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The Regulation of Caspase Activity by the Inhibitor of Apoptosis Proteins

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Molecular Pathology by

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2007
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(Co-Chair)

(Chair)

University of California, San Diego
2007
DEDICATION

To my parents, C. Cleary Eckelman and Georgia Palmer,
for their tremendous love and support
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LIST OF ABBREVIATIONS

Apaf-1: Apoptosis protease-activating factor
AICD: Activation induced cell death
Bad: BcL-XL/Bcl-2-associated death promoter
Bak: Bcl-2-homologous antagonist/killer
Bax: Bcl-2-associated x protein
Bcl-2: B cell lymphoma-2
BH: Bcl-2 homology
BIR: Baculoviral IAP repeat
CARD: Caspase Recruitment Domain
cIAP: cellular IAP
CrmA: Cytokine response-modifier A
CTL: Cytotoxic T-lymphocyte
DD: Death Domain
DED: Death Effector Domain
DISC: Death Inducing Signaling Complex
DR: Death Receptor
FADD: Fas-associated death domain
FasL: Fas Ligand
FLIP: FLICE-like inhibitory protein
FLICE: Fas ligand-interacting cell effector
IAP: Inhibitor of Apoptosis Protein
IL: Interleukin
Ipaf: ICE-protease activating factor
ICE: Interleukin converting enzyme
Kₐ: Dissociation Constant
Kᵢ: Inhibitory Constant
LRR: Leucine Rich Repeats
ML-IAP: Melanoma IAP
NACHT: Domain found in NAIP, CIITA, HET-E and TP1
NAIP: Neuronal apoptosis inhibitory protein
NFκB: nuclear factor κ B
NIK: NF-κB-inducing kinase
NK cell: Natural Killer
NLR: NOD-Like LRR Repeat
NOD: Nucleotide Oligomerization Domain
MOMP: mitochondrial outer membrane permeability
PAGE: Polyacrylamide gel electrophoresis
PYD: Pyrin domain
RING: Really interesting new gene
RIP: Receptor-interacting protein
SDS: Sodium dodecyl sulphate
SMAC: Second Mitochondrial Activator of Caspases
TLR: Toll like receptor
TNF: Tumor necrosis factor
TNFR: Tumor necrosis factor receptor
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<td>TNF-related apoptosis inducing ligand</td>
</tr>
<tr>
<td>UBC</td>
<td>Ubiquity conjugating</td>
</tr>
<tr>
<td>XIAP</td>
<td>X-linked IAP</td>
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<tr>
<td>XAF1</td>
<td>XIAP associate factor1</td>
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My family has always provided a great foundation of support that has afforded me the ability to pursue all my goals in life. Samantha Lopez has been with me for the majority of my time in graduate school and served as one of my biggest motivators and is paramount to my success.
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  Invited Talk: The Consequences of NAIP/Caspase Interactions
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  Talk: Elucidation of the Requirements for IAP-mediated Caspase Inhibition
• International Proteolysis Society Conference Quebec City, Canada October 2005
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ABSTRACT OF THE DISSERTATION

The Regulation of Caspase Activity by Inhibitor of Apoptosis Proteins

By

Brendan P. Eckelman

Doctor of Philosophy in Molecular Pathology

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Professor Guy S. Salvesen, Chair
Professor Steve L. Gonias, Co-chair

Members of the inhibitor of apoptosis protein (IAP) family have the unique ability to regulate apoptosis induced by numerous stimuli. The defining feature of the members of the IAP family is the presence of at least one baculoviral IAP repeat (BIR) domain, and elucidating the characteristics of these domains is the unifying principle of my thesis research. The X-linked IAP (XIAP) is the most well studied member of the family and has been established as a potent inhibitor of caspases-3, 7, and 9. Due to the high degree of similarity, and initial enzymatic analysis, it has often been assumed that other IAPs have the ability to inhibit caspases. Furthermore, the binding specificities of BIR domains have been
generalized to fit a simple consensus sequence. These two assumptions have lead many to assume that most IAPs have overlapping and redundant functions.

In the first aim of my thesis, I sought to determine the requirements for IAP-mediated caspase inhibition. In my dissection of these interactions it has become apparent that IAPs, other than XIAP, do not function as physiologic caspase inhibitors. I have found that the BIR domains of many IAPs are suitable for caspase binding; yet the flanking regions possess surfaces or conformations that disallow potent caspase inhibition. Therefore, the ability to potentially inhibit the catalytic activity of caspases is unique to XIAP. This marks an important revision in the field and opens several questions as to the mechanism apoptotic occlusion by these other IAPs.

Secondly, I sought to investigate the other generalization of the IAP field, that being the equivalent specificities of the BIRs domain. Many BIR domains have a surface groove that endows them with the ability to interact with caspases and IAP-antagonists. Interestingly, the interaction motif of these pro-apoptotic proteins is conserved and thus it is assumed that this groove maintains the same specificity across many BIRs. In my second aim, I developed a method to profile the binding specificity of this groove. I have found that many BIRs have binding signatures that closely resemble the classic motif. However, there are several other BIRs in which the signature is quite varied. These studies allow for predictions of cellular binding partners and may provide insight into the mechanism of apoptotic inhibition by some IAPs.
Lastly, I turned my attention toward one of the least studied IAPs, the neuronal apoptosis inhibitory protein (NAIP). Although it was the first human IAP identified little is known about the true function of this IAP. Like most IAPs, NAIP has been suggested to be a caspase inhibitor. However, based on my dissections of IAP-mediated caspase inhibition in the first aim of this thesis it is clear that the BIR domains of NAIP lack conservation of the key caspase inhibitory surfaces. Interestingly, many have suggested that NAIP plays a role in innate immune signaling, however, the only data for this is based on the mouse studies suggesting it plays a significant role in restriction of the replication of intracellular bacteria. As predicted, I have found that NAIP is unable potently inhibit caspases. Intriguingly, I found that NAIP functions as an activator of the inflammatory caspases-4 and 5. The mechanism of activation seems to be quite unique and uncharacterized within the caspase family. These finding demonstrate for the first time that NAIP indeed has a role in regulating immune signaling.

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1. INTRODUCTION
1.1 Apoptosis

Apoptosis is a tightly regulated cellular signaling pathway, conserved from worms to humans, that ultimately results in the packaging and disposal of unwanted or damaged cells. This process is crucial in development, the maintenance of proper cell number, and the prevention various pathologies. Cellular apoptosis is characterized by defined events at both the molecular and morphological levels. At the heart of all apoptotic pathways is a family of cysteine proteases, known as the caspases, the activity of which is responsible for the demise of the cell. Apoptosis results in the packaging of the dying cell's contents into apoptotic bodies that are removed by phagocytic cells. This allows for the clearance of apoptotic cells while the nearby cells are unaffected. There are various stimuli that initiate the apoptotic signaling cascades, which can be grouped into three classes, 1) the extrinsic stimuli that signal from the extracellular environment into the cell; 2) the intrinsic stimuli that have their origins within the cell; and 3) the granule-mediated stimulus elicited by nearby immune cells.

1.2 Physiological Importance of Apoptosis

The development of multicellular organisms is often characterized by the overproduction of cells that are later removed throughout the developmental program. The mechanism by which these excess cells are removed is apoptosis,
and thereby represents an essential physiological process. Regulated cell death is an important aspect in the development of all multicellular organisms (Meier et al., 2000; Vaux and Korsmeyer, 1999). The first insight into the existence of a physiological genetic cell death program was gained from studies using *C. elegans* (Ellis and Horvitz, 1986). The capacity of a human anti-apoptotic gene to block cell death in *C. elegans* suggested an evolutionarily conserved apoptotic program. Apoptosis occurs during all stages of mammalian development from the early point of inner and outer mass formation of the blastocyst to digit formation (Coucouvanis and Martin, 1995, Brison, 1997 #4230). In fact it has been observed in mice that caspase inhibitors can prevent the removal of the intradigital web between the fingers (Milligan et al., 1995). There are numerous studies demonstrating the importance of apoptosis in development. A great example, highlighting the critical nature of this cell signaling pathway is the observation that caspase-deficient mouse embryos have massive forebrains compared to their wildtype littermates, suggesting an overproduction of neural cells followed by apoptosis-mediated selective loss (Kuida et al., 1998; Kuida et al., 1996). The function of the nervous system is highly dependent on the ability of its cells to make functional connections.

Physiological apoptosis does not end at birth, as it is a vital process ongoing for the life of the organism. In the human body it is estimated that a hundred thousand cells die by apoptosis and are replaced by mitosis every second (Vaux and Korsmeyer, 1999). The removal of old, damaged, infected, or
unwanted cells is essential for maintenance of homeostasis within the body and is central to the prevention of many diseases.

Cells of the immune system are eliminated by apoptosis at two predominant points, negative selection and following clonal expansion post-activation. Negative selection, also called clonal deletion, involves the apoptotic removal of T-lymphocytes in which the T-cell receptor interacts with antigen too early in the cells’ developmental stage. This essentially suggests that these cells are recognizing a “self-antigen” and are thereby eliminated to curtail the initiation of an autoimmune state. Mature T-lymphocytes expand in numbers following “activation” by the engagement of their T-cell receptors. Most of these cells subsequently die by an apoptotic mechanism, reducing their numbers to a resting basal level. Proliferation followed by apoptosis of lymphocytes provides a mechanism to respond to antigens and maintain homeostasis. Apoptosis of lymphocytes both in response to antigen stimulation and those that are potentially “self reactive” is called activation induced cell death (AICD) as it involves the ligation of the T-cell receptor - reviewed in (Green et al., 2003).

Each time a cell divides there is a chance for an error to occur during DNA replication. Thus, cells that have replicated several times are more likely to carry mutations. Importantly, most normal cells are capable of only a finite number of replication cycles before they undergo apoptosis. In addition, severe DNA damage can directly induce apoptosis of the cell. Both of these strategies rely on functional apoptotic signaling pathways as means to mitigate the continued propagation of mutations.
A single cell gone astray can have disastrous implications for the body as a whole. For instance a cell that has acquired a myriad of mutations affording it a growth or replicative advantage could wreak havoc in the form of cancer. While a self-reactive T-lymphocyte might be capable of initiating an immune response toward the bodies own cells. Apoptosis provides an essential control mechanism to remove unwanted cells like these. Furthermore, organ remolding both during development and in response to injury proceeds through an apoptotic mechanism.

1.3 Initiation of Apoptotic Pathways

1.3.1 The Extrinsic Apoptotic Pathway

The extrinsic apoptotic pathway is initiated by the engagement of a death receptor on the cell surface (Fig 1.1). These death receptors integrate extracellular cues into a cellular apoptotic signaling pathways. Death receptors are members of the tumor necrosis factor (TNF) receptor superfamily and are characterized by the presence of cysteine-rich extracellular region coupled to a conserved cytoplasmic fold termed the death domain (DD). (Ashkenazi and Dixit, 1998; Schulze-Osthoff et al., 1998; Tartaglia et al., 1993; Walczak and Krammer, 2000). It is the DD of the death receptor that is crucial for the engagement of the cellular apoptotic machinery.

There are currently six known death receptors and much work has been done to define their ligands. For the most part death receptor ligands are structurally related belonging to the TNF superfamily. The most well studied of
these receptors are the Fas receptor/CD95 that interacts with Fas Ligand (FasL)/CD95L (Suda et al., 1993) and the TNFR1 that binds both TNFα and lymphotoxin α (Tartaglia et al., 1993; Wallach et al., 1999) (Ashkenazi and Dixit, 1998; Nagata, 1997). Other death receptors include, death receptor 3 (DR3) (also called Apo3,TRAMP, WSL-1, or LARD) that interacts with Apo3 ligand (Apo3L)/TWEAK (Chinnaiyan et al., 1996); DR4 (also called TRAIL-R1) and DR5 (also called TRAIL-R2) both bind Apo2L which as known as TNF related apoptosis inducing ligand (TRAIL) (Pan et al., 1997b; Walczak et al., 1997); CAR1 for which the ligand is unknown; and the low affinity p75, nerve growth factor (NGF) receptor, which possess a DD but , its ligand, NGF is distinct from the TNF family.

Ligation of the Fas receptor by its ligand, FasL, results in the clustering and conformational change of the receptors that allows the cytoplasmic DD to interact via a homotypic interaction with the DD of the adaptor protein, FADD (Fas-associating protein with death domain). In addition to its DD, FADD has a death effector domain (DED) that interacts with the DED of procaspase-8, again via a homotypic interaction. This multi-protein complex is called the DISC (Death inducing signaling complex) as it is here that procaspase-8 is dimerization and activated, representing a major initiation point of apoptotic signaling. (Algeciras-Schimnich et al., 2002; Enari et al., 1996)

Signaling initiated by ligation of the TNFR1 is less well understood and not exclusively apoptotic. It has been proposed that there are two multi-protein complexes that can incorporate TNFR1 upon TNFα binding (Micheau and
Tschopp, 2003). Complex I is associated with the membrane and results in the induction of the NF-κB pathway and cell survival. This complex is composed of the TNFR-associated DD protein (TRADD), TNFR associated factor 2 (TRAF2) and TRAF6, the receptor interacting protein (RIP) and the cellular inhibitor apoptosis protein 1 (cIAP1) and cIAP2 (Rothe et al., 1995). Complex II is cytosolic and thought to induce apoptosis as it includes TRADD, FADD, and caspase-8 and its closest homolog caspase-10. Interestingly, there is balance between the pro-survival signals initiated from complex I and the pro-apoptotic cues induced via complex II. TNFα also interacts with the TNFR2, however, the downstream signaling events are much less well understood (Tartaglia et al., 1993).

TRAIL binding to either DR4 (TRAIL-R1) or DR5 (TRAIL-R2) elicits an apoptotic signaling pathway seemingly similar to that induced by FasL, involving the same protein components and DISC formation (Chaudhary et al., 1997; Kelley et al., 2005; Wiley et al., 1995). Interestingly, TRAIL only seems to induce a cytotoxic signal in cancer cells. This could be due to the observation that DR4 and DR5 are more highly expressed on the surface of tumor cells than normal cells. In addition, to DR4 and DR5, TRAIL binds three receptors, decoy receptor 1 (DcR1, also called TRID for TRAIL receptor without intracellular domain), DcR2, and osteoprotegerin (OPG), which either completely lack a cytoplasmic domain (in the case of DcR1 and OPG) or have a truncated DD (as in DcR2) (Ashkenazi, 2002; Ashkenazi and Dixit, 1999; LeBlanc and Ashkenazi, 2003). Therefore, apoptotic signaling following TRAIL binding to a decoy receptor is
incapacitated. Decoy receptors may be the basis by which normal are protected from TRAIL-mediated apoptosis (Marsters et al., 1997; Pan et al., 1997a; Sheridan et al., 1997). There is great hope of exploiting the TRAIL pathway for cancer therapies and activating antibodies of DR4 and DR5 have progressed into early clinical studies.
Figure 1.1 Extrinsic Apoptotic Pathway. The most straightforward extrinsic apoptotic pathway is the Fas pathway. Fas Ligand binding to the Fas receptor induces a conformational change allowing its DD’s (grey boxes) to interact with the DDs of FADD. FADD is able to recruit caspase-8 to the receptor complex via a homotypic interaction of DEDs (black boxes). Once localized to this complex, commonly referred to as the death inducing signaling complex (DISC), caspase-8 is oligomerized and activated. Caspase-8 cleaves and activates the zymogens of the executioner caspases-3 and 7, the activity of which leads to the demise of the cell.
1.3.2 The Intrinsic Apoptotic Pathway

The intrinsic apoptotic pathway is initiated by intracellular stresses and leads to a cellular signaling cascade resulting in apoptosis (Fig 1.2). A key player in the initiation of apoptotic response is the transcription factor p53, which can signal to cycle cell arrest and senescence in addition to apoptosis. In healthy, cells p53 is constitutively turned over by proteasomal degradation due to it association with the MDM2 E3 ligase. Cellular stresses including DNA damage, aberrant growth signals, ultraviolet irradiation, and a multitude of chemotherapeutic drugs initiate various signaling pathways involving distinct kinases that lead to the phosphorylation of p53. This results in the stabilization and accumulation of p53 by disrupting its interaction with MDM2 (Vogelstein et al., 2000). p53 induces the expression of several genes many of which are involved in cell-cycle arrest, DNA repair, and apoptosis. Both the TNF-R and Fas are p53 target genes, the expression of which may potentiate p53-dependent apoptosis. Other p53 inducible genes are some of pro-apoptotic members of the Bcl-2 family, including BAX, Noxa, and Puma, which are regulators of the intrinsic apoptotic pathway.

The Bcl-2 protein family consists of approximately 25 members that have either anti-apoptotic or pro-apoptotic roles. These proteins are identified by the presence of one to four BH (Bcl-2 homology) domains. The anti-apoptotic members, Bcl-2, Bcl-XL, Bcl-w, Mcl1, and A1 possess four BH domains (BH1, BH2, BH3, and BH4). The pro-apoptotic BAX and Bak have similar BH1, BH2,
and BH3 domains, while other pro-apoptotic members only have a BH3 domain. Much attention has been given to the ability of Bcl-2 proteins to regulate the mitochondrial outer membrane permeability (MOMP). In fact, upon apoptosis induction BAX and Bak translocate to the cytoplasmic face of mitochondria and alter its integrity. It has been suggested that the anti-apoptotic Bcl-2 proteins antagonize the ability of BAX and Bak to induce MOMP. The BH3 only proteins, Bim, Bad, Bik, Bmf, Hrk, Noxa, Puma, and BID relay pro-apoptotic cues to the mitochondria. Most of these, with the exception of BID, are thought to directly disrupt the interactions between anti-apoptotic members with Bax and Bak, thereby allowing the induction of MOMP - reviewed in (Cory and Adams, 2002; Green and Kroemer, 2004). Caspase-8 cleavage of BID, causes a conformational change allowing this BH3 only protein to directly induce MOMP. Thus, BID cleavage provides means by which extracellular death stimuli can engage the intrinsic apoptotic pathway, representing a major point of crosstalk between these pathways. As the integrity of the mitochondrial membrane is compromised many proteins are released into the cytoplasm. One such protein, cytochrome c, which normally functions in the cellular respiratory chain, has an ancillary function in the cytoplasm.

Once present in the cytosol, cytochrome c interacts with Apaf-1 (Apoptotic Protease Activating Factor-1). Apaf-1 is composed of a N-terminal CARD (Caspase recruitment domain), a central NACHT or NOD (Nucleotide Oligomerization Domain), and C-terminal WD40 repeats that bind cytochrome c. In the absence of cytochrome c, Apaf-1 exists in an auto-repressed state in
which the WD40 repeats are thought to interact with and occlude the rest of the protein. Upon cytochrome c binding to its WD40 repeats Apaf-1 undergoes a conformational change that allows for oligomerization of the protein via the NACHT and recruitment of procaspase-9 via homotypic interactions of the CARDs (Acehan et al., 2002; Yu et al., 2005). This complex is known as the apoptosome and it is here that the dimerization of caspase-9 endows it with catalytic activity - reviewed in (Riedl and Salvesen, 2007). Recently, the crystal structure of a WD40 deleted Apaf-1 suggest that the NACHT domain was capable of dATP hydrolysis and in the ADP state the CARD was not fully exposed to allow for caspase-9 recruitment. Therefore, it seemed that following dATP hydrolysis there needed to be an exchange of the bound dADP for another dATP before Apaf-1 can form a functional apoptosome (Bao et al., 2005; Riedl et al., 2005). The most critical substrates of caspase-9 are the zymogens of caspases-3 and 7, which upon cleavage become active executioner caspases.
**Figure 1.2 The Intrinsic Apoptotic Pathway.** The intrinsic apoptotic pathway is initiated by intracellular stresses that cause the release of cytochrome c from the mitochondria. The bcl-2 family members are key regulators of the signaling from stress to cytochrome c release. In the cytosol cytochrome c interacts with the WD40 repeats of Apaf-1 and induces a conformational change. This allows Apaf-1 to interact with caspase-9 via a homotypic interaction of their CARDs. The NACHT of Apaf-1 is oligomerizes and the apoptosome is formed. Caspase-9 is activated at the apoptosome by an induced proximity mechanism. Once active, caspase-9 cleaves and actives the zymogens of caspases-3 and 7 resulting in eventual apoptosis.
1.3.3 The Granule-mediated pathway

Cytotoxic T-lymphocytes (CTLs) and Natural Killer (NK) cells are part of the body’s own surveillance system and patrol the body for damaged or infected cells. These immune cells are capable of inducing apoptosis of unwanted cells by two mechanisms, the extrinsic pathway through the secretion of Fas Ligand; or the granule-mediated pathway, which involves the delivery of a cytotoxic granule directly to the target cell (Russell and Ley, 2002).

Once a CTL or NK cell recognizes a target cell it secretes a granule containing, perforin, a pore forming protein, and the serine proteases granzyme A and B. It is thought that the pore-forming ability of perforin enables the efficient translocation of the granzymes into the cytoplasm of the target cell. Granzyme B has a substrate specificity similar to that of the caspases (Thornberry et al., 1997) and has been found to be capable of cleaving and activating procaspase-3 (Darmon et al., 1995; Quan et al., 1996). Therefore, once in the cytoplasm of the target cell granzyme B induces apoptosis via activation of the executioner caspase-3. It has also been reported that granzyme B is able to induce apoptosis by cleaving BID endowing it with ability to induce cytochrome c release and subsequent engagement of the intrinsic apoptosis pathway (Sutton et al., 2000; Sutton et al., 2003, Waterhouse, 2005 #4239).
1.4 Caspases

The caspases are members of the CD clan of cysteine proteases and are further classified as the C14 family (Rawlings and Barrett, 1993). The name caspase is derived from the fact that they are Cysteine-dependent Aspartate specific proteases. The catalytic unit of a caspase consists of a large and small subunit, which is associated with a N-terminal regulatory domain. These regulatory domains can be the large CARDs, or the DEDs, or simply a shorter N-terminal peptide (N-peptide) extension.

The human caspase family consists of eleven members, with the majority playing a role in apoptosis (2, 3, 6, 7, 8, 9, and 10), while a smaller group have pro-inflammatory functions (1, 4, 5) (Fig 1.3A) Caspase-14 seems to be an outlier as it is not known to have a role in apoptosis or inflammation, but rather in keratinocyte differentiation and maintaining the integrity of skin cells (Denecker et al., 2007; Mikolajczyk et al., 2004). The apoptotic caspases can been further subdivided into those involved apoptotic initiation, caspase-2, 8, 9, and 10, denoted as the apical caspases; and those that are activated downstream, caspase-3, 6, and 7, denoted as the executioner caspases. Once active, the apical caspases cleave and activate the zymogens of executioner caspases-3 and 7, which in turn are responsible for the majority of proteolytic events that ultimately result in the destruction of the cell -reviewed in (Fuentes-Prior and Salvesen, 2004; Riedl and Shi, 2004). Importantly the activation mechanisms of the apical and executioner caspases are distinct.
A single catalytic domain of a caspase, having a large and small subunit, is derived from a single procaspase molecule. Yet in their active forms caspases are obligate dimers comprised of two catalytic units, each having their own large and small subunit. Like other caspases, caspase-9 is only active in its dimeric state, yet the crystal structure has revealed that this dimer only has a single properly formed active site (Renatus et al., 2001). Caspases possess a catalytic dyad, consisting of His$^{237}$ and Cys$^{285}$, which is absolutely required for hydrolysis of the scissile bond (Fig 1.3B). Conventionally, the catalytic residues are identified based on the caspase-1 numbering system. The Cys residue represents the nucleophile of the catalytic dyad (Walker et al., 1994; Wilson et al., 1994). The substrate recognition and the stringent specificity of the caspases is achieved by the presence a very basic S1 pocket within the active site, which is well suited to fit an Asp side chain (Fig1.3B). This pocket is composed of three conserved residues, Arg$^{179}$, Arg$^{341}$, and Gln$^{283}$, that give its basic nature endowing the caspase family with a specificity for cleaving the peptide bond C-terminal to Asp residues (Fuentes-Prior and Salvesen, 2004). There are a few notable exceptions to the exquisite Asp preference of the caspase. The Drosophila apical caspase, DRONC, has the capacity to cleave itself immediately downstream of a Glu residue (Hawkins et al., 2000). Similarly, caspase-5 cleaves the Max transcription factor after a Glu (Krippner-Heidenreich et al., 2001). However, these are truly exceptional cases and generally under physiologic conditions the caspases maintain a stringent Asp specificity.
The amino acid N-terminal to a proteolytic site is denoted the P1 and is accommodated by the S1 pocket. N-terminal to P1 is the P2 residue that sits in the S2 pocket of the protease and so forth and so on. The amino acid that resides immediately C-terminal to the scissile bond is denoted as P1’ and it is N-terminal to the P2’ and so on. Thus, caspases have a specificity for Asp residues in P1. Substrate profiling studies on the caspases have revealed a preference for small residues by the S1’ pocket and a Glu by the S3 pocket (Stennicke et al., 2000; Thornberry et al., 1997). The preferences of the S2 and S4 pockets vary widely within the caspase family and these distinctions account for minor specificity differences among the caspases. The executioner caspases are optimally suited to accept Asp and Val in P4 and P2, respectively. While the apical caspases tend to prefer Leu or Ile in P4 and more bulky residues in P2. The S4 pockets of the inflammatory caspases are optimized to accept aromatic residues such as Tyr and Trp. Caspases possess some of the most stringent substrate specificities known in the protease field and only carry out limited proteolysis on their substrates.
A. Human Caspases

<table>
<thead>
<tr>
<th>Caspase</th>
<th>CARD</th>
<th>Lg Su</th>
<th>Sm Su</th>
<th>Cytokine Activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase-1</td>
<td>CARD</td>
<td>Lg Su</td>
<td>Sm Su</td>
<td></td>
</tr>
<tr>
<td>Caspase-4</td>
<td>CARD</td>
<td>Lg Su</td>
<td>Sm Su</td>
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<tr>
<td>Caspase-5</td>
<td>CARD</td>
<td>Lg Su</td>
<td>Sm Su</td>
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Caspase 3

<table>
<thead>
<tr>
<th>Caspase</th>
<th>Lg Su</th>
<th>Sm Su</th>
<th>Apoptotic Execution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase-7</td>
<td>Lg Su</td>
<td>Sm Su</td>
<td></td>
</tr>
<tr>
<td>Caspase-6</td>
<td>Lg Su</td>
<td>Sm Su</td>
<td></td>
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B.

