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GENOMIC ORGANIZATION OF THE X-LINKED INHIBITOR OF APOPTOSIS AND IDENTIFICATION OF A NOVEL TESTIS-SPECIFIC HOMOLOGUE

by

Mark Lagacé

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ABSTRACT

GENOMIC ORGANIZATION OF
THE X-LINKED INHIBITOR OF
APOPTOSIS AND IDENTIFICATION
OF A NOVEL TESTIS-SPECIFIC
HOMOLOGUE

by Mark Lagacé

Chairperson of the Supervisory Committee:Professor Robert G. Korneluk
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This thesis documents the genetic characterization of the *X-Linked Inhibitor of Apoptosis* (*XIAP*) gene, and the subsequent discovery and characterization of a novel gene, called the *Testis-specific IAP* (*TsIAP*). Genomic library screening and bulk sequencing was used to isolate and sequence the genomic region that encompasses the XIAP gene. Following this, we screened cDNA libraries, as well as performed RT-PCR and rapid amplification of cDNA ends (RACE) reactions to elucidate the full length cDNA sequence and intron/exon boundaries of *XIAP*. The XIAP gene spans a 33 kb region of chromosome X and is encoded in seven exons. The transcript is 10 kb in size, containing very large 5' and 3' untranslated regions. Four separate regions of the genome cross-react with probe derived from *XIAP* coding region sequence. These 'pseudogenes' were sequenced and characterized. Three of the pseudogenes appeared to be derived from retrotransposition events as they contained partially conserved copies of *XIAP* lacking any introns. One of these pseudogenes turned out to maintain a viable open reading frame and encoded a

novel protein with expression limited to the testis. We employed a similar library screening strategy to clone and sequence the novel testis-specific IAP gene. FISH analysis was performed to localize the gene to chromosome 19q13.4. The *TsIAP* gene was found to encode a 2kb mRNA with an open reading frame encoding a region highly homologous to the carboxy-terminal end of the X-linked IAP. Attempts were made to find a murine homologue of *TsIAP* with no success, and later work published by another group confirmed that the *TsIAP* gene is a recent evolutionary event and that no murine homologue exists. Given the unusually long 5' UTR present in the *TsIAP* gene, we tested whether the mRNA could produce a protein by using Western blot analysis on both overexpressed TsIAP in cell culture, as well as endogenous expression in testicular protein extracts. A band was detected in both cases migrating at approximately 26kDa, the predicted size of the TsIAP protein. Having confirmed that the novel gene we isolated is capable of being expressed, we proceeded to characterize its functional properties. TsIAP was able to protect against apoptosis, however, only against a limited subset of apoptotic triggers when compared to its parent gene XIAP. TsIAP could not protect against the chemotoxic drugs etoposide or adriamycin, however, it showed a strong protective effect against Bax-induced apoptosis. Surprisingly, against Bax-induced apoptosis, TsIAP was a more potent inhibitor than the corresponding region of XIAP. Smac (second mitochondrial activator of caspases) is an important inhibitor of XIAP function in the context of the mitochondrial pathway of apoptosis induced by Bax. We determined that Smac was unable to bind to the TsIAP protein in pull-down experiments and thus could not reverse the protective effects of TsIAP in a Bax-induced apoptosis model.

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DEDICATION

To my wife Heidi, for her love and support.

LIST OF ABBREVIATIONS

°C – Degree Celsius
a.a. – Amino Acid
AcMNPV – *Autographa californica* Multicapsid Nuclear Polyhedrosis Virus
AIDS – Aquired Immunodeficiency Syndrome
Apaf – Apoptosis Protease Activating Factor
Bcl – B cell lymphoma
BIR – Baculoviral IAP Repeat
BIRC – BIR Containing Protein
BMP – Bone Morphogenic Protein
bp – Base Pair
BRUCE – BIR Repeat Containing Ubiquitin Conjugating Enzyme
CARD – Caspase Recruitment Domain
CAD – Caspase-activated DNAse
Caspase – Cysteine Aspartase
CDK – Cyclin Dependent Kinase
cDNA – Complementary DNA
CpGV – *Cydia pomonella* Granulosis Virus
CRD – Cysteine Rich Domain
CrmA – Cytokine Response Modifier A
dATP – Deoxyadenosine Triphosphate
dCTP – Deoxycytidine Triphosphate
DD – Death Domain
DED – Death Effector Domain
DIABLO – Direct IAP Binding Protein with Low pI
DISC – Death Inducing Signaling Complex
DNA – Deoxyribonucleic Acid
DTT – Dithiothreitol
EDTA – Ethylenediaminetetraacetic Acid
FACS – Fluorescence Activated Cell Sorting
FADD – Fas Associated Death Domain
FISH – Fluorescent *In situ* Hybridization
GFP – Green Fluorescent Protein
GST – Glutathione S Transferase
h - Hour
HIAP – Human Inhibitor of Apoptosis
IAP – Inhibitor of Apoptosis
IBM – IAP Binding Motif
ICE – Interleukin-1β Converting Enzyme

ILP – IAP-like Protein
IRES – Internal Ribosomal Entry Site
JNK – c-Jun Amino Terminal Kinase
kb – Kilobase pairs
kDa – Kilodaltons
KIAP – Kidney Inhibitor of Apoptosis
LB – Luria-Bertani Medium
MALT – Mucosa Associated Lymphoid Tissue
MAPK – Mitogen-Activated Protein Kinase
MHC – Major Histocompatibility Complex
MIAP – Mouse Inhibitor of Apoptosis
min - Minute
ml – Millilitre
ML-IAP – Melanoma IAP
mM - Millimolar
mRNA – Messanger RNA
MTN – Multiple Tissue Northern
NAIP – Neuronal Apoptosis Inhibitory Protein
NF-κB – Nuclear Factor κB
NGF – Nerve Growth Factor
OpMNPV – *Orgyia pseudosugata* Multicapsid Nuclear Polyhedrosis Virus
ORF – Open Reading Frame
PBL – Peripheral Blood Leukocytes
PCD – Programmed Cell Death
PCR – Polymerase Chain Reaction
PFU – Plaque Forming Unit
PGC – Primordial Germ Cell
PGK – Phosphoglycerate Kinase
PMSF – Phenylmethylsulfonyl Fluoride
Rb – Retinoblastoma
RFP – Red Fluorescent Protein
RNA – Ribonucleic Acid
RPM – Rotations Per Minute
RT-PCR – Reverse Transcription PCR
RZF – RING Zinc Finger
SDS – Sodium Dodecyl Sulfate
SMA – Spinal Muscular Atrophy
SMAC – Second Mitochondrial-Derived Activator of Caspases
SMN – Spinal Motor Neuron
SSC – Standard Saline Citrate
TAE – Tris-Acetate EDTA
TCR – T cell Receptor

TGF-β – Transforming Growth Factor β
TNF – Tumour Necrosis Factor
TRADD – TNF Associated Death Domain
TsIAP – Testis-specific Inhibitor of Apoptosis
UTR – Untranslated Region
UV – Ultraviolet
XAF – XIAP Associated Factor
XIAP – X-linked IAP
YAC – Yeast Artificial Chromosome
ZF – Zinc Finger

Chapter 1

INTRODUCTION

Programmed Cell Death

This thesis documents the genetic characterization of the X-linked Inhibitor of Apoptosis (XIAP) gene, and the subsequent discovery and characterization of a novel gene, called the Testis-specific IAP (TsIAP). Given the central role of apoptosis (also known as programmed cell death) in cellular function, this introduction will describe the process of apoptosis including the characteristic morphological changes as well as the molecular pathways that underlie them. Finally, the reader will be introduced to the growing family of IAP genes along with recent data on their functions and inhibitors.

History and general aspects of apoptosis

Cell death serves important physiologic and homeostatic functions in all multicellular organisms. It is now clear that the selective removal of cells plays a critical role in such diverse functions as cell deletion during embryonic development, balancing of cell numbers in continuously renewing tissues, immune system development, hormone dependent involution in the adult, and many other physiologic processes. Furthermore, numerous pathologically induced conditions such as Alzheimer's disease, autoimmune disease, cancer, and

AIDS can trace their deleterious effects to the dysregulation of programmed cell death.

The study of cell death is not a new phenomenon. As early as 1842, Carl Vogt reported cell death in the notochord and adjacent cartilage of metamorphic toads. Subsequent discoveries included phagocytosis associated with cell death in the muscles of metamorphic toads, cell death in ovarian follicles, the programmed loss of an entire population of neurons in fish embryos, the death of scattered myocytes and myofibres in mammalian muscle, and the death of many motor and sensory neurons in chick embryos; all of which were discovered before the turn of the 19th century (reviewed in (Clarke & Clarke, 1996)). Continued study into cell death through the early and middle 1900s eventually led to the seminal paper by Kerr, Wyllie, and Currie (Kerr *et al.*, 1972) distinguishing between the type of cell death that occurs naturally in animal development and homeostasis versus the type of pathological cell death that occurs at the centre of acute lesions such as trauma. In the latter case, the cells and their organelles tend to swell and rupture in a process called cell necrosis. By contrast, when cells die during normal development they usually shrink and condense, and the organelles and plasma membrane retain their integrity (see Table 1-1). This second form of cell death is an active process, requiring new RNA and protein synthesis to proceed (Lockshin, 1969). Kerr and colleagues coined the term *apoptosis* (from Greek, meaning 'falling leaves') to describe this 'natural' form of cell death.

Table 1-1. Comparison of apoptosis and necrosis.

Comparison between the two distinct forms of cell death. Apoptosis and necrosis differ in their morphological and biochemical characteristic. See the text for a more detailed description. Based on information from (Allen *et al.*, 1997).

Table 1-1. Comparison of the features of apoptosis and necrosis (based on table 1 of (Allen *et al.*, 1997)).

	Apoptosis	*Necrosis*
Occurrence	Scattered, single cells	Massive, tissue injury
Cytoplasmic changes	Shrinkage: condensed and dehydrated; normal organelles	Swelling: ER and mitochondria swell
Nuclear changes	Chromatin condenses and masses near the nuclear envelope. DNA is fragmented.	Ill-defined structure, eventual lysis of the nucleus
Plasma membrane	Blebbing observed, formation of apoptotic bodies; re-adjustment of phospholipids	Membrane injury/lysis occurs; leakage of cellular contents into surrounding environment
Tissue response	Phagocytosis of apoptotic bodies	Immune mediated inflammatory response.

Apoptosis is characterized by a set of morphological and biochemical changes in the cell (see Table 1-1). The earliest observable change is condensation of the cytoplasm and nuclear chromatin and aggregation of the compacted chromatin beneath the nuclear envelope (Kerr, 1971). Convolutions of the nuclear and plasma membranes occur progressively, leading to a distinctive blebbed appearance of the cell. Observation of cell blebbing is restricted almost entirely to cells in culture, however, and rarely seen *in vivo* as the blebbing cells are rapidly phagocytosed. Coincident with the observed morphological changes, cells undergoing apoptosis can be characterized by distinct biochemical changes. Since apoptotic cell debris is rapidly engulfed *in vivo*, the cell death program must implement changes in the plasma membrane which allows for recognition by phagocytes. A key player in this is phosphatidylserine (PS), a normal constituent of the plasma membrane that is located on the inner leaflet in viable cells. Phosphatidylserine is translocated to the outer leaflet of the plasma membrane and has been shown to mediate phagocytic recognition by macrophages (Fadok *et al.*, 1992; Savill *et al.*, 1993). In many apoptotic cells, specific endonucleases cleave DNA to produce a characteristic 'ladder' pattern of DNA fragments when they are resolved by eletrophoresis. The detection of DNA laddering, and/or the presence of free 3' OH ends generated by DNA fragmentation was often used as an early biochemical assay for apoptosis (Allen *et al.*, 1997). Current knowledge of programmed cell death, however, has shown that DNA cleavage is a very late

marker of apoptosis and may not even occur during some types of programmed cell death (Cohen *et al.*, 1992; Tomei *et al.*, 1993).

Since the initial observations on apoptotic cell death were made, it has been recognized that apoptosis is a genetically controlled process critical to normal development as well as to the pathological process in a wide variety of tissues (reviewed in (Vaux & Korsmeyer, 1999)). During nematode (*Caenorhabditis elegans*) development 1090 somatic cells are generated and precisely 131 of these cells are removed by apoptosis (Hengartner, 1999; Horvitz, 1999). The identification of genes that regulate apoptosis in the nematode (Ellis & Horvitz, 1986) has led to the discovery of homologues in vertebrates and insects, underlining the high conservation of the process among species.

Apoptosis as a normal cellular process

Programmed cell death plays an important role in diverse aspects of development and adult life of multicellular organisms. Jacobson and collegues define four key areas in which apoptosis plays a crucial role: 1) sculpting of body parts; 2) elimination of unwanted structures; 3) controlling cell numbers; and 4) elimination of non-functional or harmful cells (Jacobson *et al.*, 1997).

The formation of digits in some higher vertebrates is a well studied example of body part sculpting whereby apoptosis eliminates the interdigital

tissue (Ganan *et al.*, 1996). If cell death is inhibited, digit formation is blocked (Zuzarte-Luis & Hurle, 2002).

Often in the course of development, animals will produce structures that are unneeded – either remnants from an ancestral species that are no longer necessary, or structures that are required in one sex but not the other. The elimination of Müllerian ducts in males and conversely the elimination of Wolffian ducts in females are examples of programmed cell death used to eliminate unneeded structures (Jacobson *et al.*, 1997).

Adjusting cell numbers is also an important role for programmed cell death. A few examples of this have been well studied. In particular, apoptosis plays a crucial role in neuronal development. During the development of the central nervous system, a surplus of neurons and oligodendrocytes are produced and nearly 50% of them are subsequently removed by apoptosis (Burek & Oppenheim, 1996; Oppenheim, 1991), apparently to match their numbers to the number of target cells they innervate. Likewise, in early male germ cell development, cells are culled by apoptosis to match the number of germ cells that the supporting Sertoli cells are able to maintain (Boekelheide *et al.*, 2000).

One of the most important aspects of normal cellular apoptosis is the elimination of potentially dangerous cells. Apoptosis functions as a quality control mechanism in animal development, removing cells that are abnormal,

misplaced, nonfunctional, or potentially dangerous to the organism. A striking example of this is seen in the vertebrate immune system, where developing T and B lymphocytes that either fail to produce potentially useful antigen-specific receptors, or produce self-reactive receptors, are eliminated by apoptosis. T cells undergo two stages of screening, failure of which leads to apoptosis. The first screening, known as positive selection, requires that the nascent T-cell receptor is able to bind to the MHC complex and thus is able to present antigen. The second stage, known as negative selection, removes those T cells that have produced receptors that are capable of binding the MHC complex strongly in the absence of antigen, or in the presence of a self-antigen (Yang & Ashwell, 1999).

A second example of apoptosis as a quality control mechanism is seen in the case of damaged or stressed cells. The p53 tumour suppressor gene is upregulated in response to a diverse array of cellular stresses, including DNA damage, hypoxia, oxidative stress, ribonucleotide depletion, and oncogene activation (Shaw, 1996). Depending on the cellular context, p53 promotes either apoptosis or cell cycle arrest allowing for repair of damaged DNA. Supporting the critical role of p53 in eliminating damaged cells is the observation that p53 is the most commonly mutated gene in human malignant cancers with over 50% of tumours showing deletions or point mutations (Wyllie *et al.*, 1999; Wyllie *et al.*, 1994).

Apoptosis in disease processes

Apoptosis is the prevalent mechanism complementary to proliferation that is critical for the normal function of multicellular organisms. In adult humans, roughly 10^{12} cells are produced every day, which needs to be balanced by apoptosis to maintain a constant cell number. Changing this balance in either direction has pathological consequences. Abnormally high rate of cell death is found in neurodegenerative disorders such as Alzheimer's and Parkinson's disease, in AIDS, and in cardiovascular disease. On the other hand, reduced cell death contributes to a wide variety of human cancers (reviewed in (Thompson, 1995)). Detailed description of the involvement of apoptosis in all of these processes is beyond the scope of this thesis, and the reader is encouraged to read any number of recent review articles on the subject (table 1-2).

Molecular machinery of Cell death

The caspases: Cellular executioners.

During genetic studies of the cell death pathways in the nematode, the *ced-3* gene was determined to be essential for all 131 programmed cell deaths that occur during hermaphrodite development (Ellis *et al.*, 1991). When ced-3 was cloned and sequenced, it was found to be a homologue of the mammalian interleukin-1β converting enzyme (ICE; caspase-1). Caspase-1 is responsible for the cleavage of pro-interleukin-1 into its proinflammatory, biologically active form (Thornberry *et al.*, 1992). While caspase-1 has no known role in the

Table 1-2. Apoptosis in disease processes.

This table shows an abbreviated list of pathological conditions in which apoptosis has been shown to play a prominent role. Recent review articles for each disease are cited and the reader is encouraged to consult the listed references for more information.

Table 1-2. Apoptosis in disease processes.

Disease	*Recent Reviews*
Cancers	(Epling-Burnette & Loughran, 2003; Soengas & Lowe, 2003; Wyllie *et al.*, 1999)
Neurodegenerative Disorders	(Friedlander, 2003; Mattson, 2000; Vila & Przedborski, 2003)
Parkinson's Disease	(Fiskum *et al.*, 2003; Lev *et al.*, 2003; Tatton *et al.*, 2003)
Alzheimer's Disease	(Behl, 2000)
AIDS/HIV	(Badley *et al.*, 2003; Gougeon, 2003; Selliah *et al.*, 2003)
Creutzfeldt-Jakob	(Ferrer, 2002)
Autoimmune Thyroid disease	(Ai *et al.*, 2003)
Rickettsia	(Walker *et al.*, 2003)
Psoriasis	(Victor & Gottlieb, 2002)
Huntington's Disease	(Hickey & Chesselet, 2003)

apoptotic pathway, it was the first identified member of a large family of aspartate-specific cysteine proteases (caspases) that are responsible for diverse roles in inflammation and apoptosis. The cleavage of all caspase substrates occurs at specific aspartate residues found at the C-terminus of a 4 amino acid long recognition sequence. The absolute requirement for aspartate is unique among eukaryotic proteases and reflects the high specificity of cleavage processes occurring during apoptosis (Stennicke & Salvesen, 1997).

Classification of caspases

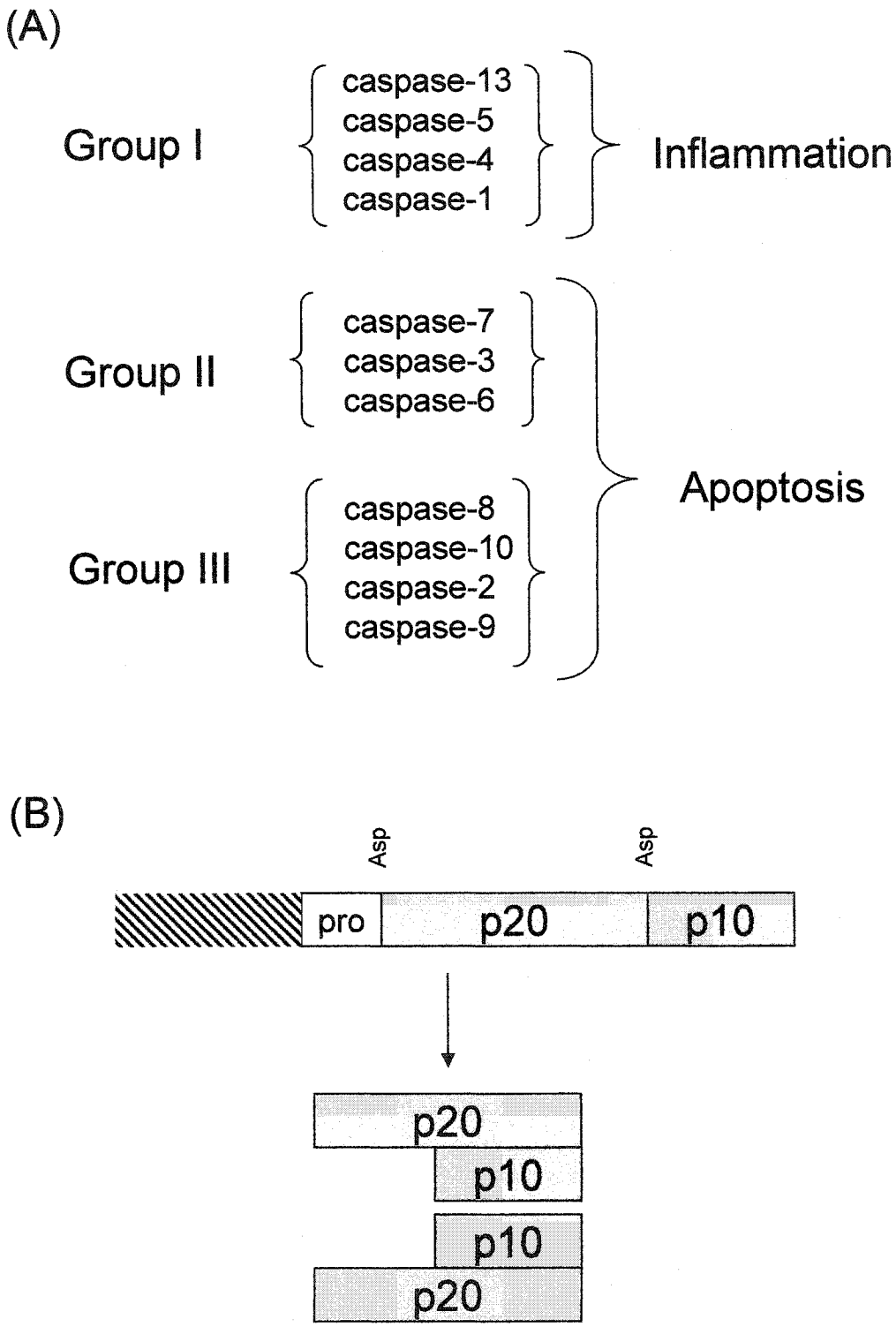
Following the discovery of ICE, a family of 13 other mammalian caspases have been identified (figure 1-1A) (Alnemri *et al.*, 1996; Cohen, 1997; Nicholson *et al.*, 1995). They fall into two major subfamilies: those most closely related to ICE (pro-inflammatory group), and those more closely related to ced-3 (apoptotic group). All of the caspases are expressed as proenzymes that contain 3 domains: an N-terminal prodomain; a large subunit containing the active site cysteine within a conserved QACXG motif; and a C-terminal small subunit (figure 1-1b). The prodomains range in length from 23 amino acids for caspases -6 and -7 to 219 amino acids for caspase-10 and are separated from the large subunit of the caspase by an aspartate cleavage site. Likewise, one or more aspartate cleavage sites are present in the linker region between the small and large subunits. Caspases are activated to form functional proteases by two cleavage events. The first proteolytic cleavage divides the proenzyme into large

Figure 1-1. The caspase family.

(A) Caspases can be grouped based on their cellular role. The pro-inflammatory caspases (1, 4, 5, and 13) form one group (Group I). The proapoptotic caspases form two distinct groups: the effector caspases (3, 6, and 7) (Group II); and the initiator caspases (2, 8, 9, and 10) (Group III).

(B) Schematic diagram of the caspase structure. Caspases are synthesized as inactive zymogens. The prodomain (pro) varies in length between the caspases, with long prodomains being associated with initiator caspases and short prodomains associated with effector caspases. Following cleavage at aspartate (Asp) sites, they form an active tetramer consisting of two small (p10) and two large subunits (p20).

Figure 1-1. The caspase family.



and small caspase subunits, and a second cleavage event removes the N-terminal prodomain. The final, active caspase is a tetramer of two large and two small subunits (Wolf & Green, 1999). The presence of aspartate at the maturation cleavage sites is consistent with the ability of caspases to auto-activate or to be activated by other caspases as part of an amplification cascade.

