BIR 1
BIR 2
BIR 3
C2 Domains
Caspase Homology Domain
Ig-like Domain
Caspase Inhibition (?)
TRAF2 Binding
NFκB Activation
NFκB sites
api2 exons
malt1 exons
Increased c-iap2-MALT1 transcription

translocation (Chu *et al.*, 1997; Erl *et al.*, 1999; Hong *et al.*, 2000). The c-IAP2-MALT translocation establishes a positive feedback loop for c-IAP2 by activating NF-κB. This in turn transcriptionally activates the promoter for c-IAP2. In this way the AP12-MALT1 fusion induces NF- κB activation and contributes to an anti-apoptotic phenomenon by upregulating transcription of anti-apoptotic genes such as c-IAP2, A20 and Mcl-2 (Hosokawa, 2005). As a result, the lymph cells are no longer dependent on the inflammatory mechanism of NF-κB activation and eliminating the *H. pylori* is no longer sufficient to shut down inappropriate cell proliferation.

Targeting IAPs for Cancer Therapy

The issue of primary or acquired resistance to current chemotherapeutic-based treatments is a major impediment to effective cancer treatment. Although there are many genetic and biochemical alterations that occur in cancer cells, *in vitro* experiments demonstrate that the up-regulation of IAP expression increases resistance to chemotherapeutic and radiation resistance. The fundamental role of the IAPs in apoptosis regulation suggests that there is value in exploiting the inhibition of IAP expression and function as a direct therapeutic strategy.

IAP Antagonist-based Therapy

Antisense-Oligonucleotides as IAP antagonists

Applications of antisense oligodeoxynucleotides (AS ODN) as selective inhibitors of gene expression are being studied for efficacy in treating genetic disorders. In fact, many AS ODNs are being assessed both pre-clinically and in early clinical trials for several cancer

types. AS ODNs are short stretches of DNA, approximately 12-30 nucleotides long that are complementary to a specific mRNA strand. Hybridization of the AS ODNs to the mRNA by Watson-Crick base pairing prevents the target gene from being translated into protein, thereby blocking the action of the gene and results in degradation of the mRNA (reviewed in Galderisi *et al.*, 1999; Jansen and Zangemeister-Wittke, 2002; Gleave *et al.*, 2002). The specificity in the AS ODNs approach is based on the fact that any sequence of approximately 13 bases in RNA and 17 bases in DNA is estimated to be represented only once in the human genome. Whereas small-molecule drugs target a protein on the basis of its molecular domain structure, AS ODNs recognize a target mRNA based on its sequence (reviewed in Jansen and Zangemeister-Wittke, 2002; Gleave *et al.*, 2002). To find suitable sequences for the treatment of a specific cancer, genes which are differentially expressed or regulated in diseased and normal tissues need to be identified. Identifying and determining the roles of cancer-related genes in tumor development is a rapidly progressing area in cancer research. A multitude of candidate genes that are involved in apoptosis have been found in recent times, and represent potential targets for antisense-based therapies.

Recently published work shows that down-regulating XIAP gene expression via small interfering RNAs (siRNAs) increased apoptosis in cultured MCF-7 breast cancer cells and enhanced the killing effects of etoposide and doxorubicin (Lima *et al.*, 2004). Another study used short hairpin RNAs as an RNAi approach directed against XIAP, and showed that XIAP mRNA could be reduced by as much as 85% in some breast carcinoma cell lines. This reduction in XIAP dramatically sensitized these cell lines to TRAIL and to taxane-induced killing (McManus *et al.*, 2004). The therapeutic approaches discussed in the following sections will focus on how the alteration of IAP expression by AS ODNs are viable therapeutic approaches.

Antagonizing Survivin

Survivin is expressed in fetal tissues, becomes restricted during development, and is absent in healthy, adult tissues (Adida *et al.*, 1998). Although not present in differentiated tissues, Survivin is re-expressed during malignant transformation and is found in nearly all tumor types including neuroblastomas (Islam *et al.*, 2000), pancreatic, prostate, gastric, colorectal, hepatocellular, and breast carcinomas, as well as lung and bladder cancers, melanomas, B-cell lymphomas and esophageal cancer (reviewed in Altieri, 2001). The expression of Survivin in cancer is a predictor of both poor prognosis and decreased survival time, and is implicated in conferring chemo- and radio- resistance phenotypes to tumor cells (reviewed in Zaffaroni and Daidone, 2002).

Due to the ubiquitous nature of Survivin in human cancer, one therapeutic focus is the targeting of this IAP in the attempt to down-regulate and eliminate its expression. The therapeutic potential of using a survivin antisense approach was initially illustrated with the observation that EPR-1 (effector cell protease receptor-1) and Survivin were encoded by mRNAs transcribed from opposite strands of the same chromosome locus (Ambrosini *et al.*, 1998). When epr-1 cDNA was over-expressed in HeLa cells, Survivin was observed to be down-regulated, the rate of spontaneous apoptosis increased, and cell proliferation was inhibited (Ambrosini *et al.*, 1998). These experiments indicated that identifying a method to effectively down-regulate Survivin could be therapeutically beneficial. Experiments were later performed to assess the ability of 20 mer AS ODNs to down-regulate Survivin expression in human lung adenocarcinoma cell lines. These AS ODNs were observed to down-regulate Survivin protein levels in a dose-dependent manner, induce apoptosis, increase levels of Caspase-3 activation, and increase the sensitivity of cells to chemotherapeutics (Olie *et al.*, 2000; Jiang *et al.*, 2001). Similar experiments were

performed by another group in which different AS ODNs spanning the *survivin* gene were used to inhibit survivin expression in neuronal tumors. Results from these studies showed that depending on the tumor type, the AS ODN inhibition of survivin could induce cell death through either a Caspase-dependent or Caspase-independent pathway (Shankar *et al.*, 2001).

Antagonizing XIAP

In contrast to Survivin, the role of XIAP in tumor establishment, growth, metastasis, and chemoresistance is less clear; however, XIAP is the most potent of the IAPs with respect to its ability to inhibit Caspase activation and suppress apoptosis. Furthermore, XIAP is highly over-expressed in many tumor cell lines of the NCI panel (reviewed in LaCasse *et al.*, 1998) and its expression has been correlated with poor prognosis in acute myelongenous leukemia (AML) (Tamm *et al.*, 2000). These factors indicate that XIAP is an attractive target for improving treatment responses for a variety of tumor types.

One study examined the effects of XIAP down regulation using AS ODNs on human non-small cell lung cancer growth both *in vitro* and *in vivo*. In this study *xiap* AS ODNs were observed to effectively down-regulate both xiap mRNA and protein levels. As well, *xiap* AS ODNs effectively induced apoptosis on their own and sensitized the tumor cells to the cytotoxic effects of several chemotherapeutics, including Taxol®, etoposide, and doxorubicin (Hu *et al.*, 2003). Furthermore, administering xiap AS ODNs in a xenograft model of human non-small cell lung cancer showed a significant down-regulation of XIAP protein. Tumor establishment was delayed when AS ODNs were combined with doxorubicin and etoposide treatments (Hu *et al.*, 2003). Other studies showed that *xiap* AS ODNs enhanced tumor regression in conjunction with radiotherapy in a mouse model of lung cancer (Cao *et al.*, 2004). When XIAP was down-regulated in the pancreatic carcinoma cell

line, Panc-1, with a second generation, mixed backbone AS ODN (AEG 35156/GEM640) these cancer cells were also sensitized to TRAIL-induced killing (McManus *et al.*, 2004). Similarly, TRAIL in combination with another *xiap* AS ODN, xiap phosphorodiamidate morpholino oligomer, potentiated both cisplatin sensitivity and TRAIL killing in androgen-refractory prostate cancer cells (Amantana *et al.*, 2004). Overall, there are promising data indicating that when XIAP protein expression levels are reduced via RNAi or antisense approaches, a decreased apoptotic threshold to an array of chemotherapeutics can be achieved.

Adenoviral expression of dominant negative mutants of survivin

Adenoviral vectors targeting regulators of both cell cycle and/or apoptosis have been examined for potential applications in cancer therapy alone, or in combination with chemotherapy drugs (reviewed in Mesri *et al.*, 2001). One such application of adenoviral-based gene therapy is the intratumor adenoviral delivery of wild type p53 in patients with non-small cell lung cancer, thereby restoring checkpoint functions of apoptosis and/or cell cycle arrest (Swisher *et al.*, 1999). Targeting the Survivin pathway via dominant negative mutants in an adenoviral gene therapy approach may also be beneficial for cancer treatments. When initial experiments were performed using DNA plasmids to transfect Survivin mutants into melanoma cell lines, one mutant, carrying a cysteine 84-alanine (Cys^{84}-Ala) mutation in the BIR domain, significantly increased the number of apoptotic cells (reviewed in Zaffaroni and Daidone, 2002). Additional dominant negative mutants of Survivin were later constructed, one of which carries a Thr^{34}-Ala mutation. This mutation destroys a phosphorylation site for the cyclin-dependent kinase $p34^{cdc2}$. Without phosphorylation at Thr^{34}, Survivin is no longer able to associate with pro-Caspase-9 and inhibit apoptosis

(O'Connor, *et al.*, 2000). When the Thr^{34}-Ala dominant negative mutant was transfected into three different melanoma cell lines, spontaneous apoptosis and an increased sensitivity to cisplatin was observed. Conditional expression of Thr^{34}-Ala also prevented tumor formation in a subcutaneous xenograft model of melanoma (Grossman *et al.*, 2001).

A replication-deficient adenovirus (pAd-T^{34}A) was later used to over-express the Thr^{34}-Ala Survivin dominant mutant. In one study it was observed that transduction with pAd-T^{34}A caused spontaneous apoptosis in breast, cervical, prostate, lung and colorectal cancer cell lines. In contrast, pAdT^{34}A had no observable effects on the proliferation or viability of healthy cells that do not express Survivin (Mesri *et al.*, 2001). Mitochondrial release of cytochrome c, cleavage of Caspases-9 and -3, and the catalytic enhancement of Caspase-3 activity were observed in the apoptotic pAd-T^{34}A-infected cells. In combination with chemotherapeutics such as Taxol®, the extent of apoptosis in HeLa and MCF-7 cells was also increased (Mesri *et al.*, 2001). Experiments using the MCF-7 human breast cancer xenograft model showed that pAdT^{34}A transduction suppressed *de novo* tumor formation, inhibited the growth of pre-established tumors, reduced intraperitoneal tumor dissemination, and induced massive apoptosis in all transduced cells (Mesri *et al.*, 2001). Thus, adenoviral targeting of the Survivin pathway using dominant negative mutants may be a powerful tool for selective cancer gene therapy.

Negative IAP Regulators As Therapeutics

XAF1

Studies have shown that restoring XAF1 expression levels increases the sensitivity of tumor cell types to apoptotic triggers. For example, one study using gene arrays observed

that in the presence of interferon β (IFN- β), xaf1 mRNA was up-regulated in several apoptosis-resistant cancer cell lines including melanoma, leukemia, and fibrosarcoma cells (Leaman *et al.*, 2002). The melanoma cell lines used in this study were largely resistant to Apo2L/TRAIL, an important mediator of IFN- β-induced apoptosis. Pre-treatment with IFN- β sensitized the cells to Apo2L/TRAIL-induced apoptosis, potentially due to the up-regulation of XAF1. When XAF1 was over-expressed by transient transfection, it was confirmed that the constitutive expression of XAF1 sensitizes the cells to Apo2L/TRAIL to the same degree as IFN pre-treatment (Leaman *et al.*, 2002). Other studies examined the expression levels of XAF1 in human colon cancer biopsies where it was observed that there were low levels of xaf1 mRNA compared to adjacent healthy tissues. From these studies it can be concluded, that Smac, or XAF1 over-expression strongly influences cellular sensitivity to the pro-apoptotic effects of Apo2L/TRAIL.

It has been recently shown that XAF1 is able to function differentially between cancer cells and healthy cells, making this protein a potentially powerful candidate as a cancer therapeutic. Using xenograft nude mouse models in which gastric and colon cancers were established by subcutaneous injections, it was observed that XAF1 over-expression alone was able to suppress tumor formation. Further studies showed that when adenoviral-*xaf1* was delivered by intratumoral injections in combination with Apo2L/TRAIL or chemotherapeutics, there was enhanced killing by apoptosis, and eventually a complete eradication of established tumors (Tu *et al.* submitted). When tissues surrounding the tumors were examined for the presence of apoptotic cells via TUNEL staining, it was observed that apoptosis was not induced in the normal cells (Tu *et al.* submitted). XAF1 has also shown selective killing potential in other human tumor cells including pancreatic and breast cancer cell lines. When these human tumor cell lines and normal cell lines were transduced with

adenoviral *xaf1*, approximately 80% of the tumor cells underwent apoptosis compared to 5-10% in the normal cell lines (Yang *et al.*, 2003). The ability of XAF1 to selectively induce apoptosis in cancer cells leaving healthy cells unaffected makes this protein a potentially strong therapeutic candidate.

Smac

Although there are many genetic and biochemical alterations that occur in cancer cells leading to chemoresistance, *in vitro* experiments suggest that chemoresistance correlates with an upregulation of IAP expression and therefore, Caspase inhibition. Smac appears to function as a general IAP inhibitor in that it has been shown to bind to all of the IAPs tested to date, with the exception of NAIP (Du *et al.*, 2000; Verhagen *et al.*, 2000; Vucic *et al.*, 2002; Davoodi *et al.*, 2004). Furthermore, when Smac is over-expressed in a variety of tissue culture cell lines, the sensitivity to apoptotic triggers, such as chemotherapeutics, is increased (Du *et al.*, 2000; Verhagen *et al.*, 2000; Vucic *et al.*, 2002; Hunter *et al.*, 2003; McNeish *et al.*, 2003). Structural analysis of Smac-XIAP binding to XIAP indicates that the amino-terminal Smac tetrapeptide recognizes a groove on the BIR3 domain surface, indicating that peptides or small molecules modeled on this binding motif might serve as prototypes for drugs capable of mimicking Smac activity (Kipp *et al.*, 2002).

Resistance to the commonly used chemotherapy drugs, etoposide, doxorubicin, paclitaxel, and Apo2L/TRAIL has become a standard problem in the clinic. As an example, Apo2L/TRAIL, having an apparent selectivity for human tumor cells, was once thought to be a promising cytotoxic agent in the treatment of cancer; however there are recent reports of tumor cell resistance to this type of therapy (Fulda *et al.*, 2002a). The delivery of either Smac-like peptides, or over-expressed Smac protein, has been shown to vastly improve the

sensitivity of these otherwise resistant cells to apoptosis and ultimately, reduce tumor burden (Fulda *et al.*, 2002a; Fulda *et al.*, 2002b; Guo *et al.*, 2002; Ng *et al.*, 2002).

Summary

Apoptosis is controlled at multiple steps, each of which is influenced by pro-and anti-apoptotic proteins. The equilibrium between the cell death inducing Caspase cascade and the IAPs constitutes one such fundamental decision point. The recurrent up-regulation of IAP expression in cancer cell lines and tumors indicates that this decision point is important in determining cell fate. The IAPs not only control cell death, but also influence signal-transduction pathways, protein turnover, and progression through cell cycle. Still, many aspects of IAP function in all these processes remain to be clarified. With the recognition of apoptosis suppression as a fundamental aspect of human cancer, the IAPs and other anti-apoptotic proteins are now acknowledged as being outstanding therapeutic targets.

Thesis Outline

Traditional cancer therapies were designed to specifically target the inappropriate proliferative characteristics of cancer cells relative to the surrounding normal tissues (reviewed in Lippman *et al.*, 1998; Kelloff, 1999). Many of the standard therapies, which include gamma-radiation or drugs that impair DNA replication, directly or indirectly damage the DNA sufficiently such that cancer cells become non-viable. Paradoxically, these treatments which are supposed to be eliminating the cancer cells may actually be generating a pool of proliferating cells with extensive chromosomal rearrangements, deletions, and point mutations that will develop into tumors that are both aggressive and resistant to treatment. It is important to note, however, that cancer is not only a disease of deregulated proliferation, but also a failure of apoptosis. Furthermore, mutation events that act to suppress apoptosis appear to be limited to regulatory genes, and in fact, the cellular machinery of apoptosis remains intact in most, if not all, cancers (Evan and Vousden, 2001; Zornig *et al.*, 2001; Green and Evan, 2002). Intuitively, therapy strategies that restore normal apoptotic regulation will thus be safer, less toxic, and ultimately more effective.

Practically all known apoptosis signal pathways converge on the capases, which are the ultimate effectors of apoptotic cell death. The Inhibitor of Apoptosis (IAP) genes encode a family of proteins that bind and inactivate key Caspases involved in both the initiation (Caspase-9) and execution (Caspases -3 and -7) of this casade (reviewed in Creagh and Martin, 2001; Zimmerman *et al.*, 2001; Salvesen, 2002). Although additional proteins have been identified that modulate the activity of initiator Caspases, the IAPs remain the only identified cellular proteins that can function to inhibit the effector stage of the apoptotic cascade. Negative regulators of the IAPS, such as Smac, have been shown to restore the

endogenous ability of the cell to undergo apoptosis by blocking IAP-based Caspase inhibition. Thus, it would seem reasonable that an over-expressed supply of Smac present in resistant cancer cell line would have a significant impact on decreasing the apoptotic threshold.

Previous observations show that when Smac is over-expressed in a variety of tissue culture cell lines by transient transfections, the sensitivity to apoptotic triggers is increased (Du *et al.*, 2000; Verhagen *et al.*, 2000; Vucic *et al.*, 2002). One inherent problem in simply over-expressing Smac, however, is that this will result in a protein that is targeted to the mitochondria. This will make Smac release dependent on apoptotic signals that are processed only through the intrinsic pathway via the mitochondria. By having a supply of over-expressed, mature, cytosolic Smac, the inhibitory activity of Smac on the IAPs and resulting Caspase activation will be independent of the type of apoptotic signal. In theory, this should make any cell, regardless of the type of genetic alteration that interferes with apoptosis, more sensitive to apoptotic triggers.

This thesis work outlines three independent strategies for harnessing and improving the apoptotic-inducing capacity of Smac for potential therapeutic development in the sensitization of cancer cells to apoptotic cell death. For the first part of my project I developed and characterized a ubiquitin-Smac fusion system that generates a processed, biologically active form of cytosolic Smac. With this system, I was able to show that this over-expressed form of Smac sensitizes cancer cells to cell death in the presence of chemotherapeutics. The second goal of this thesis work was to develop a strategy for increasing the affinity of Smac binding to the IAPs. By creating stronger Smac-IAP binding affinities, these protein-protein interactions would be favored over IAP-Caspase interactions, thus increasing apoptotic sensitivity. High affinity Smac-like peptides were identified using

a phage display screening method. Finally, the last component of this thesis work was to develop a Smac system that was a more potent apoptotic-inducer thereby allowing for lower-dose chemotherapeutic drug killing. The genesis of this idea was based on the pre-exisiting knowledge that Smac interactions with the IAP BIR domains may accelerate IAP auto-ubiquitination and destruction (Yang and Du, 2004). By turning Smac into a ubiquitinating protein via the addition of an carboxy terminal E3 ligase, IAP protein degradation was enhanced and there was indeed, a resultant increase in the sensitivity to apoptosis in the presence of chemotherapeutic drugs. Engineering Smac-based therapeutics to more effectively down-regulate the IAPs could potentially have sigificant impact on the treatment of cancer.

Chapter II

Material and Methods

Plasmid DNA Constructs

UbSmac

The human *ubiquitin* and mouse *Smac/DIABLO* coding regions were fused by an overlapping PCR strategy. The Ub coding region was amplified using the primers 5'd-AAAAAGCTTAAAATGAGAGGCAGCCACCACCATCACCATCACATGCAGATCTTC GTG and 5'd-CTGAGCAATAGGAACCGCACCACCTCTCAGACGCAGGAC. The Smac coding region was amplified with 5'd-CGTCTGAGAGGTGGTGCGGTTCCTATTGCTCAG and 5'd-TACTCGAGTTAGGCGTAATCAGGGACATCGATAGGATAATCTTCACGCAG GTAGGC. Ub and Smac PCR products were denatured at 95°C, annealed together at 50°C, and re-amplified with the Ub forward and Smac reverse primers. The UbSmac PCR product was TA cloned in pCR2.1 (Invitrogen) and completely sequenced. The coding region was then subcloned using HindIII and XhoI sites into a ubiquitin C promoter-containing expression vector (courtesy of Dr. Douglas Gray, Ottawa Regional Cancer Center, Ottawa, Canada). The final plasmid construct encodes a 279 amino acid (a.a.) precursor protein consisting of 86 a.a. of human ubiquitin fused to a.a. residues 54 through 237 of mouse Smac with a 9 a.a. carboxy terminal HA tag.

UbΔAVPI-Smac

Primers 5'd-CGTCTGAGAGGTGGTGCTCAGAAATCGGAGCC and 5'd-GGCTCCGATTTCTGAGCACCACCTCTCAGACG were used to delete the AVPI codons of UbSmac using the QuikChange™ Mutagenesis kit (Stratagene) according to manufacturer's instructions. The UbSmac plasmid was used as a template for PCR. Plasmid products were sequenced to confirm the twelve nucleotide deletion.

UbΔAVPIA-Smac

Primers 5'd-ATGAGGCTCCGATTTCTGACCACCTCTCAGACGCAG and 5'd-CTGCGTCTGAGAGGTGGTCAGAAATCGGAGCCTCAT were used to delete the AVPIA codons of UbSmac using the QuikChange™ Mutagenesis kit (Stratagene) according to manufacturer's instructions. The UbSmac plasmid was used as a template for PCR. Mutated plasmids were rescued by transformation and sequenced to confirm the 15 nucleotide deletion.

UbARPL-Smac

Primers 5'd -CGTCTGAGAGGTGGTGCTCGTCCGCTTGCT and 5'd-GGCTCCGATTTCTGAGCAAGCAAGCGGACGAGCA were used to change the Smac N-terminal AVPI sequence of UbSmac to an N-terminal ARPL sequence using the QuikChange™ Mutagenesis kit (Stratagene) according to manufacturer's instructions. The Ub-Smac plasmid was used as a template for PCR. Plasmid inserts were sequenced to confirm substitution of the AVPI codons with the sequences encoding ARPL.

UbSmac-RING

Two separate PCR cloning steps were required to generate the Smac-RING constructs described in this thesis. The first PCR step was the same for all constructs and involved the addition of a BamHI site to the 3' end of the Ub-Smac coding region and deletion of the stop codon usingUbSmac as the template and the primers 5'd-TAGGATCCGGCGTAATCAGGGACATCGATAGG and 5'd-AAAAAGCTTAAAATGAGAGGCAGCCACCACCATCACCATCACATGCAGATCTTC

GTG. The newly generated UbSmac-BamHI product was TA cloned and subcloned into pCDNA3 (Invitrogen) using the HindIII and BamHI restriction sites, thereby generating pCDNA3-UbSmac-BamHI (Fig 2-1).

UbSmac-XIAP-RING (SXR)

The XIAP-RING fragment was amplified from a pre-existing pCDNA3-6myc-*xiap* template using the primers 5'd-TAGGATCCTTAGAACAGAAGGGACAA and 5'd-TACTCGAGTTAAGACATAAAAATTTTTTGC. The PCR product was TA cloned and then subcloned, using newly generated BamHI and XhoI sites, into the pCDNA3-UbSmac-BamHI vector to produce pCDNA3-UbSmac-XIAP-RING (Fig 2-1)

UbSmac-HIAP1-CARD-RING (SH1R)

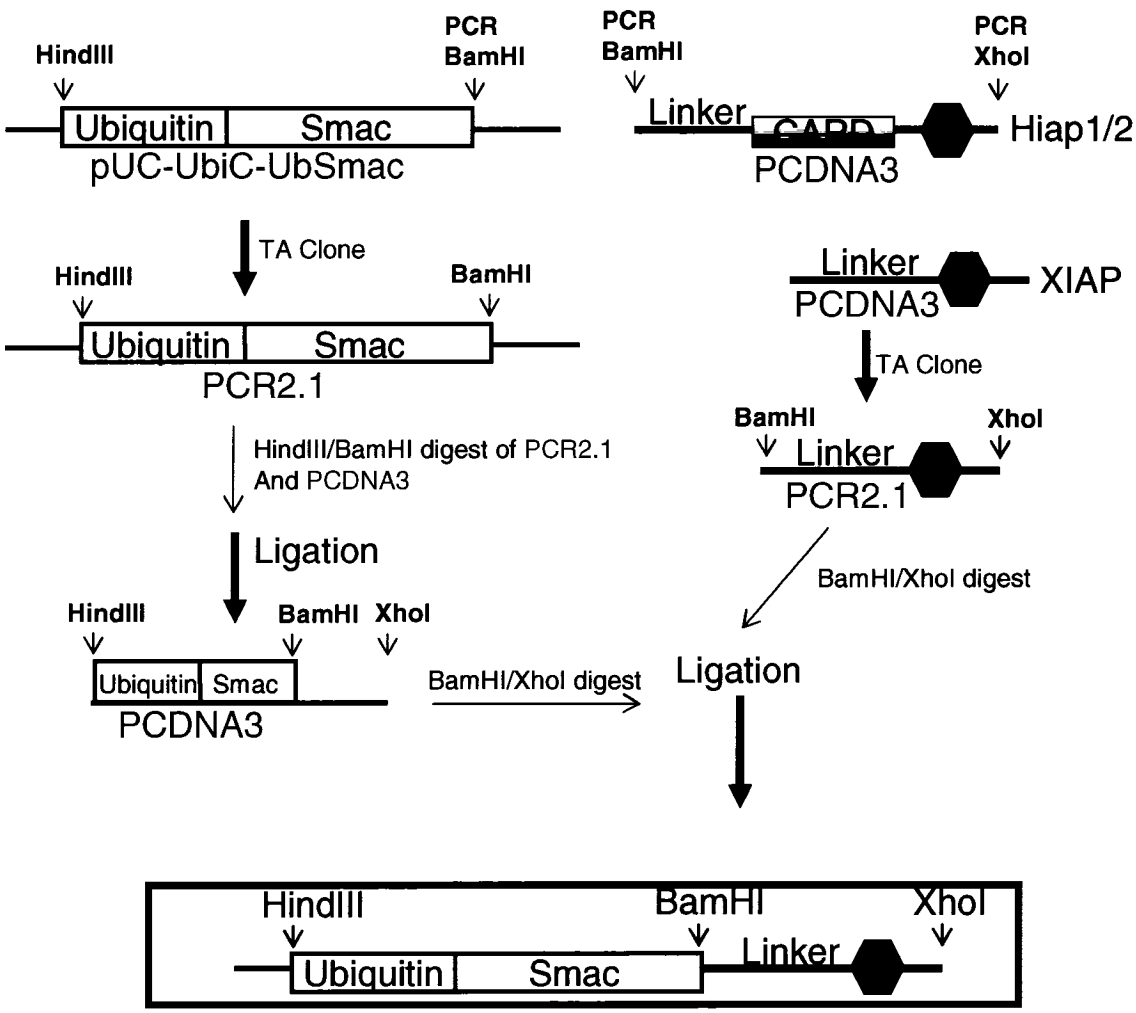
The HIAP1-CARD-RING fragment was amplified from a pre-existing pCDNA3-6myc-HIAP1 template using the primers 5'd- TAGGATCCGAATCAAATGATTTATTA TTAATCCGG and 5'd-TACTCGAGTCATGAAAGAAATGTACGAACTG. The PCR product was TA cloned and then subcloned, using newly generated BamHI and XhoI sites, into the pCDNA3-UbSmac-BamHI vector to produce pCDNA3-UbSmac-HIAP1-CARD-RING.