![Diagram of Caspase Active Sites](image)

Figure 1.3 Human Caspases. A) Cartoon representation of the human caspases, all have a large subunit (Lg.Su) and a small subunit (Sm.Su). At the most basic level, caspases are divided primarily into those having a role in cytokine maturation or apoptosis. B) The active site, caspase-3 inhibited by Acetyl-Asp-Val-Ala-Asp-methyl ketone (PDB 1CP3). Upper panel points outs the catalytic dyad (Green), His<sup>237</sup> and Cys<sup>285</sup> and the residues that provide the basic nature of the S1 pocket (Orange), Arg<sup>179</sup>, Gln<sup>283</sup>, and Arg<sup>341</sup>. The lower panel displays a surface to depict the substrate binding pockets, S1-S4.
1.4.1 The Apical Apoptotic Caspases

The activation of apical caspases marks the critical steps of both the extrinsic and intrinsic apoptotic pathways. The extrinsic apoptotic pathway involves the activation of caspase-8 and 10, whereas, the intrinsic pathway is dependent on caspase-9, and under some conditions, possibly caspase-2. Activation by forced dimerization on multimeric protein complexes is common principle in the activation of the apical caspases. Following activation these caspases are all capable of cleaving the executioner caspases-3 and 7, and thus the intrinsic and extrinsic pathways converge at this point.

Caspases-8 and 10 are at activated via death receptor signaling at the DISC. It was originally thought that oligomerization of caspases at the DISC allowed for transprocessing of the zymogen form of these caspases and subsequent activation. This assumed that the zymogen form of the caspase had a low intrinsic activity that was substantially increased as a result of cleavage between the large and small subunits (Salvesen, 1994). This model has since been refined, as it has been found that the oligomerization is actually the activation mechanism and the cleavage simply stabilizes the dimeric form of the caspase (Boatright et al., 2003; Pop et al., 2007).

Caspase-8 deficient mice have a defect in heart development and die during embryonic development. Consistent with the importance of caspase-8 in the extrinsic apoptotic pathway, fibroblasts from Caspase-8/-/- embryos fail to undergo apoptosis in response to death receptor ligation, yet remain sensitive to
stresses that induce the intrinsic apoptotic program (Varfolomeev et al., 1998). Interestingly, humans that have mutations in the caspase-8 gene leading to an inactive enzyme, develop normally yet have defective lymphocyte apoptosis and homeostasis. In addition these patients also display impairment in the activation of their T lymphocytes, B lymphocytes, and NK cells resulting in immunodeficiency (Chun et al., 2002). These findings suggest that in addition to its pro-apoptotic function, caspase-8 maintains a role in immune cell activation. In humans caspase-10 may be able to compensate for the loss of caspase-8 during development, but not in lymphocyte activation. Mice lack caspase-10 and this may explain the embryonic lethality of caspase-8-/- mice.

Caspase-9 is of primary importance to the intrinsic apoptotic pathway. The mechanism by which procaspase-9 is activated at the apoptosome has been a point of debate. It has been suggested the Apaf-1 allosterically activates the monomeric form caspase-9 (Rodriguez and Lazebnik, 1999; Shi, 2004; Shiozaki et al., 2002). However, a recent biochemical dissection of the mechanism of Apaf-1-mediated activation of caspase-9 demonstrated that direct dimerization of the caspase is required, thereby ruling out the allosteric activation model (Pop et al., 2006). This is consistent with the active form of caspase-9 being a dimer in the crystal structure. Like caspase-8, caspase-9 cleaves and activates the zymogen forms of caspases-3 and 7. Caspase-9 deficient mice display enlarged and malformed forebrains as a result of attenuated apoptosis during brain development and usually die perinatally. Thymocytes from these mice are
resistant to numerous intrinsic apoptotic stimuli, highlighting the importance of this protease intracellular apoptotic signaling (Kuida et al., 1998).

Although of seemingly less importance in canonical apoptotic signaling, caspase-2, has been proposed to play a role in apoptosis following DNA damage (Lassus et al., 2002) and as a result of Amyloid-β toxicity, and neurotrophic factor deprivation (Troy et al., 2000). The pro-domain of caspsae-2 has the conserved CARD fold that is important for the recruitment of the caspase to the multi-protein complex known as the PIDDosome (Tinel and Tschopp, 2004). The PIDDosome is composed the adaptor protein, RAIDD, containing a CARD and a DD, and PIDD (p53 induced protein with a death domain). The PIDDosome has been suggested of being capable of generating pro-apoptotic signals through caspase-2 activation and pro-survival cues mediated through NF-κB activation following genotoxic stress (Cuenin et al., 2007). The differentiation between these two signaling events of the PIDDosome has been reported to be dependent on the auto-proteolysis of PIDD itself (Tinel et al., 2007). Importantly, caspase-2 seems to play a minor and specialized role in intrinsic apoptotic signaling, whereas caspase-9 has a more global function in this signaling pathway.

1.4.2 The Executioner Apoptotic Caspases

The executioner caspases-3 and 7 represent the point of convergence of most apoptotic signaling pathways. Unlike the apical caspases, caspase-3 and 7 do not posses large N-terminal prodomains and are not associated with multimeric protein activation platforms. Caspases-8, 9, 10 and Granzyme B
cleave these executioner caspases in the interdomain linker between the large and small subunits resulting in their activation. Caspase-8 mediated cleavage of procaspase-3 results in an increase in the catalytic activity of caspase-3 by over four orders of magnitude (Stennicke et al., 1998).

Analysis of crystal structures of caspase-7 has provided much insight into proteolytic-dependent activation mechanism of the executioner caspases. The structure of the zymogen form of caspase-7 reveals that the uncleaved interdomain linker partially occupies the central cavity and the substrate binding loops are disordered (Chai et al., 2001b; Riedl et al., 2001a). A comparison of cleaved caspase-7 in the presence and absence of a peptide-based inhibitor demonstrates that when active site is unoccupied, the catalytic machinery is misaligned and only properly formed when the active site is occupied (Chai et al., 2001b; Wei et al., 2000). This suggests that substrate induced activation may be an additional component of the activation mechanism of caspases-3 and 7 - reviewed in (Fuentes-Prior and Salvesen, 2004). Significantly less is known about caspase-6. However, it does lack a large prodomain and shares a high degree of similarity within its catalytic domain with caspases-3 and 7, and thus it is most likely an executioner caspase.

Two independently derived caspase-3 deficient mouse strains yielded severely different developmental phenotypes. In one case the lack of caspase-3 caused a perinatal lethality as these mice had enlarged forebrains as a result of failed apoptosis of the neuronal progenitor cells. Conversely, another strain deficient in caspase-3 develops to adulthood with minimal neuronal abnormalities
(Leonard et al., 2002). Mice lacking caspase-6 and 7 do not demonstrate an overt developmental defect (Zheng et al., 1999). However, the conflicting phenotypes of caspase-3 deficient mice demonstrate that it is inappropriate to assign developmental and physiological roles of caspases based solely on gene ablation studies in mice.

1.4.3 The Inflammatory Caspases

Caspases do not only play roles in apoptosis, in fact the first member of the caspase protease family, caspase-1, was identified to have a role in the processing of the cytokine proIL-1β, to the mature IL-1β, a necessary step prior to secretion of this inflammatory mediator (Thornberry et al., 1992). Caspase-1 has also been demonstrated in the proteolytic activation of another cytokine, IL-18 (Ghayur et al., 1997). In addition, caspase-5 has been reported to have a role in the processing of IL-1β and associates with caspase-1 (Martinon et al., 2002). However, this caspase along with caspase-4 are much less well characterized and are classified as inflammatory caspases primarily on sequence similarity and substrate preference. The activation mechanism of the inflammatory caspases is thought to be analogous to that of the initiator apoptotic caspases-8 and 9, requiring the recruitment to multi-protein complexes that enforce activation via induced proximity of the zymogens (Gu et al., 1995; Martinon et al., 2002). The activation complex for inflammatory caspases is termed the “inflammasome” and it seems that there are several subsets of this activation platform composed of
slightly different protein constituents (Martinon et al., 2002; Martinon and Tschopp, 2004).

The fundamental component of an inflammasome is a member of the NLR (also termed NACHT-LRR or NOD-LRR, - reviewed in (Inohara et al.) protein family, which have pro-inflammatory roles in innate and adaptive immunity. Characteristic of the NLR family is the presence of C-terminal LRRs (Leucine Rich Repeats) that function as the sensors of intracellular pathogens much like the LRRs of the Toll-like receptors (TLR) that sense extracellular pathogens. In addition the central NACHT domain is present in all members of this protein family and functions as a nucleotide oligomerization domain, which is related to AAA+ NTPases (Inohara et al., 2005; Martinon and Tschopp, 2004). NLRs possess additional domains that serve as the effector regions of protein and usually contain either a CARD or a Pyrin domain (PYD). CARDs and PYD are classified as death fold domains and characteristically, undergo homotypic interactions with other proteins in multi-protein complexes (Reed et al., 2004).

The inflammasome-mediated caspase activation mechanism is proposed to be proximity-induced oligomerization. The oligomerization of an inflammasome is mediated through the NACHT domain of the NLR protein, while the caspase is recruited via interactions at the CARD or PYD. A PYD associates with caspase-1 through the adaptor protein, ASC. ASC is composed of a CARD capable of interacting with the CARD of caspase-1 and PYD that interacts with the PYD of some NLR proteins. The NALP proteins, with the exception of NALP1, only have a PYD and require ASC to tether to caspase-1. However,
adaptor proteins are not required for all inflammasomes. For instance, Ipaf (ICE protease activating factor, also known as CARD12 or CLAN), another member of the NLR family, possesses a CARD that interacts directly with the CARD of caspase-1 (Damiano et al., 2004; Mariathasan et al., 2004; Poyet et al., 2001). The fact that there are several NLR family members, suggests that each one may be able to sense and respond to a distinct set of ligands.

1.5 Natural Caspase Inhibitors

The activation of the initiator apoptotic and inflammatory caspases can be circumvented by disrupting the recruitment to their respective activation platforms. For example the decoy, CARD only proteins, ICEBREG, COP, CARD-8, and INCA interact with the CARD of caspase-1 or the CARD of the protein that recruits caspase-1 to an inflammasome and thereby inhibits the association of the caspase with its activation platform (Druihle et al., 2001; Humke et al., 2000; Lamkanfi et al., 2004; Lee et al., 2001; Razmara et al., 2002). Analogously, the short form of FLIP (Flice-like inhibitory protein) (FLIPs), which is composed of two DEDs similar to those of caspase-8, prevents caspase-8 activation by competing for binding at the DISC (Irmler et al., 1997).

Viruses have evolved exquisite mechanisms to prevent apoptosis of host cells so as to allow for viral replication. This is achieved by targeting the apoptotic machinery of the host cell. Some herpesviruses and molluscipoxviruses encode proteins similar to FLIPs that endows them with the ability to inhibit the extrinsic apoptotic signaling pathway. Other viruses are capable of direct inhibition of
caspases. The p35 and p49 proteins of the baculovirus potently inhibit many members of the caspase family (Bump et al., 1995; Jabbour et al., 2002; Ryan et al., 2002; Zhou et al., 1998). Cytokine response-modifier A (CrmA) is a serpin from the orthopox viruses that directly inhibits several caspases (Kamada et al., 1997; Zhou et al., 1997). p35 and CrmA are structurally distinct, yet both have long extended loops that are proteolytically cleaved by caspases. Following cleavage these inhibitors covalently bind the active site of the protease trapping it in an inactive conformation (Stennicke et al., 2002).

In multicellular organisms there are no inhibitory proteins capable of covalent interactions with caspases. In fact, there are relatively few endogenous proteins that are capable of direct caspase inhibition. Members of the inhibitor of apoptosis protein (IAP) family have often been reported to function as physiological inhibitors of caspases. However, only X-linked IAP (XIAP) has been well characterized in its ability to inhibit caspases (discussed below). A major aim of my thesis was to determine the requirements of IAP-mediated caspase inhibition.

1.5.1 The Inhibitor of Apoptosis (IAP) Protein Family

The inhibitor of apoptosis protein (IAP) family was discovered when a baculoviral gene was found to be sufficient to complement the loss of p35 (Crook et al., 1993). This IAP gene encodes a Zn-Finger like motif. Later, this motif has been denoted as the Baculoviral IAP repeat (BIR) domain and it is now the defining feature of the IAP family, members of which having one to three BIR
domains. IAP family proteins have since been found in many organisms ranging from flies to humans (Duckett et al., 1996). The IAPs have the unique ability to attenuate apoptosis induced both intrinsically and extrinsically. Therefore, the point of occlusion of the apoptotic pathway by the IAPs appears to be downstream of the convergence point of this pathways and most likely at the level of the caspases.

The human IAP family consists of eight members, most of which have been demonstrated to interact with caspases (Fig 1.4). However, the exact consequences of these interactions are relatively unknown, with the notable exception being the X-linked IAP (XIAP), which directly inhibits the proteolytic activity of the caspases. It seems that other IAPs may affect caspase activity by targeting them for proteasomal degradation, thereby mitigating the bio-effects of these proteases. BIR domains have been identified as the regions essential to the IAP/caspase interaction, and in the case of most IAPs, are necessary for their anti-apoptotic activity. Many BIR domains have a conserved surface groove that has preference for binding the extreme N-terminus of short peptides of defined sequence (Salvesen and Duckett, 2002; Shi, 2002). The peptide sequence preferred by this surface groove is reported to be of the consensus \((\text{NH}2)\text{A}\Phi\text{P}\Phi\), where \(\Phi\) represents a small hydrophobic amino acid and the \(\alpha\) amine group on the Ala is unblocked. This epitope is termed the IAP binding motif (IBM) and hence the surface groove is called the IBM-groove.

There are many proteins that possess potential IBMs, however, this binding motif is usually only exposed after proteolysis. A classic example of IBM-
containing proteins are the caspases. The activating cleavage within the interdomain linker of caspases-3 and 7 generates an IBM at the neo-amino terminus of the small subunit (Scott et al., 2005). Similarly, the autocatalytic processing of the same region of caspase-9 generates an IBM (Srinivasula et al., 2001). Thus IAPs are only capable of binding caspases that have been cleaved.

In addition to caspases, IAP-antagonists, such as the Second Mitochondrial Activator of Apoptosis (SMAC) (Du et al., 2000; Verhagen et al., 2000) and HtrA2 (Verhagen et al., 2002), possess the conserved IBM sequence and can disrupt IAP-caspase interactions (Srinivasula et al., 2001). Determining the exact peptide binding preference of distinct BIR domains was another major goal of my thesis project. The results of this aim provide insight into the natural binding partners of various IAPs.
Figure 1.4 The human IAP family. Schematic of the domain composition of the human IAPs. The BIR is the defining domain of this of protein family (Salvesen and Duckett, 2002).
1.5.1.1 X-Linked Inhibitor of Apoptosis Protein (XIAP)

XIAP (X-linked IAP), the most well characterized member of the IAP family, consists of three BIR domains and C-terminal RING domain. XIAP is capable of inhibiting caspases-3, 7, and 9. The mechanism of inhibition of caspases by XIAP has been determined at biochemical level, pinpointing the residues essential for physiologic caspase regulation (Chai et al., 2001a; Huang et al., 2001; Huang et al., 2003; Riedl et al., 2001a; Scott et al., 2005; Shiozaki et al., 2003; Sun et al., 1999; Sun et al., 2000). Tight inhibition of the executioner caspases-3 and 7 by XIAP requires two surfaces; the IBM-groove within the BIR2 which binds to the neo-N-terminus of the small subunit of the caspase; while linker region immediately N-terminal to this BIR domain binds across the active site cleft of the caspase to cause inhibition by steric occlusion (Fig 1.5) (Riedl et al., 2001b; Chai et al., 2001a; Huang et al., 2001).

XIAP uses its BIR3 domain for inhibition of caspase-9. Although the anchoring interaction of the neo-N-terminus of the small subunit of the caspase and the IBM-groove within the BIR domain is conserved, the mode of inhibition is not. A helix that resides immediately C-terminal to the BIR3 domain of XIAP packs against the dimmer interface of caspase-9 and induces monomerization/prevents dimerization, thereby reversing the activation mechanism of this protease (Shiozaki et al., 2003) (Fig 1.6).
Figure 1.5 Executioner Caspase Inhibition by XIAP-BIR2. Shown here is complex of caspase-3 (blue, surface) and XIAP-BIR2 (green, ribbon) (PDB 1I30) (Riedl et al., 2001b). The IBM-groove defining residues of BIR are shown in magenta, while the key caspase-inhibitory residues located in the N-terminal peptide strand presented in orange. Occupying the IBM-groove is the N-terminus of the small subunit of a neighboring caspase-3. This in The N-terminal peptide strand of the BIR binds across the caspase-3 active site (yellow) and prevents substrate access.
Figure 1.6 Caspase-9 Inhibition by XIAP-BIR3. Shown is the complex of caspase-9 (blue, surface) and XIAP-BIR3 (green, ribbon) (PDB 1NW9) (Shiozaki et al., 2003). The IBM-groove defining residues are displayed in magenta. The N-terminus of the small subunit of caspase-9 occupies the IBM-groove. Highlighted in orange are the residues within the distal helix of XIAP-BIR3 that interact with the dimer interface of caspase-9. This interaction induces monomerization of the caspase. XIAP-BIR3 does not interact with the active site (yellow) of caspase-9.
The BIR1 domain of XIAP does not function as a caspase inhibitor, and considerably less is known about this domain. The crystal structure of XIAP-BIR1 demonstrates the lack of the conserved IBM-groove and suggests this BIR is a dimerization domain (Lin et al., 2007). Recently it has been suggested that BIR1 may have a role in signaling through the NF-κB pathway. This function seems to rely on an interaction with Tab1 and signaling through the downstream kinase, Tak1 (Lu et al., 2007).

XIAP does not seem to maintain an essential role in development as knockout mice develop normally with no overt phenotypes. (Harlin et al., 2001). Interestingly there appears to be a compensatory role played by other members of the IAP family, as cellular IAP1 (cIAP1) and cIAP2 are highly upregulated in these mice. A common hypothesis regarding the physiological function of XIAP is that it acts like a safety catch, inhibiting caspases that are activated by mistake.

Further studies on XIAP deficient mice suggest that XIAP has a protective role in post-mitotic cells including neurons and cardiomyocytes (Potts et al., 2005; Potts et al., 2003). Human patients have been identified that have mutations in the gene encoding XIAP that causes a deficiency in XIAP protein expression. These individuals develop X-linked lymphoproliferative syndrome (XLP) usually following Epstein-Barr virus infections. Analysis of the lymphocytes of these patients demonstrates the importance of XIAP in immune
cell homeostasis, as these cells are more sensitive to a variety of apoptotic insults. XIAP deficient patients have a drastically reduced NKT cell population, demonstrating the importance of this apoptotic regulator in the viability of these cells in particular. Therefore, in humans XIAP seems to be required for the survival and possibly the differentiation of lymphocytes (Rigaud et al., 2006).

The RING of XIAP has been reported to function as an E3 ubiquitin ligase. In contrast to its BIR domains, the function of the RING of XIAP is less well characterized in terms of specific targets and consequences of the ubiquitin modification. It has been suggested that XIAP can mediate the ubiquitination of caspases-3 and 9 and the IAP-antagonist SMAC (MacFarlane et al., 2002; Morizane et al., 2005; Suzuki et al., 2001b). Interestingly, XIAP-mediated ubiquitination of SMAC did not cause it to be degraded by the proteasome (MacFarlane et al., 2002; Wilkinson et al., 2004). XIAP is also capable of auto-ubiquitination (Yang et al., 2000), however, the importance of this is yet to be elucidated (Shin et al., 2003). More recently, XIAP has been shown to ubiquitinate another IAP family member, Survivin, through the association with XIAP-associate factor 1 (XAF1) (Arora et al., 2007). The ubiquitination of pro-apoptotic molecules by XIAP, may contribute to its anti-apoptotic function, however, this seems to be of secondary importance compared to its role in direct inhibition of caspases.

1.5.1.2 Cellular Inhibitor of Apoptosis Proteins 1 and 2 (cIAP1 and cIAP2)

The closest paralogs to XIAP, cIAP1 (cellular IAP) and 2, also have three tandem BIR domains and C-terminal RING domain. Unlike XIAP the cIAPs have
CARD located between the third BIR and the RING domain. Both cIAP1 and cIAP2 have been demonstrated by several groups to ablate apoptosis triggered by multiple stimuli when over expressed in mammalian cells (Clem et al., 2001; Orth and Dixit, 1997; Roy et al., 1997; Simons et al., 1999; Uren et al., 1996). Substantially, much less is known about the mechanisms by cIAP1 and cIAP1 obstruct apoptosis. Due to their high degree of similarity with XIAP and the observed anti-apoptotic phenotype when overexpressed it is often reported that cIAP1 and cIAP2 are direct caspase inhibitors (Deveraux et al., 1998; Roy et al., 1997), albeit less potent than XIAP. The BIR2 and BIR3 domains of the cIAPs contain the consensus IBM-groove and should be capable of interacting with pro-apoptotic molecules including caspases and IAP-antagonists.

The cIAPs were initially identified as being associated with TRAF proteins as part of the TNF-receptor signaling complex (Rothe et al., 1995; Shu et al., 1996). It is through their associations with TRAF2 that the cIAPs are able to regulate receptor-mediated apoptosis seemingly through the NF-κB pathway. However, the mechanism by which this is achieved is not entirely straightforward. The RING domain of the cIAPs have been reported to be important for mediating these signaling events, pointing toward a critical role of the E3 ligase activity. Recently, the BIR1 domain of the cIAPs has been shown to be required for their association with TRAF2 (Samuel et al., 2005; Varfolomeev et al., 2006). Within the TNFR2 complex the cIAP1 has been reported to ubiquitinate TRAF2 causing its degradation (Li et al., 2002; Wu et al., 2005). In the TNFR1 complex both cIAP1 and cIAP2 are able to ubiquitinate RIP (Park et al., 2004b), and the NF-
κB-inducing kinase (NIK) (Varfolomeev et al., 2007). In the case of TNFR1 these ubiquitination events seem to dampen downstream signaling pathways. The TNFR1 is associated with two complexes composed of similar protein constituents, with apparently opposite functions in terms of apoptotic signaling (Micheau and Tschopp, 2003). Thus the consequence of ubiquitination of the components of TNFR1 is not resolved. Interestingly, NIK has been shown to induce the non-canonical NF-κB pathway that leads to an upregulation of TNFα and autocrine activation of caspase-8. cIAP-mediated ubiquitination of NIK attenuates this pathway and prevents a caspase-8-dependent apoptosis (Petersen et al., 2007; Varfolomeev et al., 2007; Vince et al., 2007).