The apoptotic caspases are subdivided into groups based on the length of their prodomain and their substrate specificity. Caspases with long prodomains (caspase-2, -8, -9, -10) contain sequence motifs such as caspase recruitment domains (CARD) or death effector domains (DED) that promote their interaction with cellular signalling molecules (Ashkenazi & Dixit, 1998; Hofmann *et al.*, 1997). These caspases generally act upstream of the small prodomain-containing caspases and are thus termed 'initiator' caspases. The short prodomain caspases (caspase-3, -6, and -7) are termed 'effector' or 'executioner' caspases, and are largely responsible for activating the cellular responses that result in the classic apoptotic morphology (Zimmermann *et al.*, 2001). Grouping of the caspases based on substrate specificity results in three groups of caspases. Analysis employing a positional scanning combinatorial substrate library reveals that the major determinant for sequence specificity is the amino acid at position four (P_4) of the substrate tetrapeptide (Rano *et al.*, 1997; Thornberry *et al.*, 2000; Thornberry *et al.*, 1997). Group I caspases (caspase-1, -4, -5, -13) have hydrophobic amino acids (tryptophan, tyrosine) at this position with a general

substrate recognition sequence W/LEHD. These caspases cleave cytokines thus modulating the inflammatory response. Group II caspases (caspase-3, -6, -7) have a strict requirement for aspartate on P_4 and recognize DEVD as their specific cleavage site. The group II caspases form the 'effector' caspases in apoptotic pathways. Group III caspases (caspase-2, -8, -9, -10) recognized aliphatic amino acids at P_4 such as those found in the zymogen form of group II caspase molecules. Thus the group III caspases are the 'initiator' caspases that are able to cleave and activate group II caspases.

Activation of caspases

The crystal structure has been determined for several caspases complexed with specific tetrapeptide inhibitors bound in the substrate binding site (Blanchard *et al.*, 1999; Chai *et al.*, 2001b; Chou *et al.*, 2000; Schweizer *et al.*, 2003; Walker *et al.*, 1994). The active enzyme is a tetramer composed of two p20 subunits surrounding two adjacent p10 subunits. The two active sites are in a head to tail orientation on opposite ends of the enzyme. Most of the contacts between the dimers, as well as the major determinants of substrate specificity are mediated by amino acids within the p10 subunit. Activation of a caspase requires the cleavage of the procaspase zymogen into its two subunits, followed by assembly of the active caspase tetramer. The mechanism by which the caspases are activated uses the unusual property of the caspase zymogen to autoprocess into an active form; a model known as "proximity-induced caspase-activation"

(Salvesen & Dixit, 1999). The process involves the assembly of a multi-protein complex in response to death stimuli, including recruitment of the procaspases. As a consequence of the increased local concentration, the procaspases can proteolytically cleave each other at an aspartate cleavage site in the junction between the large and small subunits to yield fully active caspases. In support of this model, the oligomerization of procaspase-8 at the plasma membrane has been shown to be sufficient for its auto-activation (Muzio *et al.*, 1998).

Caspase substrates during apoptosis

The executioner caspases (group III), in particular caspase-3, are responsible for the majority of the apoptotic phenotype. This is effected by cleavage or degradation of several important substrates. For example, high and low molecular weight DNA fragmentation is caused by the action of caspase-3 on a complex of caspase-activated DNAse (CAD)/DNA fragmentation factor 40 (DFF40); and its inhibitor iCAD/DFF45 (Enari *et al.*, 1998; Liu *et al.*, 1997; Mukae *et al.*, 1998). In non-apoptotic cells, CAD is present in an inactive form complexed with iCAD. During apoptosis, caspase-3 cleaves the inhibitor, allowing CAD to function and cleave the chromatin. Other DNA structure and repair targets include poly-ADP ribose polymerase (PARP) and the DNA-dependent protein kinase (DNA-PK) (Sanghavi *et al.*, 1998). Blebbing is achieved through the cleavage and activation of gelsolin, p21-activated kinase-2, and fodrin to dissociate the plasma membrane from the cytoskeleton (Kothakota *et al.*, 1997;

Rudel & Bokoch, 1997). Proteins implicated in the regulation of cell cycle and proliferation are also common targets for caspases, including $p21^{WAF1/CIP1}$, $p27^{KIP1}$, and p^{RB} (Cohen, 1997). Likewise, proteins whose cleavage impact apoptosis directly are also substrates, including the caspases themselves, the cellular survival factors Bcl-2 and Bcl-XL, as well as proapoptic proteins Bid and Bax (Grandgirard *et al.*, 1998).

The list of cellular proteins that are cleaved by the caspases during apoptosis continues to grow, and clearly illustrates that many integral aspects of normal cellular function are affected by caspase cleavage. Cell-cycle progression is halted, DNA and protein repair mechanisms are disabled, and cell structure is altered to facilitate engulfment by neighbouring cells and macrophages. While no single enzyme could be held responsible for all of the changes that occur in apoptotic cells, clearly the effector caspases are central to the event, setting in motion the cellular changes that have been observed since the initial descriptions of programmed cell death nearly 30 years ago.

Induction of apoptosis

Extrinsic versus intrinsic pathway

Whereas the caspases represent a class of enzymes that are important in the execution phase of apoptosis, it is clear that one or more regulation processes upstream of caspase activation must be in place to modulate the apoptotic

response. There are two major pathways which converge upon activation of the effector caspases and progression of apoptosis (see figure 1-2) (Salvesen & Duckett, 2002). The first of these pathways, the extrinsic pathway, relies on signals external to the cell that are transmitted through cell surface receptors. Numerous cell surface receptors have been implicated in triggering cell death under specific conditions. One of the best studied of these is the family of tumor necrosis factor receptors including TNFR1, and Fas (CD95). Exposure of cells expressing the Fas receptor to fas ligand or agonistic monoclonal antibody results in rapid activation of caspases and cell death. This is mediated through the recruitment of initiator caspases directly to the Fas receptor and is described in more detail below.

The second path to activation of the effector caspases lies entirely within the cell (intrinsic) and is ruled by the mitochondria. Cellular stresses, including DNA damage, heat shock, and oxidative stress can lead to the release of apoptotic factors from the mitochondria. This in turn, leads to the formation of an apoptosome complex that is capable of activating downstream caspases and promoting cell death. The key regulators of this pathway appear to be members of the Bcl2 family of proteins, described later in this chapter.

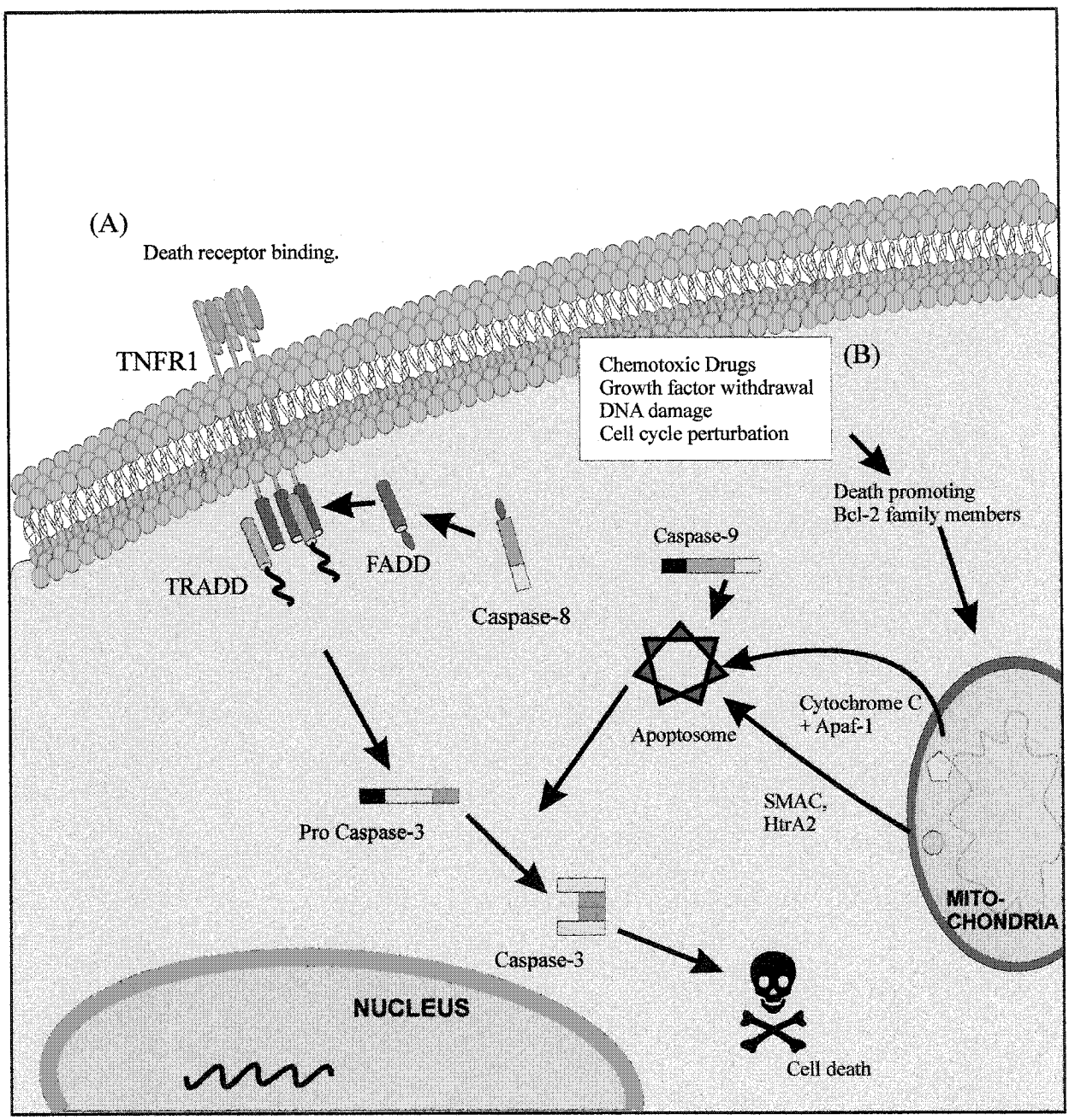
Extrinsic pathway – the Fas:Fas-ligand interaction

Fas is a glycosylated cell surface molecule of approximately 50 kDa in size (355 amino acids). It is a type 1 transmembrane receptor that can also be found

Figure 1-2. Pathways to caspase activation in apoptosis.

There are two major pathways which converge upon activation of the effector caspases and progression of apoptosis. (A) The first of these pathways, the extrinsic pathway, relies on signals external to the cell that are transmitted through cell surface receptors such as the TNF receptor. Engagement of the plasma membrane-associated death receptor results in recruitment of caspase-8 via DD containing adaptor proteins such as TRADD or FADD. Because of the trimeric nature of the death receptor ligands, receptor engagement is thought to result in aggregation of caspase-8 molecules which triggers their autoactivation, which leads to the downstream activation of caspase-3 molecules and the progression of apoptosis. (B) The second path to activation of the effector caspases lies entirely within the cell (intrinsic) and is mediated by the mitochondria. Cellular stresses, including DNA damage, heat shock, and oxidative stress can lead to the release of apoptotic factors from the mitochondria including cytochrome C, SMAC, and HtrA2. This in turn, leads to the formation of an apoptosome complex consisting primarily of Apaf-1, caspase-9 and cytochrome C that is capable of activating downstream caspases and promoting cell death. The key regulators of this pathway appear to be members of the Bcl2 family of proteins. See the text for a more detailed description.

Figure 1-2. Pathways to caspase activation in apoptosis.



in soluble form. Fas is expressed on several different cell types, including cells of immune origin such as B and T lymphocytes, thymocytes, macrophages; as well as certain cells of the spleen, lung, testis, brain, heart and ovaries. In contrast, expression of Fas ligand (FasL) is more tightly regulated and is limited to activated T and B lymphocytes, natural killer cells, and to immune priviledged sites such as the testis and the eye (Nagata & Golstein, 1995).

Ligation of Fas by FasL (or agonistic monoclonal antibodies) results in the rapid oligomerization of the receptor and formation of a death-inducing signalling complex (DISC). This complex formation is mediated by the presence of a cysteine rich domain in the cytoplasmic tail of Fas termed the death domain (DD) which can interact with other DDs present on intracellular proteins (Yuan, 1997). The Fas-associated DD (FADD) protein is rapidly recruited to the receptor's DD and in turn recruits caspase-8 to the DISC. FADD/caspase-8 interaction is mediated by domains similar to the DD of Fas, called death effector domains (DEDs) (Ashkenazi & Dixit, 1998). Thus FADD acts as an adapter protein, containing both a DD for Fas interaction, and a DED for caspase-8 interaction. The close proximity between the multiple procaspase-8 molecules in the DISC permits the proximity-induced self-activation of caspase-8 (Muzio *et al.*, 1998). In turn, the fully activated caspase-8 enzymes directly cleave the effector caspases -3 and -7 leading to the characteristic chain of events seen in apoptosis.

The induction of apoptosis through formation of the DISC can be impaired by the FLICE inhibitory protein (c-FLIP). FLIP resembles procaspase-8, but does not have an active proteinase site. Thus, it gets recruited to the ligated Fas signalling complex, but it can not cleave caspase-8 to propagate the death signal (Irmler *et al.*, 1997). Interleukin-2 has been shown to down-regulate FLIP expression and thus is able to sensitize activated T-cells to Fas (Van Parijs *et al.*, 1999). On the other hand, FLIP may be more than just an inhibitor of Fas-induced cell death. Fas-recruited FLIP also interacts with TNF-receptor associated factors 1 and 2, as well as with the kinases RIP and Raf-1, resulting in the activation of the NF-kappaB and extracellular signal regulated kinase (Erk) signaling pathways. Thus FLIP is able to both block cell death signals, as well as promote cellular survival signals (Kataoka *et al.*, 2000).

Intrinsic pathway – the role of the mitochondrion

While the mechanism by which caspase activation was achieved through death receptor binding was well studied, Liu and colleagues made an interesting observation that did not fit in with receptor induced cell death. They noted that mitochondria derived cytochrome c could drive caspase activation in cell free extracts (Liu *et al.*, 1996). While initially surprising, this observation was rapidly confirmed by other groups (Kluck *et al.*, 1997). Analysis of the kinetics of programmed cell death showed that the mitochondria undergo major changes in membrane integrity before classical signs of apoptosis become apparent. These

changes involve both the inner and outer mitochondrial membrane and correlate with an uncoupling of electron transport from ATP production and eventual release of intermembrane proteins through the outer membrane into the cell cytoplasm (Green & Reed, 1998). A large body of evidence now suggests that the mitochondria act as important conduits for signals associated with diverse cellular stresses, including heat shock, DNA damage, and oxidative stress (Kroemer & Reed, 2000).

The apoptosome

The efflux of cytochrome c from the mitochondria is a pivotal event in apoptosis as it drives the assembly of a high-molecular weight caspase-activating complex in the cytoplasm, termed the mitochondrial apoptosome (Cain *et al.*, 1999; Zou *et al.*, 1999). Cytochrome c interacts with a cytoplasmic protein known as apoptosis protease activating factor 1 (Apaf-1). In its quiescent state, Apaf-1 is a compact molecule with the N-terminal CARD domain tucked between the two WD40 repeat motifs. Cytochrome c (which is roughly the same size as a CARD domain) displaces the CARD of Apaf-1 allowing the molecule to stretch out into a more linear conformation which polymerizes upon addition of ATP (see figure 1-3). The resulting apoptosome is a wheel-like particle with seven spokes radiating from a central hub that contains the caspase-9 recruitment domain of Apaf-1 (Acehan *et al.*, 2002). Similar to the situation seen with procaspase-8 in the DISC, the aggregation of procaspase-9 in the apoptosome can trigger

Figure 1-3. Apaf-1 and the formation of the apoptosome.

(A) The relative positions of the CARD, CED-4 homology, WD40 repeats and linker regions are shown on a linear map of Apaf-1.

(B) In its quiescent state, Apaf-1 is a compact molecule with the N-terminal CARD domain tucked between the two WD40 repeat motifs. Cytochrome c (which is roughly the same size as a CARD domain) displaces the CARD of Apaf-1 allowing the molecule to stretch out into a more linear conformation which polymerizes upon addition of ATP. The resulting apoptosome is a wheel-like particle with seven spokes radiating from a central hub that contains the caspase-9 recruitment domain of Apaf-1.

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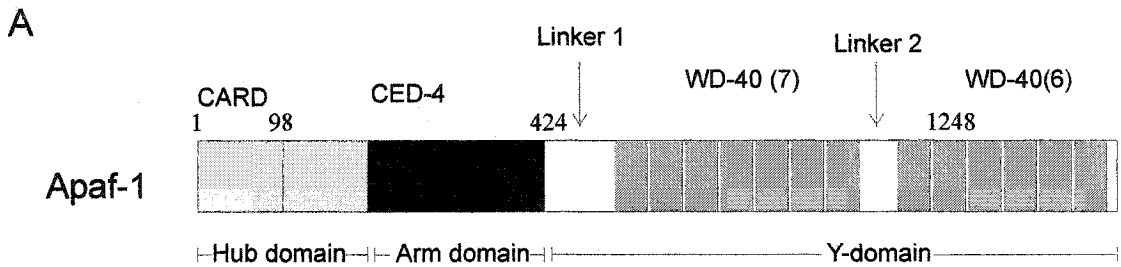

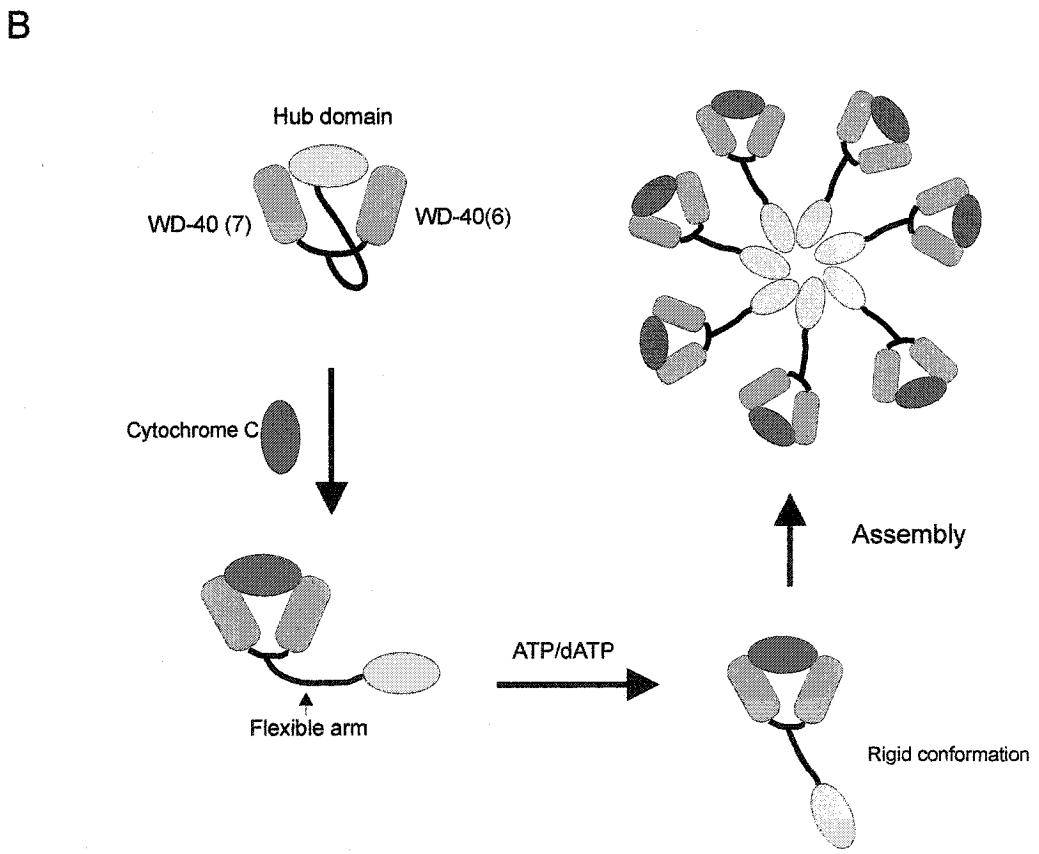

autocatalytic cleavage of the zymogen into active caspase-9 dimers. However, unlike other caspases, the cleavage of procaspase-9 does not appear to be necessary for its activation. In the presence of the apoptosome, a mutant form of procaspase-9 lacking the cleavage site between the large and small subunits was still able to function with high efficiency in cleaving procaspase-3 (Acehan *et al.*, 2002). Thus, binding of caspase-9 to Apaf-1 appears to induce a conformational change in the molecule permitting enzyme function.

Bd-2 family

The control of apoptosome activation depends on the release of cytochrome c from the mitochondria into the cytosol. The mechanisms by which this release is achieved have been the subject of intense scrutiny. Uncoupling of the mitochondrial oxidative phosphorylation is commonly observed during apoptosis, resulting in the loss of mitochondrial transmembrane potential (Green & Reed, 1998). This loss of potential has been linked to the opening of the mitochondrial permeability transition pores (PTPs), which could provide one means by which cytochrome c might escape from the intermembrane space (Zamzami *et al.*, 1995). More recent studies, however, suggest that PTP opening is a secondary, caspase-dependent event and that cytochrome c release precedes PTP opening (Bossy-Wetzel *et al.*, 1998). Thus the release of cytochrome c appears to be a specific permeabilization event, unconnected to the generic membrane depolarization that is seen in a dying cell. Indeed, evidence suggests

that members of the Bcl2 family of proteins are the key mediators of cytochrome c release in the context of apoptotic stimuli (Kluck *et al.*, 1997).

Bcl-2 was first discovered as a proto-oncogene in follicular B-cell lymphoma. Subsequently it was identified as a mammalian homologue of the apoptosis repressor ced-9 in *C. elegans*. Since then, at least 19 other Bcl-2 family members have been discovered in mammalian cells. The key determinant of a Bcl-2-related protein is the presence of at least one of four conserved Bcl-2 homology (BH1-BH4) domains. The Bcl2 family members can be divided into three categories of proteins according to their structure and function (figure 1-4) (Zimmermann *et al.*, 2001):

1. Anti-apoptotic members such as Bcl-2, Bcl-X_L, and Bcl-w, usually containing all 4 BH domains.
2. Pro-apoptotic members such as Bax, Bak, and Bok which lack the BH4 domain.
3. BH3-only pro-apoptotic members, which include Bid, Bad, Bim, Bik, Blk, and Hrk and as their name implies, only contain the BH3 homology region.

The pro-survival members of the Bcl-2 family potently inhibit apoptosis in response to many cytotoxic insults. Their hydrophobic carboxy-terminal domain (TM) helps to target them to the outer mitochondrial membrane, the

Figure 1-4. Bcl-2 family members.