UbSmac-HIAP2-CARD-RING (SH2R)

The HIAP2-CARD-RING fragment was amplified from a pre-existing pCDNA3-6myc-HIAP2 template using the primers 5'd- TAGGATCCGCATCAGATGATTTGTCA TTAATTCGG and 5'd- TACTCGAGTTAAGAGAGAAATGTACGAACAGTACC. The PCR product was TA cloned and then subcloned, using newly generated BamHI and XhoI sites, into the pCDNA3-UbSmac-BamHI vector to produce pCDNA3-UbSmac-HIAP2-CARD-RING.

Figure 2-1. Construction of the Smac-RING expression plasmids.

The UbSmac expression vector was modified at the 3' end of the Smac coding region via the addition of a BamHI restriction site and deletion of the stop codon. The generated UbSmac-BamHI fragment was subcloned into pCDNA3. IAP-RING domains were PCR amplified with 5' BamHI and 3'XhoI restriction sites and were subcloned into the pCDNA3-UbSmac construct.


HindIII
PCR
BamHI
Ubiquitin
Smac
pUC-UbiC-UbSmac
PCR
BamHI
PCR
XhoI
Linker
CARD
Hiap1/2
PCDNA3
TA Clone
HindIII
BamHI
Ubiquitin
Smac
PCR2.1
Linker
PCDNA3
XIAP
TA Clone
BamHI
XhoI
Linker
PCR2.1
HindIII/BamHI digest of PCR2.1
And PCDNA3
Ligation
BamHI/XhoI digest
HindIII
BamHI
XhoI
Ubiquitin
Smac
PCDNA3
BamHI/XhoI digest
Ligation
HindIII
BamHI
XhoI
Ubiquitin
Smac
Linker

UbSmac-HIAP1-CARD-RING*(SH1R*)

The HIAP1-CARD-RING mutant with an amino acid change from His^{547} to Ala, was generated using the QuikChange™ Mutagenesis kit (Stratagene) according to manufacturer's instructions. The pCR2.1-HIAP1-CARD-RING (obtained from Dr. Herman Cheung) template and primers 5'd-GTGTTTATTCCTTGTGGTGCTCTAGTAGTATGC and 5'd-ACCACAAGGAATAAACACTATGGACACTTC were used. The plasmid product was sequenced to verify the correct nucleotide changes and then subcloned, using newly generated BamHI and XhoI sites, into the pCDNA3-UbSmac-BamHI vector to produce pCDNA3-UbSmac-HIAP1-CARD-RING*.

UbΔAVPI-Smac-HIAP1-CARD-RING* (ΔAVPI -SH1R*)

Primers 5'd-CGTCTGAGAGGTGGTGCTCAGAAATCGGAGCC and 5'd-GGCTCCGATTTCTGAGCACCACCTCTCAGACG were used to delete the N-terminal AVPI codons in the UbSmac portion of pCDNA3-UbSmac-HIAP1-CARD-RING (SH1R), using the QuikChange™ Mutagenesis kit (Stratagene) according to manufacturer's instructions. The pCDNA3-UbSmac-HIAP1-CARD-RING plasmid was used as a template for PCR to generate UbΔAVPI-Smac-HIAP1-CARD-RING. BamHI and XhoI restriction digests were performed on both pCDNA3-UbΔAVPI-Smac-HIAP1-CARD-RING and pCDNA3-UbSmac-HIAP1-CARD-RING* (SH1R*). Ligations of pCDNA3-UbΔAVPI-Smac and the HIAP1-CARD-RING* regions generated the UbΔAVPI-HIAP1-CARD-RING* (ΔAVPI -SH1R*) construct.

pGEX-XIAP, -XIAP BIR1, -XIAP BIR2, - XIAP BIR3

To generate pGEX-XIAP, the *xiap* coding region was excised from a pre-existing pcDNA3-based plasmid, pCDNA3-6myc XIAP using BamHI and XhoI restriction sites. The

xiap DNA fragment was then subcloned into the pGEX-4T3 vector (Amersham Pharmacia) to generate a construct encoding a GST-tagged XIAP fusion protein. Individual BIR domains of *xiap* were PCR amplified and TA cloned using the following primers: BIR1 – 5'd-CAGGATCCACCATGACTTTTAACAGTTTTGAAGG and 5'd-GACTCGAGCT AGCTTCCCAGATAGTTTTCAAC. BIR2: 5'd-CAGGATCCATGAGAGATCATTTT GCCTTAGAC and 5'd-GACTCGAGCTATTCACTTCGAATATTAAGATTCCG. BIR3: 5'd-CAGGATCCATGTCTGATGCTGTGAGTTCTG and 5'd-GACTCGAGCTAAGT AGTTCTTACCAGACACTCCTCAAG. The *xiap* BIR domain fragments were subcloned into pGEX-4T3 using BamHI and XhoI.

pGEX-HIAP1,-HIAP1 BIR1, -HIAP BIR2,-HIAP1 BIR3

To generate pGEX-HIAP1, the *hiap1* coding region was excised from a pre-existing pcDNA3-based plasmid, pCDNA3-6myc HIAP1 using BamHI and XhoI restriction sites. The *hiap1* DNA fragment was then subcloned into the pGEX-4T3 vector to generate a vector encoding a GST-tagged HIAP fusion protein. Individual BIR domains of *hiap1* were PCR amplified and TA cloned using the following primers: BIR1 – 5'd-GGATCCATGAACAT AGTAGAAAACAGCATATTC and 5'd- CTCGAGTCAAACGAATCTGCAGCTAGGA TAC. BIR2: 5'd-GGATCCATGCAGAGTCTAAATTCCGand 5'd-CTCGAGTTATATAA ATGGGCATTTGGGAAAATGTC. BIR3: 5'd-CAGGATCCATGGAAAATCAGCTTCA AGAC and 5'd-GAACAGCTGCATTCCACATCATAACTCGAG. The *hiap1* BIR domain fragments were subcloned into pGEX-4T3 using BamHI and XhoI.

pGEX-HIAP2, -HIAP2 BIR1, -HIAP2 BIR2, -HIAP2 BIR3

To generate pGEX-HIAP2, the *hiap2* coding region was excised from a pre-existing pcDNA3-based plasmid, pCDNA3-6myc HIAP2 using BamHI and XhoI restriction sites. The *hiap2* DNA fragment was then subcloned into the pGEX-4T3 vector to generate a vector

encoding a GST-tagged HIAP fusion protein. Individual BIR domains of *hiap2* were PCR amplified and TA cloned using the following primers: BIR1 – 5'd-GGATCCATGCACA AAACTGCCTCCCAAAGAC and 5'd-CTCGAGTCAATAAAGCTACAGCTACAGCTA GGATATAGC. BIR2: 5'd-AGATCTATGCAGAATCTGGTTTCAGCTAG and 5'd-CTCGAGTCAAAAAATGGACAGTTGGGAA. BIR3: 5'd-GGATCCATGGAAAATTCT CTAGAAACTG and 5'd-GAACAGCTGTTGTCAACTTCATAACTCGAG. The *hiap2* BIR domain fragments were subcloned into pGEX-4T3 using BamHI and XhoI.

pGEX-TsIAP

To generate pGEX-TsIAP, the *tsiap* coding region was excised from a pre-existing pcDNA3-based plasmid, pCDNA3-6myc TsIAP using BamHI and XhoI restriction sites. The *tsiap* DNA fragment was then subcloned into the pGEX-4T3 vector to generate a GST-tagged construct of *tsiap*.

Phage Display (Panning)

As previously described, XIAP BIR3, HIAP1 BIR3, and HIAP2 BIR3 were cloned into pGEX, a Glutathione S-Transferase gene fusion vector, which was used for the expression and purification of GST-fused proteins. The GST-IAPs were expressed by innoculating 5 ml of an overnight culture into 45 ml of LB broth , containing Ampicillin (100 μg/ml) and 1 mM Zn Acetate. Cells were incubated for 1 hr, and were then induced by the addition of 0.1 M IPTG for 3 hrs at 30° C. GST-IAPs were purified and isolated using glutathione beads (Amersham Pharmacia) with an estimated purity of $\geq$ 95% by coomassie blue staining. Panning was performed using the Ph.D.-7 Phage Display Library Kit (New England Biolabs), according to manufacturer's instructions. Ph.D. -7 phage was added to each sample of GST-IAP (1μg of protein) and allowed to incubate for 30 min, followed by

10 washes in STE. Following each round of panning 20 plaques were selected and characterized by DNA sequencing. Three rounds of panning were performed to ensure that the phage, with IAP binding peptides, was selected. After the phage were characterized by DNA sequencing, the peptide sequences which have an affinity for the IAPs were identified (Fig 2-2).

Cell Culture and Transient Transfections

Human embryonic lung (HEL) 299 cells and 293A embryonic kidney cells were maintained at 37° C and 5% CO_2 in Dulbecco's minimal essential medium (DMEM) (Gibco BRL), supplemented with 10% heat-inactivated fetal calf serum (FCS) (Gibco BRL), penicillin and streptomycin (Gibco BRL). Cultures were maintained in 100 mm tissue-culture grade dishes (Corning) and passaged every 72 hrs. Transfections were performed using either Lipofectamine 2000 ™ or Lipofectamine Plus™ (Invitrogen) as outlined in the manufacturer's protocol. Plasmid DNAs were prepared using the Qiagen Maxiprep™ kit (Qiagen) according to the manufacturer's protocol.

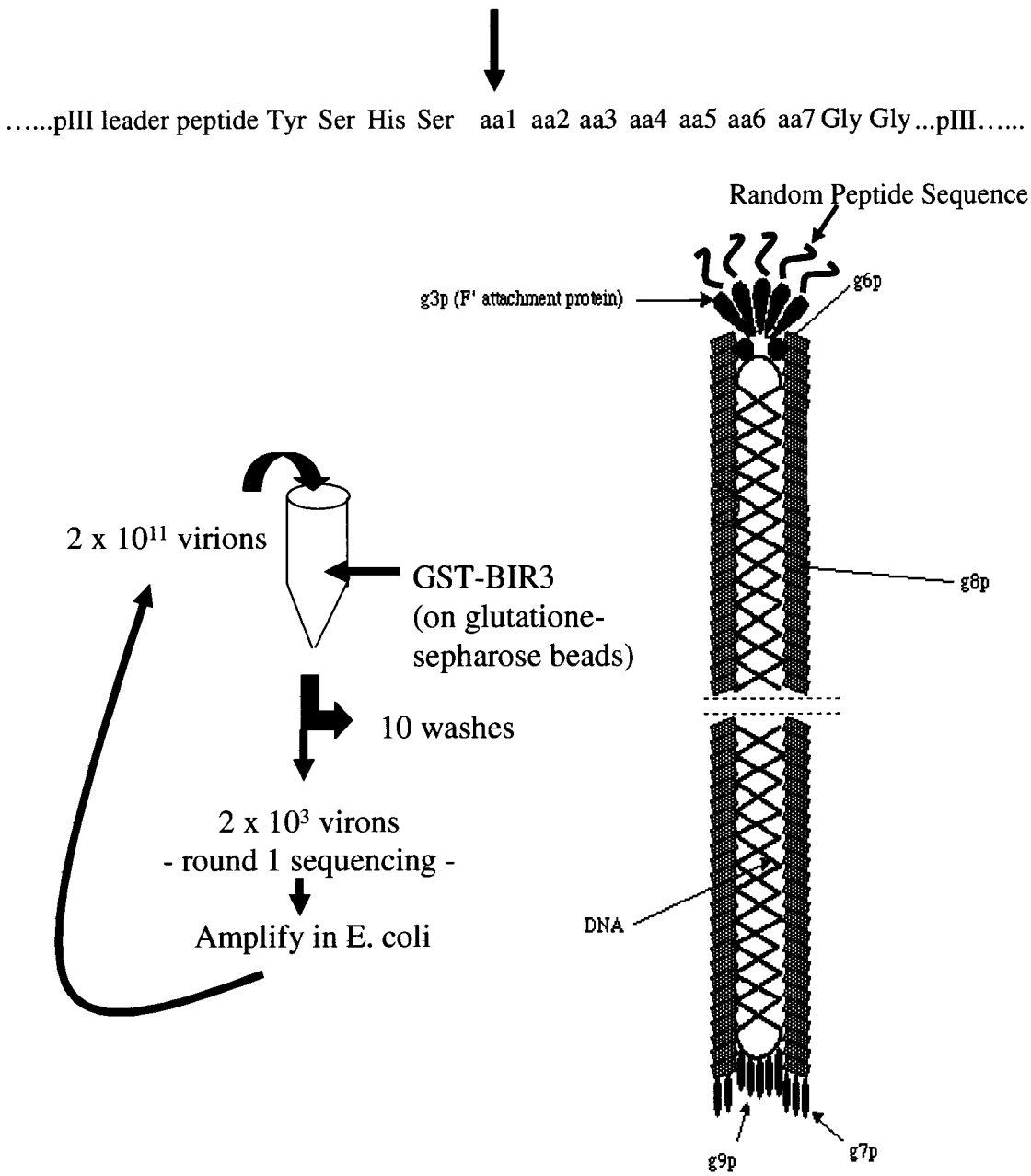
Immunoprecipitation

100 mm dishes of transfected and untransfected cells were rinsed 2X in phosphate buffered saline (PBS). 500 µl of immunoprecipitation buffer (10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 150 mM NaCl,) containing protease inhibitors (10 µM Aprotinin, 100 µM Pepstatin A 10 µM Leupeptin and 100 µM phenylmethylsulfonyl Fluoride (PMSF)) (all from Sigma Aldrich) was added to each dish and the cell lysates were scraped into Eppendorf tubes. The lysates were then sonicated 2 X 30 sec using a probe sonicator (Sonics and Materials, Inc.). Lysates were spun at 17 000g for 10 min. The supernant was removed and placed in a fresh Eppendorf tube and the pellet was discarded. A total of 3 µg of anti-RIAP3

Figure 2-2. Schematic of Phage Display Peptide Library Screening.

A peptide from a random library is expressed as a fusion with the p3 coat protein of bacteriophage M13. The peptide is displayed as a fused protein on the exterior of the phage virion, with the DNA encoding the fusion in the virion. Incubating a library of phage-displayed peptides with purified GST-IAP BIR domains in the presence of glutathione beads gives a physical linkage between the library of random peptide sequences, to the DNA encoding each sequence.

Phage Display Peptide Library Screening (NEB PH.D.-7 kit)



(rat XIAP) was added to the supernant and the samples were incubated for 1 hr on a rotating shaker at 4° C. Following the 1 hr incubation, 40 µl of a Protein A Sepharose (Amersham) bead slurry was added to the samples. The samples were incubated for 1 hr on a rotating shaker at 4° C, and then washed 3X in immunoprecipitation buffer. The beads were syringe dried using a 28 gauge needle, 2X SDS-PAGE sample buffer (BIORAD) was added, and samples were separated on SDS-PAGE gels.

Metabolic Labelling and Immunoprecipitation

293A cells were seeded in 6-well plates at a density of 2.5 x 10^5 cells per well and the next day were co-transfected with 0.5 µg of 6myc-IAP plasmid and 0.5 µg of either: UbSmac, Smac-RING, or pCDNA3 plasmid. At 24 hrs post-transfection, 35 µCi per well of S^{35}-Methionine and -Cysteine (Amersham Pharmacia) was diluted in labelling media (Cysteine-free and Methionine-free Dulbecco's Modified Eagle's Medium (DMEM)) (Invitrogen) containing 1% FCS (Invitrogen). Labelling media was added to each well of cells and incubated for 4 hrs at 37° C and 5% CO_2. Cells were then washed in 1X PBS and lysed in Cell Lytic Buffer™ diluted 1:2 with STE buffer (50 mM Tris- HCl, pH 8.0, 150 mM NaCl, 10 mM EDTA) containing protease inhibitors (10 µM Aprotinin, 100 µM Pepstatin A, 10 µM Leupeptin and 100 µM PMSF) (all from Sigma Aldrich). Cell lysates were pre-cleared with a 50:50 slurry of Protein A Agarose beads in STE buffer (Amersham Pharmacia) and were incubated on a rocking platform for 30 min at 4° C. The lysate-bead mix was centrifuged at 17 000g for 5 min. The supernatant was collected in a fresh tube and 40 µl of Protein A Agarose bead slurry was added to each sample, along with 2 µg anti-Myc antibody (Sigma Aldrich). Samples were incubated on a rocking platform for 1 hr at 4° C. After 1 hr the samples were spun at 17 000g for 5 min and washed six times in STE buffer.

Following the last spin, beads were syringe dried with a 28 gauge needle and 50 µl of 2X SDS-PAGE loading buffer (BIORAD) containing β-mercaptoethanol (Sigma Aldrich) was added to each sample. Samples were boiled for 5 min and sonicated in a bath sonicator for 10 min. Samples were separated on 12% SDS-PAGE gels. Gels were coomassie stained and destained (40% methanol; 10% Glacial Acetic Acid) and soaked in Amplify™ (Amersham Pharmacia) for 30 min. Gels were then dried and exposed to X-AR5™ film (Kodak) for 24 hrs at -80° C.

Cell Fractionation

HEL299 cells were seeded in 100 mm dishes at a density of $5x10^6$ cells/dish. 24 hrs following UbSmac transfection, the cells were washed 2X in PBS, trypsinized and pelleted at 1500g at 4° C. Cell pellets were resuspended in ice-cold RSB (10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5) and transferred to a 2 ml Dounce homogenizer. After allowing the cells to swell for 7 min, the cells were broken open with several strokes of the pestle. One half a volume of 2.5X MS buffer (210 mM Mannitol, 70 mM Sucrose, 5 mM Tris-HCl, pH 7.5, 1mM EDTA pH 7.5) was immediately added to the homogenate to give a final concentration of 1X MS and the homogenate was mixed by inversion. The homogenate was then transferred to an Eppendorf tube and spun at 1300g for 5 min to remove nuclei and large membrane fragments. The supernatant was spun one more time at 1300g and was then transferred to a clean Eppendorf tube. The supernatant was spun at 17 000g for 15 min to obtain the mitochondrial pellet. The mitochondrial pellet was washed in 1X MS buffer and the 17 000g sedimentation was repeated. The mitochondrial pellet was resuspended in a small volume of TE (10 mM Tris-HCl, 1 mM EDTA). Following the first 17 000g spin, the supernatant was processed further by ultracentifugation (Beckman TL-100) at 100 000g for 1

hr at 4° C. From this spin the cytosolic fraction (supernatant) and light membrane fraction (pellet) were obtained. The pellet was resuspended in a small volume of TE buffer. To all samples, an appropriate volume of 2X sample buffer (BIORAD) was added to give a final 1X concentration. Samples were boiled 5 min prior to electrophoresis on SDS-PAGE gels.

GST-fusion Purification and Pull-downs

Overnight cultures of pGEX–XIAP-BIR1, -BIR2 and -BIR3 were diluted 1:10 in fresh media and allowed to grow for 1 hr prior to induction. 50 μM Zn Acetate was then added to the cultures along with 0.1 mM IPTG and the cultures were induced at 28° C for 2.5 hrs. Bacteria were pelleted and resuspended in STE buffer (50 mM Tris- HCl, pH 8.0, 150 mM NaCl, 10 mM EDTA) containing protease inhibitors (10 μM Aprotinin, 100 μM Pepstatin A, 10 μM Leupeptin and 100 μM PMSF). DTT was added to 1 mM and the pellet was vortexed before adding 100 μl of lysozyme (20 mg/ml stock) (Roche). The suspension was then incubated on ice for 15 min. 1/50 the volume of 10% taurocholic acid (Calbiochem) was added along with final concentrations of 0.1mM DNase (Sigma) and 0.1 mM $MgCl_2$. The suspension was incubated on ice for 10 min, sonicated 3 X 20 sec using a probe sonicator with an amplitude of 20%, and then centifugated at 14 000g for 15 min. The supernatant was filtered through cheesecloth and 1/50 the volume of 20% Triton X-100 (Sigma) was added. Glutathione sepharose bead slurry (Amersham) was added to the lysate and incubated at 4° C for 1 hr on a rotating platform. Bead-protein complexes were collected and washed 4X in NETN buffer (2 M Tris-HCl, pH 8.0, 4 M NaCl, 20% Triton X-100, 0.5 M EDTA). The bead complexes were then stored in 200 μl of NETN buffer and 50 μM Zn acetate until further use. GST-fusion proteins were quantified by SDS-PAGE using bovine serum albumin (BSA) as a protein standard prior to performing pull-down analysis.

Cell lysates for pull-down analysis were prepared by lysing 100 mm dishes of untransfected, UbSmac and UbΔAVPI-Smac transfected 293A cells in lysis buffer (50 mM Tris-HCL, pH 8.0, 150 mM NaCl, 0.1% Triton X-100, 10 mM EDTA) and protease inhibitors. Lysates were collected in Eppendorf tubes, incubated on ice for 20 min and spun at 17 000 g for 5 min. For pull-down analysis, 100 μg total cell lysate and 2.5 μg of GST-fusion protein were mixed in an Eppendorf tube and 500 μl lysis buffer was added to allow for adequate mixing on a rotating shaker. After a 2 hr incubation at 4° C, the protein-bead complexes were washed 3X in ice cold lysis buffer. Beads were syringe dried and resuspended in a small volume of 1X sample buffer prior to SDS-PAGE.

Cell Death Assays

WST-1

HeLa cells were plated and transfected simultaneously in 96 well dishes. Plasmid DNA (320 ng/well) was diluted in 25ul/well Opti-MEM (Gibco-BRL) and dispensed in 96 well plates. 0.4 μl/well of Lipofectamine 2000 was diluted in 25 μl/well OptiMEM and added to each well. HeLa cells were trypsinized, counted, adjusted to 5 X 10^5 cells per ml, and 100 μl of cell suspension added to each well. At 24 hrs post-transfection, the media was replaced with DMEM-10%FCS containing etoposide (Sigma) at varying doses in triplicate. Cell viability was determined 48 hrs post-exposure to etoposide using water soluble tetrazolium (WST-1) (Boerhinger-Mannheim).

Trypan Blue Exclusion

293A cells were plated in 12 well plates in triplicate at a density of 1 X 10^5 cells per well. The following day the cells were transfected with 1 μg per well of UbSmac or UbSmac-RING plasmids. At 24 hrs, the cells were treated with 1 ng and 10 ng of TRAIL

(R&D Systems) diluted in DMEM and 2.5% FCS. At 24 hrs the cells were trypsinized and resuspended in 1 ml of DMEM media containing 10% FCS and counted using the automated Vi-Cell™ Cell Viability Analyzer (Beckman Coulter).

Annexin V

293A cells were plated in 12 well plates in triplicate at a density of 1 X 10^5 cells per well. The following day the cells were transfected with 1 μg per well of UbSmac or UbSmac-RING plasmids. At 24 hrs, the cells were treated with 1 ng and 5 ng of TRAIL (R&D Systems) diluted in DMEM and 2.5% FCS. At 24 hrs the culture media, PBS wash and trypsinized cells were collected and pooled together and spun at 1 500g for 7 min at 4° C. The cell pellet was washed 2X in cold 2% BSA diluted in PBS. Cells were Annexin V and Propidium Iodide stained using an Annexin V labeling kit (R&D Systems) according to manufacturer's protocol. Cells were analyzed by Fluorescence Activated Cell Sorting (FACs), using the BDFACSCanto™ Flow Cytometer (BD Biosciences).

SDS-PAGE and Western Blotting

Protein samples in 1X sample buffer were electrophoresed on 12% polyacrylamide gels and electroblotted onto Immobilon-PVDF™ membrane (Millipore) at 15V for 30 min using a semi-dry electrotransfer apparatus (Hoefer Semiphor). Blots were immediately placed into 5% skim milk in PBS-0.1% Triton X-100 (PBST) and incubated overnight. This was followed by a 1hr incubation in primary antibody diluted in 2% skim milk in PBST. Blots were washed 3 x 5 min in 2% skim milk in PBST then incubated for 1 hr in HRP-conjugated secondary antibody diluted in 2% skim milk-PBST. Antibody binding was detected by enhanced chemiluminescence (ECL) (Amersham) using Kodak film.

Immunofluorescence Microscopy

Cells grown on coverslips were rinsed briefly in PBS and were then fixed in 4% paraformaldehyde for 5 min. The coverslips were then rinsed 3 x 5 min in PBS and the cells permeabilized in 0.2% Triton X-100 (Pierce) for 20 min. The coverslips were again rinsed 3 X 5 min in PBS. Immunofluorescence labeling was performed sequentially at room temperature. All antibody incubations lasted 45 min, with 3 X 5 min washes in PBS between each application of antibody. Following the incubation of the secondary antibody, cells were washed 3 X 5 min in PBS and were then counterstained with 1 mg/ml Hoescht (Molecular Probes) diluted 1:5000 to visualize nuclei. Cells were then washed 30 s in PBS and mounted in Vectashield mounting medium (Vector). Immunolabeled samples were visualized using a Zeiss Axiophot epifluorescence microscope equipped with a 100- W mercury arc lamp. Images were digitally recorded with an AxioCam CCD camera using AxioVision v3.1 software.

Antibodies

Mouse monoclonal anti-mitochondrial heat shock protein-70 (HSP70) (Affinity Bioreagents Inc.) was used at 1:100 for immunofluorescence labeling and 1:200 for Western blotting. Rat monoclonal anti-HA (Roche) was used at 1:500 for Western blotting. Mouse monoclonal anti-Smac/DIABLO (Zymed) was used at 1:100 for immunofluorescence labeling and 1:500 for Western blotting. Rat monoclonal anti-Smac/DIABLO (Calbiochem) was also used at 1:500 for Western blotting. 5 µl of rabbit polyclonal anti-RIAP3 (rat XIAP) was used for immunoprecipitation and diluted 1:2000 for Western blotting. The rabbit polyclonal anti-RIAP1 (rat HIAP1) antibody was diluted 1:2000 for Western blotting. The mouse monoclonal anti-myc antibody was used for immunoprecipiations (2 µg) and was also

diluted 1:10 000 for Western blotting. The secondary antibodies used included goat polyclonal anti-mouse IgG $(Fab')_2$ conjugated to Alexa 488 (Molecular Probes) used at 1:400 for immunofluorescence microscopy, goat polyclonal anti-mouse IgG $(Fab')_2$ conjugated to indocarbocyanine (CY3; Jackson) was used at 1:400 for immunofluorescence microscopy. For Western blotting, a goat anti-mouse IgG conjugated to horseradish peroxidase (HRP; BIORAD) was used at 1:5000. Goat anti-rat IgG conjugated to HRP (Amersham) was used at 1:5000 for Western blotting. As well, goat anti-rabbit IgG conjugated to HRP (Amersham) was used at 1:5000 for Western blotting.