Like XIAP, the cIAPs have also been shown to ubiquitinate caspases and SMAC (Hu and Yang, 2003; Huang et al., 2000). Interestingly, SMAC has also been suggested to negatively regulate the E3 activity of the cIAPs (Creagh et al., 2004) and specifically reduce protein levels of these IAPs (Yang and Du, 2004). The dimeric nature of SMAC may bridge cIAPs with each other or themselves, enabling trans-ubiquitination or auto-ubiquitination. An emerging proposition in the IAP field is that these proteins regulate the stability of each other. This evidenced by the ability of cIAP1 to promote degradation of both cIAP2 (Conze et al., 2005) and XIAP (Silke et al., 2005).

cIAP2 deficient mice demonstrate the importance of this IAP in the innate immune response. Lipopolysaccharide (LPS) stimulation of macrophages results in the upregulation of cIAP2, which is important in preventing the death of these cells in an LPS rich/pro-inflammatory environment. Mice lacking cIAP2
demonstrate an attenuated capacity to produce the inflammatory cytokines, IL-1β, TNFα, and IL-12 as macrophages of these mice readily undergo apoptosis following LPS treatment. Endotoxic shock, or sepsis, is often a result of a hyper-inflammatory response (Sriskandan and Cohen, 1995). Interestingly, mice deficient in cIAP2 are less capable of responding to infection but are also significantly more resistant sepsis. The importance of cIAP2 in the survival of macrophages in an inflammatory environment demonstrates one of the first functions elucidated to be specific to cIAP2 over cIAP1. However, this may simply be due to differences in the transcriptional regulation and not at the level of protein function.

### 1.5.1.3 Neuronal Apoptosis Inhibitor Protein (NAIP)

The founding member of the mammalian IAP family, NAIP (Neuronal Apoptosis Inhibitory Protein), was first proposed to have a neuroprotective function against the neurodegenerative disease spinal muscular atrophy (Liston et al., 1996). However, the role of NAIP in this disorder is questionable as the mutations responsible have been mapped to the adjacent SMN gene (Lefebvre et al., 1995). Furthermore, NAIP is only minimally expressed in neuronal tissue while highly expressed in white blood cells (Inohara et al., 2005).

NAIP is a unique member of the IAP family, having C-terminal LRRs and a central NACHT domain, in addition to the three BIR domains at its N-terminus. Therefore, NAIP is the only IAP, that is also a member of the NLR family (Inohara et al., 2005) and little is known as to function of it BIR domains, as they are positioned in the effector region of the protein. There are conflicting reports as to
whether the BIR2 of NAIP functions as caspase inhibitor (Maier et al., 2002; Roy et al., 1997). The third BIR domain of NAIP has been demonstrated to bind and inhibit caspase-9, however, this too is questionable (Davoodi et al., 2004). Elucidating the consequences of the interaction between caspases and the BIRs of NAIP is a third major aim of my thesis research. Therefore, prior to this thesis the word was still out as to the capacity of NAIP to function as caspase inhibitor.

The A/J mouse strain has multiple polymorphisms and deletions in the \textit{Lgn1} locus, which is comprised of the several NAIP paralogs and macrophages isolated from these mice are permissive to intracellular \textit{Legionella pneumophila} replication. These A/J mouse-derived macrophages display marked differences in \textit{Legionella} \textit{p}.-directed bacteriostatic activity when compared to macrophages derived from mice that have the restrictive \textit{Lgn1} locus (Yoshida et al., 1991). The \textit{NAIP5/BIRC1e} gene within the \textit{Lgn1} locus has been deduced as the gene responsible for these strain differences in this response (Derre and Isberg, 2004; Diez et al., 2003; Diez et al., 2000; Growney and Dietrich, 2000; Growney et al., 2000; Wright et al., 2003). The expression of the NAIP protein has been observed to be upregulated in macrophages following phagocytosis (Diez et al., 2000), providing support for a role of NAIP in the response to this pathogen. It has been reported that the restrictive isoform of NAIP5 is required for the activation of caspase-1 resulting in macrophage apoptosis (Derre and Isberg, 2004; Zamboni et al., 2006). This may represent a means by which the host organism copes with the infection, thereby removing the infected cells, so as to curtail the spread of the infection. The recognition of cytosolic flagellin has been
observed as an essential component in the restrictive response toward Legionella p. (Molofsky et al., 2006; Ren et al., 2006). Therefore, it seemed as though the ligand for the LRRs of NAIP might be flagellin and the effector caspase-1. A recent study, however, showed that NAIP5 contributed to the restrictive response through an unknown mechanism, independent of flagellin recognition and caspase-1 activation (Lamkanfi et al., 2007). Thus, the function of NAIP in response to this pathogen is not as straightforward as originally thought. A third major aim of my thesis was to investigate the function of the BIR domains of NAIP, in hope of gaining insight into a physiologic role.

1.5.1.4 Other Mammalian Inhibitor of Apoptosis Proteins

The remaining members of the human IAP family are melanoma IAP (ML-IAP), Survivin, Bruce, and IAP-like Protein 2 (ILP2). ML-IAP is highly expressed in multiple melanoma cell lines, yet minimally expressed in most tissues including normal melanocytes, thus the role of this IAP under normal physiologic conditions is not clear. ML-IAP has a single BIR domain and C-terminal RING domain and like most IAPs has been proposed to function as an inhibitor of caspases (Vucic et al., 2000). Furthermore, SMAC has been shown to antagonize the anti-apoptotic function of ML-IAP, demonstrating the importance of the IBM-groove of this IAP in blocking apoptosis (Vucic et al., 2002).

Survivin, is only expressed at the G2/M phase of the cell cycle. It associates with chromatin and has been shown to have an essential regulatory role in the chromosomal passenger complex with Aurora B kinase and INCENP, thereby mediating chromosomal replication and cell cycle progression (Honda et
Survivin may not even be truly anti-apoptotic, but may stabilize XIAP and thereby potentiate its anti-apoptotic function (Dohi et al., 2004). Bruce has a single BIR domain and UBC domain, similar to those found in ubiquitin conjugating enzymes and has been demonstrated to function as an E2/E3 ubiquitin ligase that targets the pro-apoptotic molecule SMAC (Bartke et al., 2004). The BIR domain of ILP2 is 81% identical to BIR3 of XIAP, however, lacks conformational stability and therefore its true function remains elusive. (Shin et al., 2005).

1.5.1.5 Drosophila IAP1 and 2 (DIAP1 and DIAP2)

*Drosophila melanogaster* have two IAP family members, DIAP1 and DIAP2, the former of which has two BIR domains, while the latter has three BIR domains (Hay et al., 1995). DIAP1 has been more extensively studied and its BIRs posses the conserved IBM-grooves that enable the interactions with the *Drosophila* apical caspase, DRONC, the *Drosophila* effector caspases drICE and DCP-1, and the *Drosophila* IAP-antagonists reaper (Rpr), Grim, and HID (Chai et al., 2003; Tenev et al., 2005; Tenev et al., 2002; Yan et al., 2004; Zachariou et al., 2003). Overexpression of these IAP-antagonists disrupts the IAP/caspase interaction and promotes apoptosis (Goyal et al., 2000; Hawkins et al., 2000). Evidence of direct caspase inhibition by DIAP1 or DIAP2 is lacking and it has been reported that caspases associated with DIAP1 are still catalytically active (Tenev et al., 2005). Similar to many of their mammalian counterparts, DIAPs have a C-terminal RING domain in addition to their BIR domains. It been shown that the RING is required for auto-ubiquitination of DIAP1 and DRONC, and it is
necessary for the anti-apoptotic function of this IAP (Lisi et al., 2000; Vaux and Silke, 2005a; Wilson et al., 2002). Therefore the primary mechanism of apoptotic occlusion by the DIAPs is most probably dependent on proteasomal removal of the caspases and not direct inhibition. An interesting study by Ditzel and colleagues put forth the concept that removal of caspases by DIAP1 occurs through N-end rule based degradation (Ditzel et al., 2003). In essence, the association of DIAP1 with a caspase results in proteolysis of the IAP at its N-terminus, exposing a destabilizing residue, that causes the removal of DIAP1 and the caspase to which it is bound. The N-end rule postulates that the stability of a protein is dependent which amino acid is present at its N-terminus - reviewed in (Varshavsky, 2003). Thus the importance of ubiquitination for the anti-apoptotic function of DIAP1, suggests that the E3 ligase activity of many IAPs is the evolutionarily conserved mechanism of a cell death prevention.

1.6 Apoptosis and Disease

Dysregulation of apoptotic signaling pathways has been suggested to be a component of over 70% of all human diseases (Reed, 1999). A simplified view suggests that in cases in which apoptotic signaling pathways are overly engaged results in degenerative orders. Conversely, instances of insufficient apoptosis can induce a proliferative pathology, including cancer and autoimmune states.
1.6.1 Neurodegenerative Diseases

The role of caspases in neurodegenerative diseases is relatively unclear and remains a point of investigation by several laboratories. Apoptosis of neuronal cells has been demonstrated following neurotrophic factor withdrawal during development, however, there is little evidence that suggests trophic factor withdrawal is a primary component of neurodegenerative diseases (Yuan and Yankner, 2000). Necrosis accounts for the majority of the cell death in the brain following ischemic injury as the cells in close proximity to the injury site swell and rupture. Apoptotic cells have been found in regions more distal to the injury site. This suggests that the severity of the insult determines whether the cell death response will apoptotic or necrotic in nature, with the former only occurring under more mild stress conditions. (Friedlander, 2003; Yuan and Yankner, 2000). Furthermore, it is likely that the progressive degenerative diseases have an apoptotic component, as evidenced mouse models of these diseases.

Amyotrophic lateral sclerosis (ALS) patients suffer from a progressive loss of motor neurons throughout the brain, brain stem and spinal cord. Mice expressing a mutant form of the human superoxide dismutase (SOD1) gene, found in a subset of ALS patients, develop many common features of the human disease. Intraventricular administration of the pan caspase inhibitor, zVAD-FMK seems to slow the disease progression and increase the life span of these mice (Li et al., 2000). ALS mice that express the Bcl-2 gene demonstrate prolonged
life when compared to other ALS mice, suggesting a role of caspase-9 and the mitochondrial pathway in this pathology (Kostic et al., 1997).

The presence of expanded polyglutamine tracts is characteristic of some common neurodegenerative diseases including Huntington’s disease. The presence of these polyglutamine repeats within proteins results in the formation of aggregates. Originally thought to be the nature of the neurotoxicity associated with the disease, this has been questioned in recent years and the word is still out on whether these aggregates have pathological effect on the cell (Klement et al., 1998; Saudou et al., 1998). Although, neuronal cell death associated with this disease is not solely apoptotic many reports observed caspase activation in mouse models and well as human patient samples – reviewed in (Friedlander, 2003).

The evidence for aberrant apoptosis in brains of patients suffering from Alzheimer’s diseases is limited. A key characteristic of this disease is accumulation of amyloid-β protein within patient’s brains. It has been reported that caspase-2 is involved in neuronal cell death in response to amyloid-β deposition (Troy et al., 2000). In addition, neuronal cell death may be induced by the inflammatory signaling predominant in Alzheimer’s brains. This is mediated by the activation of the microglial cells causing the production and secretion of TNF-α and potentially other cytotoxic agents. Thus, neurons are simply bystanders that are directed to undergo apoptosis as a result of increased inflammation (Tan et al., 2002).
Increased inflammatory signaling may be a common mechanism of neuronal cell death in many neurodegenerative diseases. HIV associated dementia is thought to be caused by apoptosis of neurons and astrocytes, however, the microglia and macrophages are the primary cells directly infected by the virus. Thus it is hypothesized that infection of these inflammatory cells leads to the secretion of various factors that are either directly toxic to neuronal cells, or set in play signaling events that culminate in excitotoxic insults or other apoptotic signaling pathways in neuronal cells – reviewed in (Kaul and Lipton, 2006).

Overall necrosis takes a predominant role in acute neuronal injury, while apoptosis may be more involved in the pathology of progressive degenerative diseases. However, it still remains to be determined if apoptosis plays a primary role in the onset of neurodegenerative disease or whether neuronal cells are triggered undergo apoptosis as a secondary event; a consequence of exposure to toxic agents either derived from necrotic or inflammatory cells. In the latter case, neuronal apoptosis would serve to exacerbate the pathologic state only after a non-apoptotic insult has occurred. As mentioned above caspase inhibitors have protective effect in both acute and degenerative diseases of the nervous system. However, it is not clear whether these inhibitors are dampening pathologies associated with apoptotic caspase activity or hindering the inflammatory signaling at the level of the inflammatory caspases.
1.6.2 Diseases of the Immune System

Proper function of the immune system relies on the regulated flux of lymphocyte cell number. T and B-lymphocytes proliferate in response to antigen stimulation and most of these must be subsequently eliminated to basal levels. The mechanism of this lymphocyte population control is mediated through apoptosis. Therefore, dysfunction in the apoptotic signaling pathways in immune cells can disrupt these normally tightly regulated waves in cell number.

Aberrant or uncontrolled apoptosis can result in the inability of the immune system to sense and/or respond to various infections. On the other hand insufficient apoptosis of immune cells during development or following antigen stimulation might mark the beginning of an autoimmune state. Mice with the lpr (lymphoproliferation) or gld (generalized lymphoproliferative disease) mutations have symptoms similar to those that arise from the human disease, systemic lupus erythematosus, characterized by accumulation of T-cells due to apoptotic signaling failures. The lpr mice harbor a point mutation in the DD the Fas receptor that ablates apoptotic signaling. Whereas, the gld mice posses a mutation in Fas Ligand that precludes its ability to properly interact with the receptor (Nagata, 1998). Defects in Fas signaling have also been reported in the human disease, autoimmune lymphoproliferative syndrome (ALPS). ALPS patients have serve autoimmunity due to an increased T-cell population. Autoreactive T-cells may arise as a result of failure in AICD and experimental
models demonstrate that the drugs that aid in Fas induced apoptosis could prove beneficial in deletion of these T-cells (Zhou et al., 1999).

1.6.3 Cancer

Normally cells that have genetic instability readily undergo apoptosis and therefore avoidance of apoptotic signaling is a necessary step in the transformation process. A cell that is capable of apoptotic insensitivity is well poised to become cancerous, as this creates a situation ideally suited to propagate mutations that can endow the cell with a barrage of characteristics that can drive forward a cancerous state. Through the propagation of unstable genetic material a cell can acquire the ability to resist destruction by the immune system, ignore cell cycle checkpoints, and gain the ability to grow in the absence of sufficient growth factors and extracellular matrix components.

There are several mechanisms by which cancer cells dampen their response to apoptotic signaling pathways. It is often observed that there is a dysregulation in a key component of the apoptotic machinery. Endogenous anti-apoptotic molecules are often over expressed in several cancer types. The anti-apoptotic members of the Bcl-2 protein family have been reported to be upregulated in numerous cancers – reviewed in (Cory and Adams, 2002; Fesik, 2005; Reed, 2002). These proteins hinder the intrinsic apoptotic pathway downstream of the DNA damage signaling components and thereby allow the cell to remain viable and while propagating mutations. Although the mechanism of over expression of anti-apoptotic Bcl-2 proteins is usually a result of post-
translational regulation, there have been reports that demonstrate direct activation at the transcriptional level. For instance, most non-Hodgkin’s lymphomas have a chromosomal translocation t(14:18) that causes increased expression of the bcl-2 gene (Galteland et al., 2005; Tsujimoto et al., 1985); while a retroviral gene insertion causing the increased expression of the bcl-XL gene is found in murine leukemia-derived cell line (Thomas et al., 1998). Several Bcl-2/Bcl-XL targeting therapeutics are currently under clinical development for numerous cancer types.

Another family of pro-survival proteins, members of which are often upregulated in various cancers are the IAPs. In fact survivin is one of the most commonly up regulated molecules in cancers across the board (Ambrosini et al., 1997). The expression of XIAP has been evaluated in several tumor types (discussed below). While many mucosa-associated lymphoid tissue (MALT) lymphomas demonstrate chromosomal translocations creating two fusion proteins consisting of the three BIR domains of cIAP2 and paracaspase (Uren et al., 2000). These fusion proteins are thought to induce the activation of NF-κB thereby engaging its pro-survival functions. The expression of ML-IAP is highly restricted to melanoma cells and may directly contributes to the oncogenic state (Vucic et al., 2000).

Many pro-apoptotic molecules exhibit dampened expression or attenuated function in cancer cells. There have been many reports on the loss of expression of the pro-apoptotic members of the Bcl-2 family, such as Bax and Bak, in various cancers (Kondo et al., 2000; Meijerink et al., 1998; Rampino et al., 1997).
The p53 gene is mutated in many cancers, thereby limiting the cell’s ability to initiate cell cycle arrest, DNA repair, and apoptosis (Sherr, 2004). This can accelerate the oncogenic process through the propagation of mutations. Adenoviral delivery of the p53 gene to patients with non-small-cell lung cancer or head and neck cancer is in phase II and phase III clinical trials, respectively. It will be interesting to watch the development of this treatment tactic as gene delivery therapies have received bad press over the years. However, the delivery of this central tumor suppressor gene may be the best way to reengage apoptotic signaling pathways in these cancers.

Cancer cells acquire not only the capacity to avoid apoptotic cues initiated from within, but also those from extracellular stimuli. The immune system uses death receptors to induce apoptosis of damaged and virally infected cell, both of which are ideal candidates to become cancerous cells. Mutations that result in the loss of the Fas receptor function have been observed in several cancers, endowing them with a means to resist tumor immuno-surveillance (Debatin et al., 1997; Friesen et al., 1997). CTLs also induce apoptosis through the granule secretory pathway. As there are no endogenous inhibitors of granzyme B in mammalian cells, thus a tumor cell has no defense against this protease, except at the level of XIAP. Through upregulation of XIAP, decreased expression of IAP-antagonists, or both, a cancer cell may be able to resist apoptosis induced by granzyme B through inhibition of caspases-3 and 7.
1.6.3.1 IAPs in Cancer and as Drug Targets

Many studies suggest that IAPs are upregulated in various types of cancers and in some cases it seems that they directly contribute to the oncogenic state (Adida et al., 1998; Adida et al., 2000a; Adida et al., 2000b; Krajewska et al., 2003; Lu et al., 1998; Tamm et al., 2000; Tamm et al., 2004a; Tamm et al., 2004b; Uren et al., 2000; Vucic et al., 2000; Yang et al., 2003). In cases of acute myeloid leukemia (AML), patients with high XIAP expression have shorter survival periods when compared to patients with lower XIAP expression levels (Tamm et al., 2004a; Tamm et al., 2004b). Increased expression of XIAP in clear-cell renal carcinoma correlates with a more aggressive pathology and decreased survival. However, XIAP expression is not always predictive of disease outcome and survival and in some cases tumors expressing XIAP correlates with a better prognosis (Ferreira et al., 2001). The basis for these differences is not resolved. A likely possibility is the requirement of XIAP toward the viability of some immune cells that may be vital in tumor immunosurveillance (Rigaud et al., 2006).

Although seemingly lagging behind other cancer therapeutics there are now several groups developing targeting IAP-based therapies for cancer. There two primary means of targeting the IAPs, antisense oligonucleotides and small molecules. Antisense oligonucleotides targeting XIAP have moved into early phase clinical trials and have been demonstrated to be effective in sensitizing various cancer cells to chemotherapeutic agents (Cummings et al., 2005; Hu et al., 2003). However, the mechanism of uptake of these oligonucleotides is
unclear and toxicity towards post-mitotic cells could be an issue. In addition, the ablation of the XIAP mRNA may result in the upregulation of the cIAPs as evidenced by the XIAP knockout mice (Harlin et al., 2001).

The development of small molecule IAP-antagonist is a more widely explored area. Many groups have generated SMAC-mimetics that have been shown to induce apoptosis or sensitize these cells to various apoptotic stimuli (Arnt et al., 2002; Li et al., 2004; Oost et al., 2004; Park et al., 2004a; Sun et al., 2004; Zobel et al., 2006). These compounds bind to the IBM-grooves of the IAPs and in the case of XIAP, repress its ability to inhibit caspases. An alternative strategy, implemented by other researchers, is to specifically target the executioner caspase inhibitory site on XIAP (Schimmer et al., 2004; Wu et al., 2003). These compounds might be more specific to XIAP, and should reactivate the apoptotic program further downstream.

Interestingly, recent reports demonstrate that the primary targets of SMAC-mimetics are the cIAPs. The interaction of cIAP1 and cIAP2 with various SMAC-mimetics results in auto-ubiquitination and subsequent degradation, which seems to lead to the transcriptional up regulation of TNFα and caspase-8 dependent apoptosis (Petersen et al., 2007; Varfolomeev et al., 2007; Vince et al., 2007; Wu et al., 2007). This is very unexpected and represents a very unique mechanism of action. However, it also raises a word of caution and may suggest that a better understanding of the role played by various IAPs in cancers is needed before therapies directed at these cell death regulators are fully
exploited. At this point the word is still out as what is the approach for IAP-directed cancer therapeutics.
1.7 Objectives of This Thesis

**Aim I. Dissection of the structural requirements of IAP-mediated caspase inhibition.** Due to their anti-apoptotic roles, and close similarity with XIAP, it has often been suggested that all IAPs are caspase inhibitors. In this aim, I examined the caspase inhibitory potential of several IAP BIR domains and elucidated the specific requirements for inhibition of both the executioner caspases-3 and 7 as well as the initiator caspase-9. These studies mark a revision in the IAP field, as XIAP seems to be the only member of the family capable of physiologic caspase inhibition (Eckelman and Salvesen, 2006; Eckelman et al., 2006; Vucic et al., 2005).

**AIM II. Defining the peptide binding signatures of various IAP BIR domains.** The classic IAP Binding Motif (IBM) has been stated to be (NH2)ΑΦΡΦ, where Φ represents a hydrophobic amino acid. However, this has been defined by sequence analysis of IAP-binding proteins and limited profiling studies on a few BIR domains. In this aim I sought to determine if IAP BIR domains maintained distinctions in their peptide binding preferences. To investigate this, I developed a profiling method that enables the specific analysis of IBM-groove directed peptides. These studies revealed there are distinctions in binding signatures of the BIR domains, which naturally bifurcate into classic SMAC-like signatures and unique signatures. These studies have enabled me to
predict which BIRs will have the classic preference and which will be more varied in their specificity.

**AIM III. Investigation into the consequences of NAIP/caspase interactions.** There have been conflicting reports as to the capacity of the BIR domains of NAIP to function as caspase inhibitors. Based on the knowledge I obtained in the first aim of this thesis, the BIR domains of NAIP seem ill suited to function as caspase inhibitors. I tested this and found that the BIR domains of NAIP did not function as inhibitors of the apoptotic caspases. However, the more interesting finding of this aim, was the ability of the NAIP-BIR2 to function as potent caspase-5 activator. The results of this aim provide the first direct evidence that NAIP may function as part of a specialized inflammasome.
2. MATERIALS AND METHODS
2.1 MATERIALS

2.1.1 General Equipment

- Fmax Fluorescence Plate Reader (Molecular Devices)
- Procise 492 protein sequencer (Applied Biosystems).
- UV/VIS Spectrophotometer (Spectronic GENESYS 5, Milton Roy)
- GeneAmp® PCR system 2400 (Perkin Elmer)
- SpectraMAX 340 plate reader (Molecular Devices)
- AKTA size exclusion chromatography system (GE)
- LS50B luminescence spectrophotometer (Perkin Elmer)
- LJL Analyst Fluorescence Plate Reader (Molecular Devices)
- Sonicator (Branson)
- Refrigerated Shaker (New Brunswick Scientific)
- Baffled shaker flasks (Bellco)
- Immobilon-P (PVDF) membrane (Millipore)
- Centrifuge. Eppendorf Microcentrifuge 5415C,
- Refrigerated Centrifuge (Sorval)
- Dialysis Membrane (various MWCO, Spectrum)
- Microconcentrators (Microcon, Amicon)
- Sterilizing Filter Unit (0.45 µm, Millipore)
2.1.2 Chemicals

General chemicals of appropriate grade (reagent, analytical, molecular biology) were purchased from the following companies: VWR, Fisher, Sigma-Aldrich, Bio-Rad and GIBCO-BRL.

• IPTG

• Chelating Sepharose® fast flow (Pharmacia).

• Glutathione Sepharose 4B (APBiotech Inc)

• Peptide synthesis resins

• FITC (Emd biosciences)

2.1.3 Buffers and Solutions

• **Buffer A.** 50 mM Hepes, 100 mM NaCl, pH 8.0.

• **Buffer B.** 50 mM Hepes, 500 mM NaCl, pH 8.0.

• **Buffer C.** 50 mM Hepes, 100 mM NaCl, 200 mM imidazole, pH 8.0.

• **Caspase assay buffer.** 50 mM Hepes, 100 mM NaCl, 0.1% (w/v) Chaps, 20 mM 2-Mercaptoethanol, 10% sucrose, pH 7.4.

• **3 x SDS loading buffer.** 10% (w/v) SDS, 28% (v/v) glycerol, 70% (v/v) 4 x stacking gel buffer, small amount of bromophenol blue.

• **6 x DNA loading buffer.** 30% (v/v) glycerol, 0.01 M EDTA, small amount of bromophenol blue.