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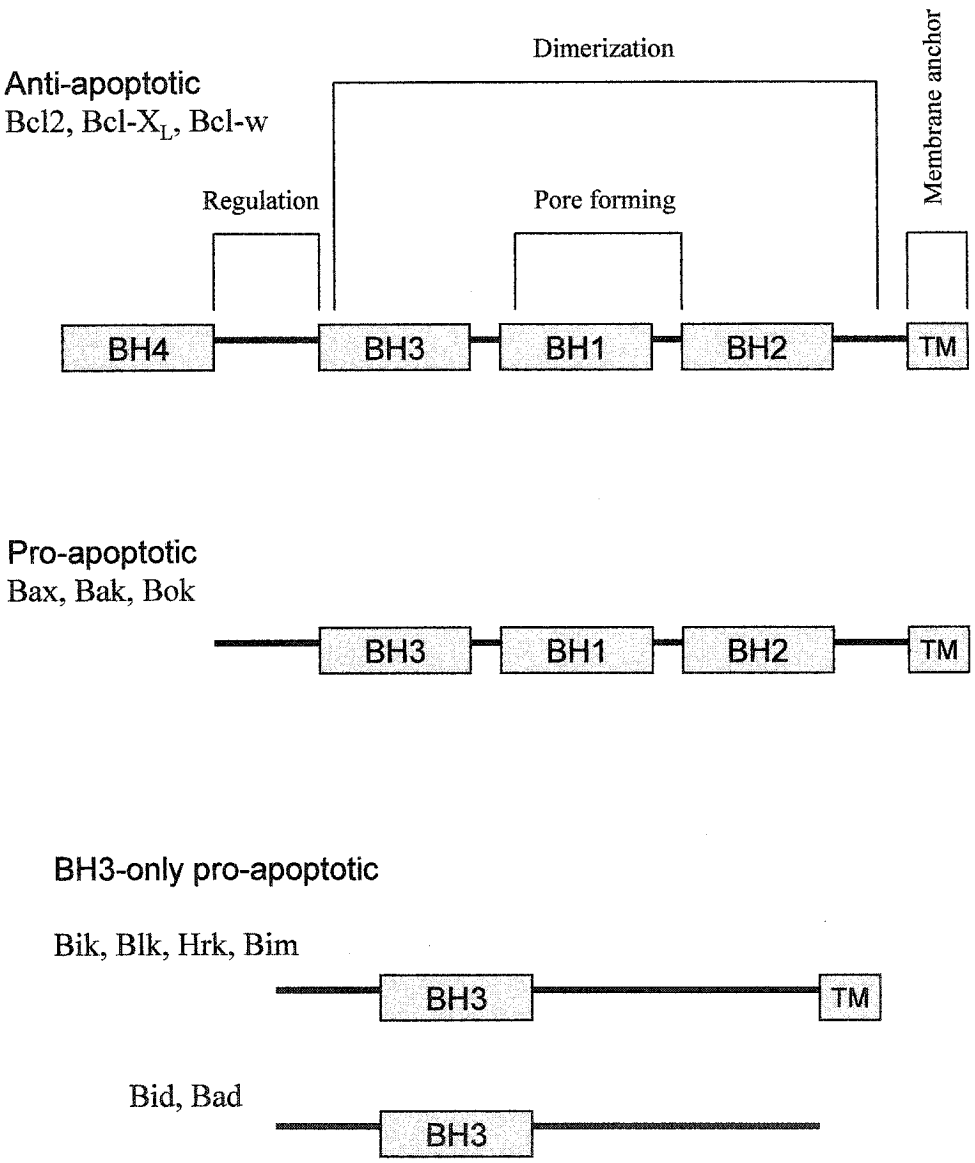
Figure 1-4. Bcl2 family members.



endoplasmic reticulum, and the nuclear envelope. Bcl-2 is an integral membrane protein in healthy cells, where as Bcl-X_L and Bcl-w only become tightly associated with the membrane after an apoptotic signal (Cory & Adams, 2002).

The pro-apoptotic proteins Bax and Bak are essential for apoptosis in many cell types as mice lacking both genes show a severe developmental phenotype (Lindsten *et al.*, 2000). Bax is a cytosolic monomer in healthy cells, but it changes conformation during apoptosis and integrates into the outer mitochondrial membrane and oligomerizes (Gross *et al.*, 1998). Bax and Bak oligomers contribute to the permeabilization of the outer mitochondrial membrane, permitting efflux of cytochrome c (Gross *et al.*, 1999). The mechanism by which they do this, however, is still under debate. One hypothesis suggests that Bax and Bak can directly form channels. Consistent with this suggestion is the fact that Bax oligomers can form pores in liposomes that allow passage of cytochrome c (Saito *et al.*, 2000).

The BH3-only members of the Bcl-2 family seem to be the initiator molecules in the mitochondrial pathway of apoptosis. They trigger apoptosis in response to developmental cues or intracellular damage (Huang & Strasser, 2000). For example, Noxa was identified as a BH3-only protein whose expression is induced by p53 in response to DNA damage (Oda *et al.*, 2000). Bad and Bim are expressed in healthy cells, but sequestered in a non-functional form. Bad is

phosphorylated on multiple sites resulting in binding to the 14-3-3 scaffold proteins (Gross *et al.*, 1999). Bim is sequestered in the microtubule complex by binding to dynein light chain LC8, a component of the dynein motor comples (Puthalakath *et al.*, 1999). In response to growth factor deprivation, however, they are released and can translocate to intracellular membranes where they neutralize the anti-apoptotic Bcl-2 homologues. The BH3-only proteins, however, cannot kill in the absence of Bax and Bak, and hence must function upstream in the same pathway (Wei *et al.*, 2001; Zong *et al.*, 2001).

Cross-talk exists between the intrinsic and extrinsic pathways

One member of the BH3-only containing Bcl-2 family members provides a link between the extrinsic (e.g. receptor triggered) cell death pathway, and the intrinsic (mitochondria-based) cell death pathway. Bid is present in most cells in an inactive form, which gets activated by proteolytic cleavage. The enzyme responsible for activation of Bid, is caspase-8. Thus activation of caspase-8 through the DISC leads to the cleavage of Bid into a truncated form which is then capable of translocating to the mitochondrial membrane and initiating the release of cytochrome c and other pro-apoptotic factors (Li *et al.*, 1998b). Thus, the extrinsic pathway is able to influence the intrinsic pathway of programmed cell death.

The opposite also holds true. In some cellular systems, activation of caspase-8 even with the release of cytochrome c is not sufficient for cell death. Recently, a pair of related proteins has been identified that are released from mitochondria along with cytochrome c during apoptosis. Smac/DIABLO (Du *et al.*, 2000; Verhagen *et al.*, 2000) and Omi/HtrA2 (Hegde *et al.*, 2002) are both able to enhance cell death signals by relieving the inhibition on caspases by the binding of cellular inhibitors. More details on the functions of these two proteins and the proteins that they inhibit will be described later in this chapter.

Inhibition of caspase function

General mechanisms of caspase inhibition

Regulation of the activity of the caspases is crucial to the proper functioning of cells. The first level of caspase regulation is seen in their structure and activation. As has been described, caspases are synthesized as inactive zymogens and are only activated as a result of strictly controlled pathways. A second level of regulation involves the specific inhibition of active caspases by naturally occurring inhibitors.

Protease inhibitors work by preventing hydrolysis of substrate by the enzyme, and almost all natural protease inhibitors achieve this by steric hindrance preventing access by substrates to the catalytic center of the protease. Usually, inhibitors bind in the same orientation and dock onto the same sites as substrates

do on the enzyme surface (Laskowski & Kato, 1980). Interestingly, none of the known caspase inhibitors use this conventional mechanism. To date, members of three protein families have been found capable of inhibiting caspase activity *in vivo*. The first identified caspase inhibitors were found in viruses and have evolved as mechanisms to evade immune-mediated cell death which would interfere with their replication cycle (Zhou & Salvesen, 2000).

Viral inhibitors

Baculovirus p35

Inhibitors belonging to the p35 family of proteins are highly specific caspase inhibitors able to block the function of almost all known caspases, including distant relatives of the caspases such as gingipain. Homologues of p35 are found in a small selection of baculoviruses, but as of yet, no mammalian homologues (or homologues in mammalian viruses) have been found (Stennicke *et al.*, 2002). The fundamental mechanism of inhibition of caspases by p35 results from the interaction of the DQMD motif in the reactive loop of p35 with the catalytic domain of the target caspase. The p35 loop undergoes normal substrate cleavage, but this results in a conformational change leading to the formation of an energetically stable covalent thiol-ester bond. The p35 reactive side loop is extremely large and flexible, which allows it to interact with and inhibit a broad range of caspases (Riedl *et al.*, 2001b).

Cowpox CrmA

The cytokine response modifier A (CrmA) is a Cowpox viral protein that has been shown to inhibit granzyme B, as well as caspases-1, -4, and -8. Inhibition of granzyme B, caspase-1 and caspase-4 can suppress the host inflammatory response to the virus, while blocking of caspase-8 prevents cell death through immune mediated death receptor signalling (Zhou & Salvesen, 2000). CrmA is a member of the serpin family of protease inhibitors. Although the origin of the name serpin suggests that these proteins are inhibitors of serine proteases, recent studies have seen them develop into a family of cross-class inhibitors capable of inactivating both serine and cysteine proteases. Two serpins have been shown to inhibit caspase activity: the endogenous serpin PI-9 (Annand *et al.*, 1999) and CrmA (Zhou *et al.*, 1997). Studies have shown that, like p35, the CrmA molecule is cleaved by the target caspase (Simonovic *et al.*, 2000). Cleavage of CrmA results in a conformational change that stabilizes its interacting with the target caspase and irrevesibly prevents further hydrolysis.

Inhibitor of Apoptosis (IAP) proteins

The prototype IAP was described in baculovirus by Lois Miller and colleagues (Crook *et al.*, 1993). They employed a functional rescue assay to find proteins that could replace the known viral apoptotic inhibitor p35. Mutations in the p35 gene of the *Autographa californica* multicapsid nuclear polyhedrosis virus (AcMNPV) result in a strain of virus that rapidly kills its host SF-21 insect cell line, termed the 'annihilator' strain. These mutant strains were co-transfected

with fragments of DNA from other baculoviruses to find genes that restored the wild-type replication cycle to the annihilator strain of AcMNPV. Using this method genes from *Cydia pomonella* granulosis virus (CpGV) (Crook *et al.*, 1993) and *Orgyia pseudosugata* nuclear polyhedrosis virus (OpMNPV) (Birnbaum *et al.*, 1994) were isolated that encoded a novel type of protein not structurally related to p35. These novel proteins, termed Cp-IAP and Op-IAP were shown to functionally replace p35 by inhibiting apoptosis (Clem *et al.*, 1996; Clem & Miller, 1994). Each contained a carboxy-terminal RING zinc finger as well as two novel N-terminal cys/his zinc binding motifs ($X_3RX_{20-23}GX_{11}CX_2CX_{16}HX_6CX_3$) termed the baculovirus IAP repeat (BIR). The BIR domain is an approximately 70 residue zinc-binding domain that is the defining characteristic of an IAP family member.

The underlying mechanism by which the IAP proteins inhibit apoptosis has only recently been elucidated. XIAP, the human X-linked IAP (described in the next section) has been found by multiple research groups to be a potent but restricted inhibitor targeting caspases-3, -7 and -9 (Deveraux *et al.*, 1997). BIR containing proteins (BIRCs) likely have functions in addition to caspase inhibition since they have been found in organisms such as yeast which have no caspases (Uren *et al.*, 1998), however, it is clear now that one mechanism by which the IAPs inhibit apoptosis is through direct caspase inhibition.

In the case of XIAP, regions encompassing the second BIR domain (BIR2) specifically target caspases-3 and -7, and regions encompassing BIR3 target caspase-9 (Fesik & Shi, 2001). Surprisingly, the crystal structures of BIR2 in complex with caspases 3 (Huang *et al.*, 2001; Riedl *et al.*, 2001a) and 7 (Chai *et al.*, 2001a) have revealed that the BIR domain itself has very little direct role in the inhibitory mechanism. All of the critical inhibitory contacts are made by the flexible region preceding the BIR domain. The inhibitory region of XIAP binds in an antisense orientation across the substrate binding site of the caspase molecule, effectively excluding access by any other substrate. The primary function of the BIR domain in this case may be to align and stabilize the inhibitory interactions.

The mechanism by which XIAP binds to caspase-9 is entirely different to the manner in which it binds caspase-3 and -7. A pocket in the BIR3 domain of XIAP binds to the small subunit of caspase-9 through a conserved 4 amino acid sequence at the N-terminus of the cleaved caspase-9 enzyme. Caspase-9 functions in a dimer as part of the 'apoptosome' complex that includes the molecules Apaf-1 and cytochrome-C and it is thought that by binding caspase-9, XIAP is able to sequester caspase-9 in a monomeric form and prevent the formation of the active apoptosome complex (Shiozaki *et al.*, 2003)

Mammalian BIR containing (BIRC) proteins

The first mammalian IAP, neuronal apoptosis inhibitory factor (NAIP), was identified in a positional cloning effort seeking the causative mutation for spinal muscular atrophy (SMA). The defining characteristic of SMA is a progressive loss of motor neurons leading to the wasting of voluntary muscles. Analysis of the complex SMA locus mapped to chromosome 5q13 led to the discovery of two candidate genes for SMA: survival motor neuron (SMN) (Lefebvre *et al.*, 1995) and neuronal apoptosis inhibitory protein (NAIP) (Roy *et al.*, 1995). It is now evident that the central and essential molecular defect in SMA is the loss or mutation of SMN (Rodrigues *et al.*, 1996), however, the discovery of NAIP initiated a flood of research into mammalian IAP genes.

Following the discovery of NAIP (BIRC1), seven additional BIR-containing proteins have been identified in humans: X-linked inhibitor of apoptosis (XIAP / MIHA / hILP / BIRC4); human IAP-1 (HIAP1 / cIAP2 / MIHC / BIRC3); human IAP-2 (HIAP2 / cIAP1 / MIHB / BIRC2) (Duckett *et al.*, 1996; Liston *et al.*, 1996; Rothe *et al.*, 1995; Uren *et al.*, 1996); Survivin (BIRC5) (Ambrosini *et al.*, 1997); BIR-containing ubiquitin conjugating enzyme (BRUCE / Apollon / BIRC6) (Chen *et al.*, 1999; Hauser *et al.*, 1998); Livin (Livin / KIAP / ML-IAP / BIRC7) (Kasof & Gomes, 2001; Lin *et al.*, 2000; Vucic *et al.*, 2000); and Testis-specific IAP (Ts-IAP / hILP2 / BIRC8) described in this thesis (Lagace *et al.*, 2001; Richter *et al.*, 2001). Mouse homologues of most human

IAPs have been reported, as well as several other mammalian species including rats and pigs, as well as in non-mammalian species such as chicken, fruit fly, nematode and even yeast (Deveraux & Reed, 1999; LaCasse *et al.*, 1998).

Structural features

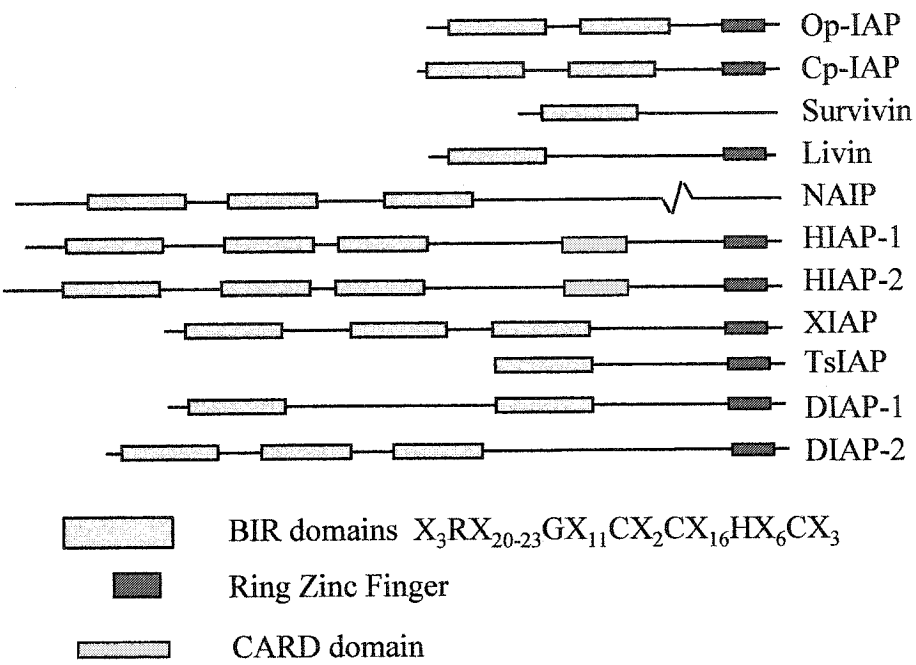
The presence of at least one BIR domain is the defining characteristic of the IAP protein family. The baculoviral IAPs both possess two BIR domains, the human IAPs possess either one or three tandem BIR domains, while the drosophila version has both two and three BIR motifs (figure 1-5A). BIR domains are numbered sequentially from the N-terminus of the IAP protein, thus the most N-terminal BIR domain is called BIR1. Sequence alignment of the BIR2 domains of several IAP proteins (figure 1-5B) shows a high degree of conservation of certain amino acids resulting in a consensus sequence of $X_3RX_{20-23}GX_{11}CX_2CX_{16}HX_6CX_3$. Nuclear magnetic resonance structures of the BIR2 and BIR3 domains of XIAP (Sun *et al.*, 1999; Sun *et al.*, 2000) have been determined. The BIR2 domain of XIAP is comprised of a classical Zinc finger structure with a three-stranded antiparallel β-sheet and four α-helices. Three conserved cysteine residues and a histidine stabilize the overall fold by chelating zinc. The BIR3 domain of XIAP has the same connective patterns and structural composition as the BIR2 but exhibits a larger central β-sheet architecture. The BIR3 domain of

Figure 1-5. The IAP family.

The presence of at least one BIR domain is the defining characteristic of the IAP protein family. (A) The baculoviral IAPs (Op-IAP, Cp-IAP) both possess two BIR domains, the human IAPs possess either one (Survivin, Livin, TsIAP) or three tandem BIR domains (XIAP, HIAP1, HIAP2, NAIP) while the drosophila has both two (DIAP1) and three (DIAP2) BIR proteins. Along with the BIR domains, the several IAPs contain a RING zinc finger domain at the C-terminus. HIAP 1 and 2 both possess a caspase recruitment domain (CARD) in the linker region between the BIRs and the RZF. (B) Sequence alignment of the BIR2 domains of several IAP proteins shows a high degree of conservation of certain amino acids resulting in a consensus sequence of $X_3RX_{20\text{-}23}GX_{11}CX_2CX_{16}HX_6CX_3$.

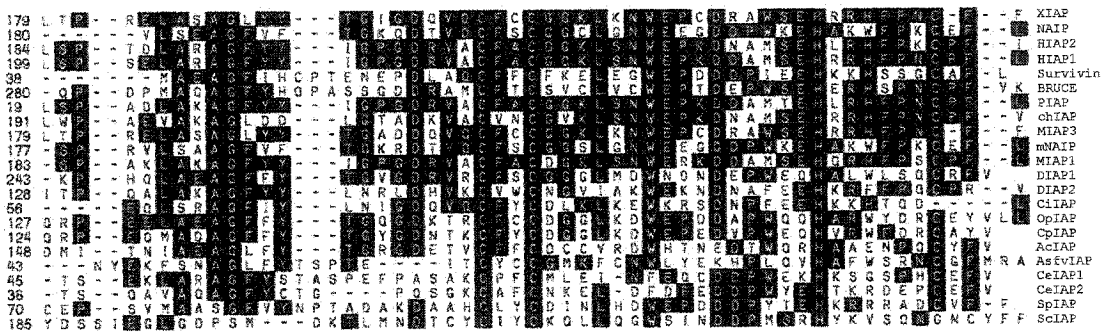
Figure 1-5. The IAP family

(A)



Category	Example
1 BIR	Survivin, BRUCE
1 BIR + 1 RING	CiV IAP, TsIAP, Livin
2 BIRs	CeBIR1, SpBIR1, ScBIR2
2 BIRs + 1 RING	OpIAP, CpIAP, DIAP1
2 BIRs + 1 CARD + 1 RING	PIAP
3 BIRs	NAIP
3 BIRs + 1 RING	DIAP2, XIAP
3 BIRs + 1 CARD + 1 RING	HIAP1, HIAP2, chIAP

(B)



HIAP2, unlike that of XIAP, lacks the formation of a β-sheet (Hinds *et al.*, 1999), which is a surprising difference considering the high sequence homology among the BIR domains of IAP family members. X-ray crystallographic studies of human and mouse survivin (Chantalat *et al.*, 2000; Muchmore *et al.*, 2000; Verdecia *et al.*, 2000) and ML-IAP (Franklin *et al.*, 2003) have revealed BIR domains that most closely resemble the BIR2 and BIR3 of XIAP respectively, although survivin appears to function in a dimeric arrangement which has not been seen with the other IAP proteins.

Most mammalian IAPs, with the exception of survivin, BRUCE and NAIP, contain another zinc binding motif at their carboxy terminus termed the RING zinc finger. RING fingers are present in diverse proteins and are characterized by a set of invariant metal-binding residues (C_3HC_4) that coordinate two zinc ions (Freemont, 1993). Structurally, the equine herpes virus protein RING has been examined using NMR spectroscopy and shown to consist of an amphiphatic α-helix next to a triple stranded β-sheet (Barlow *et al.*, 1994). In the case of the IAPs, the RING domain has been shown to have ubiquitin ligase activity (MacFarlane *et al.*, 2002; Suzuki *et al.*, 2001b; Yang *et al.*, 2000).

In addition to the BIR and RZF, certain IAPs contain other protein motifs that are able to modulate their function. The HIAP1 and HIAP2 proteins contain a caspase recruitment domain (CARD) that is present between the third

BIR and the RZF (figure 1-5). The CARD domain is a protein structure consisting of 6 α-helical folds (Vaughn *et al.*, 1999) and is a member of the death domain family along with death domains (DD), death effector domains (DED), and Pyrin domain. This domain is dispensible for the anti-apoptotic function of HIAP1 and 2 (Roy *et al.*, 1997), however, it does mediate interactions between the CARD containing IAPs and other CARD containing cellular proteins such as Apaf-1 and RIP2 (McCarthy *et al.*, 1998). Other domains of potential interest in IAP family proteins include a functionally intact ubiquitin conjugating domain in BRUCE, a large 528 kDa BIR-containing protein (Hauser *et al.*, 1998), and an ATP binding P-loop consensus sequence in NAIP (Roy *et al.*, 1995).

Expression and anti-apoptotic function

The tissue expression and anti-apoptotic function of the IAPs varies and may be important in determining their roles in diverse cell types and in response to different apoptotic stimuli.

NAIP, the first mammalian IAP discovered, was identified as a candidate gene defective in spinal muscular atrophy. The human *NAIP* gene is composed of 17 exons spanning a genomic region of approximately 56 kb. One full length copy of NAIP exists at the telomeric end of 5q13.1 and as many as 6 other tandem truncated copies are present in the same region. By Northern blot analysis, *NAIP* is only detected in the liver and placenta, however, RT-PCR also

reveals the presence of *NAIP* mRNA in spinal cord (Roy *et al.*, 1995). Immunohistochemical staining for NAIP expression in the rodent brain shows an expression pattern that appears to overlap specific neuronal populations lost in humans with severe SMA (Xu *et al.*, 1997b). In humans, motor neurons from the anterior horn which degenerate by apoptosis during the progression of SMA express high levels of NAIP (Pari *et al.*, 2000).

High NAIP levels have been shown to be protective against excitotoxic and hypoxic damage in neuronal structures. For example, the NAIP expressing mesencephalic trigeminal neurons are resistant to kainic acid induced apoptosis. Likewise, cholinergic interneurons of the striatum which express NAIP are able to survive transient forebrain ischemia. The surviving cells appear to elevate expression of the NAIP protein (Xu *et al.*, 1997a). NAIP expression levels in specific cholinergic neurons in the thalamus, striatum, hippocampus and spinal cord can be artificially increased by treatment of neuronal cells with K252a, a bacterial alkaloid which has been shown to be protective in various neurodegenerative models (Knusel & Hefti, 1992; Xu *et al.*, 1997a). Structures such as the CA1 region of the hippocampus, which have previously been shown to be highly sensitive to ischemic events, were partially protected in animals treated with K252a and had almost twice the number of NAIP expressing neurons (Xu *et al.*, 1997a). Likewise, injection of adenovirally expressed NAIP was able to protect rat motorneurons from injury following sciatic axonomy

(Perrelet *et al.*, 2000). While it is clear now that the causative genetic defect in SMA patients is the deletion or mutation of the *SMN* gene (Pellizzoni *et al.*, 1998; Rodrigues *et al.*, 1996), the expression pattern and anti-apoptotic function of the *NAIP* gene strongly suggests a potential role in modifying the severity of the disease as well as roles in other neuronal disorders (Gendron & MacKenzie, 1999).

Contrary to the specific expression pattern of *NAIP*, expression of the human *XIAP* mRNA is ubiquitous among all adult and fetal tissues tested by Northern blot analysis (Liston *et al.*, 1996) with the possible exception of peripheral blood leukocytes. XIAP has been reported to inhibit cell death in response to a large variety of apoptotic stimuli including ultraviolet radiation, tumour necrosis factor, Fas ligand, menadione (a free radical inducer), and etoposide (a topoisomerase II inhibitor) (LaCasse *et al.*, 1998; Liston *et al.*, 1996; Takahashi *et al.*, 1998). The core of XIAP's ability to block cell death are the N-terminal BIR domains which have been shown to directly inhibit both initiator and effector caspases. The wide tissue expression of *XIAP* mRNA has led to the suggestion that it may be the IAP family member that provides a 'housekeeping' role in the prevention of apoptosis in healthy cells. Expression of *XIAP* appears elevated in many cancer cell lines (Liston *et al.*, 1996), reinforcing the idea that XIAP expression level may control the apoptotic threshold of cells.