Statistical Analysis

All quantitative determinations were performed on triplicate samples within each experiment. Three complete and independent experiments were performed in all cases and the data was then pooled and plotted as the mean $\pm$ standard deviation (SD). Results were analyzed using paired two-tailed Student's t-test. p values < 0.05 were considered statistically significant.

Chapter III

Characterization Of The Ubiquitin-Smac Fusion System

Introduction

Smac is a mitochondrial protein that is proteolytically processed and released during apoptosis along with cytochrome c and other proapoptotic factors. Once in the cytosol, Smac protein binds to inhibitors of apoptosis (IAP) proteins and disrupts the ability of the IAPs to inhibit Caspases, -3, -7 and -9. The ability of Smac to negatively regulate the IAPs makes this protein an excellent candidate to be used as a part of a therapeutic strategy in the treatment of cancer. Cancer cells, however, typically display deregulated apoptotic pathways, which results in the over-expression of not only anti-apoptotic proteins such as the IAPs, but quite frequently, Bcl-2. Consequently, Bcl-2 over-expression inhibits the release of cytochrome c and Smac. Thus, the ability to circumvent the requirement for mitochondrial processing and release is critical to developing Smac as a possible gene therapy payload in cancer chemosensitization.

The consequences of Smac expression have been complicated by the absolute requirement for an alanine at the amino terminus of the mature protein. Microinjection or transfection of Smac-like peptides, modeled after the amino terminus of the mature protein, recapitulate some, but not all of the effects of Smac (Srinivasula *et al.*, 2000). Transfection of plasmids encoding the full length Smac open reading frame results in the accumulation of excess Smac in the mitochondrial intermembrane space. Although it has been demonstrated that Smac overexpression sensitizes cells to apoptotic triggers, such as UV light, death ligands, or chemotherapeutic drugs (Ekert *et al.*, 2001; Li *et al.*, 2002; Guo *et al.*, 2002) it is difficult to distinguish the effects of Smac release from those caused by other pro-apoptotic factors that are released concurrently.

As an approach to dissecting the consequences of mature Smac in the cytosol of cells, we developed a novel system using ubiquitin (Ub) fusion proteins. Ubiquitin is synthesized in cells as a precursor polyprotein, in which the Ub coding region is fused to itself and to ribosomal protein subunits (reviewed in Glickman and Ciechanover, 2002). All cells possess ubiquitin specific proteases, which hydrolyse the isopeptide bond between Ub-Ub polymers or Ub-protein substrates. No sequence requirements are present downstream of the Ub peptide, thereby allowing the generation of any desired amino terminus for the down-stream fusion partner. We thus used this system to generate mature Smac protein initiating with the correct AVPI amino terminus in the cytosol of transfected cells, bypassing the normal requirement for mitochondrial processing.

Results

Ubiquitin-Smac Protein is Rapidly and Completely Processed to Yield Mature Smac

In order to bypass the mitochondrial requirement for Smac processing, we developed a ubiquitin fusion system. As shown schematically in Fig.3-1A, the coding region for human ubiquitin (76 codons) was fused in frame to the 184 codons corresponding to processed, mature mouse Smac/DIABLO. In addition, an HA tag was added to the carboxy terminus to allow distinction between the transgene encoded and the endogenous Smac proteins. Cleavage of the 31.4 kDa precursor protein by the endogenous ubiquitin specific proteases was predicted to occur immediately following the carboxy-terminal Gly-Gly dipeptide of ubiquitin, generating the correct Ala-Val-Pro-Ile amino terminus of processed, cytosolic Smac.

Plasmid transfection in HeLa cells resulted in the rapid and complete processing of the expressed Ub-Smac fusion protein. As seen in Fig.3-1B, no unprocessed UbSmac (31.4 kDa) protein was detectable by Western blot with either anti-Smac or anti-HA (data not shown). The endogenous Smac protein was also visible by Western blot, both the unprocessed, mitochondrial precursor form and the mature cytosolic form (Fig. 3-1B, lane 2). In transfected cells, the processing of Ub-Smac was predicted to generate a 21.6 kDa mature Smac protein, slightly larger than the endogenous mature human Smac (due to the presence of the HA tag), but smaller than the endogenous Smac precursor protein.

In order to determine whether the processed Smac protein was capable of binding to XIAP protein, we performed GST pull-down analysis. Protein lysates were prepared from Ub-Smac transfected cells, allowed to bind to recombinant, glutathione bead-bound XIAP protein fragments, and washed extensively. Western blot analysis with anti-Smac (Fig. 3-

1B) or anti-HA (not shown) demonstrated that the transfected Smac protein bound to XIAP BIR3 and BIR2, but not to BIR1. Although GST pull down analysis is not quantitative, there was an apparent and consistently observed enhanced XIAP BIR3 binding relative to BIR2, as has been described in the literature (Liu *et al.*, 2000). We next confirmed interaction between XIAP and Smac *in vivo*. Endogenous XIAP protein was immunoprecipitated with rabbit polyclonal anti-RIAP3 (the rat homolog of XIAP) antibody. Western blot analysis was then carried out with anti-HA. As seen in Fig. 3-1C, processed U-Smac bound to XIAP.

Smac Protein Derived from the Ub-Smac Precursor Protein is Cytoplasmic

The principal advantage of this system is the ability to express mature Smac in the cytoplasm. To confirm that the expressed Smac resides in the cytoplasm, we performed immunofluorescence microscopy. Anti-Smac immunofluorescence microscopy of untransfected cells shows a punctate staining pattern with some generalized cytoplasmic staining (Fig. 3-2A). We interpreted this as mitochondrial localization (as previously reported; Du *et al.*, 2000; Verhagen *et al.*, 2000) with perhaps some leakage of Smac into the cytoplasm. An antibody to mitochondrial HSP70 was then used to illustrate the mitochondrial staining pattern, as seen in Fig. 3-2B. Labeling of UbSmac transfected cells with anti-Smac antibody showed a clearly cytoplasmic staining pattern with relatively little mitochondrial labeling, due to the endogenous Smac protein (Fig. 3-2C). Unfortunately, the commercially available anti-HA antibodies do not function well in immunofluorescence microscopy, which would have allowed us to distinguish the UbSmac derived protein from the endogenous Smac. Confirmation that the UbSmac derived, mature Smac was localized to the cytoplasm was obtained by cell fractionation, in which mitochondrial, cytoplasmic, and light membrane (endoplasmic reticulum and Golgi) fractions were prepared. As seen

Figure 3-1. The ubiquitin-Smac expression system.

(A) Schematic diagram of the UbSmac expression vector. The cleavage site at the end of the ubiquitin peptide sequence is indicated, which generates the correct amino-terminus corresponding to mature Smac released from the mitochondria during apoptosis.

(B) GST-Pulldown and Western blot analysis with anti-Smac antibody. Endogeneous Smac is present in two forms, one possessing the 55 amino acid mitochondrial targeting peptide (mt-Smac), and cytosolic Smac in which this leader peptide has been removed (cyto-Smac).

(C) Co-immunoprecipitation of Smac-transfected cell lysates using anti-RIAP3 (rat XIAP). Western blot analysis using the anti-HA antibody shows overexpressed UbSmac present in cell lysates (left panel). Following immunoprecipitation with anti-RIAP3, Western blot analysis shows that UbSmac co-immunoprecipitated with XIAP (right panel).

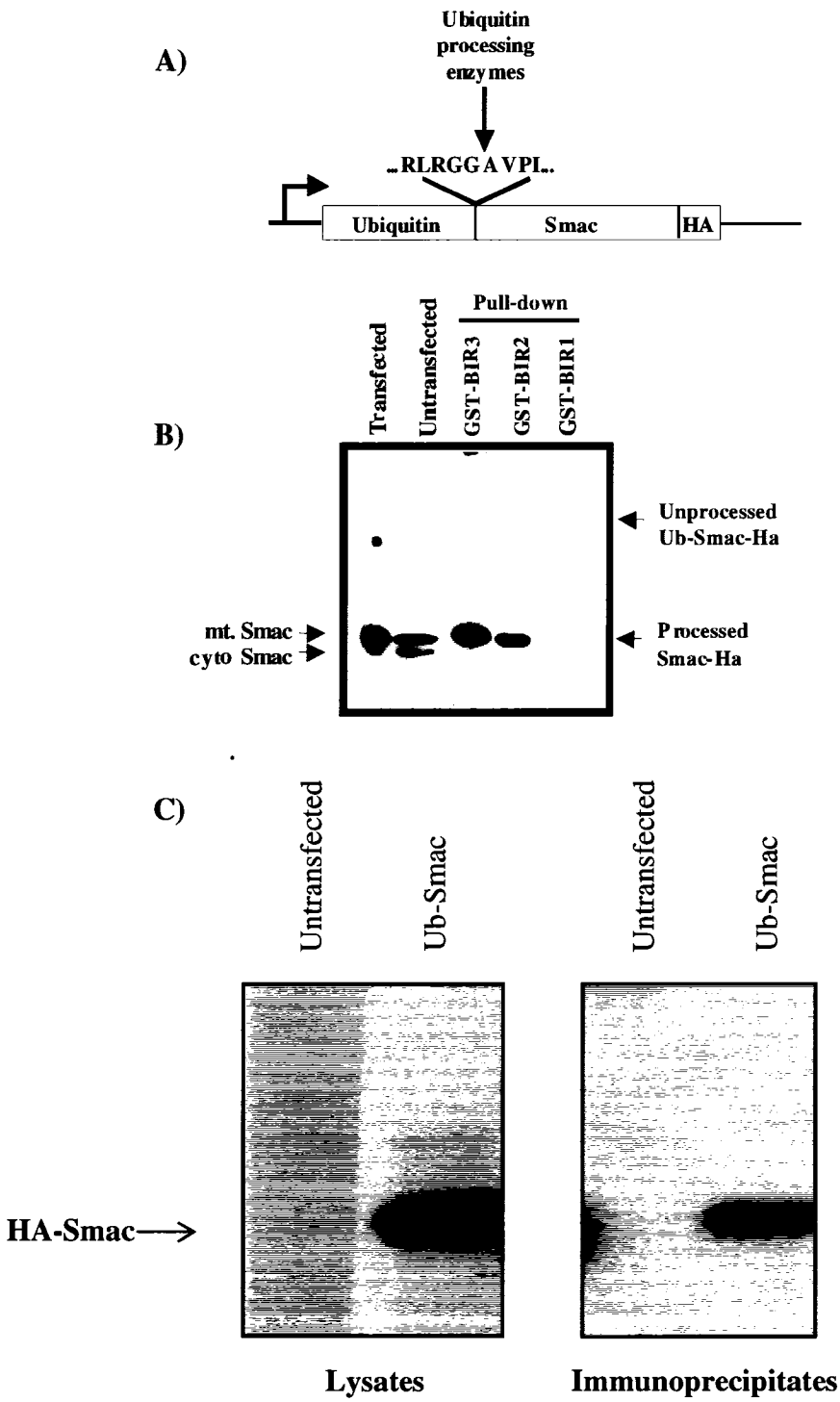
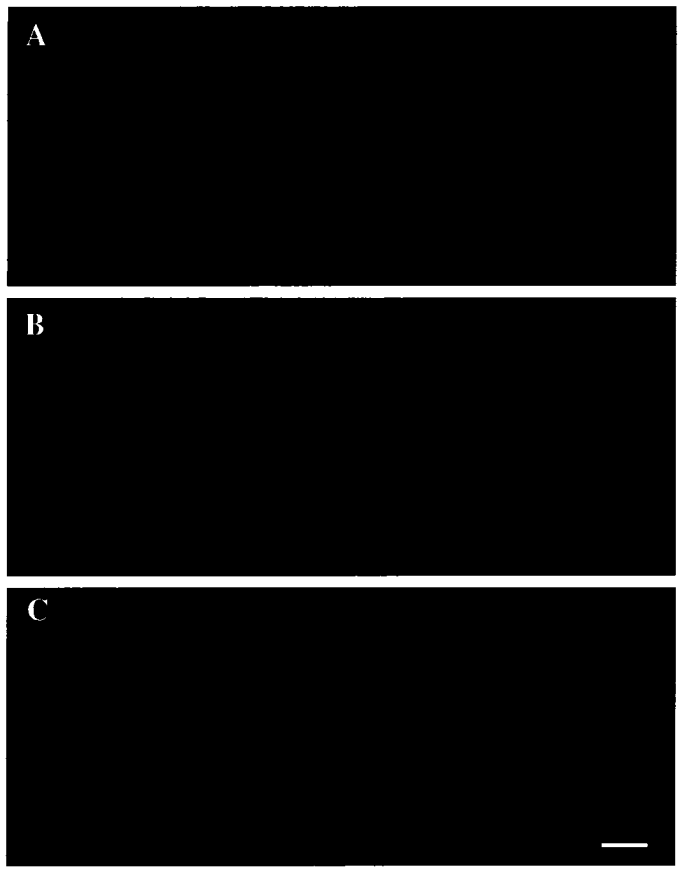
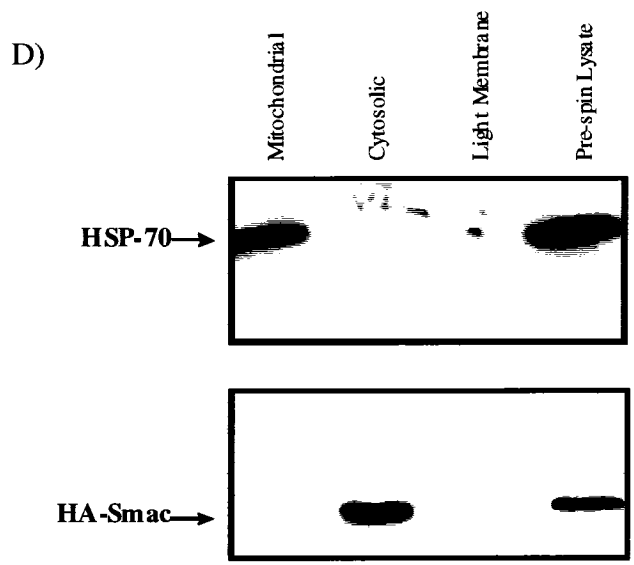

A)
Ubiquitin processing enzymes
..RLRGGAVPI..
Ubiquitin
Smac
HA
B)
Transfected
Untransfected
Pull-down
GST-BIR3
GST-BIR2
GST-BIR1
Unprocessed Ub-Smac-Ha
mt. Smac
cyto Smac
Processed Smac-Ha
C)
Untransfected
Ub-Smac
Untransfected
Ub-Smac
HA-Smac
Lysates
Immunoprecipitates

Figure 3-2. UbSmac expression generates cytoplasmic, mature Smac protein.

A) Immunofluorescence labeling of endogenous Smac in untransfected HEL299 cells **B)** mitochondrial HSP70 and **C)** overexpressed UbSmac in transfected HEL299 cells. Each micrograph shows the merged image of both the DAPI and the immunostaining. Bar represents 100 uM.

(D) Cell fractionation of UbSmac transfected HEL299 cells. Western blot analysis using anti-HSP70 (upper panel) is a control for the cell fractionation technique and shows mitochondrial protein HSP70 localized to the mitochondrial fraction. Western blot analysis using anti-HA shows overexpressed UbSmac localized to the cytosolic fraction (lower panel). Pre-spin lysate shows the presence of HSP70 and UbSmac prior to the fractionation procedure.


A
B
C


D)
Mitochondrial
Cytosolic
Light Membrane
Pre-spin Lysate
HSP-70
HA-Smac

in Fig. 3-2D, mitochondrial HSP-70 partitioned cleanly in the mitochondrial fraction, whereas the Smac protein (detected with the anti-HA antibody) localized exclusively in the cytoplasmic fraction.

Smac Function is Dependent on the Amino-Terminal AVPI Tetrapeptide

Next, the contribution of the Ala-Val-Pro-Ile amino terminal tetrapeptide of mature Smac to the Smac-XIAP interaction was examined. Site-directed mutagenesis was performed on the UbSmac plasmid such that the 12 nucleotides encoding the first four amino acids of mature Smac were deleted. HeLa cells were transfected with UbSmac and UbSmac-ΔAVPI expression plasmids, and expression was confirmed by both anti-Smac (not shown) and anti-HA Western blot (Fig. 3-3). Pull-down analysis was performed with GST-XIAP-BIR1, -BIR2, and -BIR3 recombinant proteins. As seen in Fig.3-3, Smac bound to XIAP BIR3 most strongly, and to a lesser extent, to BIR2, and not to BIR1. In contrast, the Smac-ΔAVPI protein failed to bind to any of the recombinant XIAP BIR domains.

The consequences of expressing cytosolic, mature Smac on cell viability was examined in both HeLa and 293A cells. No decrease in cell viability was observed either short (24 hrs post transfection) or longer term (5 days post transfection), as assessed relative to reporter-gene transfected cells (LacZ or GFP; Fig. 3-4). To determine whether mature Smac predisposes cells to undergo apoptosis, etoposide was used as a trigger. Cells were transfected in 96 well plates. At 24 hrs post transfection, etoposide was added to triplicate samples in doses ranging from 5 μM to 500 μM. Viability was evaluated by colorimetric assay based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells, as well as by cell count using trypan blue exclusion. As seen in Fig. 3-5,

Figure 3-3. Smac interaction with XIAP is dependent on the AVPI tetrapeptide sequence.

GST pull-down analysis was used to demonstrate that UbSmac protein binds to XIAP BIR3>BIR2, and not to BIR1. In contrast, UbΔAVPI-Smac does not bind to any of the recombinant XIAP BIR domains (right panel). Western blot analysis using anti-HA antibody shows both overexpressed UbSmac and UbΔAVPI-Smac were present in cell lysates prior to performing the GST pull-down analysis (left panel).

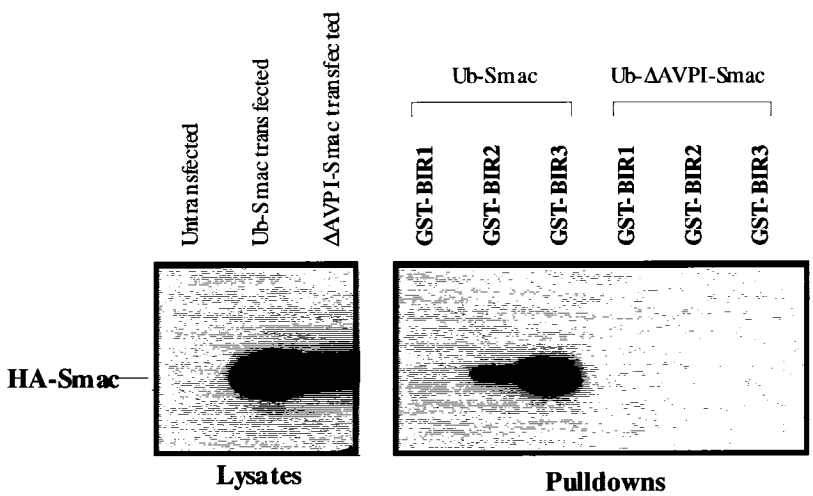
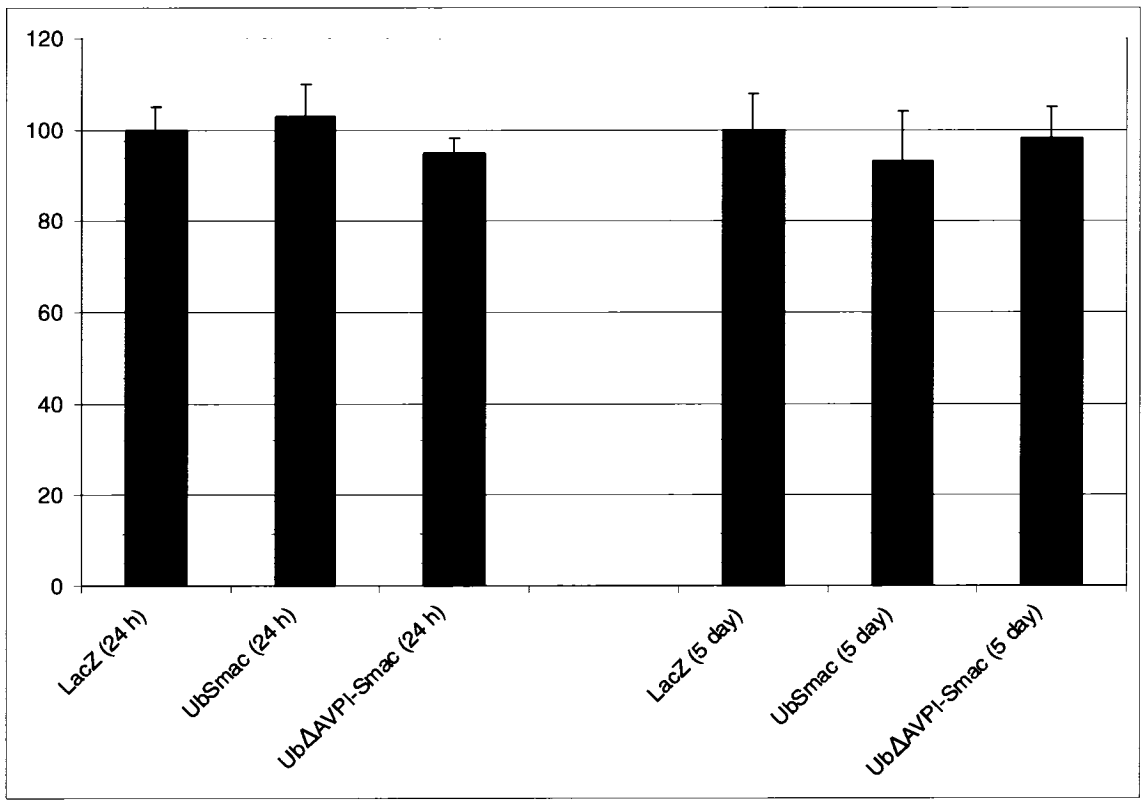

Untransfected
Ub-Smac transfected
ΔAVPI-Smac transfected
Ub-Smac
Ub-ΔAVPI-Smac
GST-BIR1
GST-BIR2
GST-BIR3
GST-BIR1
GST-BIR2
GST-BIR3
HA-Smac
Lysates
Pulldowns

Figure 3-4. UbSmac expression does not kill cells.

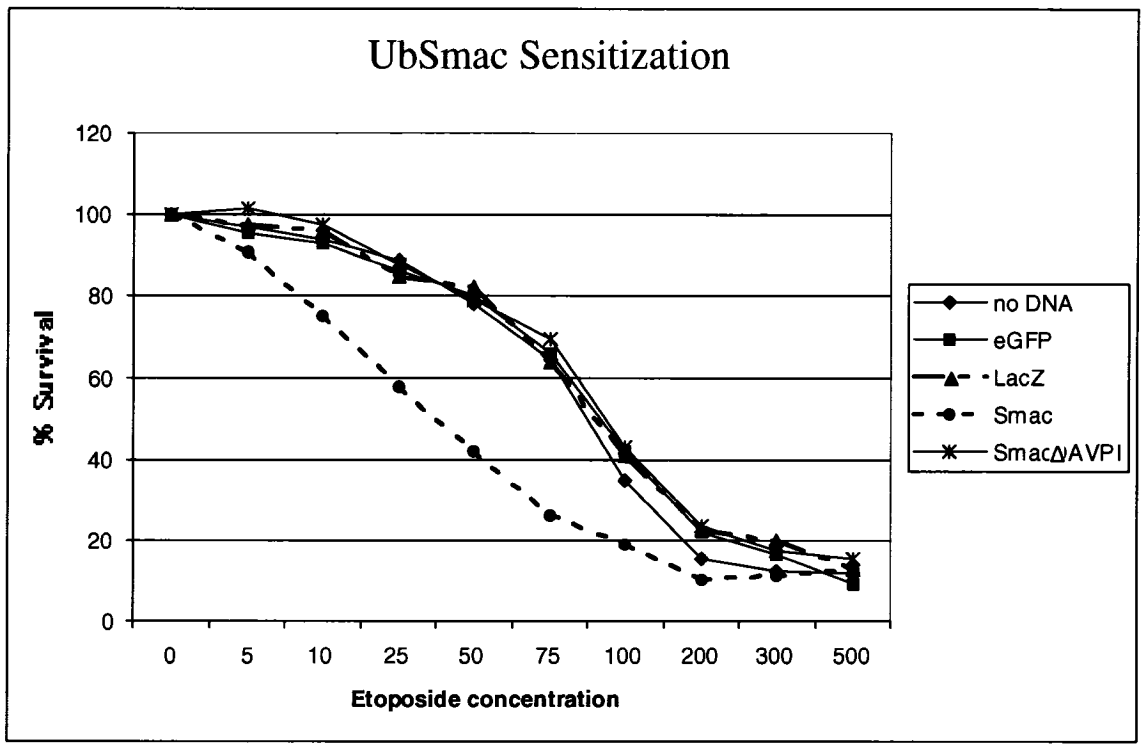
Triplicate samples of HeLa cells transfected with plasmids encoding LacZ, UbSmac or UbΔAVPI-Smac were harvested and viable cell counts were determined by trypan blue exclusion at 24 hrs and 5 days post transfection. Transfection efficiency was estimated at greater than 90%, based on X-gal staining.


120
100
80
60
40
20
0
LacZ (24 h)
UbSmac (24 h)
UbΔAVPI-Smac (24 h)
LacZ (5 day)
UbSmac (5 day)
UbΔAVPI-Smac (5 day)

control plasmids expressing GFP or LacZ had no effect on cell viability . In contrast, cytosolic Smac greatly sensitized cells to etoposide mediated killing at all exposure levels. Deletion of the Ala-Val-Pro-Ile tetrapetide sequence in UbSmac-ΔAVPI transfected cells completely abrogated the effect of Smac overexpression (Fig. 3-5).

Figure 3-5. UbSmac expression sensitizes HeLa cells to etoposide mediated apoptosis.

HeLa cells were transfected with the indicated plasmids and exposed to etoposide in doses ranging from 5 to 500 μM. Survival was assessed by WST-1 colorimetric assay and expressed as % survival relative to cells exposed to 0 μM etoposide.