• **Blocking buffer 1.** 5% Milk, in PBS with 0.1% Tween-20 (for western bolts)

• **Blocking buffer 2.** 3% BSA, in TBS with 0.1% Tween-20 (for
Streptavidin blots)

- **Transfer buffer for blotting.** 10 mM CAPS (pH 11.0), 10% (v/v) methanol
- **PBS (10x).** 0.58 M Na$_2$HPO$_4$, 0.17 M NaH$_2$PO$_4$·H$_2$O, 1.37 M NaCl.
- **PBS-T.** 1 x PBS, 0.1% Tween-20.
- **Stacking gel acrylamide solution.** 12.8% (w/v) acrylamide, 0.8% (w/v) bisacrylamide.
- **Stacking gel.** 1 ml H$_2$O, 1 ml 50% sucrose, 1 ml 4 x stacking gel buffer, 1 ml stacking gel acrylamide solution. Mix well, add 20 µl TEMED and 20 µl 10% ammonium persulfate (APS).
- **TBE (10x).** 20 mM Tris/HCl, 5.5% (w/v) boric acid, 50 mM EDTA (pH 8.0).

### 2.1.4 Molecular biology reagents

- Restriction enzymes and buffers (New England Biolabs)
- Thermal-stable DNA-polymerase (Expand High Fidelity DNA polymerase, Taq DNA polymerase) (Roche)
- Oligonucleotides for PCR (Roche)
- T4 ligase (Roche)
- Alkaline phosphatase (Roche)
- DNA standard (Fischer)
- SDS-PAGE standard BIO-RAD
• Oligonucleotides for PCR (Roche)

2.1.5 Plasmid Vectors

• pET15b. E. coli expression vector (Novagen). -T7-lacZ promoter, Ampicillin resistance (AmpR) and a N-terminal His6 or His8 tag.

• pET23b(+). E. coli expression vector (Novagen). -T7 promoter, AmpR and a C-terminal His6 tag.

• pGEX-4T-1 E. coli expression vector (APBiotech). -lacZ promoter, thrombin cleavage site, AmpR and a N-terminal GST-domain.

• pcDNA3 and pCDNA6 Mammalian expression vector (Invitrogen) –CMV promoter AmpR HygromycinR, FLAG or V5/His tag

2.1.6 E. coli strains

• BL21(DE3): E. coli B-ompT[lon] hsdSB (rB-mB-) (DE3) (1 prophage carrying the T7 RNA polymerase gene) (CmR) ∆(lac-proAB). Expression host for pET and pGEX-4T-1 constructs.


2.1.7 Synthetic enzyme substrates and inhibitors

2.1.7.1 Fluorogenic substrates

• acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (Ac-DEVD-AFC) (Anaspec)

• acetyl-Asp-Glu-Val-Asp- p-nitroanilide
(Ac-DEVD-pNA)
- acetyl-Leu-Glu-His-Asp-7-amino-4-trifluoromethylcoumarin (Ac-LEHD-AFC) (Anaspec)
- acetyl-Trp-Glu-His-Asp-7-amino-4-trifluoromethylcoumarin (Ac-WEHD-AFC) (Anaspec)

2.1.7.2 Irreversible inhibitors
- The pan-caspase irreversible inhibitor benzoxy carbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-FMK) (Anaspec)
- Biotinyl-Glu-Val-Asp-[(2,6-dimethylbenzoyl)oxy]methylketone (Biotinyl-EVD-aomk), was a kind gift of Dr. Matt Boygo (Stanford University)
- acetyl-Trp-Glu-His-Asp –ketone Ac-WEHD-CHO (Anaspec)

2.1.8 Antibodies and Detection Reagents
- Caspase-7 -Cell Signaling Technology #9494
- Caspase-3 -Santa Cruz Biotechnology #H-277
- Caspase-9 – made in house
- HRP-conjugated Streptavidin
2.2 METHODS

2.2.1 Polymerase Chain Reaction (PCR)

The basic PCR protocol used was as follows: nanogram amounts of template DNA (in colony PCR, a single colony in H₂O is used as the template), 100 µM dNTP, 1 µM each of the two primers, 1-2 mM MgCl₂ and 1 unit of thermal-stable DNA polymerase in the PCR buffer supplied by the manufacturer, in a total volume of 50 µl.

Thermal profiles were varied and optimized according to the components, but typically include a 25-30 cycles at 94°C for 45 seconds to denature, 50-60°C for 30 seconds for primer annealing, and 68°C for 1 minute/kilobase for extension. The annealing temperature (Tm) was of the primers determined using the operon website (https://www.operon.com/oligos/toolkit.php). Usually the annealing temperature set to be 5°C less than the Tm of the primers.

Site directed mutagenesis was achieved using overlap PCR. In short, a set of four primers were required, forward and reverse flanking primers each containing a restriction site and forward and reverse internal primers that contain the desired mutation. Two PCRs are carried out, 1) the forward flanking primer with the reverse internal primer and 2) the forward internal primer with the reverse flanking primer. This PCR typically uses the standard method as above and the template is the respective plasmid containing the cDNA that is to be changed. The PCR products are then subjected to electrophoresis on a TBE gel.
with 1\% Agarose and the bands are excised and purified. A second PCR reaction is then carried out in which 1 \( \mu l \) of each purified PCR product is used as the template. This reaction is carried out for five cycles in the absence of a primers, after which the forward and reverse flanking primers are added and the PCR is subject to an addition 20-25 cycles. Following electrophoresis and purification of the band the PCR product is digested with the appropriate restriction enzymes and ligated into the vector.
### 2.2.2 Cloning

#### 2.2.2.1 IAP constructs

**Table 2.1. Cloning Strategy for IAP constructs.**

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<tr>
<th>Name</th>
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<th>Vector</th>
<th>RE Sites</th>
<th>Domain Limits</th>
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Table 2.2 Primers for IAP constructs

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Table 2.3 Caspase-5 cloning strategies

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Table 2.4 Caspase-5 Primers

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2.2.4 Recombinant Protein Expression

Recombinant proteins were synthesized in *E. Coli* strain BL21. Cells were grown at 37°C and protein expression was induced with the addition of 0.4 mM IPTG when cells reached an OD$_{600}$ of 0.6. Cultures were incubated 4 hours at 30°C following IPTG induction. Proteins were purified using chelating Sepharose (APBiotech Inc.) charged with NiSO$_4$ in buffer containing 50 mM HEPES and 100mM NaCl at pH 7.4. For binding assays, proteins were left on beads and were washed three times in each of the following buffers: 1) 50 mM HEPES, 100mM NaCl, 20mM Imidazole pH 7.4, 2) 50mM HEPES, 100 mM NaCl, and 1%-TritonX-100 pH 7.4, and 3) 50 mM HEPES, 100 mM NaCl pH 7.4. For fluorescence polarization proteins were eluted in a buffer contain 50 mM HEPES, 100mM NaCl, 200mM Imidazole pH 7.4.

2.2.5 Determination of Protein Concentration

The absorbance at wavelength of 280nm (A280) was measured using spectrophotometer (Spectronic GENESYS 5, Milton Roy). The estimated extinction coefficient of the protein was determined using the ExPASy ProtoParam tool (http://ca.expasy.org/tools/protparam.html) on the primary amino
acid sequence. Using the equation: \((A_{280}/\text{Extinction Coefficient})\times10^6\) the protein concentration was calculated in \(\mu\text{M}\).

### 2.2.6 Caspase Inhibition/Activation Assays

Recombinant caspases were pre-activated with 20 mM 2-mercaptoethanol in buffer containing 50mM HEPES, 10% Sucrose (w/v) and 0.1% CHAPS with 100nM NaCl for caspases-1, 2, 3, 4, 5 and 7 or without NaCl for caspase-9 at pH 7.4 for 10 minutes) at 37°C. A range of BIR domain concentrations was incubated with the activated caspases for 5 minutes. Caspase activity was determined by cleavage of Ac-Asp-Glu-Val-Asp-AFC (Enzyme Systems Products) for caspase-3 and 7; Ac-Leu-Glu-His-Asp-AFC (Enzyme Systems Products) for caspase-9; Ac-Val-Asp-Val-Ala-Asp-AFC (Enzyme Systems Products) for caspase-2; or Ac-Trp-Glu-His-Asp-AFC (Enzyme Systems Products) for caspases-1, 4, 5; as measured in an Fmax fluorescence microplate reader (Molecular Devices. Sunnyvale, CA). Inhibitory constants for each enzyme-inhibitor pair was determined from the uninhibited rate (\(v_0\)) and inhibited rates (\(v_i\)), such that a plot of \((v_0/v_i)-1\) vs. \([I]\) gives a slope of \(1/K_i(\text{apparent})\).

Inhibition of endogenous executioner caspase activity by BIR2 was determined using HEK293A cell-free lysates pre-activated for 15 minutes as described above followed by incubation for 5 min with selected recombinant BIR2 domain proteins. Residual activity was determined as for recombinant caspases. Inhibition of endogenous caspase-9 activity by BIR3 was also determined using
HEK293A cell-free lysates. In this case cytochrome c/ATP activation was carried out in the presence of recombinant BIR3 proteins and activity was measured by cleavage of Ac-Asp-Glu-Val-Asp-pNA (Bachem Torrance, CA) on a SpectraMax 340 spectrophotometric plate reader (Molecular Devices), as a measure of the ability of caspase-9 to activate executioner caspases.

### 2.2.7 293A Cytosolic Lysates

HEK293A cells were grown at 37°C in 15cm plates to 70% confluence, harvested by scraping in PBS, washed and allowed to swell for 30 min on ice in hypotonic buffer (20mM PIPES, 10mM KCl, 2mM MgCl2, and 4mM DTT at a pH of 7.4) as previously described. Cells were sheared using a 27 gauge needle, centrifuged at 500xg for 30min, and the supernatants were collected and stored at -80°C. Lysates were activated for cell-free apoptosis by the addition of 10µM cytochrome c (Sigma, St. Louis) and 0.5mM dATP(Sigma) for 45 min at 37°C. Following activation lysates were assayed for caspase activity in the presence of BIR domains as described below, or treated with 100µM Z-Val-Ala-Asp-fmk (Enzymes Systems Products, Livermore, CA) for 30 min at room temperature to prevent further caspase activity.

### 2.2.8 Caspase pull down assays

GST-tagged BIR domains produced in E. coli were bound to glutathione Sepharose beads for 1hour at room temperature. Beads were washed three times in PBS-Triton X 100 (1% v/v), resuspended at 50% (v/v), and 5µl of beads
was incubated with 400ng (total protein) of cytochrome c activated HEK293A cell lysate in a total volume of 100µL overnight at 4°C. Beads were washed three times in hypotonic buffer and proteins eluted by boiling in SDS sample buffer containing 10mM DTT. Equal volumes of input (untreated and cytochrome c treated) BIR bound, and supernatant (depleted lysates) were loaded on an 8–18% linear gradient acrylamide SDS gel for electrophoresis. Samples were either transferred to PVDF and immunoblotted or stained with Gel Code Blue Stain Reagent (Pierce Biotechnology Inc. Rockford, IL).

2.2.9 Immunoblotting

Caspase-7 was detected using a rabbit caspase-7 polyclonal antibody (Cell Signaling Tech #9492). Caspase-9 was detected using a caspase-9 polyclonal antibody (Stennicke et al., 1999). Secondary anti-mouse IgG and anti-rabbit IgG conjugated with horseradish peroxidase were purchased from APBiotech Inc.

2.2.10 Protein Stability Assays

Unfolding studies were carried out by incubating protein (dialyzed into 50 mM Tris/HCl, 100 mM NaCl, pH 8.0), in the presence of incremental increases in guanidinium chloride (GdmCl, 0–6.35 M) for 1 hour at room temperature. Emission spectrum was measured from 300 to 400 nm after excitation at 280 nm using a PerkinElmer LS50B luminescence spectrometer coupled with the FL Win Lab software (PerkinElmer, Northwalk, CT, U.S.A.) using a 1-cm path length
cuvette (Helma). The emission and excitation slit widths were 5 nm and 7 nm respectively. Protein unfolding was indicated by a shift in the fluorescence emission peak. After collecting all the emission spectra, the area under the curve was calculated by summing the product of wavelength increment and emission intensity for each recorded wavelength-intensity pair. To calculate the average wavelength for each respective GdmCl concentration, the product of emission intensity and corresponding wavelength was calculated for each recorded wavelength-intensity pair and divided by values of the area under the curve. The change in average wavelength from native state (0M GdmCl) at each GdmCl concentration were normalized to the denatured states (6.35 M GdmCl) and plotted as relative change in the average emission wavelength (AEW).

2.2.11 Size exclusion chromatography.

Proteins were separated based on their native molecular weight using an AKTA FPLC (GE) system with a Superdex 200 column.

2.2.12 BIR peptide profiling assay.

Recombinant BIR domains immobilized on Ni-charged resin were incubated at a concentration of 20 µM with the peptide library at 2 mg/ml in H₂O. Following a 30 min incubation at room temp with rocking, beads were washed three times with 20 mM imidazole, 50 mM Hepes, 100 mM NaCl, pH 7.4 to remove non-specific interactions. Beads were the washed an additional five times in the same buffer without imidazole. Peptides were eluted using 30%
acetic acid and dried by speed vac. Dried peptides were redissolved in H₂O and incubated 30 min with the IBM-groove mutant BIR domain immobilized on Ni-charged resin, to remove any non-IBM-groove directed peptides. The supernatant was subsequently dried by speed vac and redissolved in H₂O. Peptide sequences were determined by Edman degradation N-terminal sequencing in a Procise 492 protein sequencer (Applied Biosystems). The quantity of each amino acid from the selected peptide sample was determined by subtracting the background of each amino acid from a control sample in which the respective BIR domain was incubated with H₂O instead of the peptide library. The adjusted quantity of each amino acid was normalized to yield a ratio of relative abundance at each position. Binding enrichment in each cycle was determined by multiplying this ratio by 18 (the number of individual amino acids in each position) to display positive binding events as values above 1.0.

2.2.13 Binding Recovery Analysis.

Total quantity of peptide recovered after binding was determined using an Fmax spectrophotometer (Molecular Devices) at excitation of 485 nm and emission of 538nm for the FITC tag and compared to the standard curve.

2.2.14 Fluorescence Polarization Assays.

Fluorescence Polarization studies were carried on a SMAC-8mer peptide conjugated with FITC. 5 nM peptide was used and the concentration of the BIR domain was varied from 1 nM to 27 µM. The ratio of bound ligand to free ligand
was determined using a LJL Analyst (Molecular Devices) with excitation at 485 nm and emission at 530 nm. KD was determined using a nonlinear fit of the curve generated by plot of millipolar (mP) vs. BIR concentration. The equation used was $mP = Af + [BIR](Ab-Af)/(K_d+[BIR])$ where $mP$ is the measured millipolar events, $Af$ is the free fluorescent molecule, $Ab$ is the bound fluorescent molecule, $K_d$ represents the dissociation constant of the BIR and peptide.

2.2.15 **Protein Crosslinking Assays.**

100 µl of protein(s) was added to a 100 µl of a serial dilution of Glutaraldehyde, typically 0.032-500 mM, and incubated for 5 minutes at room temp. 2M NaBH$_4$ in 1M NaOH (made fresh) was added and samples were subsequently incubated at 37°C. Proteins were precipitated with 25% TCA and washed with acetone. Crosslinked proteins were subjected to SDS-PAGE and stained with the comassie blue.
3. DISSECTION OF THE REQUIREMENTS FOR IAP-MEDIATED CASPASE-INHIBITION.
3.1 Introduction

XIAP is the most thoroughly studied member of the IAP family, and was the first identified endogenous caspase inhibitor. Many of the other IAPs have been proposed to function as caspase inhibitors, albeit less potently than XIAP. The first major aim of my thesis project was to elucidate the requirements of IAP-mediated caspase inhibition and determine whether IAPs, other than XIAP, were *bona fide* caspase inhibitors.

I began my investigation into the requirements of IAP-mediated caspase inhibition, through a functional comparison of XIAP, a potent caspase inhibitor, ML-IAP that has a single BIR domain, and cIAP1 and cIAP2, which are the closest paralogs to XIAP. The BIR2 and BIR3 of the cIAPs and the single BIR of ML-IAP posses the residues that define the conserved IBM-groove (Fig 3.1A). Sequence alignment of the IAPs reveals that in addition to the conserved surface groove, cIAP1 and to a lesser extent cIAP2, maintain most of the residues in the peptide strand N-terminally flanking the BIR2 domain, reported to be crucial to XIAP-mediated inhibition of caspases-3 and 7 (Fig 3.1B). It is the conservation of the surface groove of BIR2 and the seemingly conformational similarity of the N-terminal linker that was the basis for my prediction that the cIAPs are capable of executioner caspase inhibition. The BIR domain of ML-IAP, and the BIR3 domains of the cIAPs, only maintain only one of the four residues in the C-terminal helix that have been demonstrated essential to the XIAP-BIR3/caspase-9 inhibitory interaction (Fig 3.1C). It is this lack of conservation of these key...
residues in this distal helix of the cIAPs and ML-IAP that lead to my hypothesis that these IAPs are not caspase-9 inhibitors.
Figure 3.1 Caspase interaction sites within or flanking the BIR domains of IAPs. A) Sequence alignment of several IAP BIR domains at the region near the IBM-groove. The black bars highlight the residues that characterize the conserved IBM-groove. Notably XIAP-BIR1 does not possess this groove. B) Sequence alignment of the peptide strand N-terminal to BIR2 domains. The black bars point out the residues of XIAP that have been shown to critical to inhibitory interaction with caspases-3 and 7. C) Sequence alignment of the region C-terminal to BIR3, or the single BIR in the case of ML-IAP. In XIAP this is a helix and the black bars display residues that have been shown to interact with the dimer interface of caspase-9.
3.2 Expression and Purification of IAP-BIR domains.

The cDNA encoding the BIR2 or BIR3 domains of cIAP1 and cIAP2 were cloned into a modified pET15b or pGEX4T-1 vectors to add a N-terminal 8xHis or GST tag, respectively. Recombinant proteins were expressed in *E. coli* BL-21 strain and subsequently purified on NiSO₄–charged or glutathione sepharose resin depending on the specific tag. Figure 3.2 presents representative purifications of His (A) and GST-tagged (B) cIAP BIR domains. Of note, The BIR1 of XIAP does not contain the IBM-groove (Lin et al., 2007), and does not interact with caspase or IBM-antagonists (Deveraux et al., 1999; Huang et al., 2003; Takahashi et al., 1998). Therefore the IBM-groove mutant BIR domains were generated through mutation of two of the key residues that characterize this groove (Glu²³⁹ and His²⁴³ in cIAP1-BIR2 and Glu325 and Trp3²⁹ in cIAP1-BIR3) to the corresponding residues in XIAP-BIR1, Arg⁸² and Val⁸⁶. These mutations in have been shown to ablate caspase binding without compromising the structural integrity of the BIR itself (Eckelman and Salvesen, 2006; Scott et al., 2005).
Figure 3.2 Protein expression of cIAP BIR domains. A) GST-tagged BIR2 and BIR3 domains from cIAP1 and cIAP2. WT: wildtype RV: IBM-groove mutant cIAP1-BIRs, E239R/H243V in BIR2 and E325R/W329V in BIR3. B) His-tagged BIR2 and BIR3 domains of cIAP1 and cIAP2. X-cIAP1 is the chimeric protein composed of the N-terminal peptide strand of XIAP-BIR2, fused to the BIR2 domain of cIAP1. X-cIAP1-RV is the IBM-groove mutant of this chimeric protein. cIAP1-GHL has the mutations R342G/Q349/G350L. Following SDS-PAGE proteins were stained with comassie blue reagent.
3.3 The BIR2 and BIR3 domains of cIAP1 and cIAP2 bind to caspases-7, and 9.

Binding to caspases is a necessary step in their inhibition by XIAP. To determine the capacity of the cIAPs to bind caspases, I used GST-tagged BIR2 and BIR3 domains and analyzed binding to endogenous caspases 7 and 9 from cell free lysates induced to develop apoptotic activity. This was performed by initiating cell-free apoptosis by the addition of cytochrome c to hypotonic cytosolic extracts of HEK 293A cells. Following the induction of the intrinsic apoptotic pathway, recombinant GST-BIR domains immobilized on glutathione sepharose beads were incubated with the lysates. BIR domains and bound material were collected by centrifugation and removal of the supernatant. Following several washing steps, bound material was analyzed by western blot using caspase specific antiserum. Both the BIR2 and BIR3 domains of cIAP1 and cIAP2 were found to bind caspases-7 and 9, with the notable exception of cIAP2-BIR3, which failed to bind caspase-9 (Fig 3.2). The caspase/IAP interaction was found to be dependent on the IBM-groove maintained within the BIR domain as mutagenesis of key residues that characterize these grooves in cIAP1 (E239R and H243V in BIR2 and E325R and W329V in BIR3) ablated caspase binding. The cIAP BIR domains interacted with two forms of caspase-7. This is consistent with a recent study demonstrating that caspase-7 possesses an IBM at the N-terminus of the large subunit, in addition to the canonical IBM located neo-N-terminus of the
small subunit. Both of these IBMs are exposed by proteolysis, either within in the interdomain linker (Asp^{198}) or following N-peptide removal (Asp^{24}) (Denault and Salvesen, 2003; Scott et al., 2005; Tenev et al., 2005). The large and small subunits of caspases remain associated following proteolysis. Therefore, the location of the IBM (large or small subunit) of the lower p20 band cannot be determined. The cIAP BIRs interacted with a single form of caspase-9, most likely possessing the IBM exposed after proteolysis at Asp^{330} within its interdomain linker.
**Figure 3.3 clAP-caspase binding.** Association of endogenous caspase-7 (top panel) and caspase-9 (bottom panel) was observed with wild type (WT) BIR2 (lane 3) and BIR3 (lane 5) of clAP1 but not the mutants (Mut; lanes 4 and 6). Two forms of caspase-7 associated with each BIR domain (lanes 3 and 5). Depleted indicates the post-binding supernatant and demonstrates the ability of the wild type domains to deplete the entire endogenous pool of caspase-7 and approximately half of the caspase-9 (lanes 7-10 top and bottom panels). The cartoons to the left illustrate the various processed caspase forms, with the black bars representing the neo-epitope IAP binding motifs (IBM).
3.4 The BIR2 domains of cIAP1 and cIAP2 are poor inhibitors of the executioner caspases-3 and 7.

The ability of XIAP to inhibit the catalytic activity of the executioner caspases is dependent on two surfaces, the IBM-groove and the peptide extension immediately N-terminal to the BIR2 domain. My binding studies demonstrate that the cIAP BIR domains have maintained the conserved IBM-groove dependent anchoring interaction with the executioner caspases. Sequence alignments reveal that cIAP1 and to a lesser extent cIAP2, possess many of the residues in the N-terminal peptide extension that are essential to the ability of XIAP-BIR2 to inhibit caspases-3 and 7 (Fig 3.1B). Therefore, I hypothesized that cIAP1-BIR2 and possibly cIAP2-BIR2 should be capable of executioner caspase inhibition.

To test this hypothesis, I titrated recombinant cIAP1 and cIAP2 BIR2 domains with the respective N-terminally flanking linkers (residues 144-260 of cIAP1, residues 129-246 of cIAP2, equivalent to the caspase-3 and 7 inhibitory unit of XIAP) against recombinant caspases-3 and 7. These BIR domains displayed virtually no inhibition (apparent Kᵢ values greater than 5 µM, Fig 3.4A and 3.5A cIAP2 data not shown). XIAP-BIR2 is a tight binding inhibitor of caspases-3 and 7 with Kᵢs in the low-to-sub nanomolar range. Slight inhibition of caspase-7 by cIAP1-BIR2 was observed at concentrations in excess of 20 µM (data not shown). Therefore, the cIAPs maybe capable of weak executioner
caspase inhibition *in vitro*, but this is unlikely to be a physiologically relevant event.

These findings were quite surprising as cIAP1 and cIAP2 are the closest homologs to XIAP and possess most of the key caspase inhibitory residues. I wanted to investigate why cIAP1 and cIAP2 were such poor inhibitors of these caspases. The BIR domains of the cIAPs possess a functional IBM-groove as evidenced by the ablation of caspase binding when these regions were specifically mutated. Thus, it does not seem that the poor inhibitory capacity I observed for these BIRs was due to their inability to properly associate with caspases. Therefore, I predicted that the N-terminal peptide strand was the basis for the poor inhibition. This strand, although conserved with XIAP at the key caspase interacting residues, may have an altered conformation causing a misalignment of these residues with the caspase active site.