On the other hand, the XIAP transcript has very long 5' and 3' UTRs which could provide a means of regulating the levels of XIAP protein independent of transcriptional control of the mRNA. Long 5' UTRs are rarely found in eukaryotic cells and interfere with the normal ribosome scanning method of translation (Kozak, 1991). To overcome this, a number of eukaryotic genes with long 5' UTRs have been shown to translate their proteins through a cap-independent internal ribosome entry site (IRES). IRES elements were first described in picornavirus RNA. Picornaviral protease 2A cleaves cap-binding proteins, inhibiting the normal cellular translational machinery, yet the viral proteins are still able to be efficiently translated. This is due to the presence of an ill-defined RNA element that permits ribosome binding near the translational start site of the message. The human XIAP 5' UTR has been shown to exhibit IRES activity (Holcik & Korneluk, 2000; Holcik *et al.*, 1999). The presence of an IRES element in XIAP permits its expression in cells under stress conditions that normally block cap-dependent translation. Thus XIAP protein may be preferentially upregulated relative to other cellular proteins under those same conditions. Indeed, under conditions of low dose γ-irradiation, XIAP translation is upregulated in an IRES dependent manner (Holcik *et al.*, 2000). In addition to translational control of XIAP protein levels, some evidence exists that XIAP message may be more strictly regulated than initially believed. Two groups have

confirmed that XIAP expression levels are upregulated by the stress responsive transcription factor NF-κB (Stehlik *et al.*, 1998; Tang *et al.*, 2001).

HIAP1 and 2 were initially identified through screening for TNF-receptor associated proteins (Rothe *et al.*, 1995). A high degree of conservation exists between the two proteins (72% aa identity) and indeed the genetic organization of the *HIAP1* and *2* gene loci strongly suggests they arose through a gene duplication event (Young *et al.*, 1999). The HIAP1 and 2 genes are present in a back to back fashion within 7 kb of each other on chromosome 11q22-23. The HIAP1 gene encodes an 8.7 kb transcript from 10 exons whilst the HIAP2 gene transcript is 4.5 kb in size and encoded in 9 exons. Expression of HIAP1 and 2 is highest in the lymphoid tissues such as thymus, peripheral blood and spleen, and is lowest in the central nervous system. Although the tissue distributions of HIAP1 and HIAP2 are similar, the relative expression of HIAP2 appears to be generally higher (Young *et al.*, 1999). The expression of both HIAP1 and HIAP2 are increased following activation of the NF-κB transcription factor by the TNF receptor and they may have a role in protecting cells from TNF-induced apoptosis (Wang *et al.*, 1998a). Like XIAP, overexpression of both HIAP1 and HIAP2 block apoptosis induced by a variety of cell death triggers including serum withdrawal, menadione, and etoposide by directly inhibiting caspase activity (Liston *et al.*, 1997; Roy *et al.*, 1997).

Unlike the more widespread distribution of XIAP, HIAP1, and HIAP2 mRNA, the expression of survivin appears to be very tightly regulated. The survivin gene was initially identified as a coding region that overlaps with the effector cell protease receptor 1 (EPR-1) gene in the inverse orientation (Ambrosini *et al.*, 1997). Survivin mRNA is not detected in adult tissues, being restricted to embryonic tissues and cancer cells (Ambrosini *et al.*, 1998). The assessment of survivin in human tumor specimens included both *in situ* RNA hybridization and immunohistochemical analysis, confirming expression in tumour cells but not in the stromal cells or neighbouring normal tissues. This expression pattern, however, may be an artefact of the rapid rate of cell division seen in cancer and fetal tissues, as survivin appears to be regulated in a cell-cycle dependent manner. The survivin promoter contains four copies of G1 repressor elements that have been implicated in controlling cell cycle periodicity in some G2/M-regulated genes as well as a cyclin homology region (CHR). In addition, a pair of cycle dependent elements (CDE) are present downstream of the transcriptional start site (Li & Altieri, 1999). When studied in reporter gene assays, the survivin promoter exhibits typical M phase inducible activation, suggesting that survivin expression may be induced in dividing cells. Indeed, in HeLa cells, survivin mRNA is upregulated 40 fold at the G2/M junction (Li *et al.*, 1998a). In mouse fetal tissues, survivin expression was prominent and nearly

ubiquitous at embryonic day 11.5, whereas by E15-21 survivin expression was limited to only a few locations (Adida *et al.*, 1998).

Like the other IAPs, survivin is able to inhibit apoptosis induced by a variety of triggers including IL-3 withdrawal from B-cells (Ambrosini *et al.*, 1997); Fas ligand, Bax overexpression, and etoposide (Tamm *et al.*, 1998); and treatment with the microtubule stabilizing agent taxol (Li *et al.*, 1998a). The latter activity is related to the direct binding of survivin with polymerized microtubules, as deletion of a carboxy-terminal coiled-coil region eliminated microtubule binding and abrogated survivin's ability to protect against taxol-induced apoptosis. The BIR domain of survivin is able to bind and inhibit caspase-3 and -7 directly (Shin *et al.*, 2001). Thus the anti-apoptotic properties of survivin can be attributed to direct inhibition of caspases by the BIR domain, as well as indirect protection mediated by the carboxy-terminus affecting microtubule function.

Less is known about the more recently identified IAP known as Livin (Kasof & Gomes, 2001), KIAP (Lin *et al.*, 2000) or ML-IAP (Vucic *et al.*, 2000). Initially thought to be separate genes, Livin, and KIAP were found to be splice variants of the same gene and were renamed Livin α and Livin β (Ashhab *et al.*, 2001). The difference between the two splice variants is in the use of an alternative splice acceptor site for exon 6 that results in an in-frame 18 amino acid deletion in the shorter splice variant (Livin β). The deletion in Livin β does not

affect either the BIR domain or the RZF. Surprisingly, the two splice variants display different tissue distribution and antiapoptotic characteristics. Livin α was not detected in any of the tested fetal tissues. On the other hand, high levels of Livin β were detected in fetal kidney, and lower levels were seen in fetal heart and spleen. In adult tissues, elevated levels of both splice forms were detected in heart, placenta, lung, spleen and ovary. Low levels of only Livin α were detected in brain, skeletal muscle and peripheral blood lymphocytes. Adult kidney expressed low levels of only Livin β. In cancer tissues, extremely high levels of both splice variants was seen in melanoma and colon cancers, with lower levels in most other cancer cells tested (Ashhab *et al.*, 2001).

Overexpression of either Livin isoform blocks apoptosis triggered through the TNF or Fas pathway or by expression of pro-apoptotic proteins such as Bax (Kasof & Gomes, 2001; Lin *et al.*, 2000; Vucic *et al.*, 2000). On the other hand, the two Livin isoforms behave differently with the apoptotic triggers staurosporine and etoposide. Only Livin α was protective against the kinase inhibitor staurosporine, whereas only Livin β could protect against the topoisomerase II inhibitor etoposide (Ashhab *et al.*, 2001). As with the other IAPs, the BIR domain of Livin β is able to bind and inhibit caspases directly (Kasof & Gomes, 2001). The sum total of the anti-apoptotic properties of Livin cannot be attributed entirely to the BIR domain however, as there is no difference between the BIRs of the two splice isoforms yet they respond

differently to apoptotic triggers. Sanna and colleagues (Sanna *et al.*, 2002a) show that prevention of TNFα-induced apoptosis by Livin is dependent on the selective activation of the c-Jun kinase (JNK1) pathway. Overexpression of catalytically inactive JNK1 blocks the anti-apoptotic ability of Livin on TNF induced apoptosis, but has no effect on its anti-caspase ability. This does not, however, account for the difference between the two Livin isoforms as both are able to block TNFα induced apoptosis.

IAPs in cell signalling events

Complementary to their roles in inhibiting caspases, several IAPs have been associated with cellular receptors and their associated signalling cascades. As previously mentioned, HIAP1 and HIAP2 were isolated due to their association with the TNF-receptor associated factors TRAF1 and TRAF2. The recruitment of HIAP1 and 2 to the TNFR2 complex requires the presence of both TRAF1 and TRAF2 (Rothe *et al.*, 1995) and is mediated by the interaction between the BIR domains of the IAPs and the TRAF domain of TRAFs (Roy *et al.*, 1997). The TNFR1 complex is also able to recruit HIAP2 through its interaction with TRAF2 via the adaptor molecule TNF-receptor associated death domain protein (TRADD). The TNF-receptor can mediate both survival and death signals. Survival signals are thought to be transduced by TRAF2/HIAP2 interactions, whereas death signals are mediated through TRADD/FADD binding and activation of caspase-8 (Fotin-Mleczek *et al.*, 2002). Oddly, a recent

report showed that the RING zinc finger domain of HIAP2 is able to ubiquitinate and promote the degradation of TRAF2 (Li *et al.*, 2002b). In this situation, HIAP2 is more likely to sensitize to, rather than protect from apoptosis as TRAF2 is generally required for pro-survival signals through its NF-κB inducing properties (Wang *et al.*, 1998a). Further compounding this paradox is the identification of a cleaved form of HIAP2 that lacks the BIRs but contains the RZF and shows a pro-apoptotic effect (Clem *et al.*, 2001). The proapoptotic effect of the RZF of HIAP2 may be due to its ubiquitination and degradation of TRAF2, however, this is unlikely as the IAP-TRAF interaction is mediated through the BIR domain of HIAP2. A form of HIAP2 that lacks the BIRs would not be expected to interact with TRAF2 so would not likely be able to promote its ubiquitination.

The biological properties of XIAP as well are not limited to caspase inhibition. In a two-hybrid screening experiment, XIAP was pulled out as a binding partner for TAB1, a member of the transforming growth factor β (TGF-β) signalling pathway (Yamaguchi *et al.*, 1999). Members of the TGF-β superfamily, which include activins and the bone morphogenic proteins (BMPs), regulate cell growth, differentiation, and morphogenesis. The TGF-β family members transmit signals through heteromeric receptor complexes consisting of type I and type II serine/threonine kinase receptors (Liu, 2003). XIAP has been shown to bind the type-I BMP receptor (Yamaguchi *et al.*, 1999), as well as the

type-I TGF-β receptor (Birkey Reffey *et al.*, 2001) in complexes that contain the mitogen-activated protein kinase kinase kinase (MAPKKK) TAK1 and its co-activator TAB1. Overexpression of XIAP has been shown to augment TGF-β and BMP responses. In the case of TGF-β, three distinct signalling pathways have been reported to be triggered after receptor activation: NF-κB, c-Jun amino terminal kinase (JNK) and Smad dependent transcription; and XIAP has been shown to activate all three (Birkey Reffey *et al.*, 2001; Sanna *et al.*, 2002a; Sanna *et al.*, 1998; Tang *et al.*, 2001). XIAP, however, cannot be absolutely required for TGF-β signalling as the XIAP knock-out mouse show very few phenotypic differences from the wild-type (Harlin *et al.*, 2001) whereas TGF-β knockouts show very severe effects (Chang *et al.*, 2001).

IAPs in cell cycle control

The timing and order of cell cycle events are monitored during cell cycle checkpoints that occur at the G1/S phase boundary, in S phase, and during the G2/M phases (McDonald & El-Deiry, 2000). The cell cycle control system is chiefly based on two protein families: the cyclin-dependent protein kinases (Cdks) and the cyclins. Cdks allow progression through the different phases of the cell cycle by phosphorylating substrates (McDonald & El-Deiry, 2000). Their kinase activity is dependent on the presence of activating subunits known as cyclins. The abundance of specific cyclins increases during the phase of the cell cycle in which they are required and decreases during phases in which they are not needed

(Murray, 1994). The expression of these proteins is in turn regulated by numerous proteins, including p53, $p21^{WAF1}$, $p16^{INK4}$, and cdc25. Downstream targets of cyclin-cdk complexes include pRb and E2F, proteins that are critical for progression through S-phase.

Immunolocalization has provided evidence that survivin associates with mitotic spindle microtubules, centrosomes and the cytokinetic remnant – a filamentous structure representing the last bit of connecting material joining two daughter cells at the end of telophase (Li *et al.*, 1999). This association with microtubules is mediated by the c-terminal (non-BIR) portion of survivin, and there is evidence that via this interaction survivin monitors mitotic spindle integrity. It is hypothesized that survivin controls the elimination by apoptosis of those cells with aberrantly formed mitotic spindles (Li *et al.*, 2000; Reed & Bischoff, 2000).

The expression and degradation of survivin is tightly controlled by cell cycle components. Survivin expression is upregulated during the G2/M phase and is rapidly degraded during the G1 phase of the cell cycle. The degradation of survivin is mediated by the ubiquitin proteasome pathway, but the E3 ligase responsible for transfering the ubiquitin moiety onto survivin has yet to be identified (Zhao *et al.*, 2000). Survivin expression has recently been shown to be downregulated by the actions of p53, suggesting a mechanism by which p53 is

able to induce apoptosis in damaged cells (Hoffman *et al.*, 2002). Other critical cell cycle proteins affect, and are affected by, survivin. Survivin is phosphorylated in the G2/M phase by the cdc2/cyclin B1 complex prior to its association with mitotic spindles (O'Connor *et al.*, 2000). At the G1/S transition, suvivin has been shown to translocate into the nucleus and bind cdk4 leading to Cdk2/Cyclin E activation and Rb phosphorylation (Suzuki *et al.*, 2000a). This results in two effects. First, it initiates S phase by blocking the association of cdk4 and $p16^{INK4a}$ (Suzuki *et al.*, 2000a). Secondly, as a result of Survivin/Cdk4 complex formation, $p21^{WAF1}$ is released from its complex with Cdk4 and is able to interact with mitochondrial procaspase 3 (Suzuki *et al.*, 2000b; Suzuki *et al.*, 1998). Thus, survivin supports procaspase 3/$p21^{WAF1}$ complex formation as a result of interaction with Cdk4 resulting in suppression of cell death signalling. These studies show the important interplay between apoptotic regulation and cell cycle control and demonstrate yet another function of the BIR family of proteins.

Regulation of IAP function

With the wide variety of critical cell functions performed by the IAP proteins, the regulation of the IAP function is of paramount importance. As described previously, the different IAP genes are subject to tight transcriptional control (e.g. cell-cycle dependent expression of survivin) and translational control (e.g. IRES element of XIAP). In addition to these regulatory mechanisms, IAPs

are subject to post-translational control in the form of protein stability, and inhibitory molecules.

IAP protein degradation via ubiquitination

Ubiquitination involves a series of sequential reactions in which the carboxyl terminus of free ubiquitin is conjugated to a ubiquitin-activating enzyme (or E1) through the formation of a thiol-ester bond. The activated ubiquitin moiety is then transferred to a ubiquitin conjugating enzyme (or E2). The transfer of ubiquitin from the E2 to the target protein is then catalysed by a ubiquitin ligase enzyme (or E3). The E3 proteins provide the bulk of the target specificity in the ubiquitin system (Weissman, 2001). As previously mentioned, the carboxy-terminal RZF domain of several IAPs possess ubiquitin ligase activity (Huang *et al.*, 2000; Li *et al.*, 2002b; Suzuki *et al.*, 2001b; Yang *et al.*, 2000).

The ability of XIAP and HIAP2 to promote ubiquitination was initially described by Yang and colleagues (Yang *et al.*, 2000). They noted that in glucocorticoid or etoposide treated thymocytes XIAP and HIAP2 were lost in a proteasome-dependent manner before observation of classic signs of cell death. Their experiments revealed that the IAPs were capable of catalyzing their own ubiquitination *in vitro*, an activity that was dependent on the presence of the RZF domain. Notably, overexpression of XIAP lacking the RZF resulted in higher levels of protein and better protection from etoposide-induced apoptosis thus

suggesting the existence of a self-regulating process of IAP levels in cells and their degradation in response to apoptotic stimuli (Yang *et al.*, 2000). On the other hand, IAPs have been shown to mediate the ubiquitination of other proteins including caspase-3 and -7 (Huang *et al.*, 2000; Suzuki *et al.*, 2001b), TRAF2 (Li *et al.*, 2002b), and the IAP inhibitor Smac (MacFarlane *et al.*, 2002) described below, suggesting that the role of the RZF may be more complex than merely controlling self-degradation in response to apoptotic stimuli.

Inhibitors of the inhibitor

An alternate way to modify IAP function requires the specific binding of proteins to the IAPs resulting in an impairment of their apoptosis inhibitory function. It is therefore not surprising that several cellular inhibitors of the IAPs have been recently identified.

Using a two-hybrid screening strategy with XIAP as bait, a novel 34 kDa protein was isolated and termed XIAP-associated factor 1 (XAF1) (Liston *et al.*, 2001). XAF1 encodes a 301 amino acid protein that potentially forms 7 zinc fingers, and has a predominantly nuclear localization. Overexpression of XAF1 effects the relocalization of XIAP from the cytoplasm to the nucleus, and is able to reverse the inhibitory effects of XIAP on caspase-3 function *in vitro*, as well as reverse the protective effects of XIAP on etoposide and serum-withdrawal induced cell death in cell culture systems (Liston *et al.*, 2001). The *xaf1* gene

consists of seven exons spanning 18 kb and was localized by fluorescence in situ hybridization to chromosome 17p13.2, approximately 3 cM distal to p53. Microsatellite analysis of the xaf1 locus using a 60 cell line panel revealed significantly decreased heterozygosity at the XAF1 locus, suggesting that allelic loss of the xaf1 gene is prevalent in cancer cell lines. Examination of the same NCI cell line panel for xaf1 RNA expression demonstrated that cancer cell lines exhibited very low levels of mRNA relative to normal human liver. In contrast, XIAP mRNA levels were relatively high in the majority of cancer cell lines tested. Given that XAF1 is ubiquitously expressed in normal tissues, but low or absent in cancer cells, this would suggest a function as a constant antagonist of IAPs, the loss of which could sensitize cells to tumorigenesis.

A second IAP interacting protein was simultaneously discovered by two separate groups and given the name Smac (Du *et al.*, 2000) and DIABLO (Verhagen *et al.*, 2000). Du and colleagues (2000) used a purification process to isolate factors from the mitochondrial membrane fraction of HeLa cells that enhanced caspase-3 activation. Peptide sequencing of the 25 kDa protein revealed a previously uncharacterized protein which they named Smac for Second Mitochondrial Activator of Caspases (the 'first' mitochondrial activator of caspases being cytochrome C). Verhagen and colleagues (2000) on the other hand, immunoprecipitated several proteins which bound to mouse-XIAP and identified one of them as DIABLO, for Direct IAP binding protein with low pI.

The human homologue of DIABLO was isolated through database searches and found to encode a protein with the identical sequence as Smac. Smac/DIABLO is a 239 amino acid protein that is encoded in the nucleus but, in non-apoptotic cells, is localized to the inter-membrane space of mitochondria due to an amino terminal mitochondrial-targeting signal. Overexpression of Smac/DIABLO can not directly induce apoptosis, however, it enhances apoptosis induced by many triggers by blocking the ability of XIAP to inhibit caspases (Chai *et al.*, 2000; Ekert *et al.*, 2001).

Smac/DIABLO can be released from mitochondria, apparently along with cytochrome c, in response to apoptotic stimuli. On its release from mitochondria, the amino terminal leader sequence is removed and the resulting mature Smac/DIABLO is able to bind to several of the IAPs, including XIAP, HIAP1, HIAP2, Livin, and Survivin (Du *et al.*, 2000; Srinivasula *et al.*, 2000; Verhagen *et al.*, 2000; Vucic *et al.*, 2002). Removal of the mitochondrial leader sequence is not only necessary for the release of Smac from the mitochondria, but also crucial to its ability to bind IAPs. The four N-terminal amino acids in the mature Smac protein (AVPI) show a high homology to the IAP interacting domain at the N-terminus of the small caspase-9 subunit (ATPF) and has been termed an IAP-binding motif (IBM) (Srinivasula *et al.*, 2001; Wu *et al.*, 2000). The four-amino acid IBM binds tightly to a surface groove in certain BIR domains, thus suggesting a mechanism by which caspase-9 is sequestered by the IAPs.

Smac competes for binding with caspases in that surface groove and thus is able to free the caspase molecule from the IAP protein (Wu *et al.*, 2000). Similar motifs exist among the drosophila death promoting proteins Hid, Grim, Skl, and Reaper suggesting that these proteins function in a similar manner (Christich *et al.*, 2002; Wright & Clem, 2002).

A second mammalian IBM containing protein, Omi/HtrA2, has been identified and shown to interact with XIAP (Hegde *et al.*, 2002; Martins *et al.*, 2002; Suzuki *et al.*, 2001a). Originally identified as a homologue of the *E. coli* heat shock inducible serine protease HtrA, the Omi/HtrA2 protein consists of an N-terminal mitochondrial targetting sequence, a conserved serine protease domain and a single PDZ domain at the C-terminus (Faccio *et al.*, 2000; Gray *et al.*, 2000). Similar to Smac/DIABLO, Omi/HtrA2 contains an amino-terminal IBM that is exposed upon cleavage of the mitochondrial targetting sequence. After an apoptotic stimulus, mature Omi/HtrA2 is released from the mitochondria and is able to bind to and inhibit IAP function (Hegde *et al.*, 2002; Martins *et al.*, 2002; Suzuki *et al.*, 2001a). On the other hand, deletion mutants of Omi/HtrA2 lacking the IBM were still able to induce apoptosis despite having abrogated IAP binding. Omi/HtrA2 mutants with defective serine protease activity were unable to promote apoptosis (Li *et al.*, 2002a). Thus it appears that the serine protease activity of Omi/HtrA2 is critical for its ability to induce apoptosis and the IAP binding may act to potentiate this effect.

Finally, a third mammalian IBM-containing protein has been recently discovered. GSPT1/eRF3 is a mammalian homologue of the yeast GST1 gene (Hoshino *et al.*, 1989), which functions in mRNA translation as a polypeptide releasing factor (Hoshino *et al.*, 1998). The mammalian GSPT1 functions as the eukaryotic releasing factor 3 (eRF3) by interacting with eRF1 in translation termination. This function requires only the C-terminal domain of GSPT1 which is homologous to elongation factor-1α. The N-terminal domain appears to interact with polyadenylate-binding protein, which binds the poly(A) tail of mRNA and associates with the eukaryotic initiation factor-4G (Uchida *et al.*, 2002). GSPT1 has recently been shown to be proteolytically processed into an IAP binding protein (Hegde *et al.*, 2003). Like Smac and HtrA2, the processed GSPT1 contains a conserved IBM (AKPF) at its N-terminus which is exposed after cleavage of a 69-residue leader sequence. The processed GSPT1 is able to interact with IAPs and potentiate caspase activation, as well as ubiquitination of the target IAPs (Hegde *et al.*, 2003). The exact physiological relevance of GSPT1 cleavage is unclear as of yet. Processing of GSPT1 may be triggered by ER stress or other cellular stress conditions, resulting in its release from the ER to potentiate apoptotis. Alternatively, it may be cell cycle dependent as GSPT1 expression is highest at the G1/S phase of the cell cycle (Hoshino *et al.*, 1989).

Thesis outline

Following the identification of NAIP as the first characterized mammalian member of the IAP family of proteins, we isolated the X-linked IAP (XIAP) gene. This thesis documents the genetic characterization of the XIAP gene, and the subsequent discovery and characterization of a novel gene, called the testis-specific IAP (TsIAP). We began with a genomic library screening and bulk sequencing approach to isolate and sequence the genomic region that encompasses the XIAP gene. Following this, we screened cDNA libraries, as well as performed RT-PCR and rapid amplification of cDNA ends (RACE) reactions to elucidate the full length cDNA sequence and intron/exon boundaries of XIAP. In the course of these experiments, various XIAP 'pseudogenes' were discovered and characterized. One of these pseudogenes turned out to maintain a viable open reading frame and encoded a novel protein with expression limited to the testis. We employed a similar library screening strategy to clone and sequence the novel testis-specific IAP gene. FISH analysis was performed to localize the gene to chromosome 19q13.4. The TsIAP gene was found to encode a 2kb mRNA with an open reading frame encoding a region homologous to the carboxy-terminal end of the X-linked IAP. Attempts were made to find a murine homologue of TsIAP with no success, and later work published by another group confirmed our suspicion that the TsIAP gene is a recent evolutionary event and that no murine homologue exists. Given the

unusual 5' UTR present in the TsIAP gene, we tested whether the mRNA could produce a protein by using Western blot analysis on both overexpressed TsIAP in cell culture, as well as endogenous expression in testicular protein extracts. Having confirmed that the novel gene we isolated is capable of being expressed, we proceeded to characterize its functional properties. TsIAP was able to protect against apoptosis, however, only against a limited subset of apoptotic triggers when compared to its parent gene XIAP. Surprisingly, against Bax-induced apoptosis TsIAP was a more potent inhibitor than the corresponding region of XIAP. This was determined to be due to the inability of XIAP's natural inhibitor, SMAC, to bind and inhibit TsIAP function.