UbSmac Sensitization
120
100
80
60
40
20
0
% Survival
0
5
10
25
50
75
100
200
300
500
Etoposide concentration
no DNA
eGFP
LacZ
Smac
SmacΔAVPI

Discussion

Cancer continues to be one of the leading causes of morbidity and mortality in Western countries. It is now widely accepted that deregulated apoptosis is a fundamental characteristic of cancer cells (reviewed in Hanahan and Weinberg, 2000), necessary for the suppression of normal cell death that would occur due to deregulated proto-oncogene expression. Furthermore, cancer cells encounter severe environmental stresses, including reduced oxygen levels in solid tumor masses, loss of trophic factor support, and detachment from the extracellular matrix, any of which would trigger apoptosis in a normal cell. Finally, chemotherapeutic drugs and radiation therapy act by triggering apoptosis, and treatment failure may be in part due to further mutations in critical cell death pathways. Indeed, virtually all components of the endogenous and exogenous apoptosis pathways are mutated or inactivated in at least some cancers (reviewed in Igney and Kramer, 2002). Numerous strategies are under investigation to reestablish normal apoptosis in cancer cells, thereby triggering outright apoptosis or sensitization to treatment.

The IAPs control the activity of strategic Caspases involved in the initiation and execution phase of apoptosis, and are frequently over-expressed in cancer cell lines and primary biopsy tumor samples (Tamm *et al.*, 2000; Hofmann *et al.*, 2002; Gordon *et al.*, 2002; McElney *et al.*, 2002). IAP- mediated Caspase inhibition contributes to chemotherapeutic drug and radiation resistance. Interestingly, while Caspase-8 and -10 gene inactivation has been documented (Teitz *et al.*, 2000; Harada *et al.*, 2002), no cases of dual Caspase-3 and -7 gene deletion or silencing have been reported, suggesting that these Caspases play indispensable roles in cell processes in addition to apoptosis (reviewed in Los *et al.*, 2001). If one presupposes that Caspases -3 and -7 remain functional, albeit latent, in

all cancer cells, then the IAPs constitute the final point of deregulation which cancer cells can employ to suppress apoptosis. It is therefore reasonable to propose that strategies that interfere with IAP expression or function will find utility in increasing the susceptibility of cancer cells to drug and radiation therapies. Smac-based peptide therapy was recently demonstrated to increase the efficacy of TRAIL ligand in triggering tumor regression in a mouse xenograft model of malignant glioma, illustrating the therapeutic potential of IAP inhibitors (Fulda *et al.*, 2002a).

Cancer cells frequently over-express anti-apoptotic Bcl-2 family members or delete/inactivate pro-apoptotic Bcl-2 family members, thereby suppressing cell death by blocking the release of apoptogenic factors, such as cytochrome c and Smac. Expression strategies that bypass the mitochondrial control point are advantageous both for the precise dissection of the contributions of various apoptotic factors, as well as potentially in the development of cancer gene therapy payloads. Previous efforts to characterize cytoplasmic Smac have employed either micro-injection of recombinant protein (Deshmukh *et al.*, 2002) or the use of plasmid expression vectors encoding the Smac cDNA lacking the mitochondrial targeting sequence (Guo *et al.*, 2002; Fulda *et al.*, 2002a). Presumably, some degree of initiation methionine removal occurs, thereby generating the necessary amino terminal alanine, but the extent of this processing has not been reported. In this chapter, a unique expression system that generates mature, cytoplasmic Smac protein without requiring mitochondrial permeabilization was characterized. The mature Smac protein derived from the ubiquitin fusion protein interacts with XIAP, and is localized to the cytoplasm of the cell. Finally, Smac mediated IAP inhibition was not demonstrated to be sufficient to trigger outright cell death, but did however, sensitize cells to apoptotic triggers.

The mechanism of action of Smac is still controversial. Splice isoforms of Smac (Smacβ, Smacγ, Smacδ) have been identified that lack the mitochondrial targeting sequence and the IAP binding motif, and localize to the cytoplasm. Furthermore, Smacβ, as well as additional deletion mutants, were reported to promote apoptosis as efficiently as full length Smac (Roberst *et al.*, 2001). These results were interpreted as an indication that the primary means by which Smac triggers apoptosis is via its carboxy terminal alpha-helical bundle domain, and that IAP binding is a secondary effect. In contrast, several studies have suggested that the amino terminal seven amino acids of Smac are sufficient to sensitize cells to apoptotic triggers (Srinivasula *et al.*, 2000; Guo *et al.*, 2002; Fulda *et al.*, 2002a). This study directly addressed the contribution of the AVPI tetrapeptide. Site directed mutagenesis was used to delete this sequence. No binding of the Smac-ΔAVPI protein, either *in vitro* using GST fusion pull-downs, or *in vivo* by co-immunoprecipitation analysis (data not shown) was observed. Furthermore, in these assays, the deletion of the IAP recognition motif completely abrogated sensitization to etoposide mediated killing. Taken together, these results suggest that the primary mechanism of Smac's pro-apoptotic activity is via the inhibition of IAP function. Finally, the ubiquitin fusion method is readily amenable for the study of other non-methionine initiating proteins, such as Omi, and was recently employed in the characterization of the Jafrac2 protein, a Drosophila IAP antagonist that is processed and released from the endoplasmic reticulum (Tenev *et al.*, 2002).

Chapter IV

Smac-based peptides

Introduction

The crystal structure of Smac indicates that the protein consists of three extended alpha helices bundled together to form an arc-shaped structure, exposing an unstructured amino terminus. Smac homodimerizes through an extensive hydrophobic interface and this homodimerization is essential for its activity (Chai *et al.*, 2000). Following an apoptotic signal a newly generated amino terminus in mature Smac makes critical contacts with both XIAP BIR2 and BIR3, thereby mediating XIAP inhibition. Thus, the first four amino-terminal residues of mature Smac, AVPI, are absolutely required for Smac function and any modifications to this sequence abolishes Smac-IAP interaction (Wu *et al.*, 2000; Chai *et al.*, 2000; Liu *et al.*, 2000; Hunter *et al.*, 2003).

The structure determinations of XIAP in conjunction with Caspases and Smac lead to the identification of a critical pocket and groove on the surface of each BIR domain. Within XIAP BIR3, the binding pocket interacts with Caspase-9, and can be disrupted by the presence of Smac protein, which competes for the same site. It was established that four amino acid residues, AVPI, at the amino terminus of Smac binds to a well-defined binding pocket, or surface groove, in the XIAP BIR3 domain (Fig 4-1) (Liu *et al.*, 2000; Wu *et al.*, 2000). This four amino acid sequence, known as the IAP binding motif (IBM), is present at the amino terminus of the small subunit of Caspase-9 and is structurally similar to the IBMs present in Smac, Omi, and GSPT1, as well as in the *Drosophila* IAP inhibitors, Reaper, Hid, and Grim (Fig 4-2) (Liston *et al.*, 2003).

Peptides or small molecules modeled on the IBM may serve as prototypes for drugs whose activity may complement that of Smac (Wu *et al.*, 2000; Kipp *et al.*, 2002). Initial studies did in fact show that a short 7-residue peptide derived from the Smac N-terminal was able to promote the activation of pro-Caspase-3 *in vitro*, raising the possibility of using small

peptides or peptide mimics to treat cancer cells (Chai *et al.*, 2000). Other research groups showed that when Smac peptides composed of the first 4-8 amino-terminal residues are delivered to MCF-7 breast cancer cells, they can interact with XIAP, c-IAP1, (Arnt *et al.*, 2002; Fulda *et al.*, 2002a) and the single BIR domain of ML-IAP (Vucic *et al.*, 2002). As well as being fully competent in interacting with the IAPs, these peptides significantly enhanced the induction of apoptosis and long term anti-proliferative effects of a range of chemotherapeutics including paclitaxel, etoposide, and doxorubicin. Furthermore, Smac-like peptides delivered in combination with potential cancer therapeutics such as Apo2L/TRAIL dramatically decreased the apoptotic threshold (Arnt *et al.*, 2002; Fulda *et al.*, 2002a).

The treatment of cultured tumor cell lines with Smac-peptides sensitizes these cells to chemotherapeutic drug-induced apoptosis (Guo *et al.*, 2002; Arnt *et al.*, 2002). This enhanced sensitization is credited to the alleviation of IAP suppression of Caspases, thus making a Smac-peptide delivery approach a promising development strategy for cancer therapeutics. Caspase-9 and Smac both possess an IBM, making them competitors for IAP binding. Furthermore, Smac is able to compete with Caspases-3 and -7 for IAP binding. Thus, it is reasonable to propose that Smac-like peptides, possessing a higher affinity IBM than the wild type AVPI Smac-tetrapeptide, should be better competitors for IAP binding than Caspases. This, in effect, would allow for an even greater sensitization to chemotherapeutic-triggered apoptosis than the endogenous Smac sequence. In this study, a phage display library screening approach was used to identify novel, high-affinity amino acid sequences, which would share homology with Smac sequences. Ultimately these novel Smac-like peptides could be used to prevent IAP/Caspase interactions, thereby potentiating apoptosis.

Results

Smac Peptide Screening

To identify novel, high-affinity Smac-like amino acid sequences, a phage display peptide screening method was employed. With this method, a peptide from a random library is expressed as a fusion with the p3 coat protein of bacteriophage M13. The peptide is displayed as a fused protein on the exterior of the phage virion, with the DNA encoding the fusion in the virion. Incubating a library of phage-displayed peptides with purified GST-IAP BIR domains in the presence of glutathione beads gives a physical linkage between the library of random peptide sequence, to the DNA encoding each sequence (Fig 2-1). When the phage is characterized by DNA sequencing, the peptide which has the highest affinity for IAP BIR domains can be identified. In this study, a 7 mer peptide library was screened using GST-fusion proteins encoding BIR3 of XIAP, HIAP1, and HIAP2 to identify peptide sequences. Screening with XIAP BIR3 identified peptides with positively charged amino acids in P2 (Arg, His, Lys), which was not expected from our existing knowledge of XIAP-Smac, or XIAP-Caspase-9 interactions (Fig 4-3). The remaining amino acids identified in the P2 position were uncharged residues (Fig 4-3). Furthermore, all of the peptide sequences that bind to the BIR3 domains of the IAPs tested contain an AxPx sequence, in which the amino terminal alanine position 1 (P1) and the proline in position 3 (P3) are known to be absolutely required (Fig 4-4) (Wu *et al.*, 2000). In contrast, both HIAP1 and HIAP2 BIR3 pulled out only peptides with hydrophobic residues in P2 (Fig 4-4). The physiological relevance of the AxPx sequences identified for HIAP1 and HIAP2 is difficult to interpret, given that little is known about how these IAPs mediate Smac interactions. Therefore,

Figure 4-1. 3D structure of XIAP BIR3, indicating the Smac binding pocket.

This surface map of the XIAP BIR3 domain models the interaction with the first four amino acids of mature Smac. The blue color indicates basic/positively charged regions, while red indicates acidic/negatively charged areas. Analysis of the 3D structure indicates that Smac interaction with XIAP BIR3 is primarily via the four amino acid sequence Ala-Val-Pro-Ile. Key amino acids that form the alanine (A1) binding pocket (Tryptophan^{310}, Asparagine^{319}, Lysine^{307}) are indicated. Valine in position 2 (V2) interacts with Threonine^{308}). The positions 3 Proline (P3) forms the kink in the overall peptide and and may make interactions with Tryptophan^{323}. Isoleucine in position 4 (I4) interacts with Lysine^{297} and Lysine^{299}, both of which form a groove for this interaction. D^{309} is proposed to potentially make interactions with positively charged amino groups in position 2 that are identified in phage display screens. (Reprinted with permission, copyright Liu *et al.* *Nature*. (2000) 408: 1004-1008).

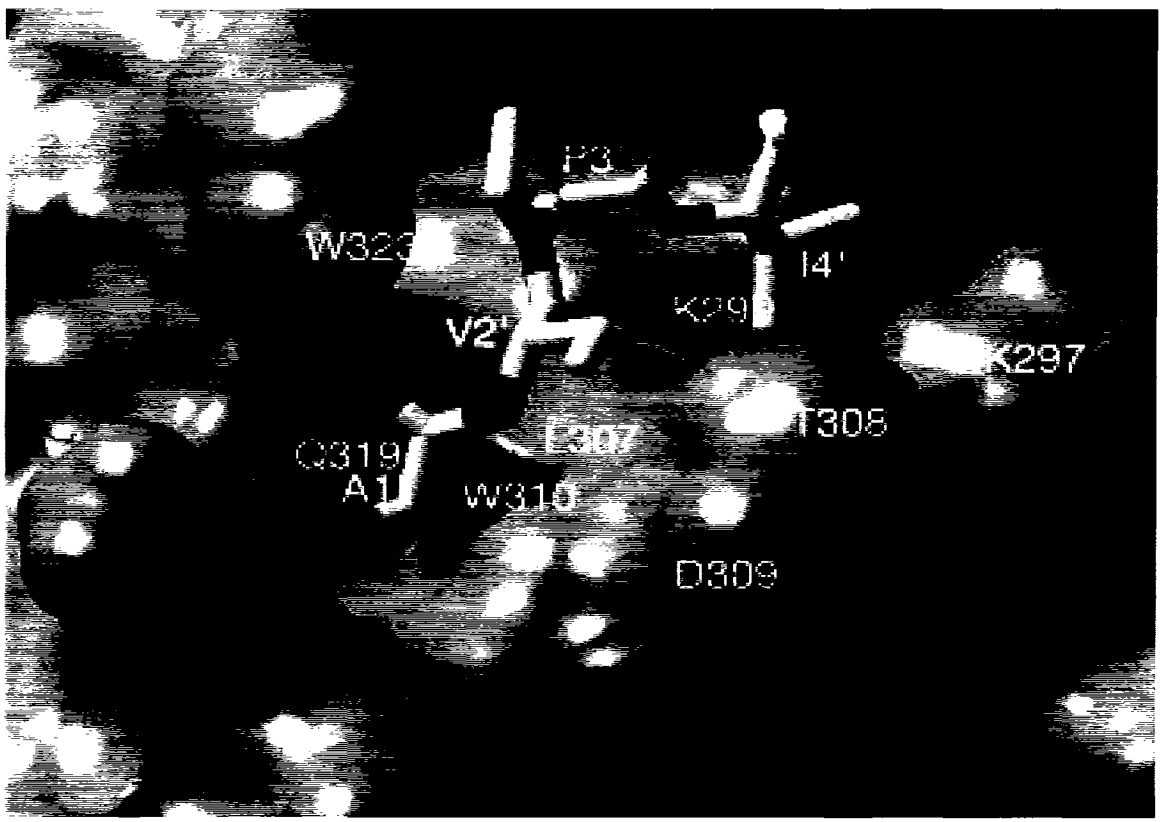

P3'
W323
I4'
K299
V2'
K297
T308
Q319
A1
W310
D309

Figure 4-2. IBM consensus sequences.

The first seven amino acids of the various mature IAP-interacting proteins are shown. Mammalian proteins are presented above the line and *Drosophila* proteins below. Note that all of these IBM-containing proteins conform to the AxPx consensus sequence.

Caspase-9:	A	T	P	F	Q	E	G
Smac:	A	V	P	I	A	Q	K
Omi:	A	V	P	S	P	P	P
Chk1:	A	V	P	F	V	E	D
GSPT1:	A	K	P	F	V	P	N
Reaper:	A	V	A	F	Y	I	P
Grim:	A	I	A	Y	F	I	P
Hid:	A	V	P	F	Y	L	P
Skl:	A	I	P	F	F	E	E
Jafrac2:	A	K	P	E	D	N	E

Figure 4-3. Summary of phage display results with XIAP BIR3.

Twenty representative phage isolates were sequenced after three rounds of affinity purification using XIAP BIR3. The majority of the residues in P2 were charged, unlike the predicted uncharged residues found in P2 of Smac or Caspase-9 (shown above the reference line).

	P1	P2	P3	P4	P5	P6	P7
Smac:	Ala	Val	Pro	Ile	Ala	Gln	Lys
Caspase 9:	Ala	Thr	Pro	Phe	Gln	Glu	Gly
	Ala	Lys	Pro	Leu	Ala	Leu	Thr
	Ala	His	Pro	Gly	Met	Pro	Gln
	Ala	Thr	Pro	Trp	Val	Asp	Gln
	Ala	Arg	Pro	Phe	Ala	Thr	Tyr
	Ala	His	Pro	Val	Met	Pro	Gln
	Glu	Met	Arg	Leu	Gly	Leu	Glu
	Ala	Val	Pro	Leu	Ser	Thr	Gln
XIAP BIR	Leu	Ser	Gly	Ala	Asn	Ser	Thr
binding peptides:	Ala	Arg	Pro	Phe	Ser	Ser	Pro
	Ala	Arg	Pro	Leu	Ser	Asn	Ile
	Ala	Leu	Pro	Leu	Ser	His	Val
	Ala	Thr	Pro	Val	Phe	Asp	Leu
	Ala	Lys	Pro	Phe	Pro	Ser	Ala
	Ala	Thr	Pro	Ile	Trp	Leu	Pro
	Ala	Asn	Pro	Phe	Leu	Ser	Asp
	Ala	Met	Pro	Tyr	Ala	Pro	Gly
	Ala	Thr	Ser	Phe	His	Asp	Ala
	Ala	Leu	Pro	Leu	Thr	Gln	Val
	Thr	Gly	Ala	Ser	His	Ala	Pro

Figure 4-4. Summary of Phage display results for IAP BIR3 domains.

The phage display screen resulted in the identification of AxPx sequences. XIAP BIR3 bound phages encoding p3 proteins with positively charged amino acids in position 2 (P2), whereas both HIAP1 BIR3 and HIAP2 BIR3 bound to phages encoding p3 proteins with uncharged, hydrophobic residues in the P2 position. All three BIR3 domains displayed an absolute requirement for alanine in P1 and proline in P3. Superscript numbers represent the total number of times the specified amino acid appeared.

XIAP BIR3

Ala [Arg^{3} / Lys^{2} / His^{2}] Pro [Leu^{6} / Phe^{5} / Ile^{1}]

HIAP1 BIR3

Ala [Ile ^{6} / Phe ^{5} / Lys ^{2}] Pro [Trp^{3} / Ile^{7} / Phe^{6}]

Asn^{1} Val^{1}

Val^{1} Tyr^{1}

Ser^{1}

Met^{1}

HIAP2 BIR3

Ala [Thr^{4} / Met^{3} / Asn^{3}] Pro [Tyr^{4} / Trp^{3} / Ile^{4}]

Phe^{2} Val^{2}

Ile^{1} Thr^{1}

Ser^{1}

His^{1}

recognizing that XIAP is the most potent IAP, the rest of this study focuses on the most prevalent AxPx sequence identified for XIAP BIR3, which was ARPL (Figs 4-3 and 4-4).

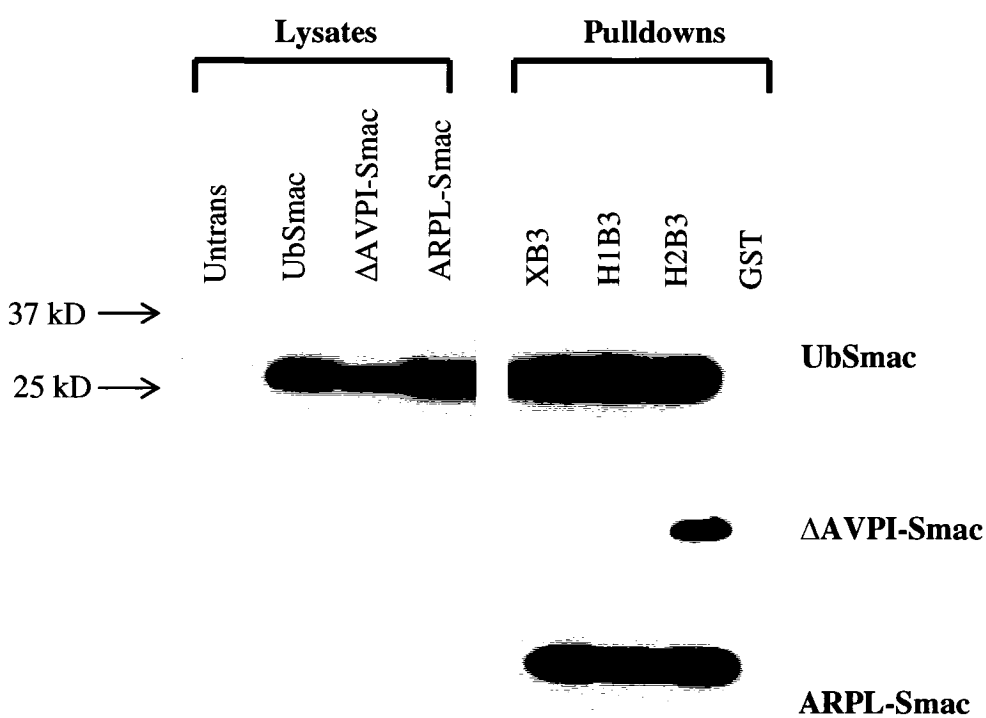
Analysis of Novel Smac Sequences

In order to determine whether the identified ARPL sequence might be of physiological relevance as a high affinity Smac therapeutic, it was necessary to first assess if Smac protein, with an ARPL substitution at the amino terminus, behaves as a true IAP binding protein. Using the ubiquitin-Smac expression system, Smac protein containing the wild type AVPI sequence (UbSmac), a negative control Smac protein with the AVPI sequence deleted (ΔAVPI-Smac), and Smac protein with the AVPI sequence substituted with ARPL (ARPL-Smac) were generated and expressed. These proteins were then used in pull-down assays to observe interactions with the BIR3 domain of XIAP, HIAP1 and HIAP2 (Fig 4-5). It was observed that UbSmac showed a strong interaction with all of the tested IAP-BIR3 proteins. As predicted from the phage display results, ARPL-Smac retained the ability to interact with XIAP BIR3, as well as HIAP1 BIR3, and HIAP2 BIR3 (Fig 4-5). This raises the possibility that the Smac binding clefts in HIAP1- and HIAP2 BIR3 may be structurally similar to that of XIAP BIR3.

The pulldown assay with the ΔAVPI-Smac protein demonstrated that the interactions with XIAP BIR3 or HIAP1 BIR3 were dependent on the tetrapeptide as predicted (Fig 4-5). Surprisingly, however, HIAP2 BIR3 appeared to retain the ability to bind Smac despite the deletion of the AVPI recognition sequence. This observation raised the possibility that Smac interaction with HIAP2 is fundamentally different from the other IAPs and does not require an AxPx sequence. Alternatively, the binding pocket of HIAP2 might be structurally

Figure 4-5. Smac encoding an ARPL IBM sequence retains XIAP binding activity.

Cell lysates were prepared from untransfected 293A cells and cells that were transiently transfected with HA-tagged- UbSmac, -ΔAVPI- Smac, and -ARPL- Smac. Pulldown analysis was performed with individual GST-IAP BIR3 domains from XIAP, HIAP1 and HIAP2. Protein complexes were separated by SDS-PAGE on 12% polyacrylamide gels. Smac interactions were assessed via Western blotting with the monoclonal anti-HA antibody. Overexpressed UbSmac, ΔAVPI- Smac, and ARPL-Smac were present in cell lysates prior to performing the GST pulldown analysis. Pulldown analysis indicated that UbSmac and ARPL-Smac interacted with all of the IAP BIR3 domains. Surprisingly, ΔAVPI- Smac shows an interaction with the HIAP2 BIR3 domain.


Lysates
Pulldowns
Untrans
UbSmac
ΔAVPI-Smac
ARPL-Smac
XB3
H1B3
H2B3
GST
37 kD
25 kD
UbSmac
ΔAVPI-Smac
ARPL-Smac

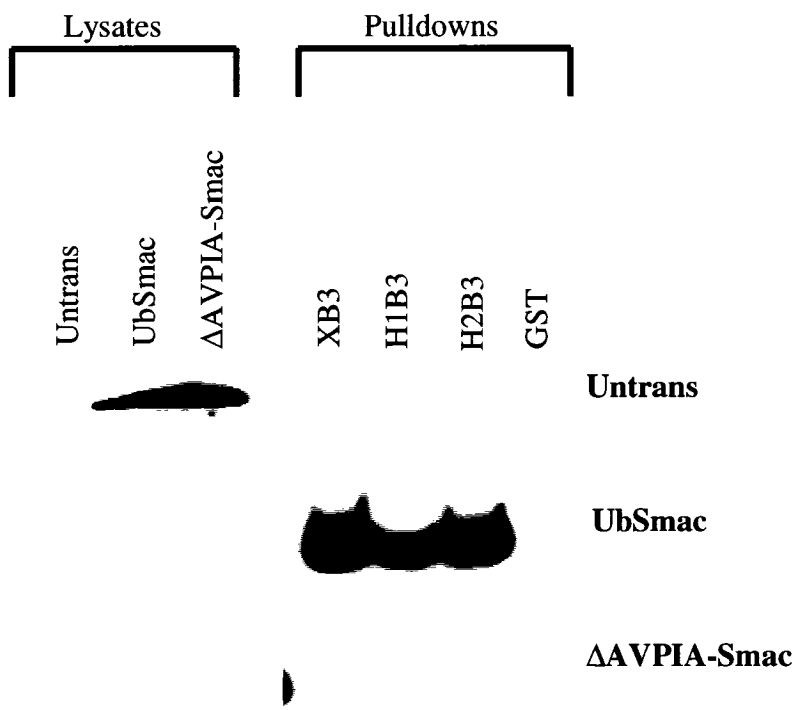
Anti-HA

more variable and only require an amino terminal alanine. To address this issue the first four amino acids of mature Smac were deleted placing the fifth amino acid at the amino terminus, which happens to be an alanine (ΔAVPIA-Smac). Pulldown analysis showed that the interaction with the HIAP2 BIR3 domain was completely ablated in the absence of the amino terminal Smac-AVPIA sequence confirming that the Smac P5 alanine residue does not make contact in the HIAP2 BIR3 binding pocket (Fig 4-6).

To assess whether ARPL-Smac is able to recapitulate all of the IAP binding characteristics of UbSmac, GST-pulldown assays were performed with the individual BIR domains of XIAP, as well as with the full-length IAP proteins. It was observed that both UbSmac and ARPL-Smac had the strongest interaction with XIAP BIR3, a much weaker interaction with XIAP BIR2 and no interaction with XIAP BIR1 (Fig 4-7). The control ΔAVPI-Smac did not show any evidence of binding. When Smac interactions with the full length proteins of XIAP, testes-specific IAP (TsIAP), HIAP1, and HIAP2 were examined, it was observed that UbSmac and ARPL-Smac bind to the all of the IAPs, with the exception of Ts-IAP (Fig 4-8). Furthermore, UbSmac and ARPL-Smac were shown to interact with XIAP not only under *in vitro* pulldown conditions, but *in vivo* as well. When co-immunoprecipitation experiments were performed, it was observed that both over-expressed UbSmac and ARPL-Smac interact with endogenous XIAP. As predicted, the ΔAVPI-Smac did not show any interactions (Fig 4-9).

Figure 4-6. Smac with a deleted AVPIA sequence does not interact with the IAPs.