To investigate whether the N-terminal peptide strand was the basis for the poor inhibition by the cIAP BIR2 domains, I implemented a gain-of-function strategy and I generated a hybrid protein consisting of the N-terminal peptide strand from XIAP (residues 123-163, replacing residues 144-183 of cIAP1) fused to the BIR2 domain of cIAP1. This hybrid protein (X-cIAP1 BIR2) was a tight binding inhibitor of caspases-3 and 7 with low-to-sub-nanomolar apparent K_i (Fig 3.4B and 3.5B). Importantly, it has been shown that this peptide strand of XIAP on its own is insufficient for executioner caspase inhibition (Scott et al., 2005; Sun et al., 1999). My findings reveals that the BIR2 domain of the cIAP1 properly anchors this IAP on to caspases in a manner that allows for inhibition.
Secondly, it reveals that the N-terminal extension of cIAPs contains residues that disallow the tight interaction seen with the corresponding region of XIAP. To further confirm that the hybrid protein was using a two-site mechanism to inhibit caspases-3 and 7, like that used by wildtype XIAP-BIR2, I disrupted the IBM-groove in the hybrid protein by mutagenesis (E239R and W243V). With an ablated IBM-groove this protein displayed markedly weaker inhibition of both caspases-3 and 7, with apparent Kis in excess of 4 µM (Fig 3.4C and 3.5C). These findings demonstrate that the conformation of the peptide strand N-terminal to the BIR2 domains of the cIAPs, disallows tight binding inhibition of the executioner.
Figure 3.4 cIAP1-BIR2 is not a potent inhibitor of caspase-3. The ability of cIAP1-BIR2 (A), the XIAP/cIAP1-BIR2 fusion protein (X-cIAP1-BIR2) (B) and the IBM-groove mutant of the fusion protein, X-cIAP1-BIR2-RV (C) to inhibit the activity of 100 pM caspase-3 was monitored continuously by the cleavage of Ac-DEVD-AFC (left panel). The right panels represent the relative activity of caspase-3 as determined by the slope of the progress curve. Relative activity given by the activity in the presence of the BIR (vi) normalized to the activity in the absence of the BIR (vo). D) Plot of vo/vi-1 vs. [BIR] was used to determine the Ki of X-cIAP1-BIR2 toward caspase-3. The apparent Ki is 1/slope of this plot, shown in the box.
Figure 3.5 cIAP1-BIR2 is not a potent inhibitor of caspase-7. The ability of cIAP1-BIR2 (A), the XIAP/cIAP1-BIR2 fusion protein (X-cIAP1-BIR2) (B) and the IBM-groove mutant of the fusion protein, X-cIAP1-BIR2-RV (C) to inhibit the activity of 350 pM caspase-3 was monitored continuously by the cleavage of Ac-DEVD-AFC (left panel). The right panels represent the relative activity of caspase-7 as determined by the slope of the progress curve. Relative activity is given by the activity in the presence of the BIR (vi) normalized to the activity in the absence of the BIR (vo). D) Plots of vo/vi-1 vs. [BIR] was used to determine the Ki of cIAP1-BIR2, X-cIAP1-BIR2, and X-cIAP1-BIR2-RV toward caspsae-7. The apparent Ki is 1/slope of this plot, shown in the box.
In order to confirm that my analysis of caspase inhibition by the cIAPs resembles what takes place in a cellular environment, I compared the inhibitory capacity of cIAP1-BIR2 and X-cIAP1-BIR2, to that of XIAP-BIR2 on endogenous caspases. To do this I used hypotonic lysates treated with cytochrome c as the source of active executioner caspases in my fluorogenic assay. I found that XIAP-BIR2 and X-cIAP1-BIR2 both were able to inhibit caspases under these conditions, whereas cIAP1-BIR2 had no effect (Fig 3.6).
Figure 3.6 cIAP1-BIR2 does not inhibit endogenous executioner caspases. Cytosolic lysates preactivated by cytochrome c and dATP, were incubated with 1μM of recombinant BIR2 proteins, and residual activity was monitored by cleavage of the substrate Ac-DEVD-AFC. A) Constant monitoring of caspase activity demonstrates the ability of X-cIAP1-BIR2 (open circles) and XIAP-BIR2 (black triangles), but not cIAP1-BIR2 (black circles) to inhibit executioner caspases. A control activation trace (grey diamonds) is representative of the caspase activity in the absence of recombinant BIR proteins. B) Relative activity is calculated from the normalization of the linear activity rate in the presence of the BIR (vi) to the linear rate in the absence of the BIR (vo). Error bars are representative to three independent experiments.
3.5 The BIR of ML-IAP is a poor inhibitor of caspases-9.

The crystal structure of XIAP-BIR3 in complex with caspase-9 demonstrates four residues (Pro325, Gly326, His343, Leu344) that seem to make important contacts with the dimer interface of caspase-9. With the exception of ILP-2, none of human IAPs has conservation of all of these residues. However, it may be the case that other residues are tolerated at these key contact sites.

The BIR domain of ML-IAP has been shown to be required for its ability to associate with caspases and block apoptosis (Vucic et al., 2000). SMAC has been shown to disrupt the ML-IAP caspase interaction and negatively regulate its anti-apoptotic function (Vucic et al., 2002). The first enzymatic assays suggested that ML-IAP functioned as a caspase-3 and 9 inhibitor. However, the lack of conservation of the N-terminal peptide strand with XIAP, and the incorporation of a GST-tag on the BIR used in these assays casts doubt as to its caspase-3 inhibiting capacity. In these studies ML-IAP was also unable to inhibit caspase-7 (Vucic et al., 2000). Modeling studies suggest that ML-IAP does posses a helix distal to it BIR domain and this may be the basis for inhibition of caspase-9. Therefore, I wanted to test the ability of ML-IAP to directly inhibit caspase-9.

Using a recombinant protein corresponding to the BIR domain of ML-IAP (residues 63-179), I found that it inhibited caspase-9 with an apparent Ki in excess of 9 µM, suggesting that direct inhibition of caspase-9 is a the physiologic function of this IAP (Fig 3.7C). ML-IAP lacks conservation at three of the four
critical caspase-9 dimer interface interacting residues within the distal helix (Fig3.7A). To determine whether the weak caspase-9 inhibitory capacity of ML-IAP was due to a divergence from XIAP at these residues I tested various ML-IAP BIR proteins in which the three divergent residues were changed to those of XIAP either pairs or all three. I found that the double mutants endowed the BIR of ML-IAP with virtually no increase in inhibitory capacities toward caspase-9, having apparent Kᵢ's ranging from 6-19µM (3.7D-F). Intriguingly, the triple mutant (S150G/Q167H/E168L) was a very tight binding caspase-9 inhibitor with a Kᵢ in the low nanomolar range (Fig 3.7H). This demonstrates that all residues in this distal helix that interact with the dimer interface of caspase-9, as seen the XIAP-BIR3/caspase-9 complex (Shiozaki et al., 2003), are required for potent inhibition. Even the slightest deviations from XIAP within the distal helix seems to preclude potent caspase-9 inhibition.
Figure 3.7 ML-IAP is poor inhibitor of caspase-9. A) Sequence alignment of the distal helix of XIAP-BIR3, and ML-IAP-BIR. Residues important for the interaction with caspase-9 are highlighted in black box and the corresponding residues of ML-IAP in the grey box. The double and triple mutants of ML-IAP-BIR are presented with the grey boxed residues of ML-IAP changed to the black boxed residues of XIAP. B) Titration curves of caspase-9 with wildtype (WT) and the various mutant ML-IAP BIRs. Relative activity is presented as activity of caspase-9 in the presence of the BIR (vi) normalized to the uninhibited rate (vo). Caspase-9 activity was determined by cleavage of the Ac-LEHD-AFC substrate. Determination of the apparent $K_i$ for wildtype ML-IAP-BIR (C); ML-IAP-BIR-HL (Q167H/E168L) (D); ML-IAP-BIR-GH (S150G/Q167H) (E); ML-IAP-BIR-GL (S150G/E168L) (F); and ML-IAP-BIR-GHL (S150G/Q167H/E168L) (G), toward caspase-9 as determined by the inverse slope of the plot of vo/vi-1 vs. [BIR]. The apparent $K_i$ is presented within the box on each graph.
3.6 The cIAPs are poor inhibitors of caspase-9

My binding studies revealed that the BIR3 of cIAP1 is capable of interacting with caspase-9 through an IBM-dependent mechanism. However, my findings with ML-IAP suggests that the BIR3 domains of cIAPs should be unable to potently inhibit caspase-9 due to the lack of conservation with XIAP-BIR3 within the distal helix (Fig 3.1C). Previous studies reported that BIR3 or cIAP1 and cIAP2 were caspase-9 inhibitors (Deveraux et al., 1998). The inhibition observed in these reports, may simply be attributed to the presence of a GST-tag, however, it was important to test this issue experimentally. To investigate the capacity of the third BIR domains of cIAP1 and cIAP2 to inhibit the catalytic activity caspase-9, I titrated recombinant BIR3 domains (residues 257-356 of cIAP1 and 243-333 of cIAP2) against purified caspase-9. At 2 µM protein, I observed no inhibition at all, suggesting that the $K_i$ would be in great excess of 5µM (Fig 3.8B,C, cIAP2 data not shown). Similar to the BIR of ML-IAP, mutation of the divergent residues in cIAP1-BIR3 (R332G, Q349H, G350L) restored tight binding caspase-9 inhibition (Fig. 3.8D,E). The findings demonstrate that the BIR3 domains of the cIAPs are not potent caspase-9 inhibitors due to divergence from XIAP at three key residues.

To confirm that my in vitro assays mimicked apoptosis driven by endogenous proteins I used cytosolic lysates activated with cytochrome c to compare the inhibitory potential of cIAP1-BIR3, the triple mutant, X-cIAP1-BIR3-
(GHL), and XIAP-BIR3. Consistent with my finding using recombinant caspases, pretreatment of the lysates with recombinant X-clAP1-BIR3 and XIAP-BIR3, but not clAP1-BIR3, ablated the ability of endogenous caspase-9 to activate downstream executioner caspases (Fig 3.9).
**Figure 3.8 cIAP1-BIR3 is not a caspase-9 inhibitor.** A) Sequence alignment of the distal helix of XIAP-BIR3, and cIAP1-BIR3. Residues important for the interaction with caspase-9 are highlighted in black box and the corresponding residue of cIAP1-BIR3 in the grey box. The triple mutant of cIAP1-BIR3 (GHL) is also presented with the grey boxed residues of cIAP1 changed to the black boxed residues of XIAP. B) Progress curve cIAP1-BIR3 titration of caspase-9. C) Relative activity of caspase-9 presented as the activity in the presence of cIAP1-BIR3 (vi) normalized to the activity of caspase-9 in the absence of cIAP1-BIR (vo). D) Progress curve cIAP1-BIR3-GHL titration of caspase-9. E) Relative activity of caspase-9 presented as the activity in the presence of cIAP1-BIR3-GHL (vi) normalized to the activity of caspase-9 in the absence of cIAP1-BIR-GHL (vo). F) Determination of the apparent $K_i$ of cIAP-BIR3-GHL toward caspase-9 as calculated from the inverse slope of (vo/vi)-1 vs. [BIR]. $K_i$ is presented in box on graph. Caspase-9 activity was determined by cleavage of the Ac-LEHD-AFC substrate.
Figure 3.9 ciAP1-BIR3 is a poor inhibitor of endogenous caspase-9. 1µM of selected recombinant BIR3 protein was incubated with cell-free lysates prior to cytochrome c activation. A) Progress curves of caspase-9-mediated activation of executioner caspases. B) Relative activity plot, data from the linear portion of each progress curve was normalized to the control in which cytochrome c dependent caspase activity was monitored in the absence of any BIR.
3.7 The BIR domains of cIAP1 are properly folded

In these studies I have used recombinant BIR domains, most of which failed to inhibit caspases. There is the formal possibility that the differences in observed inhibitory capacities among the BIRs tested is due variations in the conformational stability of the protein. In fact we found that ILP-2 that has a BIR domain highly similar to the BIR3 domain of XIAP lacks the capacity to potently inhibit caspases simply due to a lack of innate structural integrity of its BIR domain (Shin et al., 2001). This stability deficit is due to a naturally truncated N-terminus and this region may be quite important to other BIR domains. To test the possibility that conformational stability has a role in the lack of apparent caspase-3 and 7 inhibition by the cIAPs, I conducted unfolding studies on cIAP1-BIR2, the hybrid protein X-cIAP1-BIR2, and the IBM-groove mutant of the hybrid protein. The stability of the BIR domain was analyzed at varying concentrations of guanidinium chloride (GdmCl). This method is ideally suited to compare the conformational stability of similar proteins (Pace, 1986). Thermal denaturation calorimetry provides alternate means to determine protein stability. However, we have previously found that sensitivity to GdmCl denaturation directly correlates with the functionality of other BIR domains (Shin et al., 2001). These studies revealed essentially indistinguishable stabilities, ruling out domain stability as a reason for the poor inhibition of caspases-3 and 7 (Fig 3.10A). Analysis of the conformational stability of cIAP1-BIR3 and the triple mutant, cIAP1-BIR3-GHL,
demonstrated that these two were equally stable (Fig 3.10B). This confirms that the lack of caspase-9 inhibition that I observed was not due to a problem in recombinant protein folding. These BIRs were seemingly less stable than the BIR2 domains, however, they must maintain sufficient integrity, evidenced by the potent caspase-9 inhibitory capacity of cIAP1-BIR3 –GHL.
A.

Figure 3.10 Analysis of the conformational stability of clAP1 BIR domains. The data are plotted as change in the average emission wavelength (AEW) normalized to the native (0 M GdmCl) and unfolded states (6.35M GdmCl) A) clAP-BIR2, X-clAP1-BIR2, and X-clAP1-BIR2-RV demonstrate similar conformational stabilities. B) clAP1-BIR3 and clAP1-BIR3-GHL are equally stable.
3.8 The CARD of cIAP1 does not disrupt apoptosome function.

I have found that the BIR domains of the cIAPs and the single BIR of ML-IAP are unable to function as physiologic caspase inhibitors. Although the BIR domains themselves may be required for the anti-apoptotic activity of these IAPs, other regions must be important as well. Little is known as to the function of the CARD within the cIAPs. It is often observed that CARDs participate in homotypic interactions. A logical hypothesis is that the CARD domains of the cIAPs interact with that of caspase-9 or Apaf-1 and disrupt the formation of a functional apoptosome. In fact, CARD-only proteins have shown to disrupt the recruitment of caspase-1 to inflammasomes and thereby prevent its activation (Druilhe et al., 2001; Humke et al., 2000; Lamkanfi et al., 2004; Lee et al., 2001; Razmara et al., 2002). To test whether the CARD of cIAP1 can affect apoptosome formation, I expressed a recombinant protein consisting of the BIR3-CARD of cIAP1 (Fig 3.11A). Activation of cytosolic lysates with cytochrome c resulted in robust caspase activation. The presence of 10μM cIAP1-BIR3-CARD had only a very minor effect on the generation of a functional apoptosome, as evidenced by the slight delay in the activation curve (Fig 3.11). The BIR3 domains of XIAP and cIAP1 were included as the respective positive and negative controls. This suggests that the CARD of cIAP1 is unable to interact with the CARD of either caspase-9 or Apaf-1 and mechanism of apoptotic inhibition is not mediated through apoptosome disruption.
Figure 3.11 The CARD of cIAP1 cannot disrupt apoptosome formation. A) Recombinant His-tagged cIAP1-BIR3-CARD purified following E. coli expression was subjected to SDS-PAGE. Proteins were stained with comassie blue reagent. B) Cytosolic lysates were activated with cytochrome c in presence of 10µM cIAP1-BIR3 (black trace), cIAP1-BIR3-CARD (light gray trace), or XIAP-BIR3 (dark grey trace) and the activity of caspases was monitored by cleavage of Ac-DEVD-pNA. The control trace, represents the activity of a cytochrome c activated lysate in the absence of any recombinant IAP protein.
3.9 Activation of Caspase-9 by Dimeric cIAP1-BIR3

From my studies demonstrating that the cIAPs were capable of binding caspase-9 in a non-inhibitory manner, I formulated a hypothesis that if I could artificially dimerize the BIR3 domain of cIAP1, I should observe caspase-9 activation. This hypothesis was based on the knowledge that caspase-9 is naturally activated by dimerization by Apaf-1 at apoptosome (Boatright et al., 2003; Pop et al., 2006). To test this, I utilized GST-tagged BIR3, as this should be dimeric due to the fact that GST complexes with itself.

As predicted, I observed caspase-9 activation by dimeric cIAP1-BIR3 (Fig 3.12). To determine if the interaction was dependent on the IBM of caspase-9 interacting with the IBM-groove in BIR3, I utilized a non-cleavable caspase-9. Mutation of the two Asp residues (D315A, D330A) within the interdomain linker ablates any proteolytic processing. This renders the caspase unable to generate a functional IBM (Stennicke et al., 1999). I observed no activation of this non-cleavable caspase-9 by GST-cIAP1-BIR3, demonstrating this is an IBM-dependent interaction. In addition a heptapeptide analogous to the N-terminus of mature SMAC (SMAC 7) was capable of disrupting the cIAP1-BIR3-mediated caspase activation, and further confirms that activation of caspse-9 by BIR3 of cIAP1 is dependent on the IBM-groove.
Figure 3.12 Activation of caspase-9 by forced cIAP1-BIR3 dimerization. The BIR3 domain of cIAP1 was N-terminally fused to GST to artificially generate a dimeric form of the BIR domain. This protein was used to titrate fixed concentrations of caspase-9. The dimeric cIAP1-BIR3 was found to activate caspase-9. This phenomenon was found to be an IBM-dependent interaction as treatment with 10 µM SMAC 7-mer peptide and the use of a non-cleavable recombinant caspase-9 ablated the observed activation. Activity of caspase-9 was measured by cleavage of Ac-LEHD-AFC.
3.10 Summary of Aim I

These studies demonstrate that cIAP1, cIAP2, and ML-IAP have maintained the proper BIR fold and are all capable of binding specific caspases through the conserved surface grooves. However, none of these IAPs are capable of inhibiting the proteolytic activity of caspases. It should be noted that previous reports of cIAP and ML-IAP-meditated caspase inhibition (Deveraux et al., 1998; Roy et al., 1997; Vucic et al., 2000) utilized GST-tagged proteins. The dimeric nature of GST, likely artificially enhances the inhibitory capacity of these proteins. Through these studies I have dissected the requirements for IAP-mediated inhibition of caspases-3, 7 and 9. Minor deviations from XIAP in the conformation of the peptide strand N-terminal to BIR2, disallows inhibition of the executioner caspases by the cIAPs. The lack of conservation with XIAP at three key residues in the helix C-terminal to the single BIR of ML-IAP, or the BIR3 of the cIAPs, is the basis for the poor inhibitory capacity of these IAPs toward caspase-9. From these I predict that XIAP is the only member of the human IAP family capable of direct caspase inhibition. It has been reported by several groups that cIAP1, cIAP2, and ML-IAP have an anti-apoptotic function when overexpressed in cells (Clem et al., 2001; Orth and Dixit, 1997; Roy et al., 1997; Simons et al., 1999; Uren et al., 1996; Vucic et al., 2002) and it now is evident that anti-apoptotic mechanism these IAPs is not mediated by direct caspase inhibition.
Importantly, I found that although the cIAPs do not have the capacity to inhibit caspases they have maintained the ability to bind these proteases. This lead to the testable hypothesis that BIRs lacking the proper conformation for caspase-9 inhibition, if dimeric, can actually active this caspase. I am not proposing that cIAP1 has a pro-apoptotic role mediated by caspase-9 oligomerization. However, these experiments were the initial driving force for me to direct my attention toward investigating NAIP/caspase interactions discussed in chapter 5 of this thesis.
4. DEFINING THE PEPTIDE BINDING SIGNATURE OF IAP BIR DOMAINS.
4.1 Introduction

Many BIR domains possess the critical residues that define a conserved surface groove. This groove is denoted as the IAP Binding Motif (IBM) groove as it endows some BIRs with the ability to bind N-terminal epitopes of defined sequence, the IBM. However, the exact sequence requirements that constitute a functional IBM and whether they are the same for all BIRs is relatively unknown. The IBM itself has been defined by a limited number of profiling studies and analysis of IAP-interacting proteins on a few BIR domains (Franklin et al., 2003; Oost et al., 2004; Sweeney et al., 2006). The third BIR domain of XIAP has been the most well studied BIR in terms of defining the consensus IBM sequence (Denault et al., 2007; Liu et al., 2000; Srinivasula et al., 2000; Srinivasula et al., 2001; Wu et al., 2000). Due to overlapping binding capacities of well characterized BIR domains, the IBM consensus is suggested to match \( \text{A}\Phi\text{R}\Phi \), where \( \Phi \) represents a hydrophobic amino acid and the Ala at the extreme N-terminus and unblocked.

In this study I developed a method to profile the peptide binding signature of the BIR domains from XIAP, cIAP1 and NAIP. These studies revealed that BIRs that possess the conserved surface groove can be divided in to two subgroups based on the selection in the third position of the ligand. This subclassification is bifurcated primarily on the presence of either His or Trp within the IBM-groove. This has allowed me to define IBM-groove containing BIRs as
type II (BIR2-like) or type III (BIR3-like) depending on whether they possess a HIS (corresponding to H^{223} in the BIR2 of XIAP) or a Trp (corresponding to W^{323} in the BIR3 of XIAP), respectively at the conserved region within this groove (Fig 4.1). I have found that this classification matches the elucidated binding signatures, and allows for a prediction of type III BIR specificity.

BIR domains that lack the apparent IBM-groove have been shown not to interact with caspases, IAP-antagonist, or representative peptides (Deveraux et al., 1999; Huang et al., 2003; Sweeney et al., 2006; Takahashi et al., 1998). The notable exception here is NAIP-BIR3 that does not possess the conserved residues that define this groove, but has been reported to bind caspase-9 but not SMAC (Davoodi et al., 2004). SMAC and caspase-9 have very similar IBM sequences and it is unclear as whether the BIR3 of NAIP interacts with caspase-9 in a non-IBM-dependent manner. In my peptide profiling screen I only tested those BIRs that have a clearly conserved IBM-groove. In addition, I profiled NAIP-BIR3 in order to determine whether it maintains a variant groove. The results of these profiling studies provide insights into the natural binding partners of the IAP and aid in the understanding of the actual targets of IAP-antagonist that are currently being explored as cancer therapeutics.
Figure 4.1 The IBM-groove. A) Sequence alignment of several human IAP BIRs highlighting the residues that characterize the IBM-groove (black boxed residues). The residue that was mutated along with the primary residues is also highlighted (grey boxed residues). The IBM-groove mutants used in this study have the black boxed residues mutated to the corresponding residues of XIAP-BIR1. B) left, a type II BIR represented by the IBM-groove of XIAP-BIR2 with the neo-terminus of the small subunit of caspase-3 bound (PDB 1I3O); right, and type III BIR represented by the IBM-groove of XIAP-BIR3 bound to a SMAC peptide (PDB 1G73). The peptide positions are denoted as P1'-P4', with P1' being at the N-terminus.
4.2 Method for profiling BIR domain specificity.

I used a peptide library randomized in the first four positions to determine the binding signature of various BIR domains. An isokinetic mixture of all amino acids, except Cys and Trp, was included in these first four positions. Cys was excluded so as to avoid oxidation problems, while Trp was left out simply because it is not resolved by Edman degradation N-terminal sequencing. The classic IBM motif has been suggested to be a tetrapeptide with longer peptides having similar affinities as the tetrapeptides (Liu et al., 2000). Thus, I chose to add two Gly residues, adjacent to the four random positions, to function as a linker while having negligible additional interactions with the BIR. Next to the tandem Gly residues a FITC conjugated Lys included as a means to track peptides through the binding protocol. Arg was incorporated into the C-terminal position to aid in overall peptide solubility. Therefore, the peptides are a mix of H₂N-XXXXGGK(fitc)R-COOH, where X is any of the 18 amino acids.