Chapter 2

MATERIALS AND METHODS

DNA Isolation and Analysis

Plasmid constructs:

We obtained a pcDNA3 based plasmid containing a 6-myc tagged *XIAP* coding region from Dr. Peter Liston. Un-tagged *XIAP* was excised from that plasmid by a BamHI - XhoI restriction digest and subcloned into pcDNA3, pCI (Promega) and pEGFP-C1 (Clontech) vectors. A GST tagged construct of *XIAP* (in the pGEX-4T3 vector, Amersham Pharmacia) was also kindly donated by Dr. Peter Liston.

We constructed a *TsIAP* expression plasmid by PCR amplification of *TsIAP* from library clones isolated during library screening. *TsIAP* was PCR amplified using a BamHI tagged forward primer (# 4056 – CAG GAT CCA TGC ATA GTG AAG AAG CTA GAT TA) and a XhoI tagged reverse primer (# 4061 – GAC TCG AGT TAA GAC ATA AAA ACT CTT TGC TT) using Pfx Polymerase (Gibco BRL) and was cloned by TOPO-TA cloning (Invitrogen) into the PCR2.1 TOPO vector. The TOPO-TA cloned *TsIAP* was sequenced to ensure no errors were introduced in the amplification process and then further subcloned into the 6-myc pcDNA3 vector, pCI vector, and pEGFP-C1 vector.

To create a *BAX* expression plasmid, we amplified full length BAX cDNA from a human liver cDNA library (Clontech). The resulting amplicon was cloned using the TOPO-TA system (Invitrogen) into the PCR2.1 TOPO vector. Following sequencing, the BAX cDNA was then subcloned into the pEGFP-C1 vector by restriction digest and ligation.

Genomic DNA isolation:

Total genomic DNA was isolated from human peripheral blood by standard phenol/chloroform extraction (Current Protocols in Molecular Biology, Ch. 2.2). Peripheral blood was separated and cells were lysed in digestion buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 25 mM EDTA, 0.5% SDS, 0.1 mg/ml proteinase K) for 12-18 h at 50°C with shaking. The DNA solution was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (49:49:2), and again with 1 volume chloroform followed by precipitation with ½ volume 7.5 M ammonium acetate and 2 volumes 95% ethanol. DNA pellets were washed with 70% ethanol, dried and resuspended in Tris-EDTA (10 mM Tris-HCl, pH 8.0, 25 mM EDTA). The DNA concentration was determined on a Genequant spectrophotometer (Pharmacia).

Plasmid DNA isolation:

Mini-prep plasmid DNA was extracted using a standard boiling lysis method (Current Protocols in Moldecular Biology, Ch 1.6). Cells were grown in Luria Broth (LB, 1% tryptone, 0.5% yeast extract, 1% NaCl) containing the

appropriate antibiotics (50μg/ml ampicillin or 30μg/ml kanamycin) overnight at 37°C in an incubating shaker. The bacterial cells were then pelleted and resuspended in 475 μl of STET (8% sucrose, 5% Triton X-100, 50 mM Tris-HCl, pH 8.0, 50 mM EDTA, pH 8.0) with 25 μl of 10mg/ml lysozyme solution and boiled for 90 seconds. The samples were centrifuged again, and the pellet removed from the tubes using a toothpick. The DNA in the supernatant was then precipitated and resuspended as described for the genomic DNA isolation.

Larger scale preparation of plasmid DNA was done according to manufacturer's instructions using either a maxi-prep column kit from Qiagen, or equivalent maxi-prep kit from Invitrogen, or by the following caesium-chloride purification method. Briefly, a 200 ml overnight culture of bacteria was pelleted and resuspended in 4 ml of GTE (25 mM Tris-HCl, pH 8.0, 10 mM EDTA, 50 mM glucose). A small amount (1-2 mg) of lysozyme was added and the solution was incubated at room temperature for 10 minutes. Following that, 8 ml of 0.2N NaOH/1% SDS was added and the solution incubated on ice. After 5 minutes, 8 ml of 3M KOAc pH 4.8 was added and the solution was further incubated on ice for a minimum of 1 h. The solution was then centrifuged at 10000 rpm and filtered through cheesecloth into a new tube. The DNA was then precipitated using an equal volume of isopropanol, and washed twice with 70% ethanol. After the ethanol washes, the pellet was resuspended in 2 ml of water with 2.2 g CsCl and 200 μl of 10mg/ml ethidium bromide and then centrifuged in an

ultracentrifuge at 80000 x g for 20 hours. The plasmid DNA could then be seen in a clear band in the centrifuge tube and was removed carefully using a needle and syringe. Ethidium bromide was removed from the DNA by washing the DNA pellet with salt saturated butanol, and then the CsCl was removed by washes with 70% ethanol.

Lambda Phage DNA isolation:

Virus from a single plaque was eluted for 1 hour at room temperature in 100 μl of SM buffer (5.8 g NaCl, 2.0 g $MgSO_4 \cdot 7H_2O$, 50 ml 1M Tris 7.5, 5 ml 2% gelatin, per litre of distilled water). The eluted phage was then added to 500 μl of host cells (strain XL1-blue MRA (P2), or Y1090r$^-$, grown overnight in LB containing 0.2% maltose and diluted to OD_{600} of 0.5 with 10mM $MgSO_4$) and allowed to bind for 15 minutes at 37°C. The bound virus was then added to 15 ml of LB broth supplemented with 10mM $MgSO_4$ and allowed to grow overnight with vigorous shaking at 37°C. The bacteria were then lysed by the addition of 300 μl of chloroform and the debris pelleted by centrifugation at 3000 x g for 10 minutes. RNaseA and DNaseI were added to the supernatant to a final concentration of 5 μg/ml each to digest bacterial nucleic acids. After 30 minutes incubation at 37°C, an equal volume of 20% PEG-8000 in 2M NaCl was added and the virus particles were precipitated by incubation on ice for a minimum of one hour. The precipitated virus was pelleted by centrifugation and resuspended in 500 μl of SM buffer. Further debris was pelleted again by centrifugation in a

microcentrifuge and the virus containing supernatant was transferred to a new eppendorf tube. EDTA and SDS were added to the supernatant to a final concentration of 10 μM and 0.1% respectively, followed by incubation for 15 minutes at 65°C. After this, a standard phenol, phenol-chloroform, and chloroform extraction was performed to extract the viral DNA followed by precipitation and resuspension as described for the genomic DNA extraction.

Transposon facilitated sequencing:

We sequenced the complete genomic region of *XIAP* by transposon facilitated sequencing (Strathmann *et al.*, 1991) of the isolated *XIAP* phage clones. According to this methodology, the DNA fragment to be sequenced is subcloned into pBluescript SK(-) (Stratagene). Subsequent to selection and amplification in DH5α, the resulting plasmid is used to transform *E.coli* strain DPWC (Gold Biotechnology). While in this host, the engineered plasmid is randomly fused to the resident F' plasmid (Tn 1000^{+}, res^{-}). Ampicillin resistant clones (resistance derived from Bluescript) are grown on selective media then mated in a conjugation reaction with *E.coli* BW26 (Kan^{r}, res^{+}) in liquid culture. Once transferred to BW26 the combined plasmid (BS/F) is resolved leaving within the cloning vector a randomly inserted transposon. The multicopy plasmids obtained from the resulting Kan/Amp resistant clones served as sequencing templates. We performed forward and reverse sequencing of individual clones using universal primers within the transposon, followed by multiple alignment of these

sequences (Genetics Computer Group, 1991) to generate the full contiguous array.

Southern blotting:

Isolated genomic DNA (5 μg) or phage DNA (1 μg) was digested with the appropriate restriction enzyme overnight at 37°C and run on a 1% agarose gel in 1X TBE buffer overnight at 45 V. The DNA was partially depurinated by soaking the gel in 0.25 M HCl for 30 minutes and denatured in 1.5 M NaCl/0.5 M NaOH for 40 min before being transferred onto nylon membranes (Pall Biodyne B membrane, Gibco BRL). Blots were baked for 20 min at 80°C and pre-hybridized for a minimum of 1 hour at 65°C in hybridization buffer (1.5 X SSPE, 7% SDS, 10% PEG). Southern blots were probed overnight with the P32 labelled probes generated by nick translation using Redi-prime kits (Amersham Pharmacia). The membranes were washed at 65°C with buffers of increasing stringency, ranging from 2 x SSC to 0.01 x SSC, all with 0.1% SDS. The blots were exposed to film at -80°C. The probes used were as follows: 1) Full length *XIAP* probe generated by restriction digest of pcDNA3-XIAP. 2) *XIAP* 5' UTR probe generated by PCR of genomic DNA or phage clones using the primers # 3544 (TAT TTT GCC TTT GAC AAG) and # 3545 (TAT ATG TAA CAC TGC CCT CTA). 3) *XIAP* 3' UTR probe 1 generated by PCR of phage clones using the primers # 3794 (GAA TGA CAG GGC TGG AA) and # 3797 (ATG ACA ATT TTG GCT TTT TC). 4) *XIAP* 3' UTR probe 2 generated by PCR of

phage clones using the primers # 3792 (ATG CCT ATA GAT TTT TGG AG) and # 3793 (CTA CAA TGA ATG CCA GAT TA). 5) *TsIAP* specific 5' probe generated by PCR of phage clones using primers # 4393 (CGC CCG TGT CTC GTT CTT TT) and # 1227 (AAT ACG ACT CAC TAT AGG).

Phage library screening for XIAP genomic clones:

A Lambda-FIX II human male placenta genomic library (Stratagene) was probed for *XIAP* according to the manufacturer's instructions. Initially, a full length *XIAP* cDNA probe (identical to that used for Southern blots) was used to screen the library for clones. DNA was isolated from the phage clones as described previously and restriction digest maps were generated by Southern blot analysis. Fragments of interest were subcloned into pBluescript SK^- and sequenced via transposon mediated sequencing. Using the sequence thus obtained, further probes in the 5' and 3' regions of *XIAP* were generated (*XIAP* 5'UTR probe, *XIAP* 3'UTR probe 1, and *XIAP* 3'UTR probe 2, described above) and the Lambda-FIX II library was further screened to ensure the entire genomic region of the XIAP gene was covered.

Library screening for full length XIAP cDNA:

To determine the full-length cDNA sequence of *XIAP*, we probed a human liver cDNA library (Clontech) with *XIAP* coding region sequence as per the manufacturer's instructions. We isolated clones and mapped them by restriction digest and Southern blot as described, followed by subcloning into

pBluescript SK$^-$ (Stratagene) and sequencing via primer walking and transposon facilitated sequencing (Gold Biotech). Comparison of the cDNA and genomic sequences of XIAP permitted identification of intron/exon boundaries. Where ambiguities persisted, primers were designed for PCR amplification that would allow determination of the exact boundary.

5' and 3' Rapid Amplification of cDNA Ends (RACE):

By making use of 3' and 5' RACE kits from Gibco BRL we were able to extend the cDNA sequence of *XIAP* to the initiation and termination of transcription sites. We used 5 μg of total RNA from human muscle tissue, human spinal cord or human placenta (kindly provided by Dr. Natalie Gendron) as a template for the RACE reactions and designed nested primers based on genomic DNA sequences obtained previously through transposon sequencing. We cloned the resulting amplification products by TA cloning (Invitrogen) and sequenced them to determine the initiation and termination of transcription sites. The primers used were the following: # 4067 (CTC CAT ATT GCC ATC TAT CTA), # 4241 (ATT TGT AGA CTG CGT GGC ACT ATT TT), # 4242 (TGA TGC TGA AAC AGG ACT ACC ACT TG), # 3597 (GTC AAA GGC AAA ATA AGA), # 3598 (TCT CCC ACA TTG CCT GAT), # 3646 (AAT CAC AAT AAA TAA ATA GGT C).

Isolation of the testis specific IAP (TsIAP):

Given the presence of a second transcript expressed in the testis lane of the multiple tissue northern blot, we decided to screen a human testis cDNA library (Clontech) using a 1.5 kb probe derived from *XIAP* coding region. After three rounds of plaque purification, we sequenced the ends of positive clones using phage specific primers and compared the sequence obtained with known *XIAP* cDNA sequence. Of 23 clones isolated from the library, 16 were clearly *XIAP* sequence (>99% identity), while the 7 remaining clones bore only 75% to 85% identity to *XIAP*. The differences in the sequence could not be attributed to sequencing error as the 7 clones all had identical changes in their sequence when compared to *XIAP*. We chose the largest cDNA clone to sequence completely. To test whether we had isolated the transcript seen in the testis lane of the *XIAP* northern blot, we used a 200 bp region of that clone (the 5' end) that was not homologous to *XIAP* to probe a multiple tissue northern blot, a Southern blot of genomic DNA, and the various genomic phage clones isolated during the *XIAP* genomic screening. One of the previously isolated genomic phage hybridized to the probe and was sequenced with primers based on the testis specific cDNA sequence.

Fluorescence in situ hybridization:

We RNase treated an 8 kb probe generated from a genomic phage clone of *TSIAP* and labelled it with Biotin 14dCTP by random priming using a

commercially available DNA labelling kit (BRL Bioprime). The hybridization mixture was composed of 50-100 ng labelled probe, 2 μg dried cot-1 DNA, 1μg herring sperm DNA in 50% formamide/10% dextran sulphate/2XSSC in a volume of 10μl. We then denatured the probe for 10 minutes at 70°C and applied it to slides previously denatured for 2 minutes in 70% formamide/2xSSC and dehydrated in three cold ethanol washes (70%, 80% and 90%). Hybridization took place at 37°C for 16 h, and was followed by washing at 42°C in 50% formamide/2XSSC for 10 min, 2XSSC for 10 min and 5% Triton X 100/4XSSC for 5 min. To detect the biotin labelled DNA probe we used fluorescein isothyocyanate-conjugated avidin and anti-avidin (ONCOR detection kit). We counterstained the chromosomes with propidium iodide and examined them with an Olympus BX60 epifluorescence microscope. To identify the chromosomes, we stained the slides with recorded, labelled metaphases with DAPI.

Promoter analysis of XIAP:

A 1 kb region upstream of the transcription start site for XIAP was PCR amplified and cloned into XhoI site of the pCAT-Enhancer vector (Promega). Clones were obtained in both forward and reverse orientation, as determined by end-sequencing using the RV3 primer provided. Hela cells were grown in DMEM supplemented with 10% heat-inactivated fetal calf serum and transfected with the various pCAT plasmids using Lipofectamine 2000 reagent (Invitrogen). Twenty-four hours post-transfection, the cells were washed twice with PBS then

scraped in 1 mL of PBS. The cells were pelleted by gentle centrifugation at 1000 rpm for 5 min at 4 °C. CAT expression in the cells was determined using a CAT ELISA kit (Stratagene) according to manufacturer's instructions.

Protein Preparation and Analysis

Western Blotting:

Protein extracts (20 μg/lane), and broad range protein markers (New England Biolabs) were run on discontinuous SDS-PAGE gels, consisting of a lower separating gel (12% acrylamide/0.3% bisacrylamide, 375 mM Tris-HCl, pH 8.8, 0.1% SDS, 0.05% ammonium persulfate) and an upper stacking gel (4% acrylamide/0.1% bisacrylamide, 125 mM Tris-HCl, pH 6.8, 0.1% SDS, 0.05% ammonium persulfate). Proteins were electrophoretically transferred onto PTFE membranes using a semi-dry gel transfer apparatus. The efficiency of transfer and equivalence of loading were monitored using Ponceau S dye. Blots were then blocked in skim milk blotting buffer (5% skim milk/0.1% Tween20/1X TBS) for 1 hour at room temperature, or overnight at 4°C. Primary antibody was diluted in blotting buffer and the blots were incubated in this solution for 1 hour at room temperature. Polyclonal α-XIAP and α-RIAP3 antibodies were used at a dilution of 1 in 2000. Polyclonal α-SMAC was used at 1 in 500. The blots were washed with TBS/0.1% Tween 4 times for 15 min. Blots were then treated with secondary anti-rabbit or anti-mouse antibodies conjugated to horse radish

peroxide (HRP), diluted 1/2500 in blotting buffer and incubated for 1 h with shaking. Blots were washed 4 times for 15 min each before chemiluminescent processing (ECL, Amersham) and exposure to X-ray film.

Immunofluorescence staining:

Cells for immunostaining were plated thinly on glass cover slips 1-2 days prior to staining. The coverslips were transferred (cell side up) into PBS, rinsed briefly, then fixed for 5 min in 3% paraformaldehyde solution (3% PFA in PBS, pH 7.0). The cover slips were then washed twice in PBS and transferred to permeabilization solution (0.2 % Triton X-100 in PBS, pH 7.0) for 20 minutes. Following permeabilization the cells were washed 3 times for 4 minutes each in PBS. Cells were blocked for 45 minutes in 5% serum from the same host species as the secondary antibody (donkey serum for the rabbit polyclonal anti-XIAP and RIAP3, goat serum for the mouse monoclonal anti-myc antibody). Primary antibody diluted 1/50 in PBS was incubated over the cells for 1 hour at room temperature. The cover slips were washed twice in PBS and then transferred into the secondary antibody solution (CY3-conjugated donkey anti-rabbit for polyclonal XIAP, FITC-conjugated goat anti-mouse for anti-myc) for 1 hour. The cover slips were then washed 3 times with PBS and transferred into Hoechst 33258 solution (1mg/ml in PBS, pH 7.0) for nuclear counterstaining followed by a wash in PBS. The stained cover slips were mounted on slides with a drop of

VectaShield mounting medium (Vector Laboratories) and were visualized using a Zeiss Axiophot fluorescence microscope.

Cells transfected with GFP-tagged constructs were fixed, counterstained and mounted as described above, however, no antibody staining was performed.

Protein purification:

GST fusion proteins were produced overnight in a 50 ml LB culture of *E. coli* BL21 supplemented with 50 μg/ml ampicillin. The following day, the starter culture was added to 500 ml of fresh LB medium supplemented with 50 μg/ml ampicillin and 1 μM Zinc Acetate. The culture was allowed to grow in an incubator shaker at 37 °C to an OD_{600} of 0.8-1.0 (usually 2-3 hours), and then 500 μl of 0.5 M IPTG was added and the culture incubated a further 2-3 hours of incubation. Alternatively, following the addition of IPTG the culture was incubated at room temperature overnight. The bacteria were pelleted by centrifugation at 12000 x g for 10 minutes then resuspended in 30 mL of lysis buffer (50mM Tris-Cl pH 7.5, 200 mM NaCl, 5% glycerol, 0.5% Triton X-100, 1 mM DTT, 1 μM ZnOAc, 10 mM lysozyme). The mixture was supplemented with the following protease inhibitors: 300 μl of 100 mM PMSF, 300 μl of 0.1 mg/ml Aprotinin, 60 μl of 1 mg/ml leupeptin, and 30 μl of 1 mg/ml pepstatin A. The resuspended pellets were kept on ice for 1 hour, then subjected to a freeze/thaw cycle (at -80 °C). If the bacteria were not sufficiently lysed at this point, as evidenced by high viscosity of the solution, then fresh lysozyme was

added followed by room temperature incubation. Following lysis of the bacteria, DNaseI (100U) and $MgCl_2$ (to a final concentration of 1.5 mM) were added to the solution followed by incubation on ice for 1 hour. Cell debris was pelleted by centrifugation at 15000 x g for 1 hour and the supernatant was kept for the glutathione sepharose 4B column.

The glutathione sepharose column was prepared by adding 500 µl of glutathione sepharose beads (Pharmacia) to a column and allowing the beads to settle. Five mL of PBS were run through the column to pre-wash the beads. The cell supernatant was passed through the column and the eluant saved and passed through the column a second time to ensure all GST-fusion protein bound. The column was then washed with cold PBS for 1 hour at a low flow rate (1-2 ml/minute). GST-fusion proteins were eluted by resuspension of the beads in 500 µl GSH elution buffer (10 mM GSH, 50 mM Tris-Cl pH 9.6) followed by 15 minute incubation at room temperature. The elution was repeated three times and the fractions analysed by SDS-PAGE. The elutions containing the greatest amount of protein were pooled and then dialyzed overnight against dialysis buffer (60mM Tris-Cl, 80mM NaCl, 2mM DTT, 10 µM ZnAc).

Protein interaction – GST pull down:

Hela cells were plated at a density of 3 x 10^5 cells per well (in a 6-well dish) in DMEM supplemented with 10% FCS and allowed to grow overnight at 37 °C with 5% CO_2. The cells were transfected with a plasmid expressing a functional

SMAC (Hunter *et al.*, 2003) using the Lipofectamine 2000 reagent (Invitrogen). The following day, the media was aspirated and the cells were harvested and washed with PBS. The cells were then resuspended in lysis buffer (50 mM Tris-Cl pH 8.0, 150 mM NaCl, 1% Triton X-100, 10 mM EDTA, 1 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin A) and kept on ice at least 30 min. The cell debris was removed by centrifugation and the lysate was split into separate tubes for the pull down experiment.

To test interaction between GST-fused XIAP and TsIAP with SMAC, bead bound GST-fused IAPs were incubated with SMAC transfection lysate for 2 h at 4 °C. Approximately 10 μg of bead bound protein was used to pull down SMAC from the cell lysate. The beads were washed 3 times with ice cold wash buffer (50 mM Tris-Cl pH 8.0, 150 mM NaCl, 0.1% Triton X-100, 10 mM EDTA). They were then boiled for 2 minutes in SDS loading buffer and analyzed by Western blotting.

Cell viability assays

WST-1 viability assay:

Hela or 293T cells were plated in DMEM supplemented with 10% FCS at a density of $3x10^5$ cells per well of a 6-well plate. The cells were transfected the following day with pCI-XIAP, pCI-TsIAP, pCI-XIAP-reverse, pCI-TsIAP-reverse, and duplicate wells of pCI-LacZ using Lipofectamine Plus reagent (Invitrogen). Twenty-four hours later, transfection efficiency was determined by

staining one well of pCI-LacZ transfected cells for β-gal. Briefly, the cells were washed with PBS twice, and then fixed with 1% glutaraldehyde in PBS at 4 °C for 5 minutes. The cells were then washed 4 times with PBS and stained with X-gal buffer (1 mg/ml 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 0.05% triton X-100 in PBS) for 2-8 hours at 37 °C. If the transfection showed >60% efficiency, cells in the remaining wells were trypsinized, counted, and then plated at a density of 3×10^3 cells per well in 96 well microtiter plates. The cells were allowed to recover overnight, and then were treated for 4 h with varying concentrations of either etoposide or adriamycin. Cell viability was determined 18 h later by adding 10 μl of WST-1 reagent (Boehringer-Mannheim) and incubating the cells at 37°C in 5% CO_2 for 1-4 h. Formation of a coloured product, indicative of cell viability, was measured in a colorimetric plate reader at 405 nm. The percent survival of the treated cells was calculated relative to untreated cells.

PI visual staining viability assay:

We transfected 1 μg of pEGFP-*XIAP*, pEGFP-*TsIAP* or pEGFP DNA into Hela cells maintained in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% FCS using Lipofectamine Plus (Life Technologies). The next day, we treated the transfected cells with 6.4 μg/ml adriamycin for 4 h at 37 °C and 5% CO_2 then removed the media and replaced it with fresh DMEM for an overnight recovery period. We assayed for cell survival

by staining with propidium iodide (PI) and viewing the stained cells using inverted fluorescence microscopy. A minimum of 150 GFP fluorescent cells per transfection were scored positive or negative for PI.