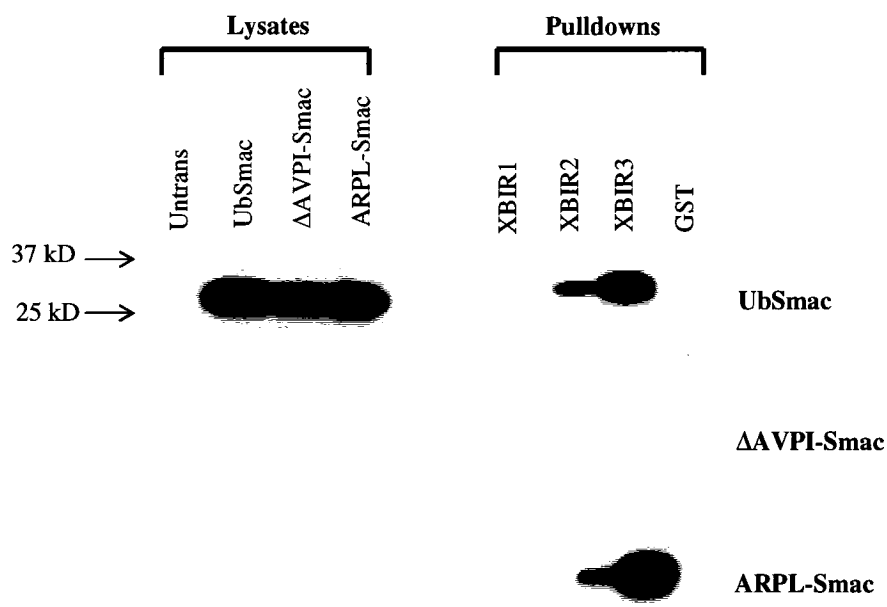
Cell lysates were prepared from untransfected 293A cells and cells that were transiently transfected with HA-tagged- UbSmac, and -ΔAVPIA- Smac. Pulldown analysis was performed with individual GST-IAP BIR3 domains from XIAP, HIAP1 and HIAP2. Protein complexes were separated by SDS-PAGE on 12% polyacrylamide gels. Smac interactions were assessed via Western blotting with the monoclonal anti-HA antibody. Overexpressed UbSmac and ΔAVPI-Smac were present in cell lysates prior to performing the GST pulldowns. Pulldown analysis indicated that UbSmac interacted with all of the IAP BIR3 domains, whereas ΔAVPIA- Smac did not show any interactions.


Lysates
Pulldowns
Untrans
UbSmac
ΔAVPIA-Smac
XB3
H1B3
H2B3
GST
Untrans
UbSmac
ΔAVPIA-Smac

Anti-HA

Figure 4-7. Wild-type Smac and Smac mutants interact with BIR2 and BIR3 of XIAP, but not with BIR1.

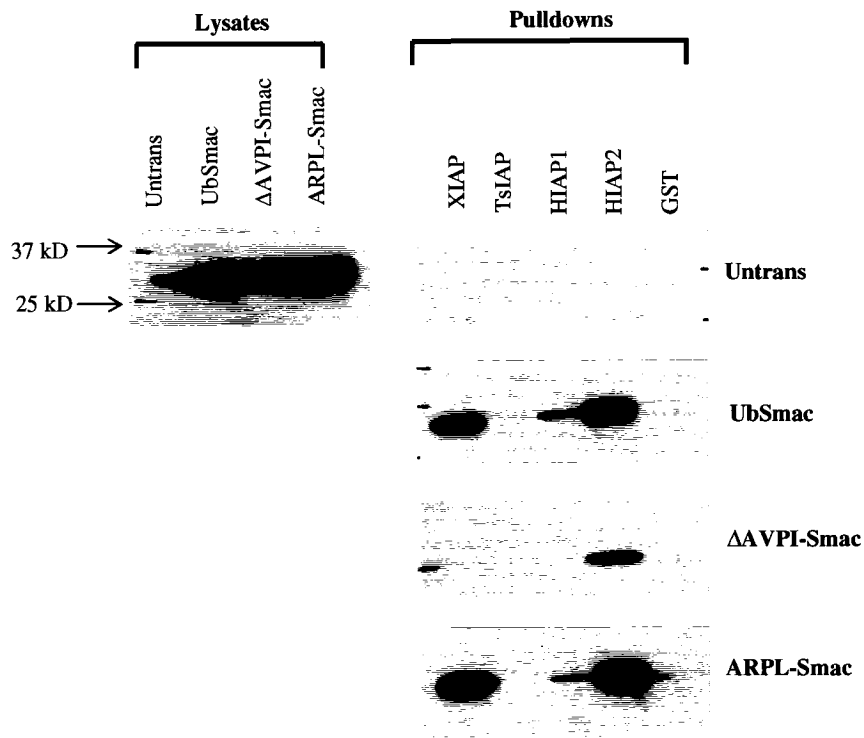
Cell lysates were prepared from untransfected 293A cells and cells that were transiently transfected with HA-tagged- UbSmac, -ΔAVPI- Smac, and -ARPL- Smac. Pulldown analysis was performed with individual GST-XIAP -BIR1, -BIR2 and BIR3. Protein complexes were separated by SDS-PAGE on 12% polyacrylamide gels. Smac interactions were assessed via Western blotting with the monoclonal anti-HA antibody. Overexpressed UbSmac, ΔAVPI-Smac, and ARPL-Smac were present in cell lysates prior to performing GST pulldowns. Pulldown analysis indicated that UbSmac and ARPL-Smac interacted with all of the XIAP BIR2 and XIAP BIR3 domains, but not with XIAP BIR1.


Lysates
Pulldowns
Untrans
UbSmac
ΔAVPI-Smac
ARPL-Smac
XBIR1
XBIR2
XBIR3
GST
37 kD
25 kD
UbSmac
ΔAVPI-Smac
ARPL-Smac

Anti-HA

Figure 4-8. Wild-type Smac and Smac mutants interact with full-length IAP proteins.

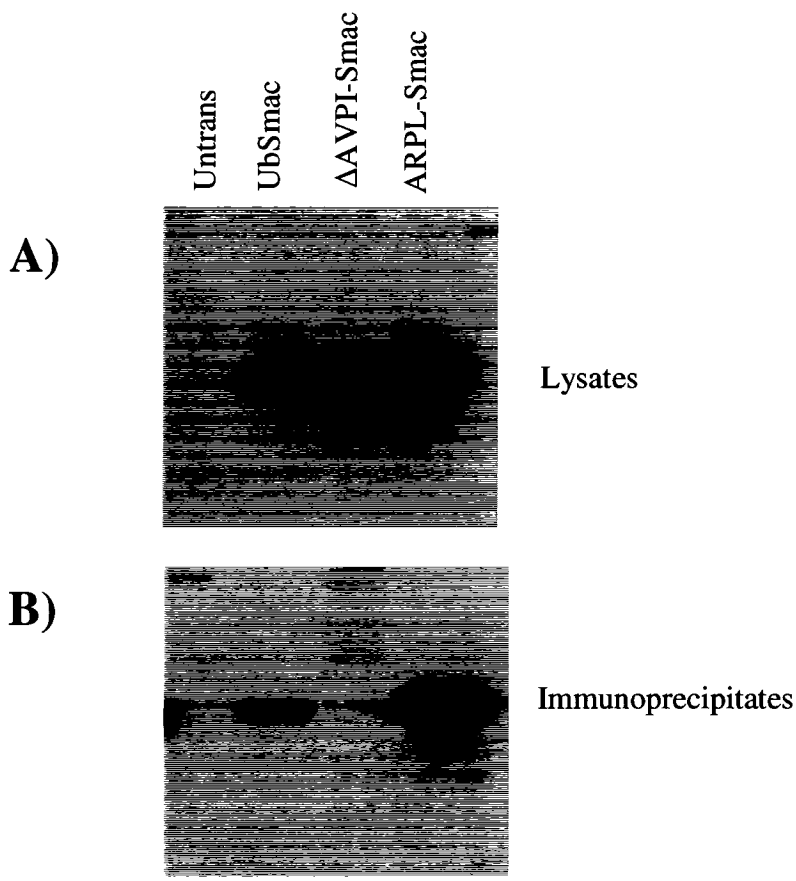
Cell lysates were prepared from untransfected 293A cells and cells that were transiently transfected with HA-tagged- UbSmac, -ΔAVPI- Smac, and -ARPL- Smac. Pulldown analysis was performed with full length GST-XIAP, -TsIAP, -HIAP1, and –HIAP2. Protein complexes were separated by SDS-PAGE on 12% polyacrylamide gels. Smac interactions were assessed via Western blotting with the monoclonal anti-HA antibody. Overexpressed UbSmac, ΔAVPI-Smac, and ARPL-Smac were present in cell lysates prior to performing GST-pulldowns. Pulldown analysis indicates that UbSmac and ARPL-Smac interacted with XIAP, HIAP1 and HIAP2, but not TsIAP. ΔAVPI- Smac shows an interaction with full length HIAP2, which was not predicted.


Lysates
Pulldowns
Untrans
UbSmac
ΔAVPI-Smac
ARPL-Smac
XIAP
TsIAP
HIAP1
HIAP2
GST
37 kD
25 kD
Untrans
UbSmac
ΔAVPI-Smac
ARPL-Smac

Anti-HA

Figure 4-9. Co-immunoprecipitation verifies *in vivo* XIAP and Smac interactions.

Cell lysates were prepared from untransfected 293A cells and cells that were transiently transfected with HA-tagged- UbSmac, -ΔAVPI- Smac, and -ARPL- Smac. Lysates were immunoprecipitated with rat polyclonal anti-XIAP antibody (anti-RIAP3). Protein complexes were separated by SDS-PAGE on 12% polyacrylamide gels. Western blot analysis with the anti-HA antibody shows over-expressed Smac protein in cells lysates **(A)** and the XIAP-Smac interactions following immunoprecipitation **(B).**


Untrans
UbSmac
ΔAVPI-Smac
ARPL-Smac
A)
Lysates
B)
Immunoprecipitates
Anti-HA

Discussion

Structural and biological studies have established that XIAP-Smac binding involves a well-defined surface groove in the BIR3 domain and the four amino acid residues Ala-Val-Pro-Ile at the N-terminus of Smac (Liu *et al.*, 2000; Wu *et al.*, 2000). The unstructured first four amino- terminal residues of Smac make critical contacts with XIAP BIR3 and mediate XIAP inhibition, making this protein an excellent candidate to model peptide-based or small molecule therapeutics.

Smac and XIAP BIR 3 Interactions

X-ray crystallography studies indicate that Smac-XIAP BIR3 complex formation is critically dependent upon hydrophobic interactions between the P1 alanine of Smac and the tryptophan^{310} (W^{310}) in the XIAP BIR3 binding pocket. In addition, critical contacts between Smac and the XIAP BIR3 residue at tryptophan^{323} (W^{323}), are allowed via the structural formation of a kink created by the P3 proline (Fig 4-1). Mutagenesis experiments show that changes in any of the XIAP BIR3 pocket residues prevents Smac binding (Wu *et al.*, 2000). As well, these same studies provide evidence for the absolute requirement of the P1 alanine and P3 proline, in Smac, for XIAP BIR3 binding. When the P1 alanine was mutated to either a glycine or methionine, Smac-XIAP BIR 3 binding is abrogated, whereas mutating the Smac P3 proline residue significantly reduces the interaction (Wu *et al.*, 2000). Thus the contacts between Smac and XIAP BIR3 are very precise and absolutely dependent on Smac-like AxPx sequences (Figs. 4-1, 4-3 and 4-4).

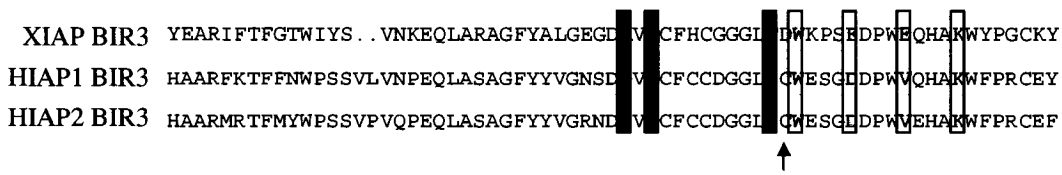
Phage Display Screening

Most of the identified IBMs possess uncharged residues at P2 of their amino terminus (Fig 4-2). The co-crystal structure of XIAP BIR3 and Smac indicates that the uncharged valine residue in P2 of Smac forms hydrogen bonds with T^{308} in XIAP (Fig 4-1). Based on this known Smac-XIAP interaction, it was surprising to find that the phage display screens identified positively charged residues at P2 (Figs 4-3 and 4-4). Looking more carefully, however, at the Smac-XIAP BIR3 model (Fig 4-1), it is plausible that these sequences are able to make ionic interactions with the aspartate residue at position 309 (D^{309}) in the XIAP cleft. It is not known, however, if this interaction would be favored under naturally occurring, physiological conditions.

Unlike the phage display results for XIAP BIR3, the HIAP1 BIR3 and HIAP2 BIR3 screens identified Smac-like sequences with uncharged, hydrophobic residues in P2 (Fig 4-3). These results were not unexpected. Although the co-crystal structures of Smac and the HIAPs have not been determined, sequence alignments between XIAP, HIAP1 and HIAP2 provide some information which may explain the absence of positively charged P2 peptides in these screening efforts (Fig 4-10). Comparisons of these alignments indicate that the D^{309} aspartyl residue in XIAP is not conserved in HAIP1 or HIAP2; rather a cysteine residue occupies this position (C^{309}) (Fig 4-10). Thus, the structural difference in the HIAP BIR3 binding pockets, may make interaction with positively charged amino acids such as Arg, His and Lys in P2 less favorable for these IAPs. Site directed mutagenesis of XIAP BIR3 might determine whether D^{309} is, in fact, critical for allowing interactions with these positively charged P2 Smac peptides. Likewise, changing the HIAP1 and HIAP2 C^{309} residue to a D^{309} may make these IAPs more likely to interact with positively charged amino acids in additional phage display screens.

Figure 4-10. Sequence comparison of the BIR3 domains of XIAP, HIAP1, and HIAP2.

Residues that form the alanine binding pocket are highlighted in green. Threonine[308], which is predicted to make hydrophobic interactions with the valine in P2, and the corresponding arginines found in HIAP1 and 2 are highlighted in red. The lysine residues that form the back of the groove are highlighted in blue. Aspartate[309], which is proposed to form ionic interactions with peptides identified in the phage display screen, is indicated by the arrow. Note that this position is occupied by a cysteine in both HIAP1 and HIAP2 BIR3.


XIAP BIR3 YEARIFTFGTWIYS..VNKEQLARAGFYALGEGD
HIAP1 BIR3 HAARFKTFFNWPSSVLVNPEQLASAGFYYVGNSD
HIAP2 BIR3 HAARMRTFMYWPSSVPVQPEQLASAGFYYVGRND

GST-Pulldown Analysis

Upon identifying the ARPL sequence in the phage library screen, it was of interest to assess whether this Smac-based sequence does in fact recapitulate and augment the behavior of AVPI Smac (UbSmac). GST-pulldown experiments showed that ARPL-Smac protein, ΔAVPI-Smac, and UbSmac all interacted with the individual XIAP BIR domains as predicted (Fig. 4-7). Unexpectedly, the pull-down analysis with the individual IAP-BIR3 domains and the full-length IAPs (Figs. 4-5 and 4-8) indicated that the ΔAVPI-Smac protein may be interacting with HIAP2. Additional studies demonstrated that the P5 Smac alanine residue was responsible for this interaction (Fig 4-6). Given that only the co-crystal structure for the XIAP–Smac interaction is known, it is possible that differences in the HIAP2 amino acid sequence (Fig 4-10) result in a Smac binding pocket that is unique from both XIAP and HIAP1. Perhaps these proposed differences in HIAP2 structure could enable the P5 alanine to mediate Smac interactions that are not possible with the other IAPs. However, co-immunoprecipitation experiments suggest that HIAP2-ΔAVPI-Smac interactions do not occur *in vivo* and thus the physiological significance of this binding is doubtful.

Potential differences in IAP binding pockets also explain the absence of Ts-IAP-Smac interactions. The Smac binding groove between Ts-IAP and XIAP may differ in that the critical threonine residue (T^{308}) in XIAP is substituted to an alanine residue in Ts-IAP. As well, the XIAP aspartic acid residue (D^{309}) is substituted to asparagine residue in Ts-IAP (Lagace, 2004, Ph.D. thesis). Thus, these differences in binding pocket residues appear to abolish any interaction between Smac and Ts-IAP (Fig 4-8).

Smac-Based Therapeutics

Upon identifying and characterizing the novel ARPL candidate Smac peptide sequence the ultimate goal was to commission peptides with this sequence and perform binding affinity experiments for the ARPL versus the wild-type, AVPI sequence. If it was determined that the ARPL sequence was of higher affinity to XIAP BIR3 than AVPI, the next step would have been to synthesize ARPL-Smac peptides as TAT transduction domain fusions. The peptide activity would then be investigated in cell death assays and eventually, in *in vivo* tumor models. However, at this point in my research program, several large pharmaceutical companies, including Novartis, Aventis, and Genentech targeted the same site using small-molecule library screening techniques and identified lead compounds that disrupt the XIAP-Caspase-9 interaction. The pharmacodynamics of these compounds are sufficiently promising that peptide-based therapeutics are unlikely to be competitive.

Initially it was believed that binding of the Caspase-9 linker peptide and Smac to the BIR3 domain of XIAP is mutually exclusive, suggesting a competition model in which Smac displaces XIAP from Caspase-9 (Srinivasula *et al.*, 2001). Smac was also predicted to bind XIAP BIR2 and disrupt Caspase-3 and -7 inhibition, possibly by steric hindrance (Chai *et al.*, 2000). Recently it was shown that Smac is unable to relieve Caspase -3 and -7 inhibition by a recombinant protein consisting of the linker-BIR2 domains of XIAP. Furthermore, Smac inefficiently relieves Caspase-9 inhibition by recombinant BIR3; however, when constructs were used that express the XIAP- BIR2 and –BIR3 domains in tandem, Smac was shown to effectively prevent IAP inhibition of both initiator and effector Caspases. Furthermore, the affinities of the BIR2 and BIR3 domains of XIAP for Smac were shown to be almost identical with each Smac dimer interacting with the BIR2 and BIR3 domains of one XIAP molecule to form a 2:1 stoichiometric complex (Huang *et al.*, 2003). Although individual

BIR domains of XIAP are adequate for inhibiting *in vitro* Caspase activity, these findings suggest that both BIR2 and BIR3 are required not only for XIAP-Smac interaction but also for subsequent liberation of Caspase inhibition (Huang *et al.*, 2003). If this model is correct, then monomeric Smac-based peptides would be incapable of forming the dimer complexes that are necessary for Caspase liberation from the IAPs.

Other limitations with using Smac peptides as a therapeutic approach have also been identified. Some studies have shown that N-terminal Smac tetrapeptides bind only to the BIR3 domain of XIAP with low affinity, are sensitive to proteolytic degradation, and have a poor capacity to penetrate cells (reviewed in Li *et al.*, 2004). Efforts to circumvent the limitations of Smac peptides led to the discovery of small molecule Smac mimetics, which are compounds that inhibit the IAPs with higher affinities than Smac peptides. Such submicromolar, small- molecule, non-peptidic antagonists were initially reported by Park *et al.* (2005). These compounds were created by individually replacing several amino acid residues while still maintaining the essential interactions necessary for binding in the Smac binding site on XIAP BIR3 (Park *et al.*, 2005). Another identified class of compounds is a series of proteolytically stable, capped tripeptides consisting of unnatural amino acids that bind to XIAP BIR3 with high nanomolar affinities. These compounds are cytotoxic in cancer cells and delay tumor growth in breast cancer xenograft models (Oost *et al.*, 2004).

An additional compound class, known as a modified oxazoline molecule was shown, after several modifications, to bind to the IAPs with a higher affinity than Smac tetrapeptides, and block IAP interaction with Caspase-9. This compound also acted synergistically with Apo2L/TRAIL and various chemotherapeutic agents (Li *et al.*, 2004). Additional studies using a high IAP-expressing breast cancer cell line, MDA-MB-231 showed that at low nanomolar concentrations, this Smac-mimicking compound significantly

sensitized these cells to both Apo2L/TRAIL- and etoposide-induced apoptosis via Caspase-3 activation (Bockbrader *et al.*, 2005). The effectiveness of this compound is due to its ability to mimic the dimeric structure of Smac protein, which, as mentioned previously, acts at the IAP BIR2-linker and BIR3 regions to liberate Caspases-3,-7, and -9 (Li *et al.*, 2004); Bockbrader *et al.*, 2005).

Other successful XIAP inhibitors include a class of compounds known as polyphenylureas which not only possess a high binding affinity for the BIR2 domain of XIAP, but also appear to have the ability to reverse Caspase-3 inhibition by XIAP (Schimmer *et al.*, 2004). Furthermore, these compounds cause XIAP to dissociate from effector Caspase-3 *in vitro*, and also restore Caspase-3 enzymatic activity (Wang *et al.*, 2004). Thus these polyphenylureas, but not their structural analogs, interact with XIAP in a mechanism distinct from that of Smac and Smac-peptide. Unlike these new compounds, Smac peptides are not able to remove Caspase-3 from XIAP because they do not interact with the linker region of XIAP. Furthermore, these compounds may be considered a significant improvement to Smac-peptide approaches in regulating XIAP inhibition because they possess the ability to reduce clonogenic survival in a broad spectrum of cancer cells while eliciting little toxicity in normal cells (Schimmer *et al.*, 2004). The observations from these studies further confirm that alleviating IAP suppression of Caspases greatly sensitizes cancer cells to apoptosis, making this family of proteins a promising target for the development of cancer therapeutics.

Chapter V

Smac-Ring Protein Degrades The IAPs And Sensitizes Cells to Apoptosis

Introduction

Cellular processes such as cell development, cell cycle progression, and apoptosis are in part regulated by ubiquitin-dependent pathways, which control the selective degradation or stabilization of intracellular proteins. Protein ubiquitination is a post-translational modification in which a 76 amino acid ubiquitin polypeptide becomes covalently attached to substrate proteins via a protein complex. This protein complex usually includes an activating enzyme (E1), a conjugating enzyme (E2), and a protein ligase (E3) (reviewed in Ciechanover *et al.*, 2000).

A number of apoptosis regulatory proteins including the Bcl-2 family member, Mule/ARF-BP1, IκB of the κB family, and some IAP family members are regulated via E3 ubiquitin ligase activity (reviewed in Zhang *et al.*, 2004; Vaux and Silke, 2005; Gao and Karin, 2005). E3 Ligase activity in some proteins, such as the IAPs, can be attributed to the presence of RING domains that possess a characteristic spacing of histidine and cysteine residues within their structure (reviewed in Vaux and Silke, 2005). This spacing has been shown to be necessary for recruiting an E2 conjugating enzyme. Upon binding to the RING containing protein, E2 enzymes catalyze the transfer of ubiquitin to the target substrate (Lorick *et al.*, 1999). The addition of four or more ubiquitin subunits target the substrates for degradation via the 26s proteasome.

The mammalian IAP family consists of eight members, five of which are known to contain a carboxy-terminal RING domain (Lorick *et al.*, 1999; Yang *et al.*, 2000). Earlier studies on the RING domains of XIAP and HIAP2 demonstrated that this domain is involved in the ubiquitination and degradation of the IAPs in response to apoptotic triggers (Yang *et al.*, 2000). Other studies showed that HIAP1 and HIAP2 participate in TNFα- induced cell death by

forming a multimeric complex with TRAF proteins, resulting in the subsequent ubiquitination and proteasome-mediated degradation of TRAF2 in response to TNFR2 signaling (Li *et al.*, 2002). Further reports indicated that XIAP and HIAP2 promote ubiquitination and degradation of Caspases-3 and -7, thereby enhancing the anti-apoptotic effects of the IAPs (Suzuki *et al.*, 2001). Thus, the ubiquitination and subsequent proteasomal degradation of both of the IAPs and their pro-apoptotic targets may be a key regulatory event in the apoptotic program.

The IAP antagonists in insects (Reaper, HID, Grim, and Jafrac2) and mammals (Smac, Omi, and GSPT1) interact directly with IAP-BIR domains via an IBM. The direct binding to the IAPs makes these proteins prime candidates for IAP-mediated ubiquitination and degradation. This was observed *in vitro*, where the *drosophila* IAPs, DIAP1 and DIAP2, were shown to mediate polyubiquitination and degradation of Reaper (Olson *et al.*, 2003). Similar *in vitro* experiments demonstrated that the mammalian homologue, Smac, was a substrate for XIAP-mediated ubiquitination, but unlike Reaper, was not subsequently degraded (MacFarlane *et al.*, 2002; Wilkinson *et al.*, 2004); however, Smac interactions with both HIAP1 and HIAP2 may accelerate IAP auto-ubiquitination and destruction (Hu and Yang, 2003). Thus, in some instances, IAP antagonists are not only targets for IAP-mediated degradation, but are also able to promote auto-ubiquitination and degradation of the IAPs themselves.

Smac appears to function as a general IAP inhibitor in that it has been shown to bind to all of the IAPs tested to date, with the exception of NAIP (Du, *et al.*, 2000; Verhagen *et al.*, 2000; Vucic *et al.*, 2002; Davoodi *et al.*, 2004) and TsIAP (Chapter 4). Furthermore, the over-expression of Smac sensitizes cells to apoptosis in the presence of chemotherapeutic drugs (Du *et al.*, 2000; Verhagen *et al.*, 2000; Vucic *et al.*, 2002; Hunter *et al.*, 2003; McNeish *et al.*, 2003). This increase in sensitivity to apoptosis is likely mediated via Smac-IAP interactions, perhaps due to the acceleration of auto-ubiquitination and destruction of the IAPs. By completely

eliminating the IAPs from the cellular environment, Caspase inhibition would be relieved allowing for amplification of the apoptotic process. Thus, increasing the sensitivity of cells to undergo apoptosis will have utility in the treatment of cancer.

The co-crystal structure of Smac and XIAP has been determined, and indicates the carboxy-terminus of Smac is poised above the BIR domains of XIAP (Fig 5-1). The goal of this study was to determine whether conferring E3 activity to Smac via the addition of a RING domain at the carboxy terminus of Smac would diminish cellular IAP levels and improve upon the existing ability of wild type Smac to sensitize cells to apoptotic stresses.

Figure 5-1. Co-crystal structure of Smac and XIAP.

The crystal structure of Smac reveals a long, bridge-like structure consisting of three extended alpha helices bundled together, and an unstructured amino terminus. Smac is a dimer in the image (indicated white asterix), with two monomeric BIR3 domains associated (indicated by yellow asterix). The co-crystal structure of Smac and XIAP showed that the carboxy terminus of Smac (indicated by white arrow) is poised above the BIR domains of XIAP. Image was downloaded using the Cn3D image analysis program and information is from the GenBank Structure Database. The image has been rotated in order to highlight the carboxy terminus of one of the Smac monomers.