The peptide library was incubated with a BIR domain immobilized on Ni-charged resin by virtue of an engineered His tag. Several washing steps were utilized to remove non-specifically bound peptides. The BIR domain and bound peptides were eluted with 30% acetic acid and dried in a speed vac. The peptides were then redissolved in H₂O, while the BIR domain remains precipitated. In developing this screen I took advantage of our BIR mutagenesis studies, demonstrating that mutation of the key residues within the IBM-groove of the BIR to the corresponding residues of XIAP-BIR1, ablates this groove yet
does not disrupt the structural integrity of the BIR (Eckelman and Salvesen, 2006; Scott et al., 2005). Here, I utilized the IBM-groove mutants as a negative selection step to remove non-IBM-groove directed peptides. This step involved incubating the peptides that interacted with wildtype BIR domain with the IBM-groove mutant BIR domain immobilized on solid support. Following centrifugation the IBM-groove directed peptides remain in the supernatant and are collected and subjected to Edman degradation N-terminal sequencing. Fig 4.2 provides a flow chart of the basic protocol used to determine BIR domain binding specificity. The quantity of each amino acid from the selected peptide sample was determined by subtracting the background of each amino acid from a control sample in which the respective BIR domain was incubated with H₂O instead of the peptide library. The adjusted quantity of each amino acid was normalized to yield a ratio of relative abundance at each position. Binding enrichment in each cycle was determined by multiplying this ratio by 18 (the number of individual amino acids in each position) to display positive binding events as values above 1.0. Notably, all BIR domains analyzed demonstrated a strong preference for Ala in the first position (P1’), and thus seemed a limiting factor in binding. Therefore, to screen the specificity in positions 2-4 (P2’-P3’), I used a second peptide library of which the first position was fixed to be Ala, and the adjacent three positions were randomized as before (H₂N-AXXXGGK(fitc)R-COOH). The use of the Ala-fixed peptides allowed me to analyze more stringent binding events and elucidate the full signatures of the BIR domains tested.
Figure 4.2 Flow chart depicting the method for the isolation of IBM-directed peptides. The peptide library is incubated with the BIR domain that is immobilized on resin. Washing steps remove non-specific peptides. A 30% acetic acid treatment removes the BIR and its bound peptides from the resin, which are then dried. The peptides are then redissolved in H₂O, while the BIR domain remains precipitated. The redissolved peptides are subjected to a negative selection step that entails incubating them with the respective IBM-groove mutant BIR domain. This will remove any peptides that are not directed toward the region of interest, that being the IBM-groove. Remaining peptides (specific IBM-groove binders) are collected and analyzed by Edman degradation N-terminal sequencing.
4.3 The binding specificity of BIR2 and BIR3 domains of XIAP.

In order to test the validity of my experimental setup I first investigated two well characterized BIR domains, BIR2 and BIR3 of XIAP. These BIRs have been profiled previously using both phage display and peptide library screening (Franklin et al., 2003; Sweeney et al., 2006) and provided a benchmark to gauge my profiling studies. The peptide binding assay revealed that these BIRs have differential binding specificities. Utilizing the peptide library randomized in all four N-terminal positions both XIAP-BIR2 and XIAP-BIR3 demonstrated strong preference for Ala in the first position (P1'). I found that utilizing the Ala-fixed library these BIR domains also had similar preferences in the second position (P2'), selecting for two β-branched amino acids Val and Ile, and also Arg. The selection for Arg in this position was not surprising as a recent study measuring the binding affinity of XIAP-BIR3 with peptides based on the natural IAP binding protein SMAC (A-V-P-I), found that this BIR actually bound better when the P2' Val was replaced by Arg (Kipp et al., 2002). The most notable difference in binding signatures between these BIR was the selection for Pro in the third position (P3') by BIR3, whereas Ala was selected for at this position by BIR2. Arg and Gly were the second most preferred residues in P3' by BIR3 and BIR2, respectively, and this is consistent with the previous studies (Franklin et al., 2003; Sweeney et al., 2006). These BIRs also had a slight difference in selectivity in P4', with Ile being most preferred by BIR3 and Val by BIR2 (Fig 4.2 and Table 4.1).
The results of this profiling study with the XIAP BIRs demonstrate that this method is capable of defining the binding signature of IBM-groove containing BIR domains. The binding signature of XIAP-BIR3 (A-I/V-P-I) very closely resembled that of the mature N-terminus of SMAC (A-V-P-I), while the signature of XIAP-BIR2 (A-I-A-V) was distinct from this classic consensus (Fig 4.3 and Table 4.1). The differential specificity in the P3’ position demonstrates that IAPs have distinct intrinsic binding selectivities.
Figure 4.3 The binding signature of XIAP-BIR2 and BIR3. The binding enrichment of each amino acid at P1'-P4' is representative of the molar fraction of a given residue above background. Values above 1.0 demonstrate a positively selected binding event. The molar fraction is the ratio of a given amino acid to the total quantity of amino acids at the given position. The BIR2 (A) and BIR3 (B) domains of XIAP demonstrated distinct preferences in the P3' position. The overall binding signature revealed from the profiling screen is provided under each graph.
4.4 The peptide binding signature of XIAP-BIR3 is physiologically relevant.

During apoptosis caspase-9 is cleaved between the large a small subunit at two distinct sites. The primary cleavage, an autocatalytic event, occurs at Asp\textsuperscript{315}, and exposes the ATPF sequence on the neo-terminus of the small subunit. It is this neo-epitope on caspase-9 that was shown in the crystal complex to interact with the IBM-groove on BIR3 of XIAP (Shiozaki et al., 2003). The second cleavage is mediated by caspase-3 and occurs downstream at Asp\textsuperscript{330} thereby removing the ATPF motif, and exposing the sequence AiSS (Fig 4.4A). There has been some debate as to the biological consequences of caspase-3 mediated cleavage of caspase-9. Originally it was demonstrated using cytosolic extracts reconstituted with XIAP and Apaf-1, that caspase-9 cleaved at Asp\textsuperscript{330} with the AiSS motif (caspase-9\textsuperscript{AiSS}) exposed failed to interact with XIAP (Srinivasula et al., 2001). Conversely, a later study that utilized purely recombinant components suggested that XIAP-BIR3 was still capable of inhibiting caspase-9\textsuperscript{AiSS} (Zou et al., 1997).

My profiling studies reveal that the ATPF motif closely mimics the optimal XIAP-BIR3 binding signature and replacement of this with AiSS should significantly attenuate the XIAP/caspase-9 interaction. This was tested this using caspase-9 depleted cell-free extracts reconstituted with either recombinant caspase-9 cleaved either Asp315 (caspase-9\textsuperscript{ATPF}) or Asp330 (caspase-9\textsuperscript{AiSS}). To
do this required the mutation of one or the other of these Asp residues, so as to allow for cleavage solely at the other site. Caspase-9 will normally cleave itself at Asp$^{315}$ during expression in *E. coli*. and thus this form of the enzyme required no additional preparation step. However, caspase-9 will not cleave itself at Asp$^{330}$. To generate caspase-$9^{AISS}$, *E. coli.* lysate expressing caspase-9 D315A was incubated with caspase-3 expressing *E. coli.* lysate prior to purification so as to generate caspase-9 cleaved at Asp$^{330}$. Importantly, the caspase-3 lacked a His tag, allowing for purification of only caspase-9 on the Ni-charged resin. In this reconstituted system XIAP-BIR3 could only effectively inhibit the activity of caspase-$9^{ATPF}$. (Fig 4.4B) Of note, the caspase-$9^{AISS}$ was slightly more active in this assay, most likely due to the ability of endogenous XIAP to inhibit only caspase-$9^{ATPF}$. The difference in inhibitory capacity of XIAP-BIR3 is quite apparent by the degree in shift of the activation curves. This suggests that the functional IBM of caspase-9 is removed when caspase-3 cleaves caspase-9 at Asp330.

The conflicting results of the previous investigations into this cleavage event can simply be attributed to experimental design. Caspase-9 expressed in *E. coli.* has very low intrinsic activity in the absence of the apoptosome. Therefore, in order to measure the catalytic activity on a fluorogenic substrate often necessitates the use of high enzyme concentrations. However, in order to properly determine a $K_i$ the enzyme concentration must be kept low compared to the inhibitor. Thus, if the concentration of caspase-9 in the assay is high then the sensitivity is lost and any difference in $K_i$ of XIAP-BIR3 toward caspase-$9^{ATPF}$
compared to caspase-9\textsuperscript{ATPF} is not apparent as the assay is saturated by caspase-9.
A. 

Figure 4.4 XIAP-BIR3 differentiates between N-terminal motifs. A) schematic representation of the cleavage sites, D315 (autocatalytic site) and D330 (caspase-3 site), within the interdomain linker of caspase-9. The neo-epitopes (ATPF and AISS) exposed after cleavage are in black and underlined. B) Cytosolic lysates depleted of caspase-9 were reconstituted with either caspase-9$^{\text{AISS}}$ (black trace) or and caspase-9$^{\text{ATPF}}$ (grey trace) and activity was monitored by cleavage of the substrate Ac-DEVD-pNA. Thin traces represent the activity of caspase-9, in the absence of XIAP-BIR3. Thick traces are indicative of caspase-9 activity in the presence of 150nM XIAP-BIR3. Arrows indicate the ability to XIAP-BIR3 to inhibit caspase-9. The activity of caspase-9 depleted lysates with and without cytochrome c addition are indicated by *. 

B. 

![Graph showing activity over time](image-url)
4.5 The binding specificity of BIR2 and BIR3 domains of cIAP1.

Relatively little is known about the BIR domains of cIAP1. I tested the peptide binding specificity of the BIR2 and BIR3 domain of cIAP1, as I have previously found these domains capable of associating with caspases in an IBM-dependent manner. Similar to the XIAP BIRs, I found that both of these BIR displayed a strong preference for Ala in P1’ and yet the preferences in adjacent positions were quite distinct. Lys was the most preferred residue in both the P2’ and P3’ and by cIAP1-BIR2, demonstrating a very unique and uncharacterized IBM preference. This BIR also selected for aliphatic residues, suggesting that the interaction is directed toward the side-chain rather than the ε-amine of the Lys. This binding signature distinguishes cIAP1-BIR2 from XIAP-BIR2 (Fig 4.5A and Table 4.1). Conversely, cIAP1-BIR3, displayed an IBM preference that was more similar to the classic IBM consensus, as it selected for Ile, Pro, and Ile in positions P2’, P3’, and P4’ respectively (Fig 4.5B and Table 4.1). These profiling studies reveal that BIR3 domains have binding signatures that resemble the classic “SMAC-like” consensus whereas BIR2 domains have more varied binding selectivities. The most notable is the difference in binding signatures between BIR2 and BIR3 domains seems to the selection of Pro in P3’.
A.

**Figure 4.5 The binding signature of clIA1P-BIR2 and BIR3.** The binding enrichment of each amino acid at P1'-P4' is representative of the molar fraction of a given residue above background. Values above 1.0 demonstrate a positively selected binding event. The molar fraction is the ratio of a given amino acid to the total quantity of amino acids at the given position. The BIR2 (A) and BIR3 (B) domains of clIA1P demonstrated distinct preferences in the P2' and P3' positions. The overall binding signature revealed from the profiling screen is provided under each graph.
4.6 Specificity determinants of cIAP1 BIR domains.

Structural analysis of several BIR domains reveals that the defining residue (His$^{223}$ and Trp$^{323}$ of XIAP) within the IBM-groove, lies in close juxtaposition with the third residue of the peptide (Fig 4.1B). I sought to determine whether this was the basis for the P3' selection by Trp-containing BIR domains. To test this I took a mutagenesis approach, using cIAP1-BIR2 and BIR3 as templates. I mutated the critical His in BIR2 to Trp and made the converse mutation in BIR3. Importantly, sequence alignment shows that in most IBM-groove containing BIR domains that if the defining residue is a His, then it is usually preceded by Arg, whereas if a Trp is present it is usually preceded Lys. This adjacent residue does not seem to interact with the peptide within the groove and thus most likely does not have a role in the specificity of the groove, yet it may play an important role in maintaining the structural integrity of the BIR itself. Therefore, I elected to make the double mutant of Arg-His to Lys-Trp and vice versa instead of the single mutants (cIAP1-BIR2 R242K/H243W and cIAP1-BIR3 K328R/W329H).

I then tested the peptide binding specificity of these mutant cIAP1 BIRs. Interestingly, I found that mutation of the His to Trp within cIAP1-BIR2 (R$\rightarrow$K/H$\rightarrow$W) conferred this BIR domain with the ability to select for Pro in P3' (Fig 4.6B and Table 4.1) The reverse mutation of Trp to His within cIAP1-BIR3 (K$\rightarrow$R/W$\rightarrow$H) resulted in the loss of Pro selection at P3' (Fig 4.6C and Table 4.1).
With the exception of the P3’ Pro selectivity there were no overwhelming differences in binding signatures of these mutant BIR domains and their respective wildtype counterparts. This suggests that the presence of a Trp within this groove mediates the selection for Pro at P3’ and therefore allows for a classification of IBM-groove containing BIR domains based on this defining residue. Here, I define His containing BIRs as type II and Trp containing BIRs as type III.
Figure 4.6 Switching the P3’ specificity. A) Mutation of R242K/H243W (R→K/H→W) in cIAP1-BIR2 endows it with a P3’ Pro specificity. B) Mutation of K328R/W329H (K→R/W→H) in cIAP1-BIR3 ablates the P3’ Pro specificity. The overall binding signature revealed from the profiling screen is provided under each graph.
Table 4.1 Peptide binding signatures of IAP BIR domains. The binding preference for positions P1’-P4’ for each peptide and the BIR type is provided.

<table>
<thead>
<tr>
<th>BIR Type</th>
<th>Signature</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>XIAP-BIR2</td>
<td>A(I,V)AV</td>
<td>II</td>
</tr>
<tr>
<td>XIAP-BIR3</td>
<td>A(I,V)PI</td>
<td>III</td>
</tr>
<tr>
<td>cIAP1-BIR2</td>
<td>AKK(V,I)</td>
<td>II</td>
</tr>
<tr>
<td>cIAP1-BIR2 R→K/H→W</td>
<td>AKPV</td>
<td>III</td>
</tr>
<tr>
<td>cIAP1-BIR3</td>
<td>AIPI</td>
<td>III</td>
</tr>
<tr>
<td>cIAP1-BIR3 K→R/W→H</td>
<td>AI(V,I)I</td>
<td>II</td>
</tr>
<tr>
<td>NAIP-BIR2</td>
<td>AR(R,P)I</td>
<td>III</td>
</tr>
</tbody>
</table>

4.7 Determination of the binding affinity of cIAP1 BIR domains for optimal peptides.

In order to validate the elucidated binding signatures I determined the affinity of the optimal peptide for the wildtype and specificity switched mutant cIAP1 BIRs. The peptide synthesized were AKKVGK(Fitc)R for cIAP1-BIR2, AKPVGGK(Fitc)R for cIAP1-BIR2 R242K/H243W, AIPIGGK(Fitc)R for cIAP1-BIR3 and AVIGGK(Fitc)R for cIAP1-BIR3 K328R/W329H. To determine the binding affinity of each peptide I used a fluorescence polarization assay in which the BIR domain was titrated against a fixed amount of peptide. I found that the BIR3 domains bound all Ala containing peptides with much greater affinity overall than BIR2 domains, demonstrating a difference in intrinsic peptide binding affinities between BIR2 and BIR3 domains (Fig 4.7 and Table 4.2).

The affinity determinations for each peptide toward each of the BIR tested (cIAP1-BIR2 and BIR3 wildtype and specificity switched mutants) are provided in
Table 4.2. cIAP1-BIR2 bound its optimal peptide AKKV, better than any other peptide tested with a $K_D$ of 4.2 µM. Although this represents relatively weak binding it is consistent with affinity of XIAP-BIR2 toward its optimal peptides (Franklin et al., 2003). When the Lys in the P3' was replaced with Pro (AKPV) the binding to cIAP1-BIR2 was weakened, displaying a $K_D$ of 7.2 µM (Table 4.2). The trend in binding affinity toward these two peptides for the specificity switched mutant cIAP1-BIR2 (cIAP1-BIR2 R242K/H243W) was in essence reversed, with the Pro containing peptide (AKPV) displaying much tighter binding compared to the peptide lacking Pro (AKKV) ($K_D$ of 4.7 µM compared to 17.5 µM). This demonstrates that the elucidated binding signatures are representative of the optimal specificity. Furthermore differences in Pro selectivity in P3' can account for the differences in the binding affinities between cIAP1-BIR2 and cIAP1-BIR2 R242K/H243W. The importance of the P3' Lys also evidenced by the trends in binding affinity when the BIR3 optimal peptides were tested on these BIR2 domains. The P3' Pro-containing, AIP1, peptide was bound tighter by the P3' Pro-selective BIR2 mutant compared to the wildtype BIR2 ($K_D$ of 4.99 µM vs. 8.40 µM). Conversely, the AIVI peptide, lacking a P3' Pro, was bound more tightly by the wildtype than the mutant cIAP1-BIR2 ($K_D$ of 5.6 µM vs. 17.3 µM) (Fig 4.7)

cIAP1-BIR3 and cIAP1-BIR3 K328R/W329H bound their optimal peptides with much greater affinity than the BIR2 domains. Importantly, the trend of the P3' Pro selection was consistent with the elucidated binding signatures. cIAP1-BIR3 that prefers Pro in P3', bound it optimal peptide (AIP1) with $K_D$ of 0.13 µM.
Replacement of the P3’ Pro with Val (AIVI) resulted in decrease in affinity to a $K_D$ of 0.75 $\mu$M. Furthermore, analysis of the BIR2 optimal peptides on the BIR3 domains confirmed the importance of the P3’ Pro selection. The peptide AKKV bound BIR3 with a $K_D$ around 2 $\mu$M representing a substantial decrease in binding affinity. BIR3 binding affinity was increased when the P3’ Lys of this peptide was replaced by Pro (AKPV), restoring a $K_D$ of 0.14 $\mu$M (Fig 4.7 and Table 4.2). Interestingly, this is quite close to the affinity of the optimal AIVI peptide, suggesting the P3 Pro is of primary importance. Importantly, P3’ Pro selection by BIR3 seems mitigate binding to non-optimal peptides rather than contribute to overall binding affinity as evidenced by peptide binding studies on cIAP1-BIR3 K328R/W329H. This mutant BIR3 that no longer selects for Pro in P3’, bound all peptides relatively tightly, maintaining the high intrinsic peptide binding affinity of BIR3 domains for Ala-peptides (Table 4.2).
Table 4.2. Binding affinities of cIAP1 BIR domains toward optimal peptides.

K<sub>D</sub> values are from non-linear fit of the fluorescence polarization data presented in Figure 4.8. The values parenthesis is the standard error derived from the fitting equation

\[
K_D (\mu M) = \frac{XXXXGGK(Fitc)R}{XXXX} = AKKV \quad AKPV \quad AIPI \quad AIVI
\]

<table>
<thead>
<tr>
<th>XXXX=</th>
<th>AKKV</th>
<th>AKPV</th>
<th>AIPI</th>
<th>AIVI</th>
</tr>
</thead>
<tbody>
<tr>
<td>cIAP1-BIR2</td>
<td>4.18 (0.25)</td>
<td>7.22 (0.53)</td>
<td>8.40 (1.3)</td>
<td>5.60 (0.46)</td>
</tr>
<tr>
<td>cIAP1-BIR2R→K/H→W</td>
<td>17.52 (2.23)</td>
<td>4.70 (0.46)</td>
<td>4.99 (0.47)</td>
<td>17.32 (2.17)</td>
</tr>
<tr>
<td>cIAP1-BIR3</td>
<td>2.09 (0.16)</td>
<td>0.135 (0.012)</td>
<td>0.13 (0.013)</td>
<td>0.75 (0.055)</td>
</tr>
<tr>
<td>cIAP1-BIR3K→R/W→H</td>
<td>0.476 (0.04)</td>
<td>0.156 (0.013)</td>
<td>0.16 (0.015)</td>
<td>0.22 (0.015)</td>
</tr>
</tbody>
</table>
Figure 4.7 Peptide binding of cIAP1 BIR domains. Fluorescence polarization assay was conducted by titrating a varying concentrations of the BIR domain (2.4 nM to 27 µM) into a 5 nM of Fitc-labeled peptide. The data of millipolarization units (mP) vs. BIR concentration was analyzed using the non-linear fitting equation, \( mP = A_f + \frac{[BIR](A_b-A_f)}{K_D+[BIR]} \), where \( mP \) is the measured millipolarization units, \( A_f \) is the free fluorescent molecule, \( A_b \) is the bound fluorescent molecule, \( K_D \) represents the dissociation constant of the BIR and peptide. The \( K_D \) values from these fits are provided in Table 4.2.
4.8 Peptide binding preference of BIR2 and BIR3 of NAIP.

The BIR domains of NAIP are unique. The BIR2 domain has an IBM-groove groove that resembles that of other BIR3 domains. Based on my previous profiling and mutagenesis studies, I predicted that NAIP-BIR2 should select for Pro in P3’ and have a SMAC-like binding signature. This was indeed the case, as NAIP-BIR2 displayed a binding preference similar to XIAP BIR3. However, this BIR also tolerated Arg in P3’ demonstrating a slight deviation from other type III BIRs (Fig. 4.8).

The BIR3 domain of NAIP has conservation of many residues near the IBM-groove, yet lacks the two groove defining residues. A Cys residue replaces the Trp/His, while the upstream Gln/Glu is replaced by Asp (Fig 4.1A). When I analyzed the peptide binding specificity of NAIP-BIR3 with both peptide libraries I found that it was unable to bind peptides with any preference (data not shown), suggesting that this BIR domain does not possess the IBM-groove.
Figure 4.9 The binding signature of NAIP-BIR2. The binding enrichment of each amino acid at P1'-P4' is representative of the molar fraction of a given residue above background. Values above 1.0 demonstrate a positively selected binding event. The molar fraction is the ratio of a given amino acid to the total quantity of amino acids at the given position. The overall binding signature revealed from the profiling screen is provided under the graph.
4.9 Summary of AIM II

I have developed a method to screen the IBM-groove of IAP BIR domains. This method enables the isolation of peptides that bind specifically to this groove, while other peptides are removed by negative selection. Through this screening method I have found that there are distinct binding specificities between BIR domains. Furthermore, my findings here have allowed me to define BIR domains that have this conserved groove, as type II or type III. This assignment of BIRs is dependent on their binding signature and closely correlates with the presence of a Trp or His at the defined position. Type III BIRs possess a Trp and have binding signatures that resemble the classic “SMAC-like” consensus. Type III BIRs are not simply BIR3 domains, as the single BIR of ML-IAP (Franklin et al., 2003) and the BIR2 of NAIP both have the Trp and display “SMAC-like” binding signatures. The type II BIRs display much more varied binding specificities. The most notable difference between type II and III BIRs is the selection for Pro in P3' by the latter. My mutagenesis studies revealed that this P3’ Pro selectivity is primarily due to the presence of the Trp within this groove. Analysis of several BIR domains.

Investigating the binding affinities of the optimal peptides of the BIRs of cIAP1, I found that the BIR3 domains bound all peptides with a greater affinity than did the BIR2 domains. This is consistent with peptide binding studies done
previously on the BIRs of XIAP (Franklin et al., 2003; Oost et al., 2004; Sweeney et al., 2006, Kipp, 2002 #4196). This demonstrates that in addition to distinctions in peptide binding signatures, there are differences in the intrinsic binding affinities for peptides, between various BIR domains. The Trp within the groove of type III domains is not the basis for the tighter overall binding affinity. This became evident in the specificity switched mutant of cIAP1-BIR3, in which the Trp was replaced by His, which lost its P3’ Pro selectivity, while its overall binding affinity for peptides was maintained. In fact it seems that the Trp is restrictive, preventing the tight binding to non-optimal peptides as this mutant bound all peptides with high affinity, whereas replacing the P3’ Pro with Val or Lys drastically decreased the affinity of the wildtype cIAP1-BIR3.

The basis for the differential intrinsic binding affinities of BIR2 and BIR3 domains is unclear. By examining the structures of several BIR domains in complex with peptides it became apparent that the type III domains posses a pocket that accepts the forth residue of the peptide (Fig 4.1B). This pocket is substantially shallower in type II BIRs, and this may be the reason for the skewed affinities between these two types of BIR domains. Interestingly, in the type III BIRs, proper positioning of P4’ into the pocket necessitates a kink in the peptide chain at the third residue. Pro in the third position is ideal to obtain the proper peptide conformation to align the fourth residue into this pocket.

Based on these profiling and affinity studies it is clear that the SMAC mimetics that are currently under preclinical development will primarily target
type III BIR domains (Arnt et al., 2002; Li et al., 2004; Oost et al., 2004; Park et al., 2004a; Sun et al., 2004; Zobel et al., 2006).
5. THE CONSEQUENCES OF NAIP/CASPASE INTERACTIONS
5.1 Introduction

NAIP is the founding member of the human IAP family and there have been conflicting reports as to whether its BIR domains function as physiological caspases inhibitors. One group claimed the BIR2 domain of NAIP was capable of inhibition caspases-3 and 7 (Maier et al., 2002), while in a previous study our group found no evidence for this (Roy et al., 1997). Furthermore, the BIR3 domain of NAIP has been suggested to inhibit caspase-9 (Davoodi et al., 2004). I have found that only the BIR2 domain of NAIP has the conserved IBM-groove as evidenced by my profiling studies described in chapter 4 of this thesis. However, I predicted that NAIP-BIR2 cannot function as an inhibitor of caspases-3 and 7. NAIP is much more divergent from XIAP than are the cIAPs in the peptide extension N-terminal to its BIR2 domain. My studies presented in chapter 3 of this thesis demonstrate and that minor deviations from XIAP in this region disallow potent execution caspase inhibition. Therefore, NAIP cannot inhibit caspases-3 and 7 via the same mechanism as XIAP. Analogously, neither the BIR2 or BIR3 domains of NAIP, possess proper residues in the distal helix to interact with the caspase-9 dimer interface in an inhibitory manner.