Bax viability assay:

We seeded 293T cells in 6-well dishes at a density of 2.5 x 10^5 cells per well in 2 ml medium (DMEM with 10% fetal calf serum). The following day we used Lipofectamine Plus to co-transfect the cells with 0.25 μg of pEGFP-Bax and 1 μg of either pcDNA3-6-myc-*TSIAP*, pcDNA3-*XIAP*ΔB1+2, or pcDNA3-LacZ. Three samples per experiment were transfected without pEGFP-Bax and set aside for protein extraction to determine expression level. Eighteen h following transfection we harvested the cells, washed once with PBS then stained with PI. We analyzed the samples for GFP and PI fluorescence in a Coulter EpicsXL flow cytometer.

To test the effects of *SMAC* on the protective properties of *XIAP* and *TsIAP* we performed a similar assay, however this time we added 1 μg of pcDNA3-Ub-*SMAC*, or 1 μg of pcDNA-LacZ (as control) to the *XIAP* and *TsIAP* co-transfections.

Caspase-9 assay:

293T cells were transfected with pcDNA3-6-myc-XIAP and TsIAP in 6 well culture trays using Lipofectamine Plus (Invitrogen) as previously described.

To prepare S-100 fractions the cells were harvested by centrifugation at 1500 x g for 5 min at 4 °C. After one wash with cold PBS, the cell pellet was suspended in 5 volumes of ice cold buffer A (20 mM Hepes-KOH pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 2 mM sodium EDTA, 1 mM DTT, and 0.1 mM PMSF) supplemented with protease inhibitors (1 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin A). After sitting on ice for 15 min, the cells were disrupted by douncing 15 times in a Kontes dounce homogenizer with the B pestle (Kontes Glass Company). The nuclei were removed by pelleting at 1000 x g for 10 min at 4 °C. The supernatant was further centrifuged at 100000 x g for 1 hour in a table top ultra-centrifuge. The resulting S-100 fraction was stored at -80 °C and used for the in-vitro caspase assays.

Caspase activity was assayed at 37 °C in 50 μl of caspase buffer (100mM MES pH 6.5, 10% PEG 8000, 0.1% CHAPS, 10 mM DTT). 30U of recombinant caspase-9 was pre-incubated with varying amounts of the S-100 fractions from transfected and untransfected cells. The reaction was initiated by the addition of 50 μl of 10mM ATP and 4 mM colorimetric substrate Ac-LEHD-pNA (BIOMOL Research Laboratories) in caspase buffer. The absorbance at 405nm was monitored continuously.

Chapter 3

CHARACTERIZATION OF THE X-LINKED INHIBITOR OF APOPTOSIS

Introduction

Apoptosis is a fundamental process of cell death required for the elimination of unwanted cells in multicellular organisms. As such, it plays a central role in growth and development, as well as in various pathologies including cancer, neurodegenerative disorders, and viral infections including acquired immunodeficiency syndrome (AIDS). Dysregulation of apoptosis is known to result in much pathology. Excessive apoptosis can result in neurodegenerative disorders such as Alzheimer's disease, Huntington's disease and cell death associated with ischemic stroke as well as being implicated in the death of mature CD4+ T cells in HIV infection. Too little apoptosis appears to be associated with cancer and autoimmune disease (reviewed in (Thompson, 1995)).

Much of our knowledge of the biochemical pathways involved in apoptosis comes from the study of viruses and their means of controlling programmed cell death. Apoptosis of virally infected cells is a major host defence mechanism against viral attacks. As such, viruses have evolved mechanisms to evade or inhibit the host cell's apoptotic response either through expression of anti-

apoptotic proteins or through regulating the expression of cellular proteins of the apoptotic pathway. The baculoviral Inhibitor of Apoptosis proteins (IAPs), CpIAP and OpIAP were identified based on their ability to restore a wild type replication cycle to a p35 deficient strain of AcMNPV (Birnbaum *et al.*, 1994; Crook *et al.*, 1993). These genes were characterized by two amino terminal cys/his motifs and a carboxy terminal RING zinc finger motif. The two amino terminal motifs, termed Baculovirus IAP Repeat (BIR) domains, are the hallmark of the IAP family of proteins and are present in one to three copies in all members discovered to date.

The first mammalian IAP (Neuronal apoptosis inhibitory protein; *NAIP*, now *BIRC1*) was identified during a positional cloning effort seeking candidate genes for spinal muscular atrophy, a neurodegenerative disorder characterized by progressive loss of motor neurons leading to wasting of the voluntary muscles (Roy *et al.*, 1995). Following the identification of *NAIP*, 6 other human IAPs (*HIAP-2/c-IAP1/BIRC2*, *HIAP-1/c-IAP2/BIRC3*, *XIAP/BIRC4*, *survivin/BIRC5*, *BRUCE/BIRC6*, *Livin/ML-IAP/KIAP/BIRC7*) and their murine homologues have since been reported, along with two *Drosophila* IAPs (*DIAP-1*, *DIAP-2*) and one chicken IAP (*ITA*) (reviewed in (LaCasse *et al.*, 1998; Verhagen *et al.*, 2001)).

At the time this project was started, knowledge of the genetic structure of the X-Linked IAP was limited to a single cDNA clone spanning the coding region and extending less than 500 base pairs into the 3' UTR (Liston *et al.*, 1996). Given the importance of apoptosis to normal and diseased cell processes we undertook the task of cloning and sequencing the complete *XIAP* gene in an effort to better understand its genetic regulation. Genomic sequencing of DNA presents unique difficulties (Olson, 1993; Smith, 1993). To be able to sequence the entire 10 kilobase transcript of *XIAP* (as seen on a northern blot) using a primer walking method of sequencing would require dozens of lengthy repetitions of generating a primer, followed by sequencing to discover new DNA sequence on which to design the next primer. It would not be economically feasible to attempt to cover the entire genomic region of *XIAP* using that same method. Instead, we chose to make use of a transposon-facilitated DNA sequencing technique that allows the introduction of sequencing priming sites throughout a target sequence by bacterial mating (Strathmann *et al.*, 1991). Here we describe the results of that sequencing effort.

Results

XIAP genomic library screening:

Probing of human genomic DNA, digested with *Eco*RI, with a *XIAP* coding region probe shows a pattern of 6 bands (Fig. 3-1A). We screened a human male placenta lambda phage genomic DNA library using probes generated from the existing *XIAP* cDNA sequence (approximately 1.5kb of sequence spanning the coding region from the initial ATG through to 200bp beyond the stop codon) (Liston *et al.*, 1996). Three rounds of library screening were performed and 33 phage clones were isolated with inserts ranging in size from 9kb to 22kb. We managed to isolate clones corresponding to each of the 6 *Eco*RI fragments seen on the genomic DNA blot and were able to obtain four overlapping phage clones that spanned the complete *XIAP* locus (Fig. 3-2A). The clones containing the 25 kb and 3.5 kb genomic *Eco*RI fragments (bands 1 and 4, Fig. 3-1A) contain the functional *X-linked IAP* locus. The other four cross-hybridizing fragments are from four separate loci containing *XIAP* cross-reactive sequences. We subcloned fragments containing *XIAP* sequences and sequenced them via transposon facilitated sequencing (Gold Biotech). Representative fragments from three of the four other loci were also cloned and partially sequenced to determine their identity (Fig 3-1B, C).

The 25 kb fragment (band 1, Fig. 3-1) spans the majority of the *XIAP* gene, encoding the 5' untranslated region (UTR) through to the beginning of the

Figure 3-1 Southern blot analysis of XIAP.

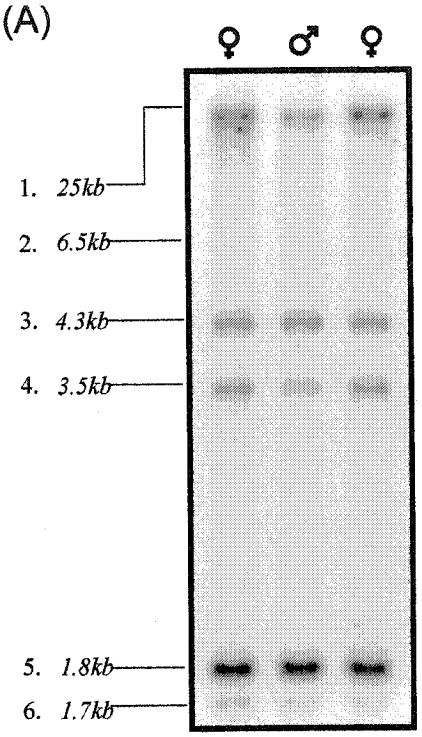
(A) Human genomic DNA digested with EcoRI and run on a 1% agarose gel was probed with full length *XIAP* coding region probe. Six bands hybridize to the probe. Two of these (25kb and 3.5kb) correspond to the X-linked IAP. Note that the X-linked bands appear at twice the intensity in the female DNA compared with the male DNA consistent with the number of X chromosomes present.

(B) Summary of the identity of each band seen on the Southern blot. Bands 1 and 4 encode the XIAP gene. Band 2 appears to contain a region of DNA that weakly hybridizes with the BIR1 domain of XIAP. Bands 3 and 5 contain retrotransposed pseudogenes with a high degree of homology to XIAP, however they do not appear to maintain any significant open reading frame. Band 6 corresponds to the Testis-specific IAP (see Chapter 4).

(C) Schematic diagram representing the regions of homology between XIAP cDNA sequence and the sequence obtained from the 4 cross-reactive genomic bands. See appendix 1 for a sequence alignment generated using Clustal version 1.82.

Figure 3-1. Southern blot analysis of XIAP.

(A)



(B)

Band	Identity	Chromosome	Comments
1	XIAP	Xq25	Start of transcription through to BIR2.
2	Pseudogene	Unknown	Weakly hybridizes to BIR1 of XIAP but nothing else.
3	Pseudogene	2q13	Shows sequence homology from BIR3 through to the 3'UTR. Contains many in-frame stop codons and short deletions.
4	XIAP	Xq25	BIR2 to the end of the coding region.
5	Pseudogene	10	Shows sequence homology from the middle of BIR3 through to the 3'UTR. No significant open reading frame maintained.
6	Testes specific IAP	19q13.4	See Chapter 4.

(C)

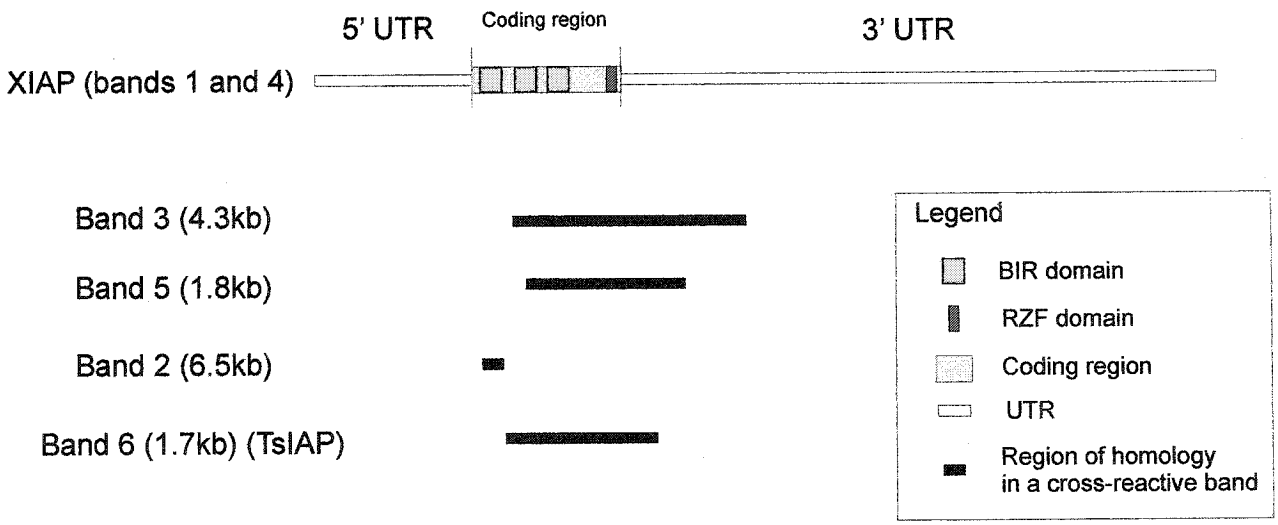
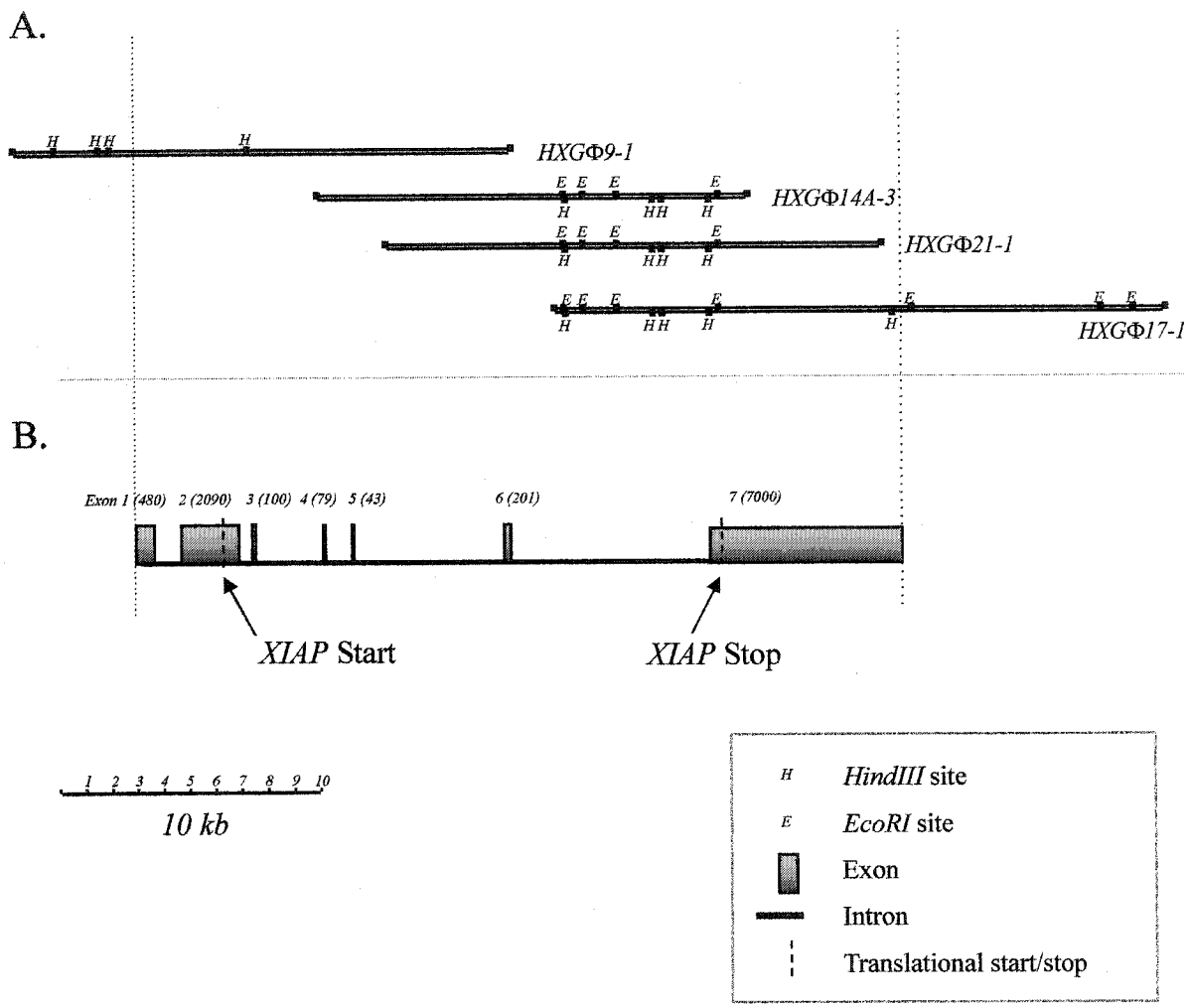


Figure 3-2 Schematic diagram of *XIAP* genomic structure.

(A) Overlapping contiguous array of phage clones spanning the BIRC4 genomic region. A small E or H denotes EcoRI or HindIII restriction sites respectively.

(B) Schematic diagram of intron/exon structure of XIAP. Exon sizes are marked in parentheses above the exons. The translational start and stop sites are marked by dashed lines.

Figure 3-2. Schematic representation of *XIAP* genomic structure.



RING zinc finger motif. The 3.5 kb fragment (band 4, Fig. 3-1) encodes the rest of the coding region and a small portion of the 3' UTR. The remaining 3' UTR is encoded in a third fragment not present on the southern blot due to the fact that the probe used was derived primarily from coding region sequence. Of the four other bands present on the southern blot, bands 3 (4.3 kb), 5 (1.8kb), and 6 (1.7kb) have been cloned and sequenced to determine their identity. The sequence obtained from the 4.3-kb band maintains rougly 90% identity with *XIAP* cDNA sequence from base 2473 through to 5024 (i.e. from the beginning of the third BIR domain and into the 3' UTR). Sequence on either side of these breakpoints diverges completely from both the *XIAP* cDNA and *XIAP* genomic sequence. Comparison of this sequence with Genbank entries reveals a match on BAC clone RP11-67L14 (gi14318395), which has been localized to chromosome 2q13. The 1.8 kb band shows a lower overall sequence homology of approximately 86% and the region of homology extends for a shorter distance from base 2559 through to 3926. Doing a BLAST search of Genbank with this sequence came up with a perfect match with clone RP11-566F5 (gi16304934) on chromosome 10. Neither of these homologous regions appears to maintain any significant open reading frame and no evidence has been found to suggest that these regions of genomic DNA are transcribed into messages either through northern blot analysis or through searching of the Genbank EST database. End sequencing of the 6.5 band (band 2, Fig 3-1) resulted in sequence from a

repetitive element and was thus not useful for Genbank screening. Probing Southern blots revealed that only probe derived from the BIR1 region of XIAP hybridized to this band. The 1.7 kb band (band 6, Fig. 3-1) was sequenced and found to contain a novel open reading frame closely related to that of *XIAP* (and described more completely in Chapter 4 of this thesis).

Promoter analysis:

Stringent computer sequence analysis of the region upstream of the *XIAP* transcription start site did not predict a classic TATA-box containing polymerase II promoter (Pedersen *et al.*, 1999), thus we cloned a 1 kb region upstream of the transcription start site into a promoter-less vector to drive the expression of the CAT reporter gene. The region cloned does show promoter activity (Fig. 3-3), although to a much lesser extent than the control plasmid pCAT-control. We did not see this promoter activity when the 1kb region upstream of XIAP was inserted in the inverse orientation.

XIAP mRNA expression:

The human *XIAP* gene appears to be expressed at the RNA level in most tissues tested, with the exception of peripheral blood leukocytes, and shows up on a northern blot analysis as a single band of approximately 10kb in size (Fig 3-4). A second band, approximately 2.2 kb in size appears solely in the testis. This band does not appear to be an artefact or degradation product since it has been corroborated by others (Richter *et al.*, 2001).

Figure 3-3. XIAP promoter function.

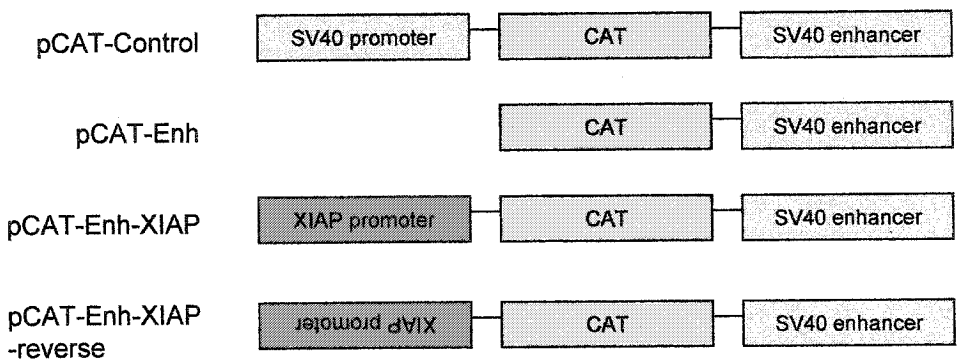
Comparison of the chloramphenicol transferase activity as measured by absorbance at 450nm.

(A) Schematic diagram of the plasmids used to test XIAP promoter function. pCAT-control plasmid contains a functional SV40 promoter and SV40 enhancer element. pCAT-Enh contains no promoter. pCAT-Enh-XIAP is the pCAT-Enh vector with the 1 kb region of DNA upstream of the start of *XIAP* transcription site inserted into the multiple cloning site. pCAT-Enh-XIAP-reverse is identical to pCAT-Enh-XIAP, with the exception that the 1 kb region was inserted in the reverse orientation.

(B) Significant promoter activity is detected when 1kb upstream of the transcription start site of XIAP is cloned into the pCAT-Enh vector. No promoter activity is seen when the promoter region of XIAP is inserted in the reverse orientation. Only the control vector (pCAT-control) and the vector containing the promoter region of XIAP in the proper orientation (pCAT-Enh-XIAP) showed CAT activity indicative of a functional promoter.

Figure 3-3. Promoter activity of XIAP

(A)



(B)

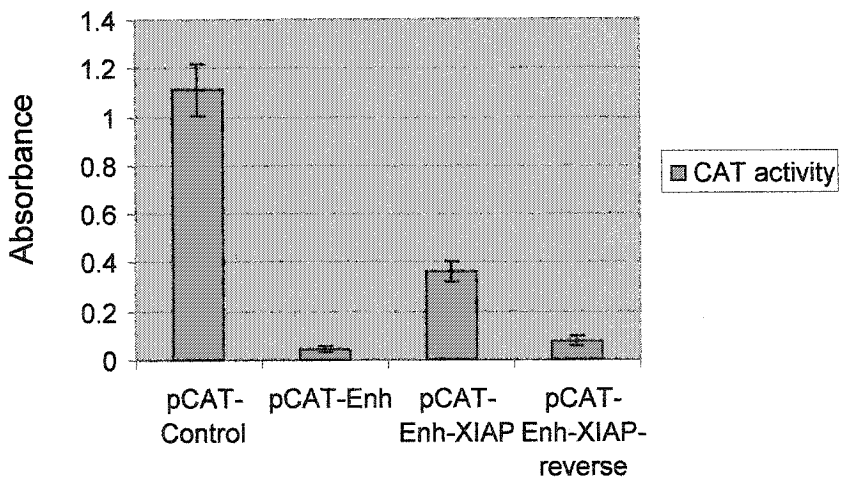
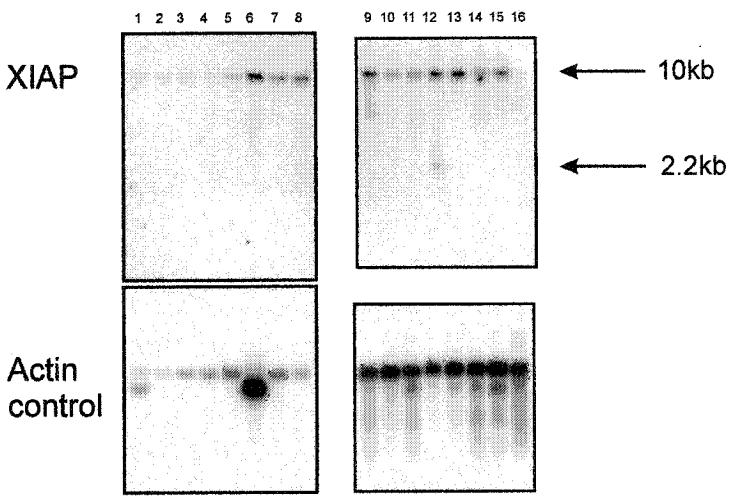


Figure 3-4. Northern blot analysis of XIAP.

Human multiple tissue northern blots probed with full length *XIAP* coding region probe. A ubiquitous 10 kb band appears in all tissues tested (except peripheral blood leukocytes). A second smaller transcript (2.2 kb) is present in the testis lane.