Results

Generation of Smac-RING fusion proteins

Proteins such as Smac are absolutely dependent on the presence of an AVPI-initiating amino terminal sequence for their ability to interact with IAP-binding partners. Our previous study reported the novel approach of using Smac as an ubiquitin fusion protein to generate mature protein with the correct initiating amino terminus. Expression of cytosolic Smac increased cellular sensitivity to etoposide-induced apoptosis (Hunter *et al.*, 2003). In this study, the UbSmac fusion system was modified to express a mature, cytosolic, Smac protein with an IAP-RING domain at the carboxy terminus, with the intention of creating a Smac system that is a more potent apoptotic-inducer. Figure 5-2 shows the schematic for the UbSmac-IAP-RING constructs. To generate the Smac-XIAP-RING construct (SXR) the coding sequences for XIAP amino acids from the end of BIR3 through to the end of the RING domain were fused in frame with the UbSmac coding region (amino acids 1022 to 1527 of XIAP). Similarly, sequences for the linker region, Caspase recruitment domain (CARD), and the RING domain from HIAP1 and HIAP2 were also fused in frame to the UbSmac coding region (amino acids1502 to 1988 of HIAP1 and 1573 to 1988 of HIAP2) to generate the Smac-HIAP1-CARD-RING construct (SH1R) and the Smac-HIAP2-CARD-RING construct (SH2R), respectively (Fig 5-2).

To verify that the Smac-RING constructs generate a corresponding protein that is the correct molecular weight and is also detectable by anti-Smac antibody, the constructs were transiently transfected in 293A cells and then cells lysates were prepared for analysis by SDS-PAGE and Western blotting (Fig 5-3). As shown previously in Hunter *et al.* (2003), UbSmac and ΔAVPI-Smac migrated at the predicted MW of 25 kDa. Based on the

Figure 5-2. Schematic of the Smac-RING constructs.

The XIAP-RING domain, HIAP1-CARD-RING domain, and HIAP2-CARD-RING domain were PCR amplified and subcloned onto the carboxy-terminus of the UbSmac construct to generate Smac-RING expression systems, SXR, SH1R, and SH2R, respectively.

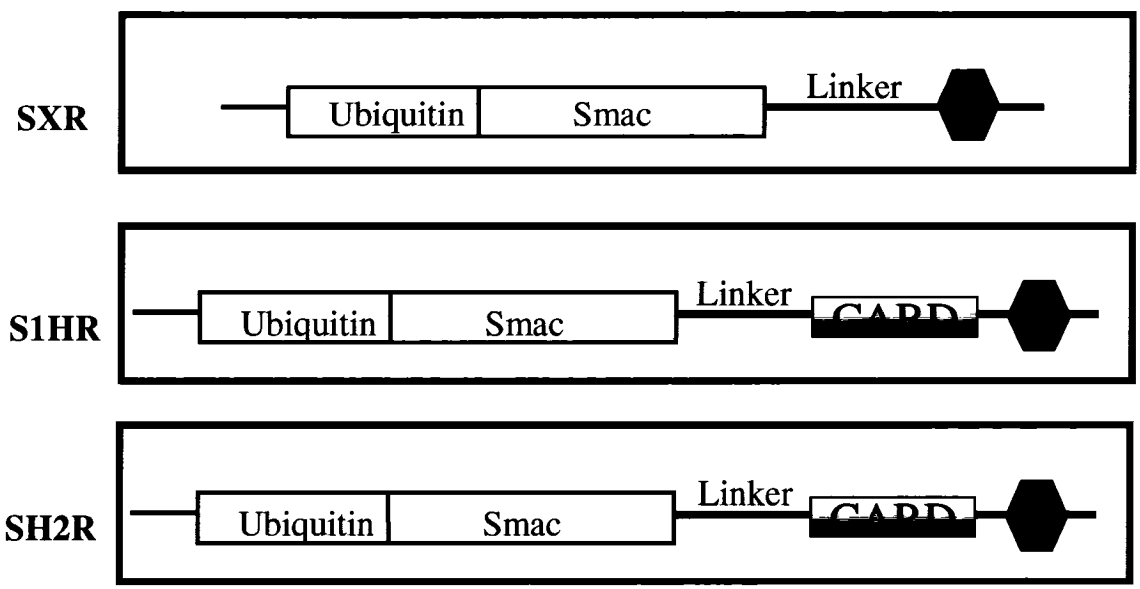
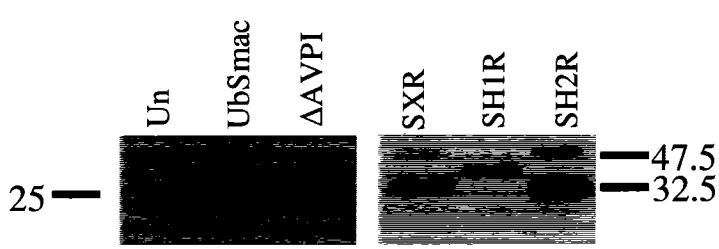

SXR
Ubiquitin
Smac
Linker
S1HR
Ubiquitin
Smac
Linker
CARD
SH2R
Ubiquitin
Smac
Linker
CARD

Figure 5-3. Western blot analysis of recombinant Smac proteins.

UbSmac, ΔAVPI-Smac, and Smac-RING constructs were over-expressed by transient transfection in 293A cells. Cell lysates were separated by SDS-PAGE on 12% polyacrylamide gels and transferred to nitrocellulose membranes for Western blotting. Smac proteins were detected using a monoclonal anti-Smac antibody.



Anti-Smac

translation of the open reading frame SXR and SH1R migrated at the predicted MW of 38.5 and 42.5 kDa, respectively. SH2R, however, does not migrate at the predicted 42.5 kDa size and may be modified by either stop codon read through or posttranslation modifications to produce the higher and lower molecular weight bands. UbSmac and ΔAVPI-Smac migrated at the predicted molecular weight of 25 kDa. Translation of the open reading frames of the SXR and SH1R constructs generated proteins with a molecular weight of 38.5 kDa and 42.8 kDa, respectively. Detection with the anti-Smac antibody verified that both of these proteins migrated at the expected sizes (Fig 5-3). Similarly, the SH2R protein was also detectable via Western blotting with the anti-Smac antibody; however the protein was not the predicted 42.4 kDa size. Repeated expression of this construct consistently produced a higher MW band with a faint signal and an intense band that migrates at approximately the same size as the SXR protein. Although this is unexpected, it is possible that the SH2R protein is somehow being modified, either by stop codon read-through or perhaps by some sort of post-translation modification, to produce the lower and higher MW bands.

Expression of Smac-RING fusions proteins alter IAP levels

The Smac-RING fusion proteins were co-expressed with a number of IAPs in 293A cells to determine their impact on IAP levels *in vitro*. Co-expression experiments showed that Smac-RING proteins generate differential effects on their ability to down-regulate IAP levels, with the SH1R protein efficiently depleting the most potent of the IAPs, XIAP. More specifically, co-expression experiments with 6myc tagged-XIAP, -HIAP1, -HIAP2, and -Survivin with SXR, SH1R, and SH2R, indicated that the SH1R protein had the greatest ability to down-regulate IAP levels (Figs 5-4 and 5-5). As shown in Fig 5-4A and 5-5,

Figure 5-4. Co-expression of Smac-RING protein with 6myc-tagged IAPs depletes over-expressed IAP levels.

6myc-tagged –XIAP, -HIAP1, and HIAP2 were co-expressed with UbSmac (S), ΔAVPI-Smac (Δ), and Smac-XIAP-RING (SXR), Smac-HIAP1-RING (SH1R), and Smac-HIAP2-RING (SH2R) via transient transfections in 293A cells. Cell lysates were separated by SDS-PAGE on 10% polyacrylamide gels and transferred to nitrocellulose membranes for Western blotting. IAP levels were detected using a monoclonal anti-myc antibody. Protein loading controls were assessed using a monoclonal anti-GAPDH antibody. Molecular weight markers are in kDa.

A) The SXR protein depletes 6myc-HIAP1 and has little effect on 6myc-tagged- XIAP or -HIAP 2 levels. 6myc-XIAP and 6myc-HIAP2 have a high molecular weight banding and smearing pattern that may be indicative of ubiquitination.

B) The SH1R protein depletes 6myc-XIAP, significantly reduces 6myc-HIAP1, and has little effect on 6myc-HIAP 2 levels.

C) The SH2R protein reduces 6myc-XIAP and depletes 6myc-HIAP2 levels. 6myc-HIAP1 levels appear unchanged and high molecular weight smearing may be indicative of ubiquitination.

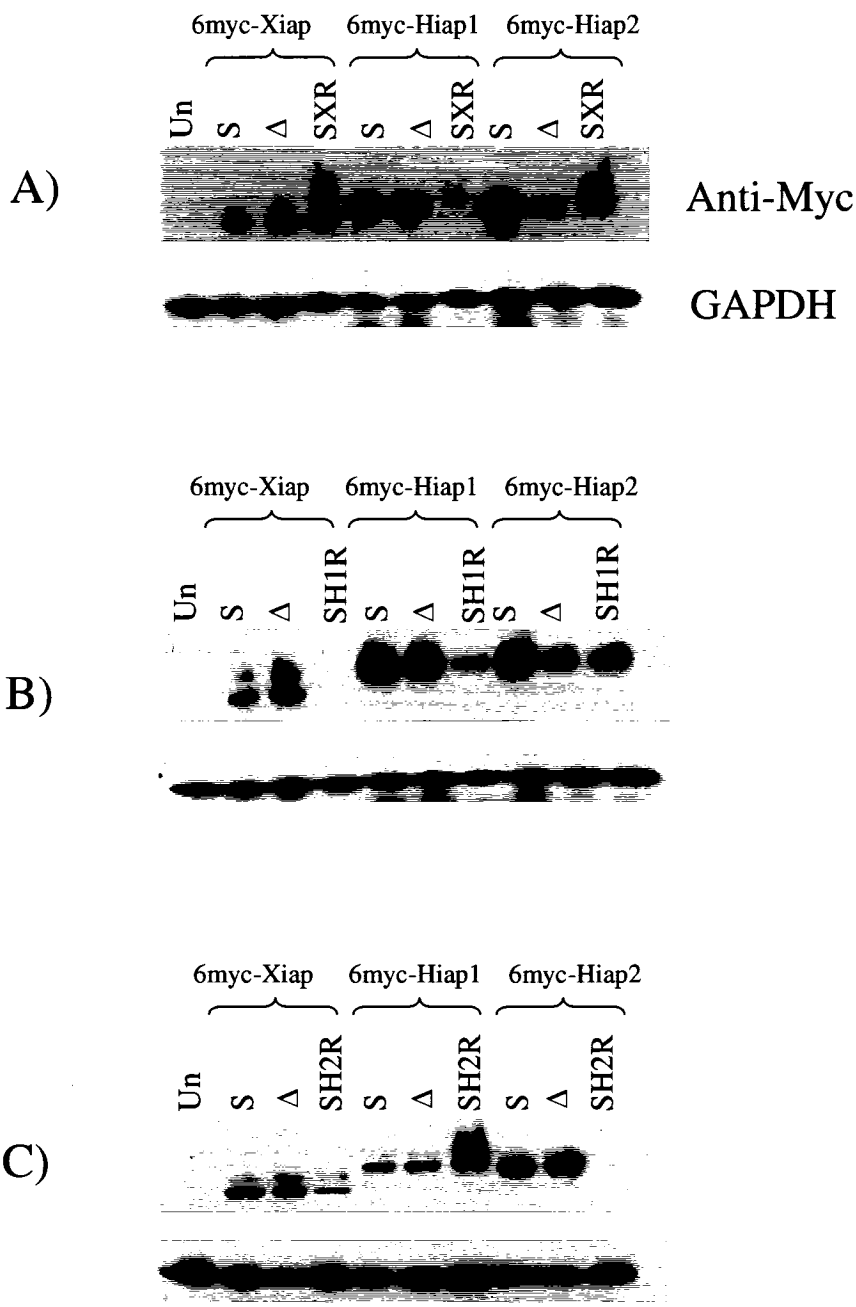
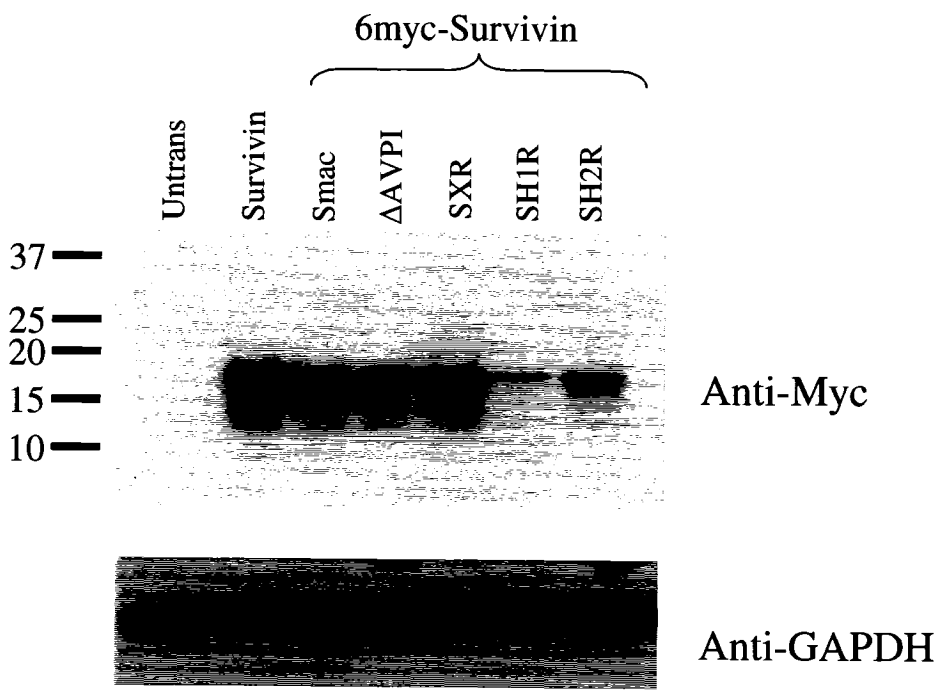

A)
6myc-Xiap
6myc-Hiap1
6myc-Hiap2
Un
S
Δ
SXR
S
Δ
SXR
S
Δ
SXR
Anti-Myc
GAPDH
B)
6myc-Xiap
6myc-Hiap1
6myc-Hiap2
Un
S
Δ
SH1R
S
Δ
SH1R
S
Δ
SH1R
C)
6myc-Xiap
6myc-Hiap1
6myc-Hiap2
Un
S
Δ
SH2R
S
Δ
SH2R
S
Δ
SH2R

Figure 5-5. Co-expression of Smac-RING protein with 6myc-tagged Survivin reduces over-expressed Survivin.

6myc-tagged –Survivin was co-expressed with UbSmac (Smac), ΔAVPI-Smac (ΔAVPI), Smac-XIAP-RING (SXR), Smac-HIAP1-CARD-RING (SH1R), and Smac-HIAP2-CARD-RING (SH2R) via transient transfections in 293A cells. Cell lysates were separated by SDS-PAGE on 15% polyacrylamide gels and transferred to nitrocellulose membranes for Western blotting. Survivin levels were detected using a monoclonal anti-myc antibody. Protein loading controls were assessed using a monoclonal anti-GAPDH antibody. The Smac-HIAP1-CARD-RING construct appears to reduce 6myc-Survivin levels, compared to the 6myc-Survivin levels following co-transfection with all the other Smac and Smac-RING constructs. Molecular weight markers are in kDa.


6myc-Survivin
Untrans
Survivin
Smac
ΔAVPI
SXR
SH1R
SH2R
37
25
20
15
10
Anti-Myc
Anti-GAPDH

expression of the SXR protein did not reduce 6myc-XIAP or –Survivin levels to a significant degree when compared to wild-type Smac protein (UbSmac) or the negative control, ΔAVPI-Smac protein; however, SXR almost completely eliminated the 6myc-HIAP1 protein and reduced 6myc-HIAP2 levels. The effects of the SH1R protein compared to the Smac control proteins showed that SH1R effectively abolished 6myc-XIAP, significantly reduced both 6myc-HIAP1 and –Survivin levels, but appeared to have no noticeable effect on 6myc HIAP2 (Figs 5-4B and 5-5). SH2R protein expression had marginal effects on 6myc-IAP levels in that 6myc-XIAP levels were reduced, -HIAP1 and -Survivin levels remained unchanged, and only -HIAP2 was completely depleted (Figs 5-4C and 5-5). Thus, the expression of Smac-RING fusion proteins either completely abolish and/or significantly reduce over-expressed IAP levels.

Smac-RING protein over-expression had similar effects on endogenous IAP levels to that observed in the co-expression studies. Although the effects of SXR expression on endogenous XIAP levels could not be assessed due to cross-reactivity of the XIAP antibody (anti-RIAP3) with the RING domain of the SXR protein (Fig 5-6A), a dramatic loss of HIAP1 was observed while HIAP2 levels appeared unchanged (Fig 5-6B). SH1R expression nearly eradicated endogenous XIAP and HIAP1, but again, had little effect on HIAP2 levels (Fig 5-6A and B). SH2R expression appeared to have little effect on endogenous XIAP (Fig 5-7A) and its effects on HIAP1 and HIAP2 could not be determined, due cross-reactivity of the anti-HIAP antibody (anti-RIAP1) with the RING domain on SH2R (Fig 5-7B). Endogenous Survivin levels did not appear to change following the expression of SXR, SH1R, SH2R, wild type Smac, or ΔAVPI-Smac, indicating that the Smac-RING proteins did not have any significant effect in depleting the levels of this IAP (Fig 5-8). Similar to the studies with over-expressed IAPs, the Smac-

Figure 5-6. Smac-RING protein over-expression depletes endogenous IAP levels.

UbSmac (Smac), ΔAVPI-Smac (ΔAVPI), Smac-XIAP-RING (SXR), Smac-HIAP1-CARD-RING (SH1R), and Smac-HIAP2-CARD-RING (SH2R) were over-expressed via transient transfections in 293A cells. Cell lysates were separated by SDS-PAGE on 10% polyacrylamide gels and transferred to nitrocellulose membranes for Western blotting. Endogenous XIAP levels were detected using rabbit polyclonal anti-RIAP3 antibody. HIAP1 and HIAP2 levels were detected using polyclonal anti-RIAP1 antibody, which reacts with both proteins. Protein loading controls were assessed using a monoclonal anti-GAPDH antibody. Molecular weight markers are in kDa.

A) XIAP levels following Smac-XIAP-RING over-expression could not be assessed using anti-RIAP3, due to cross-reactivity of the antibody with the RING protein; however, the Smac-HIAP1-CARD-RING construct reduces endogenous XIAP.

B) Compared to the Smac controls (Smac and ΔAVPI), the Smac-XIAP-RING construct and Smac-HIAP1-CARD-RING construct deplete endogenous HIAP1 levels, but endogenous HIAP2 levels remain unchanged.

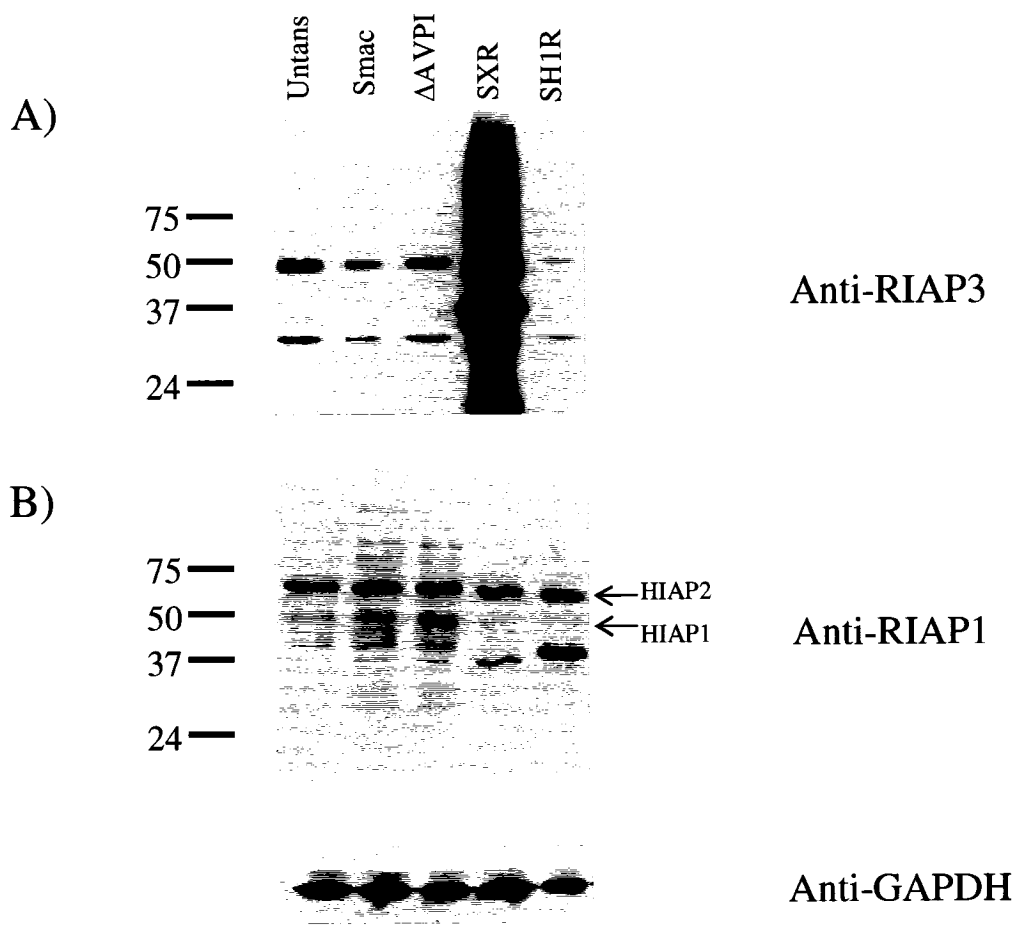

A)
Untans
Smac
ΔAVPI
SXR
SH1R
75
50
37
24
Anti-RIAP3
B)
75
50
37
24
HIAP2
HIAP1
Anti-RIAP1
Anti-GAPDH

Figure 5-7. Smac-HIAP2-CARD-RING protein over-expression does not alter endogenous XIAP levels.

UbSmac (Smac), ΔAVPI-Smac (ΔAVPI), and Smac-HIAP2-CARD-RING (SH2R) were over-expressed via transient transfections in 293A cells. Cell lysates were separated by SDS-PAGE on 10% polyacrylamide gels and transferred to nitrocellulose membranes for Western blotting. Endogenous XIAP levels were detected using the anti-RIAP3 polyclonal antibody. Both HIAP1 and HIAP2 levels were detected using the same polyclonal antibody, anti-RIAP1. Protein loading controls were assessed using a monoclonal anti-GAPDH antibody. Molecular weight markers are in kDa.

A) XIAP levels following Smac-HIAP2-CARD-RING appeared unchanged, compared to the Smac controls (Smac and ΔAVPI) as indicated by the 50 kDa MW bands. Lower MW bands are likely non-specific proteins bands that are detected by the polyclonal antibody.

B) Endogenous HIAP1 and HIAP2 levels could not be assessed using anti-RIAP1, due to cross-reactivity of the antibody with the RING domain of the fusion which appears as a multiple molecular weight laddering pattern. The faint lower molecular weight bands are likely non-specific protein bands detected by the polyclonal antibody.

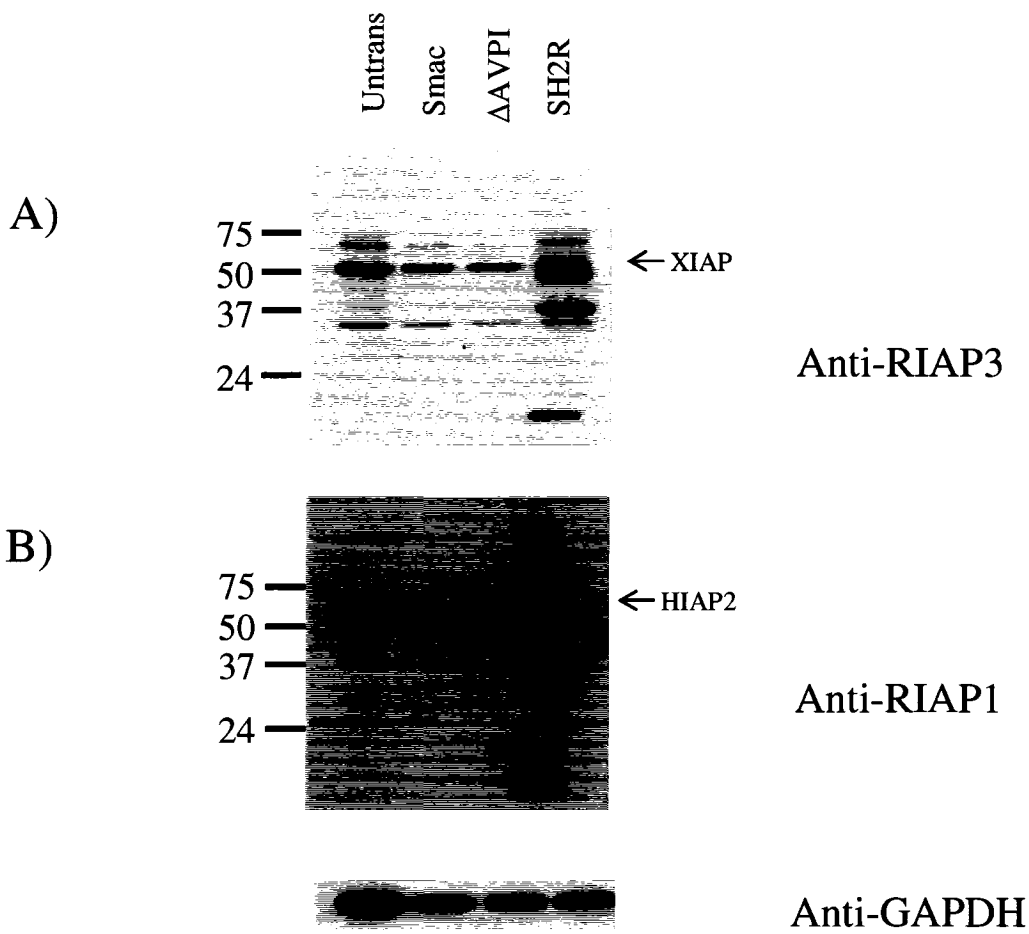
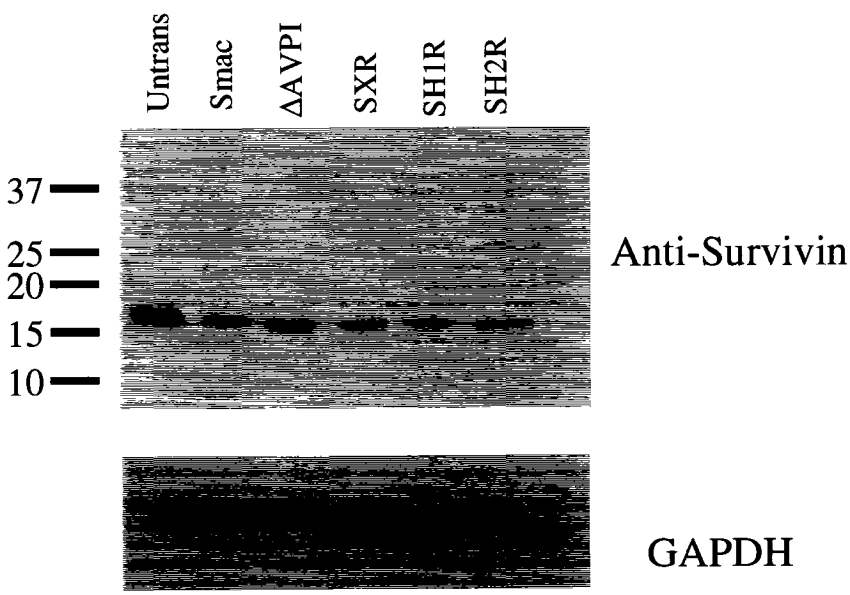

A)
Untrans
Smac
ΔAVPI
SH2R
75
50
37
24
XIAP
Anti-RIAP3
B)
75
50
37
24
HIAP2
Anti-RIAP1
Anti-GAPDH

Figure 5-8. Smac-RING protein over-expression does not reduce endogenous Survivin levels.