NAIP is a unique member of the IAP family, in addition to its three tandem BIR domains, it has a central domain that is well conserved with the nucleotide binding NACHT domains, while the C-terminus is composed of Leucine rich repeats (LRRs). This unique domain composition places NAIP in the NOD-LRR/NACHT-LRR (NLR) protein family. The members of the NLR protein family
are often able to sense and respond to various intracellular pathogens, often in a pro-inflammatory manner. The ability to detect the pathogen components is mediated through the recognition of these by binding the LRRs. The LRRs of the toll-like receptors interact with extracellular pathogens, while those of the NLR proteins bind intracellular pathogens-derived ligands. The NACHT domains are related to AAA+ ATPases and have been shown to undergo oligomerization upon ATP binding. The N-termini of the NLR proteins usually contains a recruitment domain, a Pyrin domain is found in the NALPs, a CARD in the case of the NODs and IPAF, while three BIR domains reside in this region of the NAIP protein. It is thought that most NLRs remain in an auto-inhibited state in which the LRR interact with the rest of the protein. Ligand binding to LRR induces a conformational change allowing both NACHT-dependent oligomerization and recruitment of other proteins via the N-terminal domains.

NAIP has domains conserved with those that are involved in both caspase inhibition and activation, suggesting that this protein is at a unique node in the regulation of caspases. The major open questions regarding NAIP are whether it functions as a caspase inhibitor or a unique caspase activation platform. As mentioned above, I predict that NAIP is incompetent at potent inhibition of the apoptotic caspases. Thus, I hypothesize that NAIP can function in the activation of caspases. This stems directly from a preliminary set of experiments I conducted in which I utilized an artificially dimeric BIR domain to activate caspase-9. The focus of this chapter of my thesis is to determine the consequences of NAIP/caspase interactions.
5.2 Expression and purification of NAIP BIR2 and BIR3.

The cDNA of the second and third BIR domains of NAIP were cloned into a modified pET15b vector to add an N-terminal 8xHis tag, or pGEX4T-1 to add a GST-tag. As the BIR2 is the only BIR of NAIP that appears to posses an IBM-groove it was important to include the flanking regions that may be required for caspase inhibition (domain limits: 128-248). In addition a shorter version of NAIP-BIR2 was also created (domain limits 154-236). Proteins were expressed in the *E. coli* BL-21 strain and purified on Ni-charged resin for the His-tag or glutathione sepharose for the GST-tag. Figure 5.1 presents representative protein expressions of the His-tagged version of the two variants of the BIR2 domain, the BIR3 domain, and a chimeric NAIP-BIR2/XIAP-BIR3 fusion (discussed below).
Figure 5.1 Expression of recombinant NAIP BIR domains. A) The two variants of NAIP-BIR2 (NB2) residues 128-248 and 154-236. B) the chimeric NAIP-BIR2/XIAP-BIR2 (NB2/XB3), residues 154-190 of NAIP fused to residues 290-348 of XIAP; and NAIP-BIR3 (NB3) residues 256-363, were subjected to SDS-PAGE and proteins stained with comassie blue reagent.
5.3 The BIR2 domain of NAIP binds caspases 7 but not caspase-9.

To investigate the interaction of the BIR domains of NAIP with apoptotic caspases I used a cell-free system similar to that utilized in my studies with the cIAPs. In brief, cytosolic lysates from 293A cells were induced to undergo cell-free apoptosis by the addition of cytochrome c and dATP. Following apoptotic induction lysates were incubated with the individual BIR2 and BIR3 domains of NAIP immobilized on resin via an N-terminal GST-tag. Bound material was visualized via western blot using a caspase specific antiserum. The BIR3 domain of NAIP was unable to bind any caspases, consistent with my peptide profiling studies and sequence alignments that demonstrate a lack of a conserved IBM-groove. Interestingly, the BIR2 domain of NAIP interacted with caspase-7, but not caspase-9 (Fig 3.2). cIAP1-BIR3 was included in these assays as a positive control as it is very similar to NAIP-BIR2 near the IBM-groove. It was somewhat surprising that NAIP-BIR2 interacted with caspase-7 but not caspase-9, however, neither of these proteins has an IBM ideally suited for NAIP-BIR2 binding. However, caspase-7 has been suggested to possess a second IBM at the N-terminus of the large subunit that is exposed upon removal of its N-peptide (Tenev et al., 2005). The sequence that is exposed at this second IBM is AKPD, and seems better suited than the ATPF epitope of caspase-9. The AKPD IBM closely mimics the binding signature of NAIP-BIR2 that I determined
in chapter 4 of this thesis. Notably, the Lys in P2' was the second most preferred residue, second only to the other positively charged residue, Arg.
Figure 5.2 NAIP BIR interactions with apoptotic caspases. NAIP-BIR2 interacts with caspase-7 (A), but not caspase-9 (B) in cytosolic lysates programmed to undergo cell-free apoptosis. GST-BIR domains were incubated with the lysates following cytochrome c addition. Bound caspases were visualized by western blot analysis using caspase-specific antibodies.
5.4 The BIR domains of NAIP do not function as inhibitors

Essential to our understanding of the physiologic functions of NAIP is to determine the capacity of it to inhibit caspases. Although I expect the BIR domains of NAIP not to inhibit apoptotic caspases, this must be determined experimentally as others have demonstrated inhibition (Davoodi et al., 2004; Maier et al., 2002). Importantly, these studies like the initial studies with the cIAPs utilized GST-tagged proteins that can be quite problematic in biochemical investigation of enzyme inhibition. I tested the inhibition of caspases-3, 7, and 9 using His-tagged recombinant NAIP-BIR2 and BIR3 proteins and the flanking regions that should correspond to the inhibitory regions of XIAP-BIR2 and BIR3, respectively (Fig 5.1). At a concentration of 1μM, I observed absolutely no inhibition of caspase-3, 7 or 9 by the BIRs of NAIP (Fig 5.3). This cast severe doubt on the ability of NAIP to inhibit these caspases in a cellular setting. The BIRs of XIAP and my chimeric proteins, X-cIAP1, were used as positive controls for inhibition.
Figure 5.3 *NAIP-BIR2* and *NAIP-BIR3* do not inhibit the caspases-3, 7, or 9. 1 µM recombinant BIR protein was incubated with the recombinant caspase. The activity of caspase-3 (A), and caspase-7 (B), was monitored by cleavage of the Ac-DEVD-AFC substrate. XIAP-BIR2 and the XIAP/cIAP1-BIR2 fusion (X-cIAP1-BIR2) protein were included as positive controls for potent inhibition. Whereas, cIAP1-BIR2 functioned as a negative control. The activity of caspase-9 was monitored by cleavage of Ac-LEHD-AFC. XIAP-BIR3 and the triple mutant cIAP1-BIR3 R342G, Q349H, G350L (X-cIAP1-BIR3) were included as positive controls of caspase inhibition. cIAP1-BIR3 is negative for inhibition for caspase inhibition.
5.5 Expression and Purification of caspases-4 and 5

The cDNAs encoding caspases-4, and 5 were cloned into pET21 and/or pET29 and expressed in E. coli BL21 strain. Expression of these caspases proved dubious and only low protein amounts could be obtained. During expression caspase-4, and 5, autoprocess themselves between the large and small subunits. The CARD of caspase-5 is removed during expression, generating a p25 large subunit and a p10 small subunit. I determined the cleavage site to be at Glu$^{134}$ by Edman degradation N-terminal sequencing. It is not clear whether this cleavage is due to autocatalytic activity of caspase-5 or as a result of proteolysis by an E. coli protease. Interestingly, caspase-5 has been reported to cleave the Max transcription factor at a Glu residue (Krippner-Heidenreich et al., 2001) and thus the removal of the CARD is likely due to caspase-5 itself.

In an attempt to increase protein expression yields of caspase-5, I created a truncated caspase-5 ($\Delta$CARD caspase-5) with the N-terminus ($A^{135}$) located immediately downstream of this cleavage site. When I compared the catalytic activity of $\Delta$CARD caspase-5 to that of the full length protein of which the CARD is removed during expression, the former was drastically less active (Fig 5.4A). This suggests that although the CARD is not required for the catalytic activity it may play a role in the proper folding of the protein. Therefore, for the enzyme assays presented in this chapter I utilized the full length caspase-5 and not $\Delta$CARD caspase-5.
It is not clear whether caspase-5 has a functional IBM. Cleavage at Asp\textsuperscript{330} (LEAD/S) would expose the sequence SVCK at the neo-N-terminus of the small subunit. Although this sequence is quite distinct from the peptide binding signature elucidated for NAIP-BIR2, I wanted to generate a non-cleavable form of caspase-5, so I mutated Asp\textsuperscript{330} to Ala. Expression yields of caspase-5 D330A were very poor and protein could only be detected by western blot analysis of the His-tag. The His-tag is located on the C-terminus of the protein and if cleaved the small subunit (p10 band) should be detected. If the mutation ablated interdomain cleavage of caspase-5 then a band at p35 would be detected. Western blot analysis revealed that mutation of D330A in caspase-5 attenuated the majority of processing within this region, however, there was evidence that cleavage occurred at a secondary site, albeit to a lesser extent. The small subunit band of the cleavage site mutant caspase-5 is slightly larger, implying the use of a secondary cleavage site upstream of Asp\textsuperscript{330} (Fig 5.4B). A likely candidate is Asp\textsuperscript{311} (WVRD/S) as this sequence in similar to the optimal cleavage motif determined for the inflammatory caspases (Thornberry et al., 1997).
Figure 5.4. Protein expression of Caspase-5. A) *Left panel*, protein expression of caspase-5 (wt) and ΔCARD-caspase-5, following SDS-PAGE proteins were stained with comassie blue reagent. Caspase-5 (wt) has its CARD removed during expression. Both forms of the caspase are cleaved within the interdomain linker during expression. *Right panel*, comparison the catalytic activity of caspase-5 (wt) and ΔCARD-caspase-5. The activity 100nM of each caspase was monitored on the substrate Ac-WEHD-AFC. B) Western blot analysis for the His-tag of the expressed caspase-5 (wt) and the cleavage site mutant caspase-5 (D330A). The cartoon to the right depicts the uncleaved caspase-5 and the two distinct small subunits. The grey bar is representative of the location of the His tag.
5.6 The BIR2 of NAIP activates caspase-4 and 5, but not caspase-1

Since the BIR domains of NAIP were found not to function as inhibitors of the apoptotic caspases, I turned my attention toward the inflammatory caspases. Initially, I wanted to compare the affects of the individual BIRs of NAIP to that of some other IAP BIRs. The activity of purified recombinant inflammatory caspases was monitored using the fluorogenic substrate Ac-WEHD-AFC. The presence of 1\(\mu\)M BIR2 and BIR3 domains of XIAP, cIAP1, and NAIP had virtually no effect on the activity of caspase-1 (Fig 5.5). However, under similar conditions, NAIP-BIR2 specifically enhanced the activity of caspases-4 and 5 (Figs 5.6, 5.7). The activity of caspase-5 was increased approximately ten fold, demonstrating a very robust activation event. BSA was included as a control for non-specific protein crowding, which could enhance the enzymatic activity. This was quite a surprising finding for two reasons, caspase-4 and 5 are the closest homologs to caspase-1 which was unaffected by NAIP-BIR2, and secondly I did not predict that a single BIR domain would have the ability to activate a caspase in the absence of an oligomerization domain. These findings suggest that this inflammatory caspase activation capacity is unique and specific to the second BIR domain of NAIP, as no other BIR test was competent at this task.
Figure 5.5 Caspase-1 is not affect by IAP BIR domains. A) The activity of recombinant caspase-1 was monitored by cleavage of Ac-WEHD-AFC in the presence and absence of various IAP BIR domains. B) The relative activity of caspase-1 as determined by the enzyme velocity in the presence of the BIR (vb) normalized to enzyme velocity in the absence of the BIR (vo).
Figure 5.6 Caspase-4 is activated by NAIP-BIR2. A) The activity of recombinant caspase-4 was monitored by cleavage of Ac-WEHD-AFC in the presence and absence of various IAP BIR domains. B) The relative activity of caspase-4 as determined by the enzyme velocity in the presence of the BIR (vb) normalized to enzyme velocity in the absence of the BIR (vo).
Figure 5.7 Caspase-5 is activated by NAIP-BIR2. A) The activity of recombinant caspase-5 was monitored by cleavage of Ac-WEHD-AFC in the presence and absence of various IAP BIR domains. B) The relative activity of caspase-5 as determined by the enzyme velocity in the presence of the BIR (vb) normalized to enzyme velocity in the absence of the BIR (vo), error bars represent the standard deviation of three experiments.
5.7 Determination of Dissociation constant of NAIP-BIR2 and Caspase-5

To investigate the affinity of the interaction between NAIP-BIR2 and caspase-5, I titrated the BIR from 5µM down to 0.3nM into 16nM or 3.2nM caspase-5 and measured the activity. At both concentrations caspase-5 was substantially activated by NAIP-BIR2 in a dose dependent manner that plateaued at the higher concentrations. Interestingly, it seemed that at the lower concentration caspase-5 was activated to a greater degree. To determine the $K_D$ of this interaction I used both one and two-site binding equations. The two-site binding equation provided a better fit, displaying a distinct difference in the affinities of the separate sites. The first event was rather tight with a low nanomolar affinity, while the second event was significantly weaker having a high nanomolar affinity (Fig 5.8). It is difficult to determine the nature of these seemingly distinct interactions, as there could be two forms of either the caspase or the BIR in the reaction, or the initial binding event could create a new species with a skewed affinity toward either of the inputs. Even the weaker of these two binding event is in the sub-micromolar range and could be physiologically relevant. Furthermore the weaker binding event displays a $K_D$ that is in the range of other BIR-mediated binding events.
Figure 5.8 Determination of the affinity of the NAIP-BIR2/caspase-5 interaction. The $K_D$ of the NAIP-BIR2/caspase-5 interaction was determined by incubating 16nM (A) or 3nM (B) caspase-5 with a range of BIR concentrations (0.3nM-5µM) and measuring the activity on an Ac-WEHD-AFC substrate. The data was fit to the two-site binding equation: $Y = (B_{max1} \cdot [BIR])/(K_{D1}+[BIR]) + (B_{max2} \cdot [BIR])/(K_{D2}+[BIR])$. The $K_D$ for each site is in the boxed region, and the standard deviation as determined from the fit in parenthesis.
5.8 Monomeric NAIP-BIR2 is capable of caspase-5 activation

The observation that NAIP-BIR2 can activate caspase-5 in the absence of an oligomerization domain, suggests that the recombinant BIR protein may be oligomeric and thereby simply oligomerizing the caspase. To test this, I subjected NAIP-BIR2 to size exclusion chromatography. Interestingly, NAIP-BIR2 was almost completely monomeric, running at a predicted molecular mass of 8kDa, which is actually slightly smaller than the actual molecular mass of 13kDa (Fig 5.9A). BIR domains are tightly packed domains due to the zinc coordination and this could be the basis for its slightly delayed elution from the column. It was important to determine whether this isolated monomeric form of the BIR was still able to activate caspase-5. Therefore, I titrated caspase-5 with the NAIP-BIR2 eluted from the gel filtration column and again I observed very robust caspase-5 activation (5.9B). This demonstrates that oligomerization is not the mechanism of activation.
Figure 5.9 Monomeric NAIP-BIR2 activates caspase-5. A) Elution profile of NAIP-BIR2 from size exclusion chromatography. Black bar below the graph indicates the fractions that were subjected to SDS-PAGE. Proteins were stained with comassie blue reagent. B) Titration of Caspase-5 with a range of concentrations of monomeric NAIP-BIR2.
5.9 NAIP-BIR2 generates a completely active form of caspase-5

An important question regarding NAIP-BIR2-mediated caspase-5 activation was how much of the total caspase was actually being activated? When expressed in *E. coli* caspases-3 and 7 are usually close to fully active, meaning that the concentration of formed active sites is similar to the total protein concentration. Conversely, caspases that require an oligomerization event to be active, tend to have substantially fewer formed active sites compared to the total amount of the protein, such is true of caspases-8 and 9. The number of formed active sites can be determined by titrating a tight binding inhibitor against the caspase and measuring its residual activity. Peptides that resemble the natural substrates of the caspases will only interact with these proteases if the active site residues are properly aligned. I have found that the best inhibitor to titrate caspase-5 is the peptidyl inhibitor Ac-WEHD-CHO. I titrated this inhibitor against 250 nM caspase-5, as determined by $A_{280}$, in the presence or absence of 2 $\mu$M NAIP-BIR2. In the absence of the BIR, caspase-5 displayed low catalytic activity that was not well titrated by increasing amounts of the inhibitor. This demonstrates that the active site of caspase-5 is not properly formed to allow the inhibitor to bind. In the presence of NAIP-BIR2, the inhibitor was able to effectively titrate the caspase. Although this inhibitor does not interact covalently with the caspase, it binds so tightly, that once bound, it is likely to remain
associated with that specific active site for the duration of the assay. Therefore, the inhibitor interacts with the caspase in a one-to-one manner. A linear fit of the titration enables the determination of the number of active sites. This is essentially the point in the titration were there are no more formed active sites to interact with the inhibitor, and thus the catalytic activity is zero (Stennicke and Salvesen, 1999). This analysis reveals that in the presence of NAIP-BIR2 the amount of active caspase-5 was 290 nM (Fig 5.10). This is very close to the total protein concentration that was determined by the $A_{280}$ and demonstrates that NAIP-BIR2 fully activates caspase-5.
5.10 NAIP-BIR2 is capable of activating caspase-5 completely. Titration of 250nM caspase-5 in the presence (open circles) or absence (filled circles) of NAIP-BIR2 with the inhibitor Ac-WEHD-CHO. Lower panel highlights a lower concentration range of inhibitor from the data in the upper panel. The active site concentration of caspase-5 in the presence of NAIP-BIR2 was determined to be 290nM, as this is the concentration of the inhibitor concentration at which the activity is zero.
5.10 Biotin-EVD-AOMK labeling of caspase-5 titrating NAIP-BIR2

Another way to analyze caspase activity is through the use of specific activity based probes. Similar to Ac-WEDH-CHO, the specificity of these probes is conferred through a peptide linker. These probes also have a reactive group that covalently attaches to the active site cysteine of the caspase and an affinity tag that allows for the purification or detection of the bound caspase. The probe I used for my studies incorporated a Biotin-moiety, a peptide linker composed of Glu-Val-Asp (EVD) and an acyloxmethylketone (AOMK) reactive group. This probe has been shown to be highly specific for caspases (Kato et al., 2005).

To see if NAIP-BIR2 increased labeling of caspase-5 by B-EVD-AOMK in a dose-dependent manner, I incubated 5 nM caspase-5 (estimated by A$_{280}$) with a range of BIR concentrations (5 nM-1 µM) in the presence of the probe. Following SDS-PAGE, active caspase-5 was visualized by a streptavidin blotting. Consistent with my previous findings using fluorogenic substrates, NAIP-BIR2 caused a drastic does-dependent increase in the amount of active caspase-5, even at low nanomolar concentrations (Fig 5.11). This further suggests that NAIP-BIR2 has a direct effect on the active site conformation of caspase-5.
Figure 5.11 Activity-based probe labeling of caspase-5 is dependent on NAIP-BIR2. 5 nM Caspase-5 labeled with B-EVD-AOMK is revealed by streptavidin blot. Notably, there is no detectable caspase-5 in the absence of NAIP-BIR2.
5.11 Localization of the caspase-5 activation surface on NAIP-BIR2.

5.11.1 The IBM-groove

Canonically, IAPs interact with caspases through the conserved IBM-groove located on the surface of some BIR domains. In trying to determine whether the IBM-groove of NAIP-BIR2 was important for its ability to activate caspase-5, I utilized the IBM-groove mutant NAIP-BIR2 (E216V/W220R). I found that this mutant BIR still maintained its ability to activate caspase-5 (Fig 5.12A). To further confirm that NAIP-BIR2 mediated caspase-5 activation was IBM-groove-independent, I tested the ability of NAIP-BIR2 to activate the cleavage site mutant caspase-5 (caspase-5 D330A). Although, caspase-5 does not posses an IBM that is well conserved it may be important for the interaction with NAIP-BIR2. Notably, I began these experiments prior to my profiling studies on NAIP-BIR2, thus, I was not in position to predict whether the sequence, SVCK, exposed by cleavage of caspase-5 at Asp$^{330}$ constitutes a functional interaction motif. Currently, armed with the knowledge I acquired from my profiling studies it seems that SVCK is ill suited to interact with the IBM-groove of NAIP-BIR2. Titration of NAIP-BIR2 with caspase-5D330A resulted in substantial activation, suggesting that caspase-5 does not need to be cleaved for it to be activated by NAIP (Fig. 5.12B). These findings demonstrate that NAIP-BIR2 can activate caspase-5 in a non-IBM dependent manner. This is quite logical since no other
IBM-groove containing BIR was capable of inflammatory caspase activation (Fig 5.3). and the neo-N-terminal epitope on the small subunit of caspase-5 does not resemble a preferred binding sequence of NAIP-BIR2. Therefore a region ancillary to the conserved surface groove is required for NAIP-mediated caspase activation.
Figure 5.12 NAIP-BIR2 activates caspase-5 in a non-IBM-dependent manner. A) Progress curve of caspase-5 activity in the presence of a range of concentrations of the IBM-groove mutant of NAIP-BIR2 (E216V/W220R). B) Progress curve of caspase-5 D330A in the presence of a range of concentrations of NAIP-BIR2. Caspase-5 activity was monitored by cleavage of the substrate Ac-WEHD-AFC.
5.11.2 The N-terminal region.

My findings demonstrating the IBM-independence of the NAIP-BIR2/caspase-5 interaction caused me to investigate other regions of this BIR that may be required for caspase-5 activation. The C-terminal region of this BIR seems very well conserved with other BIRs and therefore I decided to focus on the N-terminal region of the BIR. To investigate this region, I chose a gain of function approach. Here, I wanted to create a chimeric BIR domain in which I replaced a portion of a non-caspase-5 activating BIR (template) with that of NAIP-BIR2. The goal of these studies is to confer ability to activate caspase-5 to the template BIR. I selected XIAP-BIR3 as the template BIR since I am only altering the N-terminal region of the BIR and therefore it should maintain its ability to inhibit caspase-9. This provides a means to determine whether my chimeric BIR is properly folded. Modeling of NAIP-BIR2 on to the XIAP-BIR3 structure allowed me to predict a region that should have minimal secondary structure, at which to make the junction point within my chimeric BIR. I selected Gly190 of NAIP (corresponding to Gly293 of XIAP) as this junction point since it seemed be localized within a loop and did not participate in an α-helix or β-sheet. Furthermore, these two BIRs are well conserved in the regions immediately flanking this residue. Thus, I generated a chimeric protein in which residues 154-190 NAIP were fused to residues 294-348 of XIAP (NB2/XB3) (Fig 5.13). Initially, I tested the ability of the NB2/XB3 protein to inhibit caspase-9 to confirm that this BIR was properly folded. NB2/XB3 inhibited caspase-9, albeit slightly
potently than XIAP-BIR3 (Fig 5.14A). Although the distal helix may fold separately from the BIR itself, keep in mind that inhibition of caspase-9 requires a functional IBM-groove. Therefore, the ability of NB2/XB3 to inhibit caspase-9 demonstrates that the majority of the chimeric BIR protein is properly folded into the conserved fold. Next, I tested the ability of the chimera to activate caspase-5. Remarkably, I found NB2/XB3 to be capable of robust caspase-5 activation, demonstrating the importance of the N-terminal portion of NAIP-BIR2 in this activation event (5.14B).
Figure 5.13 Generation of a NAIP-BIR2/XIAP-BIR3 chimeric protein. Structure model of NAIP-BIR2 on XIAP-BIR3 (PDB 2OPY). Above is a sequence alignment of XIAP-BIR3 (black) and NAIP-BIR2 (grey), denoting the junction point in the chimeric protein (NB2/XB3). IBM-groove defining residues, (black circles) and caspase-9 dimer interface interacting residues (grey triangles) have pinpointed above. In the model the light grey portion of the strand reflects residues of NAIP and while the black region is representative of XIAP.
Figure 5.14 The N-terminal region of NAIP-BIR2 is important to caspase-5 activation. A) Caspase-9 is inhibited by XIAP-BIR3 (black circles) and NB2/XB3 (grey circles). Residual caspase-9 activity was monitored by cleavage of the substrate Ac-LEHD-AFC. B) Caspase-5 is activated by NAIP-BIR2 and NB2/XB3, but not XIAP-BIR3. Caspase-5 activity was monitored by cleavage of the substrate Ac-WEHD-AFC.
5.12 Evidence of a Caspase-5/NAIP-BIR2 complex

NAIP-BIR2 and caspase-5 directly interact as evidenced by my activation assays. In order to determine the nature of this interaction I set out to trap a NAIP-BIR2/caspase-5 complex using a crosslinking technique. I utilized glutaraldehyde at varying concentrations (16nM-250 mM) as the crosslinking reagent. Although, there are several commercially available crosslinking reagents, Glutaraldehyde has previously been shown to be effective for crosslinking of caspases (Renatus et al., 2001). Caspase-5 was crosslinked in the presence and absence of NAIP-BIR2. The association of the large and small subunits of the caspase was evidenced by the increase in a 35 kDa band that correlated with increasing glutaraldehyde concentration. In addition, the crosslinking reaction trapped some dimeric caspase-5 (70 kDa band). Importantly, in the presence of NAIP-BIR2 there was an additional band at about 45 kDa. This band is dependent on the presence of NAIP-BIR2 because it was not seen when caspase-5 was incubated with glutaraldehyde alone (Fig. 5.14A). This suggests that these two interact and this could represent a heterodimer composed of a caspase-5 monomer (35 kDa) bound to a single NAIP-BIR2 (13kDa). Further studies suggest that this is specific to NAIP-BIR2 as XIAP-BIR3 was unable to form this complex with caspase-5 (Fig. 5.14B)
**Figure 5.15 NAIP-BIR2 directly binds caspase-5.** A) Caspase-5 was incubated with increasing concentrations of glutaraldehyde (Glut) in the absence (right panel) or presence (right panel) of 2µM NAIP-BIR2. Arrow marks band unique to NAIP-BIR2/caspase-5. B) XIAP-BIR3 (XB3) is unable to induce the 45kDa complex with caspase-5, whereas NAIP-BIR2 (NB2) is.
5.13 Summary of AIM III

As predicted, I have found that the BIR domains of NAIP are not able to function as potent inhibitors of the inflammatory caspases. Interestingly, the BIR2 domain of NAIP demonstrated a unique capacity to activate caspases-4 and 5. The interaction of NAIP-BIR2 with caspase-5 seems to have a direct effect on the active site confirmation of the caspase-5. I have mapped the region required for this activation capacity to the N-terminal part of NAIP-BIR2. This is located away from the IBM-groove and does not seem to be an IBM-dependent interaction, suggesting a previously uncharacterized BIR/caspase interaction. In addition this may represent an activation mechanism very unique among the caspases. These studies provide the first evidence that NAIP has the ability to directly activate an inflammatory caspase. Importantly the biological consequences of the interaction are consistent with the previously reported functions of the NLR protein family, of which NAIP is a member (Fortier et al., 2005; Martinon and Tschopp, 2004; Martinon and Tschopp, 2005). These findings open the door for further studies on the role of NAIP in innate immune signaling.
6. DISCUSSION
6.1 XIAP is the black sheep of the IAP family

The IAP protein family rapidly caught the attention of several research groups upon the seminal discovery by Deveraux and colleagues demonstrating that XIAP functioned as a potent caspase inhibitor (Deveraux et al., 1997). A major open question in the IAP field was whether all members of this protein family functioned as caspase inhibitors. Each IAP has been suggested to be a caspase inhibitor at some point and a major aim of my research was to determine the capacity of IAPs, other than XIAP, to function as potent caspase inhibitors.