Figure 3-4. Northern blot analysis of XIAP



1. Heart
2. Brain
3. Placenta
4. Lung
5. Liver
6. Skeletal Muscle
7. Kidney
8. Pancreas
9. Spleen
10. Thymus
11. Prostate
12. Testis
13. Ovary
14. Small Intestine
15. Colon
16. Peripheral blood leukocyte

XIAP cDNA library screening:

We screened human placenta and liver cDNA libraries for *XIAP* to complete the cDNA sequence, determine intron-exon boundaries, and also to look for potential alternative splicing to account for the second band present in the testis lane of the northern blot. Initial library screening using a coding region probe failed to isolate *XIAP* clones that contained the complete cDNA, thus we designed new probes based on the most 5' and 3' sequences we had and re-screened the libraries to obtain clones containing more of the untranslated regions of *XIAP*. Complementing this, we performed 3' and 5' RACE on total RNA from human placenta and liver to delineate the exact start and stop sites of transcription. Interestingly, in the 3' UTR of *XIAP* there are several putative poly-adenylation signals. During library screening and RACE experiments, we isolated phage clones that appear to have stopped transcription at those upstream polyadenylation sites, however, no evidence of these shorter forms of *XIAP* were seen on northern blot analysis. The full length *XIAP* transcript is 10035 base pairs long and is comprised of 7 exons (6 introns) which span roughly 30 kb of genomic DNA (Fig. 3-2B). The locations of the introns (Table 1) in human *XIAP* are conserved with those in the mouse homologue (Farahani *et al.*, 1997) with the exception of the intron present in the 5'UTR of *XIAP* that does not have a corresponding intron in the mouse.

Table 3-1. XIAP Intron/Exon splice sites.

The *XIAP* gene is encoded on 7 exons (6 introns). This table lists the sequence at the splice sites for each of the introns. All of the splice junctions use the consensus GT/AG sequence with the exception of the first intron. Intron sizes are listed in base pairs (bp) in the last column of the table.

Table 3-1. XIAP Intron/Exon splice sites.

Donor site	Exon	Exon	Acceptor site	Size of intron
start of transcript	gacggggtttct..	...ttatctcatta	gcctatcaggta..	999bp
..attgcactccag	gcctgggcagca..	..tttatgctttag	gtaaactttatt..	2079bp
..gtgttttcgtag	gtgaaggtgata..	....ggtatccagg	gtaagatattta..	2520bp
.....attgtttag	gcaaatatctgt..	...gtgtctggtaa	gtctcatataat..	1402bp
..gttacaggtaag	gaactactgaga..	..ctagaagaattg	gtaaatatgctt..	7719bp
..tttttatttcag	atgataccatct..	..cattacagaaag	gtatgcattgct..	6294bp
..ctcttttgcag	agattagtact...	..end of transcript		

Subcellular localization:

To determine the subcellular localization of XIAP protein we performed basic cell fractionation experiments on HeLa cells to separate nuclear from cytoplasmic proteins. Western blot analysis of these cell fractions shows the XIAP protein clearly in the cytoplasmic fraction and not present in the nucleus (Fig 3-5A). These results were confirmed by immunohistochemical staining of endogenous as well as overexpressed XIAP protein (Fig 3-5B). We attempted to observe the localization of XIAP in apoptotic cells, however the results obtained were inconclusive. Staining for endogenous XIAP failed to clearly show XIAP localization in cells that could be identified by morphological changes to be apoptotic (i.e. a blebbing cell). When overexpressed, GFP-tagged XIAP shows a diffuse staining pattern in apoptotic cells.

Figure 3-5. Subcellular localization of XIAP.

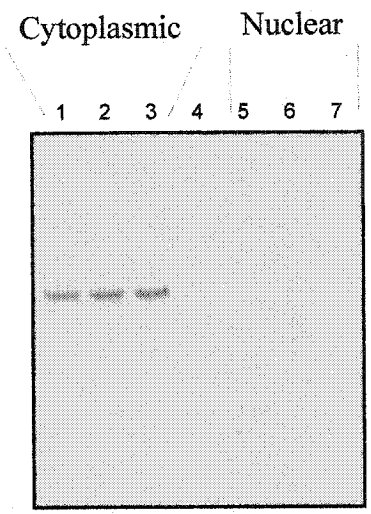
(A) Western blot of nuclear and cytoplasmic fractions of HeLa cells. Cytoplasmic and nuclear protein extracts from Hela, 293T, and Cos-7 cells were run on a 10% polyacrylamide gel and then probed with anti-XIAP polyclonal antibody. XIAP protein shows up clearly in the cytoplasmic fractions.

(B) Immunohistochemical staining of XIAP in HeLa cells. Panel (a) shows endogenous XIAP protein, as detected by polyclonal anti-XIAP antibody conjugated with CY3. Panel (b) shows the nuclear staining of the same field. Nuclei were counterstained using Hoechst 33258.

(C) Overexpressed GFP-tagged XIAP in an apoptotic HeLa cell.

Figure 3-5. Subcellular localization of XIAP

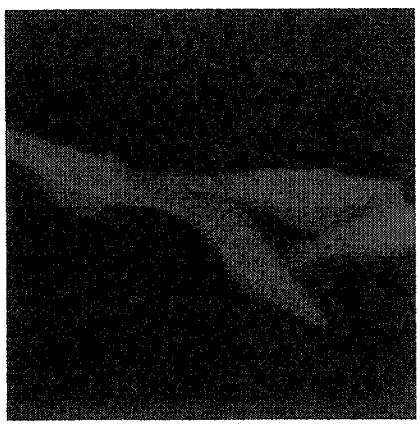
A



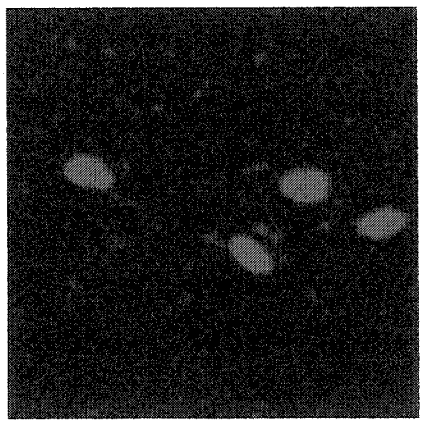
1. Cytoplasmic extract from HeLa cells
2. Cytoplasmic extract from 293T cells
3. Cytoplasmic extract from Cos-7 cells
4. Empty lane
5. Nuclear extract from HeLa cells
6. Nuclear extract from 293T cells
7. Nuclear extract from Cos-7 cells.

B

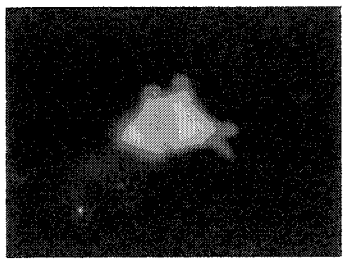
a



b



C



Discussion

XIAP is part of a multi-gene family of related anti-apoptotic proteins and shows a high degree of homology with its murine homologue and with two other closely related human IAPs, *HIAP1* and *HIAP2* (Young *et al.*, 1999). Coding region DNA of *XIAP* is also able to hybridize with several distinct loci within the human genome, with the actual *XIAP* gene present on chromosome Xq25 (Rajcan-Separovic *et al.*, 1996). Various X-linked disorders have been mapped to this region of the chromosome, including X-linked lymphoproliferative disease (reviewed in (Maia & Garwacki, 1999)). Duplication of this chromosomal region has been associated with X-linked recessive panhypopituitarism (Lagerstrom-Fermer *et al.*, 1997), and deletion of Xq25 has been associated with tumor grade and metastasis in primary breast carcinomas (Piao & Malkhosyan, 2002). Interestingly, in the case of the breast carcinomas the deletions of Xq25 were primarily from the inactivated X chromosome, suggesting that this region might escape X-inactivation. Supporting evidence for this theory is the presence of loci involved in skewing X-chromosome inactivation that have also been mapped to the region of Xq25-26 (Naumova *et al.*, 1998). Subsequent to our sequencing of the *XIAP* genomic region, the human genome project sequencing effort completed the same DNA region and the sequence was deposited under accession number AL121601. Comparison of our sequencing data to that of

AL121601 showed a sequence match across the entire region from base 9228 through 42275 of sequence AL121601.

The *XIAP* genomic region spans roughly 30kb from the beginning of the first exon to the end of the last exon. The *XIAP* gene itself is 10035bp in length, encoded in 7 exons. Only one intron-exon splice junction had a non-consensus splice acceptor site (GC instead of GT) at the 3' end of exon 1, however no evidence exists to suggest that this results in any alternative splice forms. Of note is that the first intron is entirely within the 5' UTR of the *XIAP* message.

The *XIAP* cross reactive regions of the genome all appear to encode intronless copies of the *XIAP* gene with varying degrees of sequence conservation. The presence of these *XIAP* homologous regions would be consistent with a reverse transcription event occurring with *XIAP* message and re-integration into the chromosome. Of particular interest is that one of these intronless genes appears to have maintained an open reading frame and is expressed at the mRNA level in the testis. This phenomenon appears to be limited to the human *XIAP* gene, as there is little evidence of intronless *XIAP*-like retrotransposons in the mouse (Farahani *et al.*, 1997).

XIAP is predominantly a cytoplasmic protein, in both healthy and apoptotic cells. Molecular participants in the apoptotic program are present in different subcellular compartments, including the plasma membrane, cytosol,

mitochondria, and nucleus. The interplay among these compartments and the exchange of specific signaling molecules are critical for the systematic progression of apoptosis. Healthy HeLa cells show diffuse cytoplasmic staining of XIAP with a possible concentration in the perinuclear region. Apoptotic cells show a very diffuse cytoplasmic staining pattern. This is consistent with data seen in neurons where XIAP staining became very diffuse in the cytoplasm following ischemic insult and reperfusion (Shibata *et al.*, 2002). A cytoplasmic localization is also conceptually consistent with XIAP's function as an inhibitor of caspases, as the caspase proteins are also predominantly cytoplasmic (Cohen, 1997). Likewise, the XIAP inhibitors Smac, HtrA2, and GSPT1 have been shown to be translocated to the cytoplasm from their respective subcellular compartments: mitochondria for Smac and HtrA2 (Du *et al.*, 2000; Hegde *et al.*, 2002; Verhagen *et al.*, 2000); ER for GSPT1 (Hegde *et al.*, 2003). Indeed, a potential mechanism by which the XIAP inhibitor XAF1 is thought to function is by sequestering XIAP in the nucleus, thus preventing XIAP from influencing the cytoplasmic events that occur in apoptosis (Liston *et al.*, 2001).

Human XIAP is ubiquitously expressed as seen by Northern and Western blotting. The *XIAP* gene encodes a protein that exhibits anti-apoptotic properties in a variety of apoptosis inducing conditions. In contrast, other anti-apoptotic proteins show either a more limited expression pattern (e.g. the other IAPs) or inhibition of a more limited subset of apoptotic triggers (e.g. bcl-2

family). This could imply a 'housekeeping' function for *XIAP*, requiring a minimum amount of protein in a cell to maintain viability. As more evidence emerges on the functional aspects of the IAPs it appears that, similar to the balance between pro- and anti-apoptotic BCL-2 family members, the key to IAP function is the balance between the IAPs and their respective inhibitors (reviewed in (Salvesen & Duckett, 2002)). The region upstream of the transcription start site in *XIAP* provides few clues to the transcriptional control of the *XIAP* gene. Other IAPs are subject to tight transcriptional control, such as the cell-cycle dependant transcription of *survivin* (Ambrosini *et al.*, 1997; Li *et al.*, 1998a), or the tissue specific expression pattern of *HIAP1* and *2* (Young *et al.*, 1999). *XIAP*, on the other hand, appears to be ubiquitously expressed at the mRNA level in all tissues and cells examined to date. One possibility is that transcription of the gene is promiscuous, but post-transcriptional features regulate differential expression in those tissues that require more or less XIAP protein. This theory is supported by the presence of extensive 5' and 3' untranslated regions (UTRs) in the XIAP message and the identification of an internal ribosome entry site (IRES) in the 5' UTR (Holcik *et al.*, 1999).

Consistent with the possibility of *XIAP* as an essential 'housekeeping' gene is the identification of a second homologous retrotransposon expressed in the testis. During and following meiosis of a spermatocyte, the X-chromosome is inactivated (Richler *et al.*, 1992; Salido *et al.*, 1992). Thus, for X-linked genes

required for the survival of the spermatocyte there must be some mechanism by which they evade inactivation. Some X-linked genes that are essential to the proper function of cells have a duplicate copy present on an autosome and expressed specifically in the testis (e.g. *PGK*: (Bluthmann *et al.*, 1982); *G6pd*: (Hendriksen *et al.*, 1997); *PDHA*: (Iannello *et al.*, 1995); *PRPS*: (Taira *et al.*, 1990); *GKD*: (Sargent *et al.*, 1994)). This duplicate copy is often, if not always, present as an intronless gene whose expression is limited to cells of a germ-line origin. *TsIAP*, an autosomal, intronless gene expressed solely in the testis could fill this role.

Chapter 4

CHARACTERIZATION OF THE TESTIS-SPECIFIC IAP

Introduction

Spermatogenesis:

Spermatogenesis is a dynamic and highly synchronized process that continues throughout adult life in most mammals. It occurs in seminiferous tubules containing a mixed population of germ cells and Sertoli cells, surrounded by a thin wall of peritubular cells (reviewed in (de Rooij, 1998)). Sertoli cells provide a crucial support role for the developing germ cells and are thought to coordinate important events in the process of spermatogenesis (Griswold, 1995). Sertoli cells also divide the seminiferous epithelium into two compartments: a basal compartment where cells are exposed to the surrounding milieu and a luminal compartment where cells are sequestered behind a 'blood-testis' barrier formed by junctional complexes between Sertoli cells.

The production of mature sperm is a complex process which includes multiple developmental stages. The process is initiated during early embryo development when primordial germ cells (PGC) colonize the genital ridge. Under the influence of Y-chromosome-bearing Sertoli cells, the PGC proliferate. Some undergo apoptosis while the remainder become gonocytes (Wang *et al.*,

1998b). The gonocytes remain quiescent until after birth, when they are reactivated and initiate the process of spermatogenesis.

The maturation of the initial cohort of gonocytes into spermatogonia is known as the first wave of spermatogenesis and occurs between birth and day 5 post-partum in the mouse. In the human this occurs between birth and six months of age. While some spermatogonia become self-renewing spermatogonial stem cells, most differentiate into spermatocytes and at puberty initiate meiosis. The first wave of spermatogenesis is accompanied by extensive germ cell apoptosis, which in the mouse peaks at approximately 14 days after birth (Rodriguez *et al.*, 1997). This attrition may reflect adjustment in the number of germ cells to that which can be maintained by the available Sertoli cells (Orth *et al.*, 1988). In the adult, germ cell development occurs in successive mitotic (spermatogonia), meiotic (spermatocytes), and post-meiotic differentiation (spermatids) phases, with the germ cells moving from the periphery to the lumen of the seminiferous tubule during this process.

To achieve precise homeostasis of each germ cell type in the adult, germ cell renewal, proliferation, export and apoptosis must be finely balanced. It appears that this balance is achieved through a substantial amount of apoptosis, since it is estimated that up to 75% of potential spermatozoa degenerate in the testis of adult mammals (Print & Loveland, 2000). In fact, sensitivity to apoptotic

stimuli may well be a key feature of the developing spermatozoa to ensure quality control of the gametes produced (Braun, 1998). Testicular germ cell tumors show an inherent sensitivity to apoptosis-inducing stimuli, in particular cisplatin-containing chemotherapeutics (Spierings *et al.*, 2003).

Signals that regulate germ cell survival:

During embryogenesis there are several paracrine signals that promote survival of the developing PGCs, including leukemia inhibitory factor, interleukin-4, basic fibroblast growth factor, stem cell factor, and bone morphogenic protein. In the adult, members of the BMP family promote germ cell survival in vivo (Print & Loveland, 2000). The involvement of the BMP signaling pathway is of particular note as it has been shown that the XIAP protein is integral to the signaling pathway of the BMP receptor (Yamaguchi *et al.*, 1999).

In addition to paracrine signals, germ cells also rely upon signals delivered by direct membrane contact with Sertoli cells. Disruption of the interaction between Sertoli cell membrane-bound stem cell factor (SCF) and c-kit expressed on the surface of spermatogonia leads to an increase in apoptosis in the spermatogonia and spermatocytes (Packer *et al.*, 1995). Sertoli cells may also employ the Fas system to regulate germ cell fate, as Fas-ligand is expressed on Sertoli cells while Fas is expressed on the surface of adjacent spermatogonia. Male mice that completely lack Fas are fertile, however, suggesting that while Fas may contribute to germ cell homeostasis, it is not essential (Adachi *et al.*, 1995).

The use of genetically altered mice has provided exciting insights into the intracellular mechanisms by which the fate of germ cells is decided. A summary of the various knock-out mice studied can be seen in Table 4-1. Several key players involved in the apoptotic regulation of male germ cells appear to be members of the *bcl-2* family (Sinha Hikim & Swerdloff, 1999). The pro-apoptotic Bax protein is abundant in mouse testis between one and three weeks after birth, and appears to be essential for triggering the physiological apoptosis of spermatogonia and spermatocytes which occurs during the first wave of spermatogenesis. Testes of adult *bax* knockout mice contained excessive numbers of spermatogonia and pre-leptotene spermatocytes, consistent with a failure in the early wave of apoptosis that occurs in development (Rodriguez *et al.*, 1997). The role of Bax in the adult mouse is unclear; however, it is expressed in normal adult spermatogonia and spermatocytes (Meehan *et al.*, 2001). The pro-survival protein, Bcl-w plays a different role. Bcl-w appears to be irrelevant to spermatogenesis during embryogenesis and early development. The incidence of germ cell apoptosis in the adult *bcl-w* knockout mouse, however, is greatly increased (Print *et al.*, 1998; Ross *et al.*, 1998). Other members of the Bcl-2 family are expressed in the testis, including Bcl-2, Bad, Bak, and Bim but their role in spermatogenesis has not been investigated. The roles of these endocrine and paracrine signals are summarized in Figure 4-1.

Table 4-1. Partial list of genes deleted in mice which result in apoptotic defects in spermatogenesis.

Several knock-out mouse models show defects in spermatogenesis, including Bax (Knudson *et al.*, 1995), CREM (Blendy *et al.*, 1996; Nantel & Sassone-Corsi, 1996), HR6B (Roest *et al.*, 1996), A-myb (Toscani *et al.*, 1997), Bcl-w (Ross *et al.*, 1998; Russell *et al.*, 2001), and Bcl-X_L (Meehan *et al.*, 2001). These defects are due to either excessive cell death via apoptosis, or decreased apoptosis leading to excessive cell numbers.

Table 4-1 Partial list of genes deleted in mice which result in apoptotic defects in spermatogenesis.

Gene disrupted	Phenotype
Bax	Accumulation of atypical premeiotic germ cells but no mature spermatozoa. Marked increase in germ cell apoptosis. Infertile
CREM	Late spermatids are absent and there is a significant increase in germ cell apoptosis.
HR6B	Severely impaired spermatogenesis with marked increase in germ cell apoptosis. Defects in post-meiotic condensation of chromatids.
A-myb	Arrest at pachytene spermatocyte stage. Complete absence of post-meiotic spermatids
Bcl-w	Progressive depletion of adult germ cells through accelerated apoptosis followed by loss of Sertoli cells.
Bcl-X_L	Lethal. High levels of expression in the early spermatogonia suggest it is required for survival.

Figure 4-1. Regulation of apoptosis during spermatogenesis.

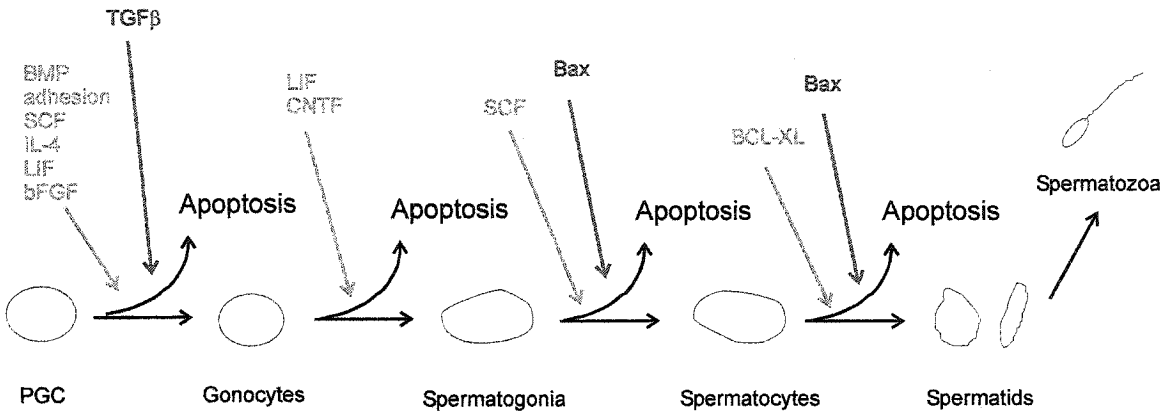
(A) Gonadal development during embryogenesis and in the first wave of spermatogenesis is normally accompanied by extensive apoptosis of all germ cell types. Signals that may regulate germ cell apoptosis are indicated by arrows and named above the cells that they influence. Green text and arrows indicate that the signal inhibits apoptosis in that cell type. Red text and arrows indicate signals that promote apoptosis. Abbreviations: BMP, bone morphogenic protein; SCF, stem cell factor; IL, interleukin; LIF, leukemia inhibitory factor; FGF, fibroblast growth factor; CNTF, ciliary neurotrophic factor; PGC, primordial germ cell.

(B) During adult spermatogenesis, germ cell apoptosis maintains appropriate numbers of each germ cell type and also selectively removes damaged cells. Again, green text and arrows indicate protective signals, red text and arrows indicate promotion of apoptosis.

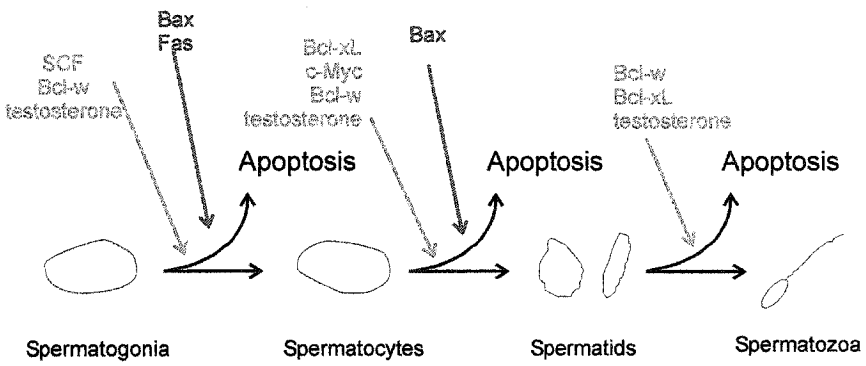
This figure is based on a diagram from (Print & Loveland, 2000).

Figure 4-1. Regulation of apoptosis during spermatogenesis

A. Apoptotic modulators of gonadal development during embryogenesis.



B. Apoptotic modulators during adult spermatogenesis



Regulation of Germ Cell Gene Expression:

The highly ordered process of spermatogenesis requires a precise and well-coordinated program that regulates the constantly changing pattern of gene expression. The development of male germ cells occurs in overlapping waves in mammals and other vertebrates, and is highly synchronized with mitotic, meiotic, and post-meiotic processes. This development is regulated by the expression of a unique cohort of genes in the male germ cells. The genes expressed only in male germ cells frequently are homologues of genes expressed in somatic cells (reviewed in (Eddy, 2002)).

It seems inefficient for male germ cells to use a new gene instead of an existing one that encodes a nearly identical protein. However, there are often significant advantages for male germ cells to have their own genes. One is that a germ cell homolog may compensate for a gene that is inactivated in male germ cells. An example of this is the phosphoglycerate kinase-1 (*pgk-1*) gene that encodes an essential enzyme in the glycolytic pathway. *Pgk-1* is located on the X chromosome, which is inactivated during the meiotic phase of male germ cell development (Richler *et al.*, 1992). The testis-specific homologue of *pgk-1*, *pgk-2*, is present on an autosome and gets activated shortly after the X-chromosome becomes inactive. *Pgk-2* lacks introns and has been hypothesized to have evolved as a functional retrotransposon of the *pgk-1* gene (Bluthmann *et al.*, 1982; Boer *et al.*, 1987).