UbSmac (Smac), ΔAVPI-Smac (ΔAVPI), Smac-XIAP-RING (SXR), Smac-HIAP1-CARD-RING (SH1R), and Smac-HIAP2-CARD-RING (SH2R) were over-expressed via transient transfections in 293A cells. Cell lysates were separated by SDS-PAGE on 15% polyacrylamide gels and transferred to nitrocellulose membranes for Western blotting. Endogenous Survivin levels were detected using a monoclonal anti-Survivin antibody. Protein loading controls were assessed using a monoclonal anti-GAPDH antibody. None of the Smac-RING constructs appeared to have any significant effect on endogenous Survivin levels, compared to the Smac controls (Smac and ΔAVPI). Molecular weight markers are in kDa.


Untrans
Smac
ΔAVPI
SXR
SH1R
SH2R
37
25
20
15
10
Anti-Survivin
GAPDH

RING proteins have differential effects on their ability to down-regulate IAP levels, with the SH1R protein efficiently depleting endogenous XIAP and HIAP1.

Smac carboxy-terminal RING activity decreases IAP levels and increases apoptotic sensitivity

Results from the Smac-RING over-expression studies indicated that compared to the other Smac-RING proteins, SH1R had the most significant effect on decreasing XIAP. Due to the potency by which XIAP inhibits Caspases and causes their ubiquitination and degradation, this IAP is considered to be the most potent of the IAPs. Therefore, SH1R was identified as the most promising Smac-RING candidate, in terms of therapeutic potential. To determine whether the observed decreases in IAP levels by SH1R was indeed due to the RING domain –conferred E3 ligase activity or simply due to protein-protein interactions, SH1R mutants were generated. The E3 activity of the HIAP1-RING domain was reported by others to be dependent on the histidine residue 574 (His^{574}) and mutating His^{574} to an alanine (Ala) was shown to diminish E3 activity (Yang and Du 2004). Additionally, the work in Chapter 4 established that the formation of HIAP1-Smac or HIAP2-Smac complexes was absolutely dependent on the presence of the IBM sequence. In this study, two mutants were generated via site- directed mutagenesis. The SH1R mutant (SH1R*) contains the His^{574}-Ala substitution, while the double mutant (ΔAVPI-SH1R*) contains the deleted Smac IBM (AVPI) and the His^{574}-Ala substitution (Fig 5-9A). These mutant constructs were transiently transfected in 293A cells and SDS-PAGE and Western blotting analysis of the cell lysates verified that the mutant proteins were detectable by anti-Smac antibody and migrated at approximately the same MW as SH1R (Fig 5-9B).

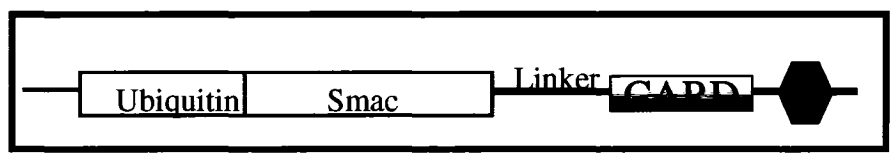
When the 6myc- tagged IAPs were co-expressed with the SH1R mutants it was observed that both SH1R* and ΔAVPI-SH1R* had negligible effects on depleting IAP levels, compared to

Figure 5-9. Schematic of the Smac-RING construct mutants.

A) Two Smac-RING mutant constructs were generated. One construct, SH1R*, has a His^{574}- Ala amino acid substitution in the RING domain to eliminate E3 ligase activity. The other construct, ΔAVPI-H1R*, has both the Smac amino terminal ΔAVPI sequence deleted and the His^{574}- Ala amino acid substitution to eliminate both IAP interaction and E3 ligase activity.

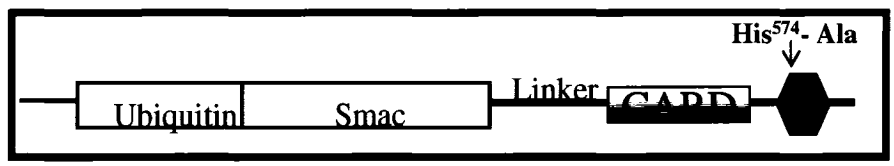
B) The Smac-RING mutant constructs were over-expressed by transient transfection in 293A cells. Cell lysates were separated by SDS-PAGE on 12% polyacrylamide gels and transferred to nitrocellulose membranes for Western blotting. Smac proteins were detected using a monoclonal anti-Smac antibody. Both mutants migrate at the predicted molecular weight of 42.5 kDa.

A) **SH1R**

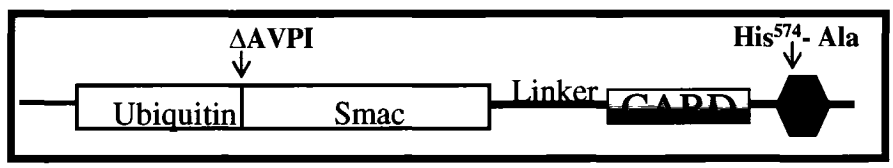


MUTANTS

SH1R*



ΔAVPI-SH1R*



B)

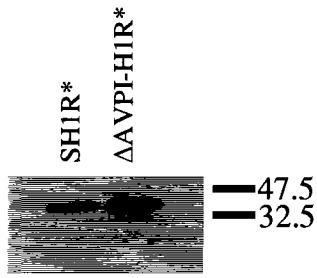
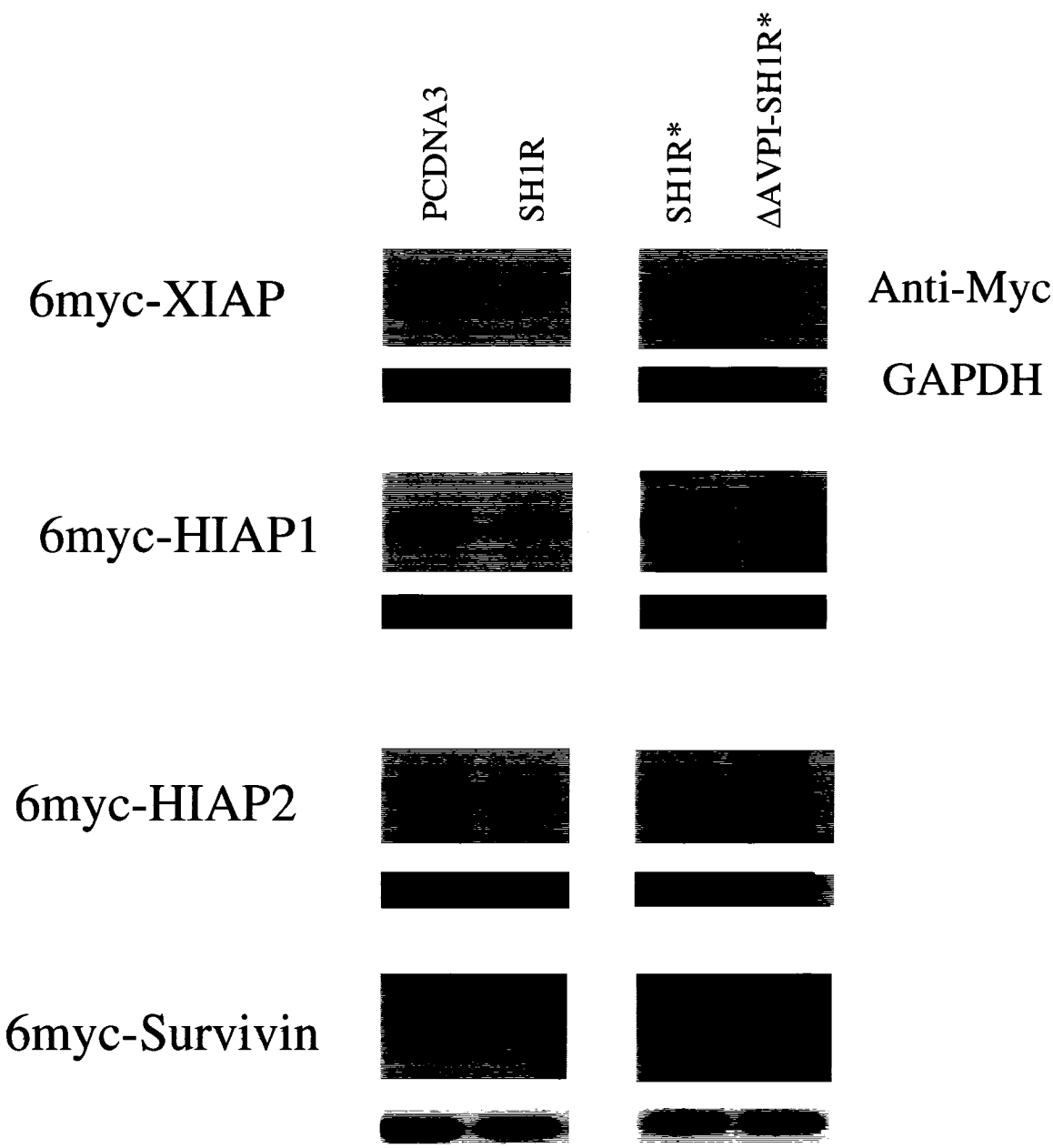


Figure 5-10. Over-expression of Smac-HIAP1-CARD-RING mutant proteins do not alter over-expressed IAP levels.

6myc-tagged-XIAP, -HIAP1, -HIAP2 and -Survivin were co-expressed with pCDNA3 (plasmid for transfection control) Smac-HIAP1-CARD-RING (SH1R), Smac-HIAP1-CARD-RING*His^{574}-Ala (SH1R*), and ΔAVPI-Smac-HIAP1-CARD-RING* His^{574}-Ala (ΔAVPI-SH1R*) via transient transfections in 293A cells. Cell lysates were separated by SDS-PAGE on 10% polyacrylamide gels. Cell lysates for Survivin detection were separated by SDS-PAGE on 15% gels. The proteins were transferred to nitrocellulose membranes for Western blotting. IAP levels were detected using a monoclonal anti-myc antibody. Protein loading controls were assessed using a monoclonal anti-GAPDH antibody.

The SH1R construct depletes 6myc-XIAP, significantly reduces 6myc-HIAP1, and has little effect on 6myc-HIAP- 2 or Survivin levels. The corresponding mutants had a slight effect on XIAP levels, most likely due to residual RING activity in SH1R*


PCDNA3
SH1R
SH1R*
ΔAVPI-SH1R*
6myc-XIAP
Anti-Myc
GAPDH
6myc-HIAP1
6myc-HIAP2
6myc-Survivin

SH1R (Fig 5-10). It is noted however, that the RING mutant, SH1R*, did exhibit a low level of activity in that 6myc-XIAP levels were marginally decreased. This agrees with published data which showed that the His-Ala mutation reduced, but did not completely abolish E3 activity (Yang and Du 2004). Overall, dramatic changes in over-expressed 6myc-IAP levels are only observed in the Smac-RING construct that possess both the IBM and unmodified RING domain (Fig 5-10). Thus, SH1R activity is proposed to depend not only on a functional RING domain, but also on protein-protein interactions mediated via the IBM.

SH1R interacts with the IAPs via the Smac IBM

To verify that decreases in IAP levels following SH1R over-expression are indeed due to direct protein-protein interactions co-immunoprecipitation studies were performed with S^{35}-labelled proteins. Figure 5-11A shows that following co-expression and immunoprecipitation of 6myc-XIAP, bands at the corresponding MW for both XIAP and Smac were identified on the autoradiograph, indicating a direct protein-protein interaction. Likewise, bands at the corresponding MW for SH1R and XIAP were also observed. Interestingly, the XIAP levels in the SH1R lane are much lower than the XIAP levels in the Smac, pCDNA3, and ΔAVPI-SH1R* lanes, implicating that both the IAP-Smac binding and the RING E3 ligase characteristics of SH1R are important in reducing XIAP levels. These co-immunoprecipitation studies showed that 6myc-Survivin does not interact with either Smac or SH1R (Fig 5-11B) which is not unexpected considering that none of the Smac-RING constructs had an effect on endogenous Survivin levels (Figs 5-8). Although SH1R may have appeared to decrease 6myc-Survivin (Fig 5-5), this is likely an artifact of the experiment, as the co-immunoprecipitation results clearly show that neither Smac, nor SH1R interact with Survivin. When 6myc-HIAP1 and -HIAP2 were co-immunoprecipated with SH1R, Smac and SH1R interactions were observed, as

Figure 5-11. Co-immunoprecipitation of S^{35}-labeled co-transfected lysates.

6myc-tagged-XIAP and -Survivin were co-expressed in 293A cells, via transient transfection, with pCDNA3 (as a plasmid, transfection control), Smac, Smac-HIAP1-CARD-RING (SH1R), and ΔAVPI-Smac-HIAP1-CARD-RING* His^{574}- Ala (ΔAVPI-SH1R*). Cell lysates were S^{35}-labeled for 4 hrs, immunoprecipitated with a monoclonal anti-myc antibody and separated by SDS-PAGE on 12% polyacrylamide gels. Gels were exposed to X-ray film for 24 hr prior to film developing.

A) Immunoprecipitation of 6myc-XIAP. Both Smac and SH1R proteins appear to have co-purified with 6myc-XIAP. XIAP levels are significantly reduced in the presence of SH1R, further confirming RING-mediated degradation of XIAP. The SH1R protein is indicated by the asterix (*).

B) Survivin does not appear to interact with Smac, and its levels are unchanged by SH1R expression.

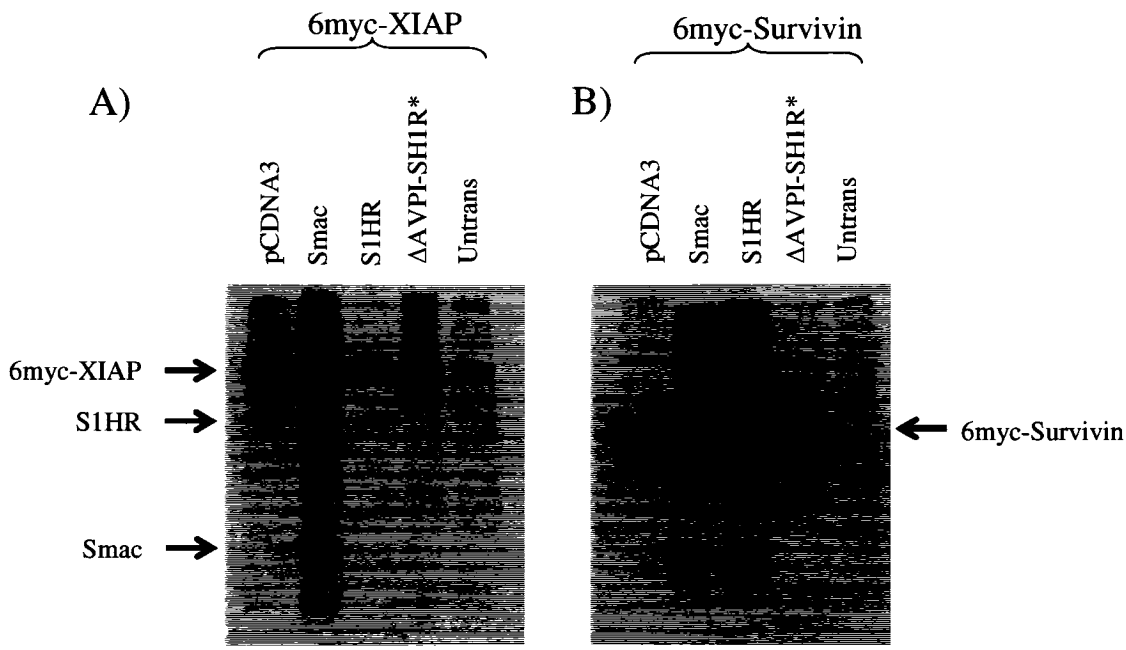
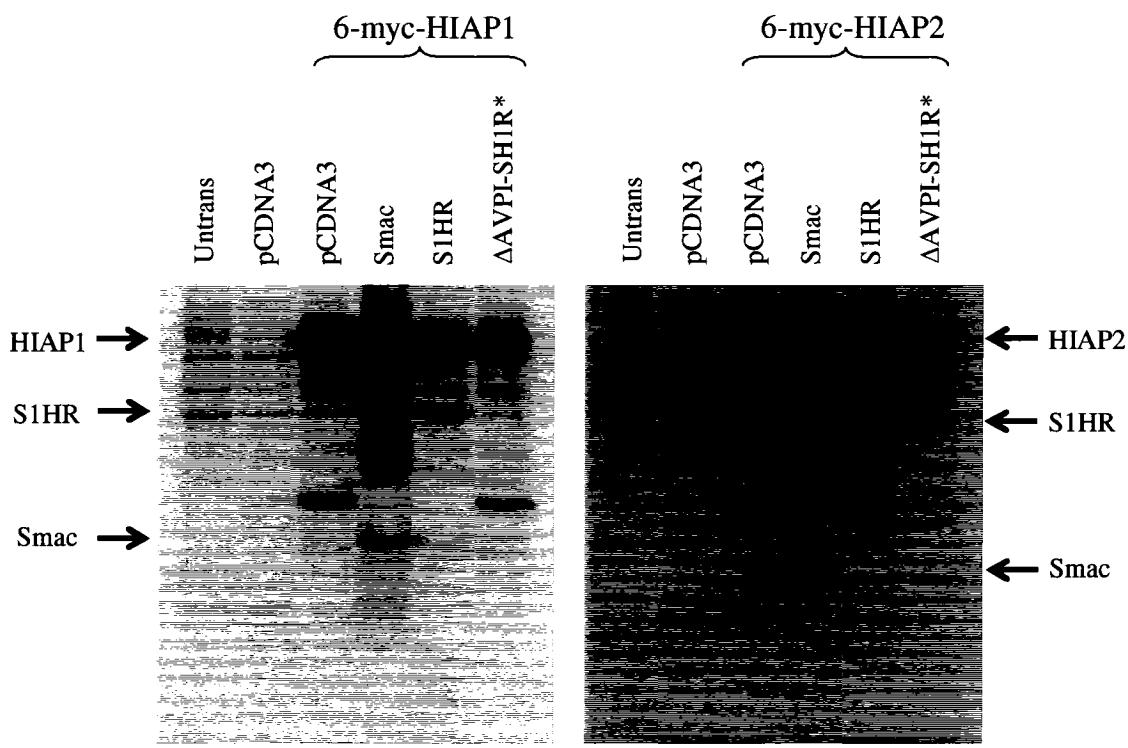

6myc-XIAP
6myc-Survivin
A)
B)
pCDNA3
Smac
S1HR
ΔAVPI-SH1R*
Untrans
pCDNA3
Smac
S1HR
ΔAVPI-SH1R*
Untrans
6myc-XIAP
S1HR
Smac
6myc-Survivin

Figure 5-12. Co-immunoprecipitation of S^{35}-labeled co-transfected lysates.

6myc-tagged –HIAP1 and –HIAP2 were co-expressed in 293A cells, via transient transfection, with pCDNA3 (as a plasmid, transfection control), Smac, Smac-HIAP1-CARD-RING (SH1R), and ΔAVPI-Smac-HIAP1-CARD-RING* His^{574}- Ala (ΔAVPI-SH1R*). Cell lysates were S^{35}-labeled for 4 hrs, immunoprecipitated with monoclonal anti-myc antibody, and separated by SDS-PAGE on 12% polyacrylamide gels. Gels were exposed to X-ray film for 24 hr prior to developing the film.

Immunoprecipitation of 6myc-HIAP1 and -HIAP2. Smac and SH1R proteins co-purified with the myc-tagged IAPs. Note the reduction in 6myc-HIAP1, but not 6myc-HIAP2. The position of the Smac and Smac-RING proteins are indicated by the asterix (*).


6-myc-HIAP1
6-myc-HIAP2
Untrans
pCDNA3
pCDNA3
Smac
S1HR
ΔAVPI-SH1R*
Untrans
pCDNA3
pCDNA3
Smac
S1HR
ΔAVPI-SH1R*
HIAP1
S1HR
Smac
HIAP2
S1HR
Smac

indicated by the presence of bands that correspond to the MW of these proteins on the autoradiograph (Fig 5-12A and B). Similar to the observations in the over-expression studies (Figs 5-4 and 5-6), the presence of a fainter HIAP1 band in the SH1R lane, compared to control lanes (pCDNA3, Smac and, ΔAVPI-SH1R*), indicates that the RING activity of SH1R may be decreasing HIAP1 levels but that this has a negligible effect on HIAP2 (Fig 5-12).

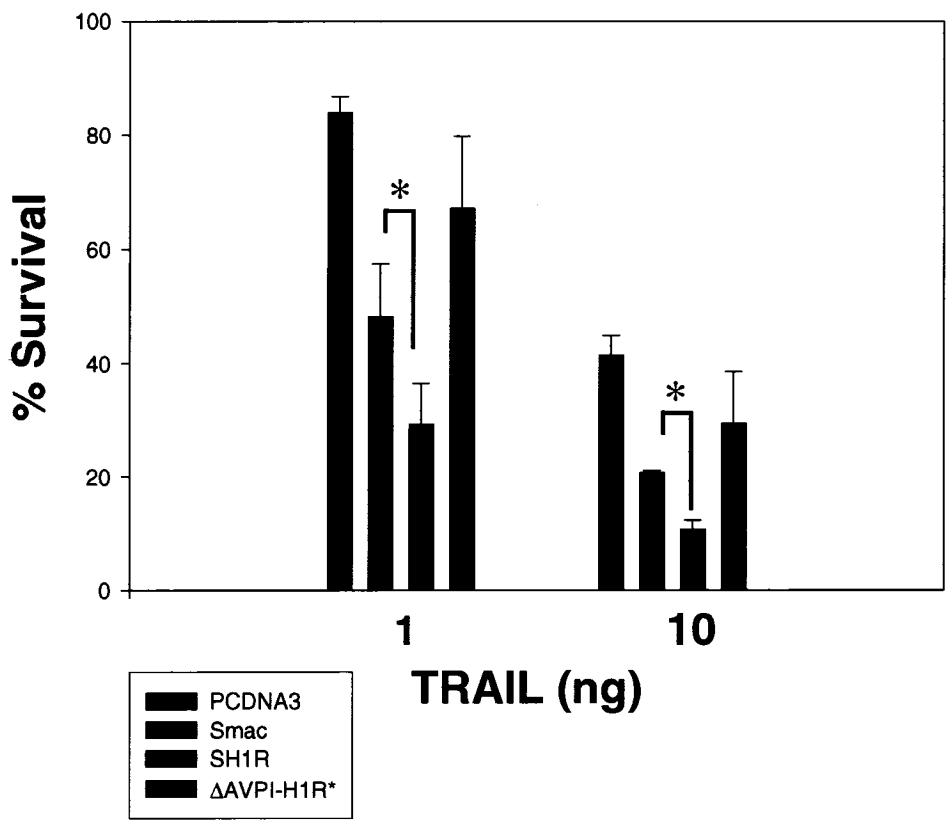
SH1R increases sensitivity to TRAIL-induced killing

Apo2L/TRAIL was considered to be a promising cancer therapeutic with an apparent selectivity for human tumor cells; however, as with most cytotoxic agents, there are reports of tumor cell resistance to this type of therapy. The combination of Smac delivery, either as an over-expressed protein or as a peptide, has been shown by several groups to significantly enhance apoptotic cell death in cancer cells that are otherwise resistant to cancer treatments such as Apo2L/TRAIL (Fulda *et al.*, 2002a; Fulda *et al.*, 2002b; Guo *et al.*, 2002; Ng *et al.*, 2002). SH1R was therefore chosen for investigation in combination with TRAIL to determine whether cell death can be enhanced above and beyond that observed with wild-type Smac delivery.

Initial cell viability experiments were performed on 239A cells transiently transfected with SH1R using trypan blue exclusion assays. The appropriate control transfections with pCDNA3, Smac, and ΔAVPI-SH1R* were also performed. Twenty-four hours after transfection, TRAIL treatments were performed for 24 hr, followed by trypan blue exclusion assays. It was observed that treatments with both low and high concentrations of TRAIL (1 ng and 10 ng, per well) resulted in a significant decrease in cell viability in the SH1R samples compared to the Smac samples ($p<0.05$, as determined by paired Student's t-test) (Fig 5-13).

Figure 5-13. Trypan blue viability assay.

pCDNA3, Smac, Smac-HIAP1-CARD-RING (SH1R), and ΔAVPI-Smac-HIAP1-CARD-RING* His574-Ala (ΔAVPI-SH1R*) were over-expressed via transient transfections in 293A cells. Twenty-four hours later, the cells were treated with 1 ng or 10 ng per well of TRAIL for 24 hr. Cell survival was measured by Trypan blue exclusion after 24 hr of TRAIL treatment. As shown by the paired Student's t-test, the Smac-HIAP1-CARD-RING construct had a significant decrease in cell survival relative to Smac alone at both 1 ng ($p<0.05$) and 10 ng TRAIL ($p< 0.05$). The results represent the mean $\pm$ one SD of three independent experiments performed in triplicate for an n=9 for each data point.


100
80
60
40
20
0
% Survival
*
*
1
10
TRAIL (ng)
PCDNA3
Smac
SH1R
ΔAVPI-H1R*

Both pCDNA3 (used to control for transfection toxicity) and ΔAVPI-SH1R* (which does not interact with or alter IAPs levels) had negligible effects on cell viability. Interestingly, SH1R appeared to have a greater impact on reducing cell viability with low doses of TRAIL, with a more generalized toxicity effect in combination with higher doses of TRAIL.