The mechanisms of caspase inhibition by XIAP have been extensively characterized through biochemical and structural studies (Deveraux et al., 1999) (Chai et al., 2001a; Riedl et al., 2001b; Scott et al., 2005; Shiozaki et al., 2003). XIAP utilizes it BIR2 domain to secure potent inhibition for caspases-3 and 7, while it uses it BIR3 to dampen the activity of caspase-9. These two BIR domains are very well conserved with each other, while the caspases all possess a very similar fold. Intriguingly, XIAP inhibits the executioner caspases-3 and 7 in a drastically distinct manner compared to means by which inhibits caspase-9. However, there is some conservation in these inhibitory mechanisms. Both the BIR2 and BIR3 domains of XIAP require a conserved surface groove within the BIR that confers the anchoring interaction with the caspase. It is the ancillary caspase interaction sites, located in the regions flanking the BIR domains that
are unique to either BIR2 or BIR3, and are the basis for their differential modes of caspase inhibition. The peptide strand N-terminal to BIR2 interacts with and occludes the active site cleft of caspase-3 and 7. Conversely, a helix C-terminal to BIR3 is required to pack against the dimer interface and induce monomerization of caspase-9, representing an entirely allosteric mechanism of inhibition.

In my studies have found that the BIR2 domains of cIAP1, cIAP2, and NAIP are unable to function as potent inhibitors of caspases-3 and 7 due to a divergence from XIAP in the conformation of the peptide strand N-terminal to the BIR. Furthermore, distinctions from XIAP at three key residues in the distal helix precludes the BIR3 domains of cIAP1 and cIAP2, and the single BIR of ML-IAP from functioning as caspase-9 inhibitors. This marks a major revision in IAP field, which through the dissection of the minimal requirements of IAP-mediated caspase inhibition, it becomes apparent that XIAP is the only member of the IAP family that is a physiologic caspase inhibitor.

A natural question that arises is how were IAP BIR domains, other than XIAP, initially reported to function as caspase inhibitors? The answer in part seems to be the specific purification tag present on the recombinant IAP protein. Initial studies on ML-IAP, cIAP1, cIAP2, and NAIP utilized glutathione S-transferase (GST) tagged protein (Davoodi et al., 2004; Deveraux et al., 1998; Maier et al., 2002; Roy et al., 1997; Vucic et al., 2000), whereas in my enzymatic assays the recombinant proteins were always His-tagged. The importance of the purification tag in enzymatic assays was recently investigated. A hybrid protein
consisting of the peptide strand N-terminal to XIAP-BIR2 fused to GFP instead of the BIR2 itself, should be a poor inhibitor of caspases-3 and 7 because it lacks the anchoring interaction mediated through the BIR2 domain. Interestingly, if this hybrid protein had a GST-tag it was a significantly more potent caspase inhibitor than if it had a His-tag (Scott et al., 2005). This is most likely due to differences in the oligomeric state of the protein. Size exclusion chromatography of GST-XIAP-BIR2 reveals that is a mixture of various oligomeric species. Conversely, His-tagged XIAP-BIR2 was determined to be solely monomeric (Fiona Scott personal communication). The ability of GST to oligomerize a protein was also evidenced by my studies in which GST-cIAP1-BIR3 was capable of activating caspase-9 through direct dimerization. Thus, a GST-tag will cause a protein to become unnaturally oligomeric and in the case of the IAPs this may result in artificial inhibition.

In the absence of a direct caspase-inhibitory function the basis of the anti-apoptotic activity of the IAPs is less clear. Importantly, many IAPs are capable of binding caspases and IAP-antagonist. Recently, it has been proposed that these IAPs might sequester SMAC upon its release from the mitochondria. IAPs functioning as SMAC sinks would effectively reduce the cytoplasmic concentration of this IAP-antagonist, hindering its ability to disrupt the XIAP-mediated inhibition of caspases (Duckett, 2005). Another, likely mechanism of apoptotic occlusion by the IAPs is the ubiquitination of pro-apoptotic molecules. IAPs have been reported to be capable of ubiquitinating caspases, IAP-antagonist, each other, and themselves – reviewed in (Vaux and Silke, 2005a;
Vaux and Silke, 2005b). The *Drosophila* IAP1, does not directly inhibit the catalytic activity of the caspases and requires a functioning RING domain to block apoptosis (Lisi et al., 2000; Tenev et al., 2005). Therefore, from an evolutionary perspective, the predominant function of the IAPs is most likely not caspase inhibition by rather involves ubiquitination. It follows that it is more likely that XIAP acquired the ability of potent caspase inhibition, rather than other IAPs evolved away from this specialized function.

### 6.2 Not all IBM-grooves are created equal

During my dissection of the IAP-mediated caspase inhibition, it was apparent that the surface groove, denoted as the IBM-groove, present on many BIRs, enabled caspase binding. As SMAC-mimetics progress toward the clinic for various cancer indications a question arises as to their specificity and mechanism of action. If XIAP is specifically targeted by these compounds over other non-caspase inhibitory IAPs the mechanism by which these compounds induce is apoptosis clear. However, the interaction of these compounds with other IAPs complicates matters. Prior to my studies presented here, there was little known about the intrinsic binding specificities of various BIR domains. The classic IBM consensus was thought to be AΦpΦ, where Φ is hydrophobic and the Ala is at the extreme N-terminus. This consensus was deduced by analysis of IAP-binding proteins and limited profiling studies of a few BIR domains, most notably XIAP-BIR3. The second major aim of my thesis work was to investigate
the peptide binding signatures of various BIR domains and I developed a method to do this.

I have found that BIR domains from various IAPs possess distinct binding signatures. Therefore, the concept of a single IAP binding motif must be refined. I have divided IBM-groove containing BIR into two subclasses (type II and type III) based on the sequence of their natural ligands, elucidated binding signatures, and which IBM-groove defining residue they posses (Trp or His). Type III BIR domains (which posses a Trp corresponding to residue 323 of XIAP) demonstrate the classic SMAC-like binding signatures, while type II BIR domains (which posses a His corresponding to residue 223 of XIAP) have more varied binding signatures. The most notable characteristic of a type III BIR domain is the selection for Pro in P'3. This selection was observed for cIAP1-BIR3, XIAP-BIR3, and NAIP-BIR2 in my profiling, as well as previous, profiling studies on XIAP-BIR3 (Sweeney et al., 2006) and the single BIR domain of ML-IAP (Franklin et al., 2003). Type II BIR domains tested, XIAP-BIR2 and cIAP1-BIR2, selected against Pro, but otherwise demonstrated no dominant specificity for P'3. I found that the basis for or against a P'3 Pro selectivity was due to the presence of the Trp or His, respectively, at the defined position within the IBM-groove.

Given the distinctions in binding specificity between type II and type III BIRs, it is natural to ask what the biological consequences are. It has been suggested that different types of BIR domains select different target proteins (Verhagen et al., 2007). Several IAPs contain tandem type II and type III BIRs, and this seems to be important in enhancing avidity toward target proteins. For
example, SMAC and caspase-9 have an IBM that is optimal for binding type III BIRs, indeed this is part of the mechanism of inhibition of caspase-9 by XIAP-BIR3 (Shiozaki et al., 2003), as well as derepression of inhibition of caspase-9 by SMAC (Du et al., 2000; Liu et al., 2000; Verhagen et al., 2000; Wu et al., 2000). The $K_D$ for SMAC and BIR3 is in the 200-700 nM range, which is above the $K_i$ for caspase-9 inhibition (Sun et al., 2000). Therefore, to be effective the $K_D$ of the SMAC/IAP interaction must be decreased in vivo. This is accomplished by the natural dimeric conformation of SMAC where one IBM interacts with BIR3 and the other with BIR2 of XIAP (Gao et al., 2007; Huang et al., 2003). This substantially decreases its $K_D$ to 0.31 nM because of the two-site interaction-driven avidity.

Although there are many published BIR domain affinity measurements, with $K_D$'s in the hundreds of nanomolar to micromolar range, these are almost always carried out on peptides and not full length (Denault et al., 2007; Franklin et al., 2003; Hell et al., 2003; Liu et al., 2000; Sweeney et al., 2006). Some full length proteins may have interactions with IAPs in addition to the N-terminal epitope (Roberts et al., 2001; Scott et al., 2005; Shiozaki et al., 2003). The property leading to a decrease in $K_D$ into the physiologic range is driven largely by two-site interactions (Eckelman et al., 2006; Huang et al., 2003). Based on the findings with SMAC, we suggest that the best candidate target proteins that interact with multi-BIR IAPs, would also likely be dimeric, or even polymeric. It follows that single BIR-containing IAPs would require an additional non-IBM directed interaction to bind target proteins in a physiological setting.
The fact that IBM-groove directed binding necessitates exposed N-terminal motifs places further restrictions on potential binding partners. There are two subsets of proteins that could possess an exposed N-terminal Ala residue. First, those that possess an Ala generated by the removal of the initiator Met by methionine aminopeptidase, which always occurs with cytosolic proteins if an Ala is present in the second position (Bradshaw et al., 1998). However, the resulting N-terminal Ala is usually acetylated in the cytosol thereby making it unavailable as an IBM (Polevoda and Sherman, 2003). The second subset is defined by proteins that have undergone an endoproteolytic cleavage, exemplified by the caspases. Mitochondrial proteins often have their signal peptides removed once that have translocated into the mitochondria. These proteins remain unacetylated and comprise the most likely pool of candidate IAP-target proteins, exemplified by SMAC and HtrA2. Figure 6.1 presents a collective set of previously identified mammalian IBM-containing proteins (Du et al., 2000; Eckelman and Salvesen, 2006; Hegde et al., 2003; Hegde et al., 2002; Martins et al., 2002; Riedl et al., 2001b; Scott et al., 2005; Srinivasula et al., 2001; Suzuki et al., 2001a; Verhagen et al., 2000; Verhagen et al., 2007; Verhagen et al., 2002), which I have divided based on whether they were shown to interact with type II or type III BIRs. The sequences of these proteins naturally fall into two categories that closely resembling the profiles I elucidated for type II and type III BIR domains. The results of my studies provide a context for understanding the natural binding mechanism of BIR domain and various IBM-containing proteins.
Hopefully, these peptide binding signatures of various BIR domains can be utilized to create tools to dissect the roles of distinct IAPs in signaling pathways, elucidate new binding partners, and provide insight into the cellular roles of the IAPs. IAP-antagonists, namely SMAC-mimetics, are currently being explored as cancer therapeutics (Arnt et al., 2002; Li et al., 2004; Oost et al., 2004; Park et al., 2004a; Sun et al., 2004; Zobel et al., 2006) and my findings here provide a focus point for knowledge based drug design in this arena.
Figure 6.1 Binding Motif containing proteins. Data collected from the literature where specific proteins have been demonstrated to interact with individual BIR domains. I have divided the interactions according to the type of domain reported to interact. Note the preference for Pro in the third position of the natural proteins that interact with type III BIRs, which is in agreement with my peptide scanning data.
6.3 Beyond the IBM-groove, specialized functions of BIR domains

I have elucidated the binding signatures of IBM-groove on several BIR domains and found interesting distinctions. These binding preferences may dictate the specificity of an interaction and are probably critical to the physiologic function of various IAPs. However, it is now apparent that the BIR domains of IAPs have specialized functions that are mediated by regions other than this groove.

My studies revealed that the BIR2 domain of NAIP functions as a robust activator of caspase-5, and to a lesser extent caspase-4. No other BIR tested demonstrated the ability to activate these caspases. This is quite surprising as the BIR domains are very similar, often sharing greater than 50% identity. Furthermore, crystal structures of several demonstrate a highly conserved fold. It is interesting to compare the interactions of XIAP with caspase-3, 7, and 9 and to NAIP with the inflammatory caspases. Although the effect on the catalytic activity of the caspase is essentially opposite it reveals what might be an underlying principle in IAP biology. I have found that the N-terminal region of NAIP-BIR2 domains provides a surface sufficient for caspase-5 activation. Modeling studies of NAIP-BIR2 demonstrate that this surface is most likely on a distal face form the conserved IBM-groove. BIR domains seem to have evolved distinct functions at sites away from this groove. Although, this groove is critical for some functions, as is the case for XIAP and its cognate apoptotic caspases, it
may be dispensable for other interactions as I have found with NAIP and caspase-5. It is these ancillary sites that provide the true specificity of the interaction. XIAP is only specific inhibitor of caspases-3 and 7 due to a peptide extension N-terminal to BIR2. Furthermore, the interaction of XIAP with caspase-9 would be of little consequence, as is the case for most BIRs, if it wasn’t for the presence of a specific helix distal to BIR3. This principle can be extended to other specific BIR interactions for instance the cIAPs and TRAF2 and TRAF6. This interaction is dependent on a unique surface found only within the BIR1 domain of the cIAPs (Samuel et al., 2005; Varfolomeev et al., 2006). These BIR does not possess an IBM-groove but the concept holds, in that this domain maintains the conserved BIR fold, however, is capable of a unique interaction that is dependent on region other than where the IBM-groove is located in most BIRs.

The BIR domain provides a seemingly efficient scaffold upon which to adopt various binding surfaces that are responsible for a multitude of specific interactions, contributing to a myriad of biological events. The IBM-groove is evolutionarily conserved and most likely represents one of the first surfaces that enabled a unique and important interaction. Evolution maintained this groove and eventually XIAP was able to utilize it as a key component of its caspase-inhibitory mechanism. Other interaction surfaces likely arose later in evolution and are thus unique to a one or a few BIRs.
6.4 NAIP the inflammatory IAP

Although it was the first human IAP identified, the cellular function of NAIP has remained elusive over the last twelve years. NAIP has a unique domain composition, that in addition to being a member of the IAP family it can also be classified as a NOD-like LRR (NLR) protein. The NLR proteins are composed of a sensing domain, usually LRRs, a nucleotide oligomerization domain, and a recruitment domain that is typically a CARD or a PYD, or in the case of NAIP a BIR domains. NLR proteins have been reported sense and respond to cytosolic pathogen associated molecular patterns (PAMPs) (Fortier et al., 2005; Martinon and Tschopp, 2004; Martinon and Tschopp, 2005). The most common reported function of the NLR proteins is the activation of the inflammatory caspases, more specifically caspase-1. The NLR family is composed of 20+ members that may have overlapping ligands and functions that may converge the limited read-outs available to us, those being the maturation of IL-1β and IL-18, and caspase-1 processing. Pan-caspase inhibitors have been useful, but cannot single out a particular caspase as being of predominant importance. To further complicate matters, it is highly likely that several NLR-dependent signaling pathways are engaged at the same time and thus elucidating the role of any one member in cellular setting is difficult at best. Mouse knockout studies coupled to mutation of potential PAMPs on the pathogen have advanced the field significantly. It is now time to utilize the knowledge acquired from the studies to tease apart these signaling pathways using isolated proteins. This type of approach is now being
applied to understand the role of the NALPs and Ipaf in inflammatory caspase regulation (Faustin et al., 2007; Martinon et al., 2002; Poyet et al., 2001).

In mice, the understanding of a physiological role of NAIP lagged, being complicated by the presence of several NAIP gene fragments. However, it is now clear that the ability to restrict intracellular replication of *Legionella pneumophila* is dependent on isoform differences in the *NAIP5/BIRC1e* gene, which resides within the *Lgn1* locus (Derre and Isberg, 2004; Diez et al., 2003; Diez et al., 2000; Growney and Dietrich, 2000; Growney et al., 2000; Wright et al., 2003). There has been some debate as to the molecular mechanisms by which this restrictive capacity is achieved. It seems that there are two essential, but distinct, signaling pathways; one involving the recognition of cytosolic flagellin and subsequent Ipaf-dependent caspase-1 activation, while the other, requires NAIP, and is much less well understood (Lamkanfi et al., 2007; Molofsky et al., 2006; Ren et al., 2006; Zamboni et al., 2006). The genetic dissection of the *Lgn1* locus has been critical in providing the first insight into a physiological role for NAIP. However, murine and human macrophages have altered capacities to restrict the intracellular replication of *Legionella p.* and in humans this pathogen is the causative agent Legionnaires disease (Asare et al., 2007). Importantly, caspase-1 is the only conserved inflammatory caspase between mice and humans, and the differences in responses to various pathogens may be a consequence of other inflammatory caspases. A recent report demonstrated that in mice NAIP restricts *Legionella p.* independently of caspase-1 (Lamkanfi et al., 2007). This thereby casts doubt onto the prediction that caspase-1 is the true
effector of NAIP signaling. Mice lack caspases-4 and 5, and have caspase-11 and this may the basis for differential response of human and mouse macrophages to *Legionella pneumophila*.

I have found that NAIP-BIR2 has the unique ability to activate the human inflammatory caspases-4 and 5, but not caspase-1. This function of NAIP fits well with the known functions of other NLR proteins. The dogma of NLR-mediated inflammatory caspase activation proposes that upon oligomerization via the NOD of NLR protein the bound caspase is activated by an induced-proximity-like mechanism. My findings that demonstrate a single monomeric domain is capable of robust inflammatory caspase activation, representing a key distinction from this dogma. However, in the context of the full length NAIP protein, oligomerization may be a key component of in the activation of caspases-4 and 5. In a cellular setting, oligomerization of NAIP, at the very least, would create a higher local concentration of the BIR2 domain, and this may be required to over come the high nanomolar $K_D$. My investigation into the consequences of NAIP/caspase interactions provides the first direct evidence for this IAP as a caspase activator. I found NAIP mediated caspase activation to be very substantial, likely representing physiologic event. To truly understand the importance of this interaction in human innate immune signaling it will be critical to determine the exact ligand of the LRRs and how this regulates the conformation of the protein as whole. Furthermore, structural studies on the caspase-5/NAIP-BIR2 complex, should provide valuable insight into the exact activation mechanism.
6.5 Future directions for the IAP world

The IAP field is at a critical transition point, translating the knowledge of basic biology into something of therapeutic benefit. As it the case for most fields that rest at this important juncture, a question of readiness arises. Has our knowledge of the biology of the IAPs advanced to a point that it can be translated into understanding the mechanisms of action of IAP-based therapeutics? XIAP is the only member of the IAP family that is a potent caspase inhibitor. Thus, specifically targeting this inhibitory interaction could reactivate the apoptotic program in cancers cells that express high levels of XIAP. In fact this strategy has demonstrated much promise as compounds that target the IBM-groove (Arnt et al., 2002; Li et al., 2004; Oost et al., 2004; Park et al., 2004a; Sun et al., 2004; Zobel et al., 2006) or other inhibitory sites (Schimmer et al., 2004; Wu et al., 2003) have been shown to induce apoptosis directly or cause a sensitization to various apoptotic stimuli or chemotherapeutics in an array cancer cells. The difference in sensitization and direct apoptotic induction can logically be attributed to whether the caspases are constitutively active and inhibited by XIAP or require stimulus to become active. In the former case these compounds should induce apoptosis in the absence of another stimulus, in the latter situation these compounds must be used in a combination so as to engage the apoptotic signaling and induce caspase activation. Thus, these compound would simply overcome XIAP’s inhibition of caspases once they become active.
For simplicity, I will denote compounds that target the IBM-groove, as type A compounds and these include all SMAC-mimetics. Consistently, compounds that have been suggested to interact with the caspase active site occluding peptide strand of XIAP, I will call type B compounds. Targeting the peptide strand N-terminal to BIR2 of XIAP would specifically disrupt the ability of XIAP to inhibit the executioner caspases and compounds have been developed in an effort to achieve this (type B compound). Targeting the IBM-groove through the use of SMAC-mimetics would most certainly interact with several BIR domains (type A compounds). So which is a better strategy? It seems logical that targeting the specific interaction of XIAP and executioner caspases would be the best as this is the last point of inhibition of the apoptotic cascade and the presence of other IAPs should be of little consequence. However, recent studies on the type A compounds have demonstrated that XIAP is not the primary target of SMAC-mimetics and antagonizing its caspase inhibitory function is of secondary importance.

Surprisingly, the SMAC-mimetics have been found to target the cIAPs and enhance their auto-ubiquitination, resulting in drastic and marked reduction of their protein levels (Petersen et al., 2007; Varfolomeev et al., 2007; Vince et al., 2007). As I found, the cIAPs are not caspase inhibitors, so what is the mechanism by which these compound induce apoptosis? The loss of cIAP1 from the TNF receptor causes stabilization in the NIK protein, leading to activation of a NFκB-dependent pathway that culminates in the transcriptional upregulation of TNFα. This results in an autocrine singling loop inducing caspase-8 activation.
Thus, SMAC-mimetics induce apoptosis through caspase-8, representing a completely serendipitous mechanism of action. How a small molecule binding to the surface groove of a BIR induces auto-ubiquitination of the cIAPs and not XIAP is unclear and somewhat remarkable. Consistently, studies have demonstrated that the SMAC protein may induce the auto-ubiquitination of the cIAPs (Yang and Du, 2004). These recent findings on the mechanism of action of SMAC-mimetics, complicate matters and may limit the usefulness of type A compounds. Instead of relieving the block in apoptotic signaling at a very terminal point in the pathway, these compounds now rely on several signaling pathways to be functionally intact. Cancer cells are quite diverse in the means by which they are transformed, and it is often difficult to determine which signaling pathways have been spared from mutation. Therefore, it will be challenging to predict which types of cancers will respond to type A compounds.

The prediction of response to type B compounds is more straightforward. The best responders will be those cancer cells that have both constitutively activated caspases and high levels of XIAP. Cancer cells that simply have upregulated XIAP and there is no evidence for caspase activation will most likely only respond to type B compounds in combination with other chemotherapeutic or apoptotic inducing agent that cause caspase activation. It is unclear at this point what percentage of cancers have constitutively activated caspases that are simply inhibited by XIAP. Thus, an investigation into this may be required before effort and resources should be directed toward the development of these type B compounds.


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