Experiments were performed to confirm that the combination of SH1R and TRAIL are decreasing cell viability via an apoptotic process. Following transfections with SH1R, Smac and the control plasmids, 293A cells were treated with 1 ng and 5 ng per well of TRAIL for 3 hr. The cells were then Annexin V labeled and PI stained for fluorescence- activated cell sorting (FACs) analysis. Analysis of the AnnexinV positive and PI positive cells revealed a significant decrease in survival in the SH1R population, compared to the Smac population at all doses of TRAIL ($p<0.05$, as determined by paired Student's t-test) (Fig. 5-14). Surprisingly, it appears that SH1R alone is able to induce apoptosis in 293A cells in the absence of TRAIL. In contrast, Smac expression does not appear to be sufficient, consistent with results presented in Chapter 3. The ΔAVPI-SH1R* protein appears to have some associated toxicity following a 5 ng TRAIL treatment. This could perhaps be attributed to residual E3 activity present in the mutated RING domain that is cytotoxic in the presence of an apoptotic trigger (Fig 5-14).

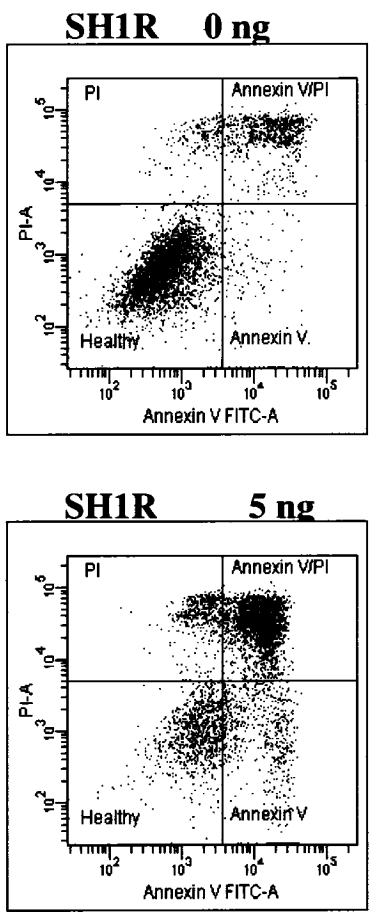
Figure 5-14. FACs analysis of Annexin V and PI Stained 293A cells.

pCDNA3, Smac, Smac-HIAP1-CARD-RING (SH1R), and ΔAVPI-Smac-HIAP1-CARD-RING* His^{574}- Ala (ΔAVPI-SH1R*) were over-expressed via transient transfections in 293A cells. Twenty- four hrs later, the cells were treated with 1 ng and 5 ng per well of TRAIL for 3 hr. Cell status was measured via FACs analysis of Annexin V/PI positive cells.

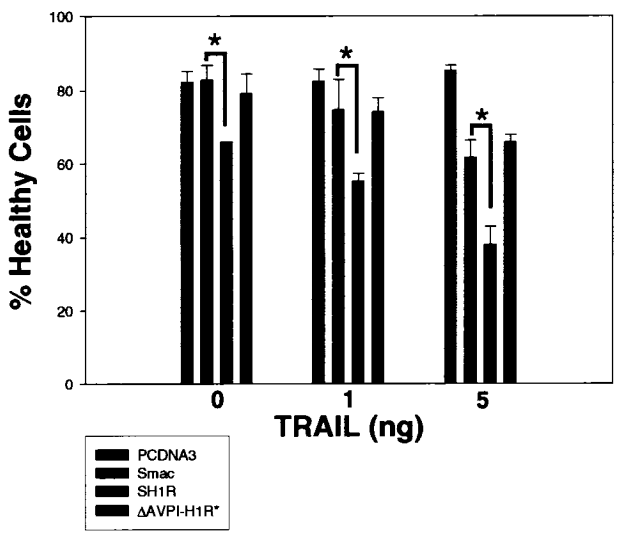
A) Representative fax profiles of SH1R transfected cells stained with Annexin V and PI after 0 and 5 ng TRAIL treatment. Healthy cells are negative for both stains (lower left quadrant).

B) Quantitation of healthy cell numbers. As shown by the Student's t-test, the Smac-HIAP1-CARD-RING construct significantly decreased healthy cell numbers, compared to Smac alone in the untreated ($p<0.05$) and in both the 1 ng ($p<0.05$) and 5 ng TRAIL-treated group ($p<0.05$). The results represent the mean $\pm$ one SD of three independent experiments performed in triplicate for an n=9 for each data point.

A)


SH1R 0 ng
PI
Annexin V/PI
Healthy
Annexin V.
PI-A
Annexin V FITC-A
SH1R 5 ng
PI
Annexin V/PI
Healthy
Annexin V
PI-A
Annexin V FITC-A

B)


% Healthy Cells
100
80
60
40
20
0
0
1
5
TRAIL (ng)
PCDNA3
Smac
SH1R
ΔAVPI-H1R*

Discussion

Proteins that interact with the IAPs and antagonize IAP inhibition of Caspases via an N-terminal motif were first identified in *Drosophila*. These insect proteins, referred to as Reaper, Hid, and Grim, were initially characterized as transcriptionally regulated proteins that are tightly associated with cell death in the developing fly embryo (reviewed in Abrams, 1999). Observations that Reaper, Hid, and Grim could also interact with mammalian IAPs and induce apoptosis in mammalian cells suggested that mammalian IAP antagonists might also exist. Smac/DIABLO was later identified as a mammalian homologue that was functionally related to the *Drosophila* proteins due to its N-terminal IBM structure and its ability to negatively regulate the IAPs.

Studies went on to show that unlike the insect IAP antagonists, Smac over-expression does not have the ability to induce cell death on its own (Verhagen *et al.*, 2000; Verhagen and Vaux, 2002). Some reports suggested that the differences in the intrinsic killing abilities between the insect and mammalian antagonists may be due to their disparate effects on cellular protein stability. Using the *Drosophila* protein Grim, as an example, it was not only observed that its over-expression outright killed both insect and mammalian cells, but also induced ubiquitination and degradation of XIAP (Goyal *et al.*, 2000; Rodriguez *et al.*, 2002; Silke *et al.*, 2004). In the case of Smac, however, studies indicated that Smac-IAP interactions cause ubiquitination of XIAP, HIAP1, HIAP2, and Livin, but only HIAP1 and HIAP2 were subsequently degraded (Yang and Du, 2004). In contrast, our studies indicate that wild-type Smac has a negligible effect on both endogenous or transfected HIAP1 and HIAP2 (Figs 5-4, 5-6, 5-7 and 5-12). Smac-IAP interactions have also been shown to trigger both Smac and IAP degradation, thus making cells more sensitive to apoptotic triggers by

neutralizing the IAPs and allowing unrestricted Caspase activation (MacFarlane *et al.*, 2002; Hu and Yang, 2003; Wilkinson *et al.*, 2004; Morizane *et al.*, 2005). In addition, Smac has been reported to function as a potent repressor of the ubiquitin ligase activities of XIAP, HIAP1 and HIAP2, thereby preventing the degradation of their target Caspases and allowing for apoptosis to proceed (Creagh *et al.*, 2004).

The Pro-apoptotic Effects of Smac Require the AVPI- IBM

The requirement for the Smac AVPI-IBM for ultimately leading to the sensitization to apoptotic triggers is controversial in the context of Smac isoforms studies. For example, the Smacβ isoform is generated by alternative splicing and is similar in structure to the wild type Smac isoform except that it lacks the four amino acid IAP binding motif (AVPI) and therefore does not interact with XIAP. Comparisons of wild-type Smac and Smacβ indicated that the association of XIAP via the AVPI- IBM in wild type Smac was essential for ubiquitination and degradation of both XIAP and Smac (MacFarlane *et al.*, 2002). However, despite the inability of Smacβ to physically interact with the IAPs it has been reported to still be pro-apoptotic. The domain responsible for this pro-apoptotic phenomenon is proposed to reside in the carboxy-terminal fragment of Smacβ and has activities beyond IAP inhibition and/or degradation (Roberts *et al.*, 2001). In this thesis work the ΔAVPI-Smac protein was not observed to induce apoptosis or sensitize cells to apoptotic triggers (Figs 5-13 and 5-14). Although similar in composition to Smacβ, it appears that the carboxy-terminal region distal to the AVPI sequence in the ΔAVPI-Smac protein does not possess pro-apoptotic activities. In fact, it appears that the function of the carboxy terminus of Smac is to promote its dimerization and to position the two respective IBMs in a spatial configuration such that contact with BIR2 and BIR3 occurs simultaneously, thereby inhibiting IAP-Caspase

interactions. Thus, in the study presented here, it is shown that an intact AVPI-IBM is required not only for engaging Smac-IAP interactions, but also for pro-apoptotic activity.

Enhancing Smac Pro-Apoptotic Activity

Intuitively, it would seem that improving upon the ability of Smac to neutralize the IAPs via an ubiquitination/degradation mechanism should decrease the apoptotic threshold and improve upon the therapeutic utility of Smac in cancer therapy. In this study, the addition of an IAP-RING domain to the carboxy-terminus of Smac promoted IAP degradation by bestowing E3 ligase activity to Smac. The Smac-RING protein possessing the HIAP1-CARD-RING domain (SH1R) has a substantial effect in depleting both over-expressed and endogenous IAP levels (Figs 5-4, 5-6, and 5-7). Although Smac-RING constructs were also generated with the XIAP RING domain (SXR) and the HIAP2-CARD-RING domain (SH2R), the SH1R protein had the most pronounced effect on XIAP levels and significantly reduced HIAP1 levels as well (Figs 5-5 through 5-8, 5-11 and 5-12). While the goal of this study was not to functionally dissect the differences between the individual IAP-RING domains, these results do indicate that perhaps not all of the IAP RINGs are created equally. Mutagenesis studies that involve amino acid swapping to make the HIAPs more XIAP like and vice versa, followed by ubiquitination assay experiments, would be one way to clarify where the functional differences are within these domains.

In these experiments, the SH1R protein was always compared against the wild type UbSmac protein to demonstrate that the Smac-RING protein is better able to enhance IAP degradation and ultimately cell death. Interestingly, UbSmac expression never caused any decrease in over-expressed or endogenous IAP levels compared to the negative control, ΔAVPI-Smac. In the study presented here, the importance of the IAP-RING domain on

ubiquitination and degradation was demonstrated, indirectly, via the generation of SH1R mutants. The SH1R* mutant has an amino acid substitution, which was proposed to eliminate the RING E3 ligase activity (Yang and Du, 2004). When SH1R* was co-expressed with the IAPs, a slight reduction in XIAP levels was observed indicating that the RING mutation still allows for some residual E3 ligase activity (Fig 5-10). This is not unexpected as it has been reported that the mutation of His^{574} to Ala does not completely abolish E3 activity, as initially thought (Yang and Du, 2004). However, when the SH1R double mutant, ΔAVPI-SH1R*, was co-expressed with the IAPs, there was no residual reduction in XIAP levels, in contrast to SH1R* expression. This indicates that the Smac IBM may have a role in IAP degradation via its direct interactions with the IAPs. At the same time, the data in this study also contradict the reports that Smac-IAP interactions result in Smac degradation (MacFarlane *et al.*, 2003), as it was observed that all of the Smac proteins levels remained unchanged in the presence of the IAPs. One explanation for these conflicting data may be that Smac-IAP interactions, in and of themselves, are not always sufficient for the degradation of either of these proteins. Rather this phenomenon may be dependent on the cellular environment and the types of stresses that are presented. Perhaps in the presence of a cell death trigger, both Smac and the IAPs may be more prone to ubiquitination and degradation as a result of global cellular ubiquitination that may be occurring in the cell.

In cancer cells the anti-apoptotic Bcl-2 family members are often over-expressed while the pro-apoptotic family members are deleted or inactivated, resulting in the suppression of cell death by blocking the mitochondrial release of apoptogenic factors such as cytochrome c and Smac. The expression of mature, cytosolic Smac as a ubiquitin fusion

can circumvent the issues of Bcl-2 over-expression by allowing pro-apoptotic Smac to bypass the mitochondria. Furthermore, the expression of UbSmac has been shown to significantly enhance etoposide-induced cell death (Hunter *et al.*, 2003). The SH1R protein possesses the features of the UbSmac fusion (Hunter *et al.*, 2003) in that it can bypass the mitochondria and interact with XIAP, HIAP1, and HIAP2, but not Survivin (Figs 5-11 and 5-12). In addition, SH1R expression proves to have an increased efficacy over wild type UbSmac in decreasing cell viability on its own (Fig 5-14) and in the presence of low doses of TRAIL, (Figs 5-13 and 5-14).

Based on the tissue culture experiments conducted in this study, SH1R would appear to be a better candidate than wild type Smac as a potential therapeutic for the treatment of cancer. The therapeutic advantage of SH1R lies in its ability to elicit a significant apoptotic response in the absence of an apopotic trigger and with lower doses of chemotherapeutic agent, than wild type Smac. *In vivo* experiments in animal tumor models are required to further validate SH1R as a promising cancer treatment strategy.

Chapter VI

General Discussion

IAP Antagonists

In mammals, as in *Caenorhabditis elegans* and *Drosophila*, Caspases are present in virtually all cell types as zymogens with low-level activity. To ensure survival of cells, proficient control mechanisms are therefore required to prevent inappropriate Caspase activation and ultimately cellular demise. The IAPs play a critical role in preventing Caspase activation in response to apoptotic stress. This is particularly important in cell types that are meant to last the lifespan of an organism, such as neurons. In situations, however, where it is necessary for cells to proceed through an apoptotic program, such as during embryonic development or in disease states such as cancer, it is critical that IAP-inhibition be relieved.

Antagonists of IAP activity were identified first in *Drosophila* as proteins that remove the "IAP brakes" on Caspase inhibition. In *Drosophila*, Reaper, Hid and Grim are three important cell death initiators whose genes are transcribed in response to diverse death-inducing stimuli (Goyal *et al.*, 2000; Rodriguez *et al.*, 2002). Transient over-expression of Reaper, Hid and Grim induce cell death in cultured insect cells, mammalian cells, and in various tissues in transgenic animals (McCarthy and Dixit, 1998). In cells that over-express Reaper, Hid and Grim, cell death can be blocked by both the Caspase-inhibitor protein, p35, and by chemical Caspase inhibitors, indicating a direct role for these antagonists in activating Caspase pathways via IAP inhibition (Goyal *et al.*, 2000; Rodriguez *et al.*, 2002). The identification of *Drosophila* IAP antagonists, and the recognition that they play such a pivotal role in regulating apoptosis prompted the search for mammalian homologues of IAP antagonists, leading to the identification of Smac.

Smac was identified as a factor in mitochondrial extracts that, together with cytochrome c and dATP, enhance the activation of Caspases in the cytosol. This protein was

purified and subsequently identified in co-immunoprecipitate-complexes with mammalian XIAP, and was shown to antagonize XIAP inhibition of UV-induced apoptosis (Du *et al.*, 2000; Verhagen and Vaux, 2002). The N-terminus of Smac is structurally similar to Reaper, Hid, and Grim, is essential for IAP interactions, and is capable of promoting apoptosis by blocking IAP-inhibition of Caspases. The Smac binding pocket formed within the XIAP BIR3 domain allows for precise Smac-IAP interactions (Li *et al.*, 2001) and many groups have searched for methods to improve Smac-IAP binding at this site with the intention of developing Smac as a therapeutic. This was the basis for the phage peptide library screening described in Chapter 4.

Mammalian and Insect IAP Antogonists Are Not Equivalent

Shortly after identifying Smac as the mammalian homologue of Reaper, Hid and Grim, it was realized that the mammalian antagonist may not be equivalent to the *Drosophila* counterparts. Unlike the *Drospophila* proteins, activity of Smac is not transcriptionally regulated, but rather is regulated via its localization to the mitochondria. In addition, Smac is constitutively expressed in many healthy cell types with no overt physiological consequence (reviewed in Verhagen and Vaux, 2002). Furthermore, unlike *Drosophila* proteins, which are potent inducers of cell death when introduced into cells, Smac was shown to be incapable of inducing cell death on its own (McCarthy and Dixit, 1998; Goyal *et al*,. 2000; Rodriguez *et al.*, 2002; Verhagen and Vaux, 2002). Initially differences in cell killing between Smac and the insect homologues was thought to be due to the fact that in healthy cells Smac was confined to the mitochondria as a mechanism to control the inappropriate induction of cell death (Verhagen and Vaux, 2002). On the contrary, the data presented in Chapter 3 show

that even when a mature form of Smac is over-expressed and localized in the cytosol, Smac is still incapable of direct killing (Hunter *et al.*, 2003).

Another difference in the mechanism of action between the mammalian and *Drosophila* IAP antagonists was identified when Reaper, Hid and Grim were shown to promote ubiquitination and destruction of their targets. Transient expression of Grim in both insect and mammalian cells was reported to not only target DIAP1 and XIAP, respectively, for degradation, but also to promote a substantial increase in total cellular ubiquitination (Silke *et al.*, 2004). Although there are reports that Smac promotes the auto-ubiquitination of XIAP, HIAP1 and HIAP2, only HIAP1 and HIAP2 actually become degraded (Yang and Du, 2004). Thus, it seems that Grim is intrinsically lethal by virtue of its ability to destabilize the most potent of the mammalin IAPs, XIAP, and also due to its ability to promote general protein instability and subsequent cellular demise (Silke *et al.*, 2004).

The Biological Consequences of IAPs in Mammals versus Insects

It has been shown in studies using knock out mice that when XIAP is deleted the levels of the remaining IAPs do not change to compensate for XIAP loss (Conze *et al.*, 2005; Conte *et al.*, 2006). In fact, the only identified case of IAP compensation has been reported with the HIAP2 knockout where there is an observed increase in HIAP1 levels (Conze *et al.*, 2005). Interestingly there are only relatively mild phenotypic differences in any of these knockout mice, compared to wild type mice. In contrast, the effect of IAP loss in *Drosophila* is very different. Loss of function mutations of DIAP 1 do not result in the up-regulation of the remaining functional IAP, DIAP2, and extensive apoptosis resulting in embryonic lethality is observed (Goyal *et al.*, 2000). Thus, the effects of removing or inhibiting IAP function in mammals compared to insects, indicates not only a redundancy in the IAP family

members in higher evolved species but also differences in the roles of the IAPs between the species.

Further differences in IAP function are observed when comparing how apoptosis is regulated between *Drosophila* and mammals. In *Drosophila,* apoptotic signals are integrated at the level of IAP function and not at the mitochrondria. For example, the *Drosophila* equivalent to Apaf-1, DARK (Drosophila Apaf-1 Related Killer) does not require cytochrome c for apoptosome formation and subsequent Caspase activation (Yu *et al.*, 2006; Dorstyn *et al.*, 2004). Rather, the only apical Caspase present in *Drosophila*, DRONC, appears to regulate apoptosis via the ubiquitination and degradation of DIAP1. Given the lack of redundancy in the *Drosophila* IAPs; the central role that DIAP1 plays in fly development; and the apoptotic regulatory function of the DIAPs, the requirement for the strict apoptotic regulation by Reaper, Hid and Grim is not surprising. Therefore, unlike Smac in mammals, the ability of Reaper, Hid and Grim to bind to DIAP1 appears to be critical for the *in vivo* regulation of apotosis during embryonic development.

Apoptotic Mechanism of Smac

Although Smac clearly has an important role in regulating apoptosis triggered through either the stress-induced pathway or the death-receptor pathway, the mechanism of Smac action is controversial. Splice isoforms of Smac exist (Smacβ, Smacγ, and Smacδ) which lack the mitochondrial targeting sequence and are therefore localized to the cytoplasm. In addition, both Smacδ and Smacβ lack an IAP binding motif; however, these Smac isoforms were found to promote apoptosis as efficiently as full length, wild type Smac (Roberts *et al.*, 2001). These results were interpreted as an indication that the primary means by which Smac triggers apoptosis is via its carboxy terminal alpha-helical bundle domain,

and that IAP binding is a secondary effect. In contrast to this one study, the majority of other studies suggest that the amino terminus of Smac is absolutely required to sensitize cells to apoptotic triggers (Srinivasula *et al.*, 2000; Guo *et al.*, 2002; Fulda *et al.*, 2002a). The contribution of the N-terminal AVPI tetrapeptide to IAP binding was addressed in Chapter 4, and it was found that ΔAVPI-Smac did not bind to XIAP, HIAP1, HIAP2, or TsIAP. Furthermore, in cell death assays, the deletion of the N-terminal AVPI tetrapeptide completely abrogated sensitization to etoposide and TRAIL- mediated killing (Chapter 3 and Chapter 5). Taken together, these studies suggest that as with the *Drosophila* homologues, the primary mechanism of the pro-apoptotic activity of Smac is via the inhibition of IAP function.

Improving the Therapeutic Potential of Smac

XIAP has been shown to target its pro-apoptotic binding partners such as Smac and pro-Caspase-9 for ubiquitination and degradation. During these interactions, XIAP also becomes ubiquitinated; however, it is not degraded. The ability of XIAP to effectively remove apoptotic proteins from the cellular environment, but itself remain stable demonstrates the potency of this IAP as an apoptosis inhibitor (MacFarlane *et al.*, 2002; Morizane *et al.*, 2005).

In this study, a Smac construct encoding a carboxy-terminal E3 ligase RING domain was generated with the expectation that the expressed protein would be capable of inducing ubiquitination and degradation of the IAPs. In Chapter 5 of this thesis it was demonstrated that the ectopic expression of Smac-RING protein was able to mimic some of the effects of the *Drosophila* antagonists, as indicated by the depletion of XIAP and significant reduction of HIAP1. While many studies have shown that wild-type Smac improves upon apoptotic

induction in the presence of a death stimulus (Du *et al.*, 2000; Verhagen *et al.*, 2000; Vucic *et al.*, 2002), the expression of the Smac-RING protein significantly enhanced the induction of apoptosis over that of wild-type Smac. Although direct comparisons to the effects of *Drosophila* protein expression were not made, the Smac-RING system also appeared to possess some intrinsic killing ability as has been reported with the insect antagonists, but not observed in this study with wild type Smac (Fig 5-14). The effect of depleting the IAPs and sensitizing cells to apoptosis indicates the valuable therapeutic potential for Smac-RING proteins in the treatment of cancer.

Smac-Based Antagonists in the Treatment of Cancer

Apoptotic Sensitivity Differs between Cancer Cells and Normal Cells

Cancer develops as a consequence of the deregulation of two fundamental and mutually dependent processes; cell proliferation and apoptosis suppression (Green and Evan, 2002; Igney and Krammer *et al.*, 2002). As a result, when tumors initially arise, they are more sensitive to apoptotic induction by DNA-damaging agents compared to their normal cellular counterparts. These newly established tumor cells are believed to be more susceptible to apoptosis in the presence of these agents due to the combination of ongoing oncogene activation in the cells and the potent pro-apoptotic signals induced by the resulting DNA damage (Green and Evan, 2002). Over time, cancer cells lose their sensitivity to apoptotic triggers. Continued expansion of the tumor mass creates an environment that lacks trophic support, and is often hypoxic due to lack of adequate vascular support. These conditions eventually make cancer cells more dependent on apoptosis suppression than

normal cells. Identifying strategies that possess the capacity to restore apoptosis sensitivity in these cancer cells would represent a therapeutic window for the treatment of cancer.

Targeting the IAPs as a Therapeutic Window for Cancer Therapy

Several lines of evidence suggest that the IAPs may play a significant role in tumorigenesis. Elevated IAP levels have been identified in a wide variety of cancer cell lines and tumor biopsy samples (Lacasse *et al.*, 1998). Over-expression of IAPs increase the resistance of cell lines to chemotherapeutic drugs or irradiation, and interfering with their synthesis leaves resistant cell lines sensitive (Sasaki *et al.*, 2000; Holcik *et al.*, 2000). More specifically, XIAP is highly expressed in many tumor cells and has been associated with refractory disease and poor prognosis (Reed, 2003; Liston *et al.*, 2003). Further evidence for the significance of IAP expression in apoptosis resistance is observed indirectly via strategies that reduce IAP levels. For example, in animal models of cancer, both anti-sense (Wittmann *et al.*, 2003; Hu *et al.*, 2003) and small molecule inhibitors of XIAP function (Kipp *et al.*, 2002; Schimmer *et al.*, 2004) have shown to significantly sensitize cancer cells to apoptosis.

Chromosome amplification of the 11q21-q23 region, which encompasses both HIAP1 and HIAP2, has been observed in a variety of malignancies including medulloblastomas, renal cell carcinomas, glioblastomas, and gastric carcinomas (Imoto *et al.*, 2001). Extranodal marginal zone mucosa-associated lymphoid tissue (MALT) B-cell lymphomas display recurrent translocation events, the most frequent of which t(11:18) encodes an in-frame chimeric protein that consists of the HIAP1 BIR domains, fused to the carboxy terminus of MALT1 (Uren *et al.*, 2000). Together, there is strong evidence to suggest that the IAPs represent important cancer therapy targets and hence, IAP antagonists are predicted to have therapeutic value in cancer treatments.

Smac Represents a Promising Therapeutic Target

In *Drosophila,* the expression of the IAP antagonists promotes the catastrophic dysregulation of Caspases and an apoptotic phenotype. Meanwhile, mammalian cells tolerate over-expressed IAP antagonists, such as mature, cytosolic Smac, making the presence of this protein relatively innocuous, until a death trigger is presented. Furthermore, *in vitro* and *in vivo* studies suggest that loss of IAP function through RNAi approaches (McManus *et al.*, 2004; Hu *et al*, 2003), or over-expression of XIAP, the XAF1 antagonist is a major determinant of cancer cell survival (Tu *et al.* submitted). These differences in cancer cells compared to normal cells represent a therapeutic window in which Smac is able to sensitize and kill cancer cells, leaving healthy cells unaffected. As shown extensively in this thesis, the development and subsequent expression of a Smac-RING protein has an increased efficiency in depleting IAP levels and sensitizing cells to apoptotic cell death, compared to wild type Smac. Thus, both the sequestering and depletion of the IAPs via Smac-based antagonists represents a powerful means of specifically sensitizing cancer cells to apoptotic cell death.

Future Experiments

Validating Smac-RING Therapeutics

The Smac-RING proteins were shown to have a significant efficacy in inducing apoptosis in the transformed 293A cell line, compared to wild type Smac (Chapter 5). In order to demonstrate the therapeutic window in which this protein works, studies in animal models of cancer need to be executed. Initial studies in normal mice must be performed to demonstrate that Adenoviral-Smac-RING delivery, alone and in combination with a

chemotherapeutic drug, is inconsequential to the health of the animal. Repeating the same experiment in mice with tumors is needed to demonstrate significant reduction in tumor burden, and an increase in lifespan.

With respect to the efficacy of Smac-RING therapy, there may be validity in targeting specific cancers such as MALT lymphomas. This cancer type has been proposed to arise as a direct consequence of the HIAP1-MALT translocation and subsequent deregulation of the NFκB cascade. Continued survival and growth of the tumor is absolutely dependent on the persistent expression and function of the fusion protein expressed by the translocated genes. Preliminary experiments have shown that co-expression of wild type Smac with the HIAP1-MALT fusion reduces NFκB activity (Dan McManus, personal communication). If Smac-RING protein specifically and completely blocks NFκB activity due to the HIAP1-MALT1 tranlocation, then an extremely precise IAP target would be identified. Therefore, selectively targeting of not only tumor cells, but also specific IAP proteins will make Smac-based antagonists, such as Smac-RING protein a promising therapeutic.

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