Another advantage of germ cell specific genes is that the encoded protein may fulfill a similar, yet distinct role as that of the somatic cell's product. For example GAPDS contains an extra proline-rich N-terminal domain that is not present in the somatic homologue GAPD (Bunch *et al.*, 1998). This N-terminal domain anchors the GAPDS protein in the sperm tail with the rest of the enzymes in the glycolytic pathway.

Here we report the cloning and characterization of *TsIAP* (*BIRC8*), a testis-specific homologue of the *X-linked IAP* gene. We show that TsIAP is a retrotransposed copy of XIAP that maintains an open reading frame homologous to the BIR3 and RZF of the XIAP gene. It is expressed both at the mRNA and protein level in human testis, but does not appear to have a murine equivalent. TsIAP is present on an autosome and thus, like pgk2, is likely to have evolved to fulfill a required function during adult spermatogenesis.

Results

Cloning of the Testis-Specific IAP (TsIAP):

On human multiple tissue northern blots of poly-A+ RNA, a 10-kb *XIAP* reactive band appears ubiquitously expressed in all tissues tested with only minor variations in intensity. Of note, however, is a second reactive species of approximately 2.2-kb in size that appears only in the testis (Fig. 3-2). To investigate the possibility of a second form of *XIAP* expressed in the testis, we screened a human testis cDNA library with full-length *XIAP* coding region probe. Interestingly, seven out of 23 clones isolated bore only 75-85% similarity with existing *XIAP* cDNA sequence. These changes could not be accounted for by sequencing error, as the 7 'different' clones all had identical changes to the *XIAP* sequence. We sequenced overlapping phage clones that contained this testis specific cDNA and compared it with the known *XIAP* sequence. The transcript contains a potential open reading frame that would encode a protein similar to the carboxy-terminal portion of *XIAP* (80% amino acid identity; 90% similarity) containing a single BIR domain corresponding to the third BIR domain of *XIAP* and the RING zinc finger (nucleotide sequence: Fig. 4-2; amino acid sequence: Fig 4-3; schematic diagram: Fig. 4-4).

Northern and Southern blots of TsIAP:

To confirm that the clones isolated from the testis cDNA library do represent the 2.2kb transcript seen on the MTN blot, we used a region of the

Figure 4-2. Nucleotide sequence alignment of XIAP and TsIAP.

Pairwise comparison between *TsIAP* (top sequence) and *XIAP* (bottom sequence) was performed using the Bestfit program of GCG (Genetics Computer Group, Inc.). The start and stop codons for XIAP and TsIAP are highlighted. The total sequence homology over the region shown is 84%.

Figure 4-2. Nucleotide sequence comparison between *XIAP* and *TsIAP*

Figure 4-3. Amino acid sequence alignment of XIAP and TsIAP.

Amino acid sequence comparison between TsIAP (top sequence) and XIAP (bottom sequence). Critical residues in the BIR domain (green) and the ring zinc finger (orange) are boxed. Residues responsible for forming the SMAC binding pocket are indicated by short arrowheads. Pairwise comparison was performed using the bestfit program of GCG. Percent identity over the entire region shown is 80.1%. Percent similarity is 90.3%. Note that the conservation of critical amino acids in the BIR and RZF domains is perfect, however, several changes are present in the SMAC binding groove.

Figure 4-3. Amino acid sequence alignment of XIAP and TsIAP

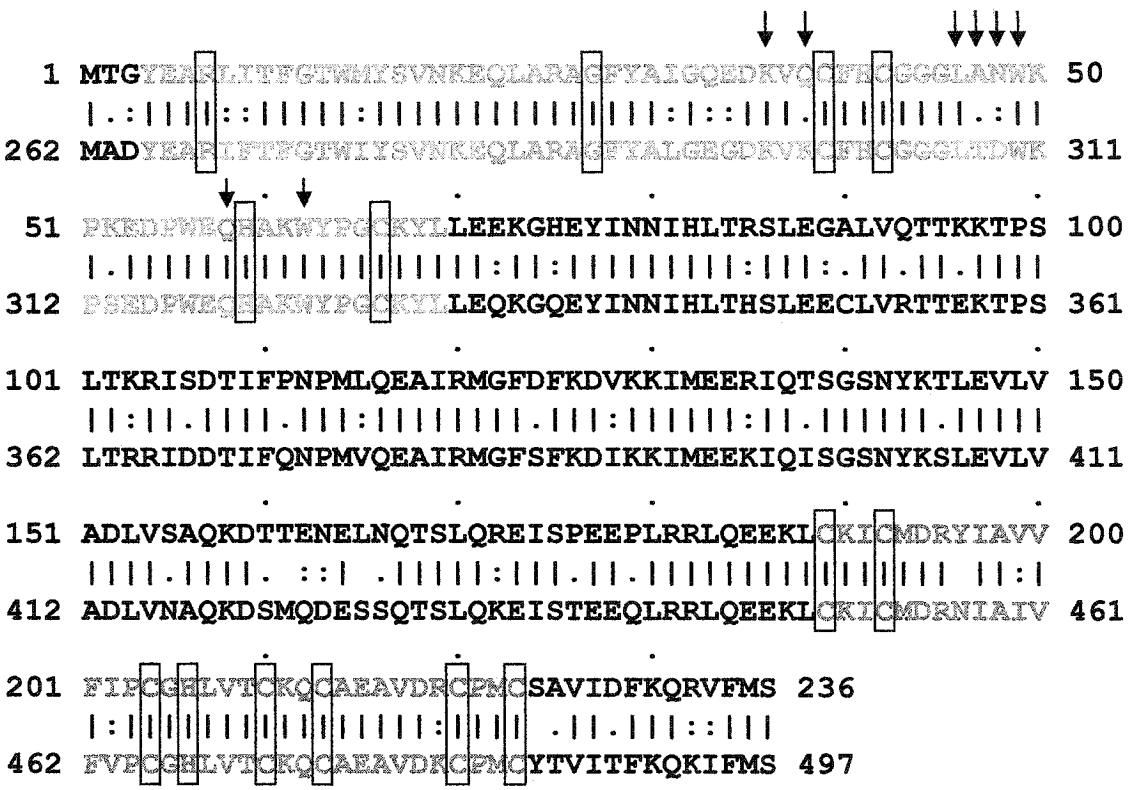
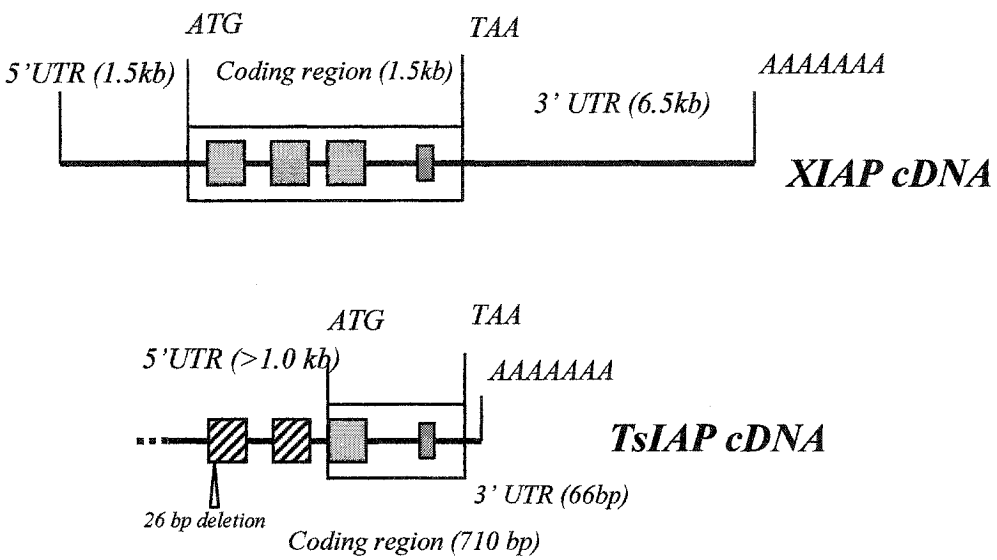


Figure 4-4. Schematic diagram of TsIAP structure.

Schematic diagram depicting a structural comparison between the *xiap* and *tiap* genes. BIR domains are shown in green, while the RING zinc finger is drawn in red.

Figure 4-4. Schematic diagram of TsIAP structure.



putative 5'UTR with no significant homology to *XIAP* to probe the original MTN blots (Fig. 4-5A). Only one band hybridized and that corresponded to the 2.2-kb band in the testis. This mRNA was not present in any other tissues tested (shown in Figure 4-5: spleen, thymus, prostate, ovary, small intestine, colon, peripheral blood leukocyte; not shown: heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas), nor was it present in a selection of transformed cell lines tested (not shown: HL-60, Hela S3, K562, MOLT-4, Raji, SW480, A549, G361). We used the same region to probe a human genomic DNA blot (Fig. 4-5B) and found that it hybridized to one *Eco*RI fragment of 1.7 kb, that appears with equal intensity in males (1X) and females (2X) suggesting it is an autosomal locus. We performed a southern blot analysis of the genomic library phage clones isolated during the *XIAP* library screening and found one of those clones hybridized with the *TsIAP* specific probe. We sequenced this phage clone with *TsIAP* specific primers and found it to contain the *TsIAP* locus.

Chromosomal location of the TsIAP gene:

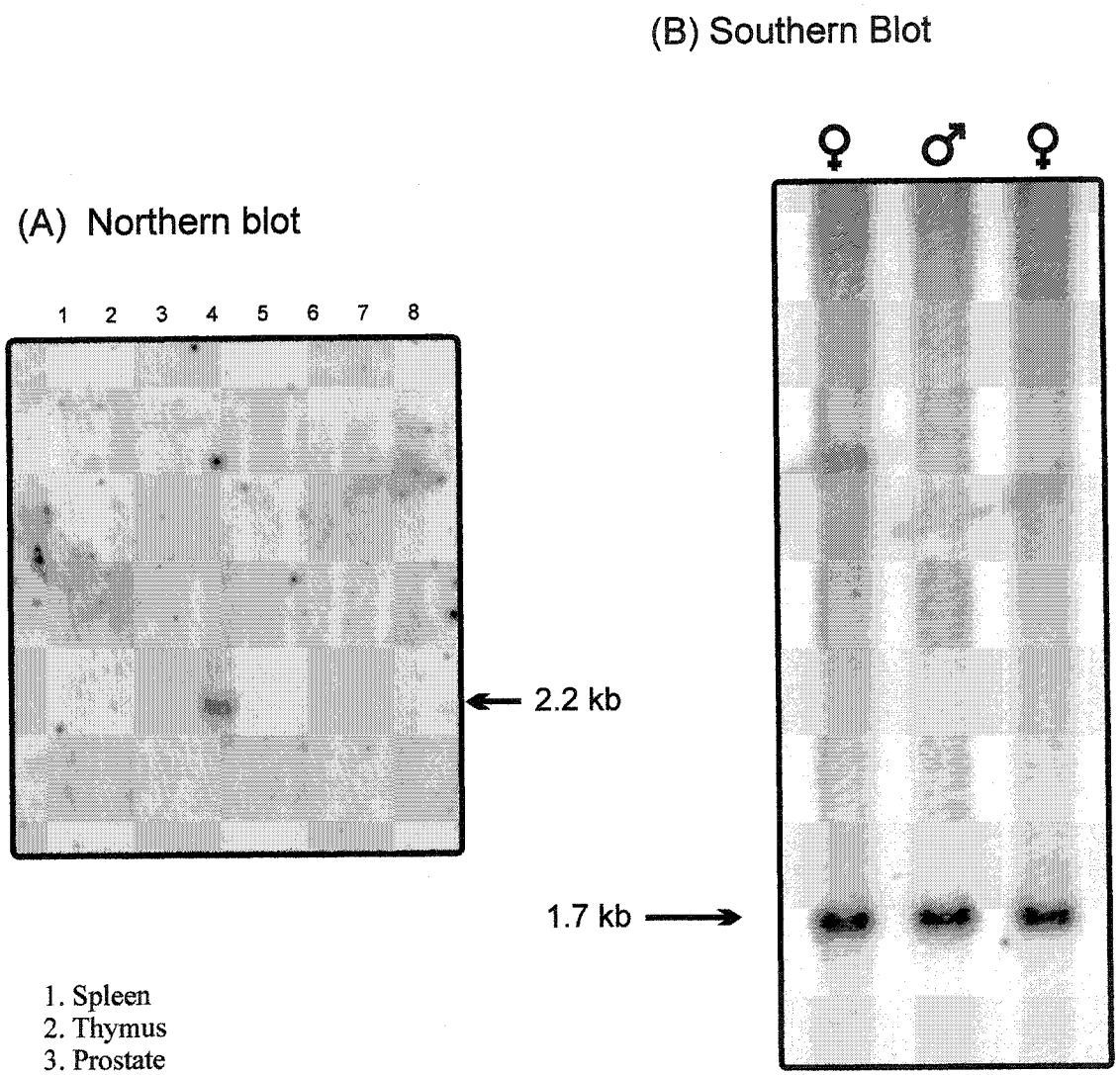
A portion of this phage clone was used in FISH analysis. The probe bound to chromosomes 19q13.4, 12q22-23, and 4p15.3 (Fig 4-6). Screening of the Genbank genomic database (htgs database, http:// www.ncbi.nlm.nih.gov / BLAST), has shown a perfect match to the *TsIAP* sequence on clone AC005780, a cosmid spanning the chromosomal region 19q13.3-q13.4. The FISH data

Figure 4-5. Northern and Southern blots of TsIAP.

(A) ClonTech human multiple tissue northern blot probed with *TsIAP* UTR sequences (no homology to *XIAP*). A single band appears in the testis lane. No other tissue type showed reactivity.

(B) Human genomic DNA digested with *EcoRI* was probed with *TsIAP* UTR sequences and resulted in a single reactive band of 1.7kb that showed no differences in intensity between males and females.

Figure 4-5. Northern and Southern blots of *TslAP*.



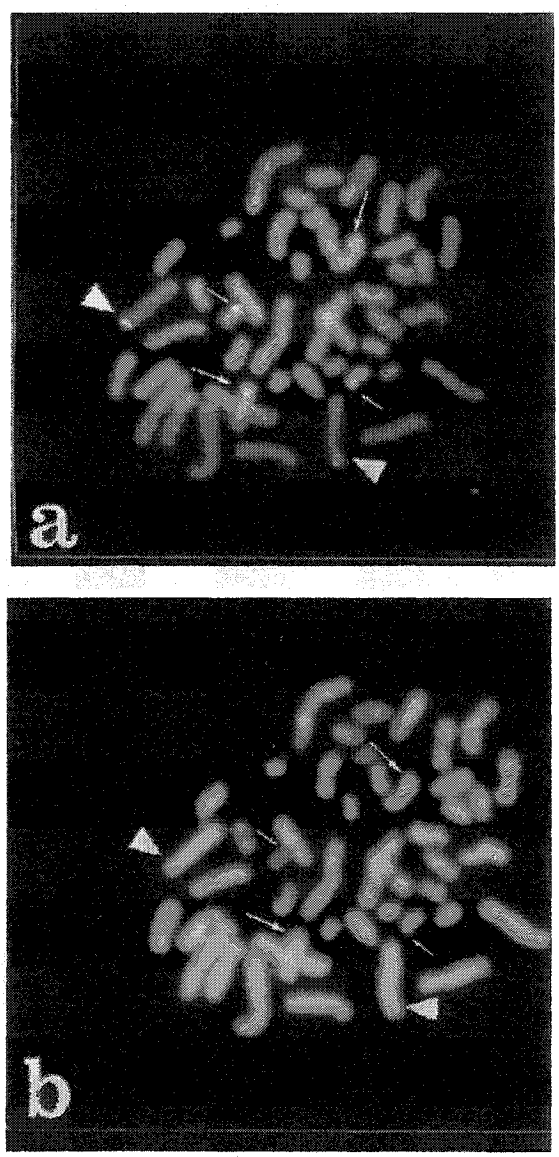
1. Spleen
2. Thymus
3. Prostate
4. Testis
5. Ovary
6. Small Intestine
7. Colon
8. Peripheral blood leukocyte

Figure 4-6. Chromosomal location of TsIAP by FISH.

(A) Metaphase spread hybridized with *TsIAP* genomic clone DNA; specific fluorescent signals were detected on 4p15.3 (arrowhead), 12q22-23 (long arrow), and 19q13.4 (short arrow).

(B) The same metaphase spread after DAPI banding. 8/32 metaphase spreads had a signal at 4p15.3, 23/32 had a signal on 12q22-23, and 19/32 had a signal on 19q13.4 on one or both chromosome homologs.

Figure 4-6. Chromosomal location of TsIAP by FISH.



together with the Genbank information strongly suggest that the gene maps to that location.

The search for the murine homologue of TsIAP:

Having determined that the *XIAP*-related transcript seen in the human testis northern blot is in fact a separate locus, we set out to find a murine homologue. Southern blot analysis performed on mouse genomic DNA using probes derived from the *TsIAP* sequence failed to show any bands. Likewise northern blot analysis on mouse testis RNA and poly-A+ enriched mRNA failed to provide any evidence of a murine *TsIAP*. A C57BL/6 mouse genomic library was screened with the *TsIAP* specific probe, but no specific clones were isolated after the third round of plaque purification. Two phage clones were isolated during library screening for the mouse homologue of *XIAP* (*MIAP3*) that did not correspond to the *MIAP3* locus and were kindly provided to us for analysis by Dr. Reza Farahani (Farahani *et al.*, 1997). Southern blot analysis and sequencing of these two clones failed to provide evidence that they encoded genes related to the IAPs. These results, along with data later published (Richter *et al.*, 2001) have led us to the conclusion that *TsIAP* is a recent evolutionary event and that no mouse equivalent exists.

Protein expression of TsIAP:

The open reading frame encoded by the *TsIAP* gene is homologous to *XIAP* from the third BIR domain through RING finger domain to the stop

codon. There is, however, a significant portion of the *TsIAP* message upstream of the start codon (thus in the 5' untranslated region) that maintains partial homology to *XIAP* cDNA sequence and could potentially interfere with translation of the message. To determine if the testis IAP open reading frame could produce a protein, we produced three plasmids: 1) A pCI based plasmid that contained just the putative open reading frame (from the start ATG to the stop codon). 2) A pcDNA3-6-myc tagged version of *TsIAP*, again cloned from the start ATG to the stop codon. 3) A pcDNA3 vector with the full length *TsIAP* transcript cloned into the multiple cloning site. This plasmid contained the full upstream UTR. Western blotting of cell lysates of transfected HeLa and probed with anti-*RIAP3* polyclonal antibody showed a non-tagged protein size of approximately 26 kDa, and a myc-tagged size of approximately 39kDa (Fig 4-7A). Probing with anti-*myc* monoclonal antibody picked up only the tagged version, at 39 kDa.

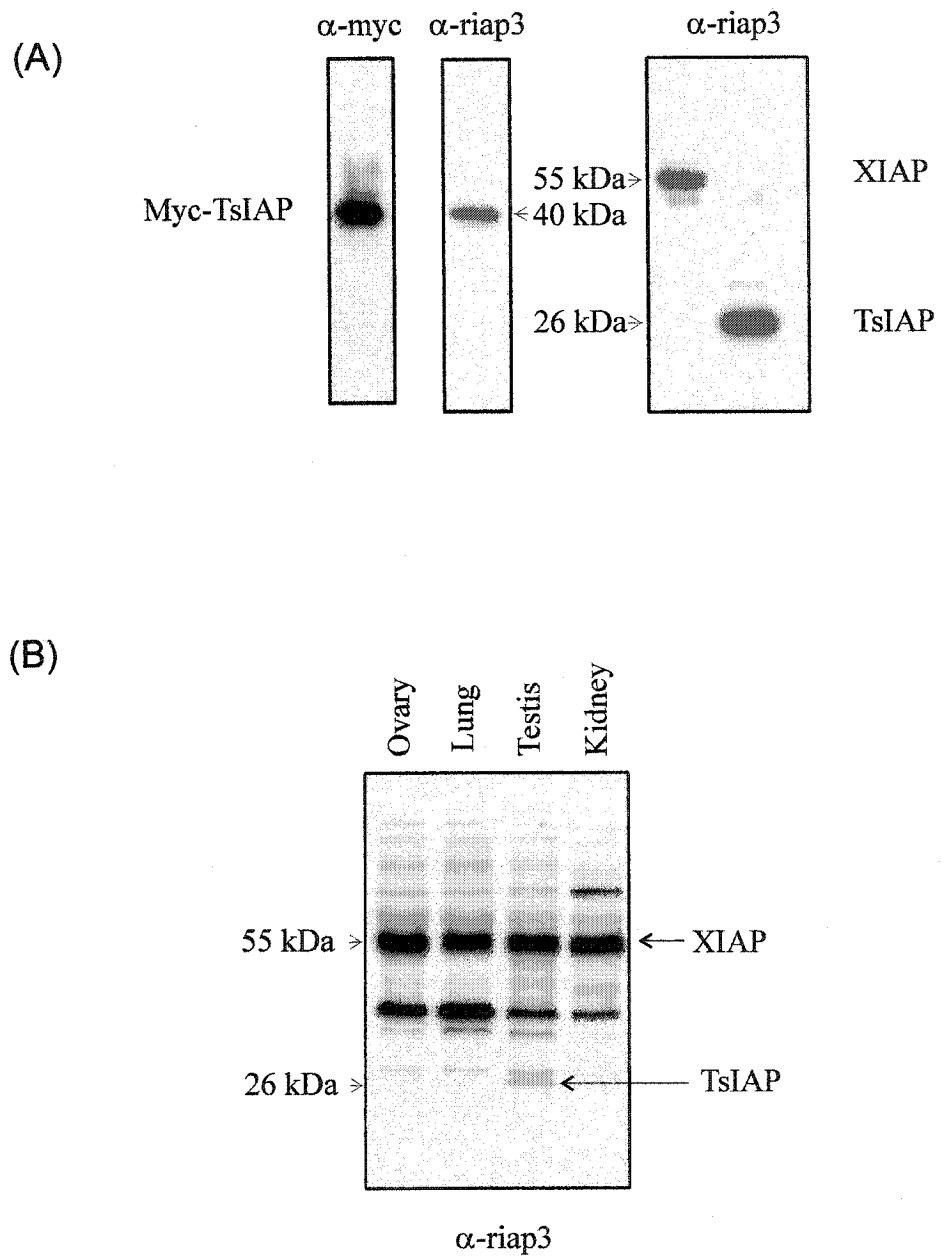
To see if we could detect endogenous TsIAP protein, we performed Western blot analysis using the anti-RIAP3 antibody on protein extracts from a variety of human tissues (obtained from Clontech). While XIAP protein was the most prominent band detected (Fig 4-7B), upon overexposure of the film weaker cross-reactive bands were seen in all of the tissues. Of note, however, is the presence of a band at 26 kDa in size that is unique to the testis protein lane (Fig 4-7B) which we believe represents the testis specific IAP.

Figure 4-7. Western blot analysis of TsIAP.

(A) Hela cells were transfected with either myc-tagged TsIAP coding region only, XIAP, or untagged TsIAP containing the full 5'UTR. The left panel shows myc-tagged TsIAP, as detected by western blot using monoclonal anti-myc antibody. The middle panel shows the same blot, stripped and probed with polyclonal anti-riap3 antibody. The right panel shows XIAP and untagged TsIAP, as detected by anti-riap3 antibody. Clearly the anti-riap3 antibody is able to strongly detect the TsIAP protein. Despite the long 5'UTR of TsIAP, it is still able to make a detectable protein.

(B) Western blot analysis using anti-riap3 antibody on proteins from human tissue samples. XIAP is clearly detected at 55kDa, however, in the testis lane there is a unique band at approximately 26kDa that corresponds to the observed size of untagged transfected TsIAP.

Figure 4-7. Western blot analysis of TsIAP



Discussion

In this chapter we describe the cloning of a novel human IAP protein, TsIAP (also known as BIRC8 (Lagace *et al.*, 2001), ILP-2 (Richter *et al.*, 2001)). Analysis of the *TsIAP* open reading frame reveals a high degree of similarity to that of *XIAP*, with the most significant difference being that *TsIAP* lacks the two amino terminal BIR domains which are present in *XIAP* (Fig 4-2). The coding sequence of TsIAP is very similar to that of the carboxy terminal portion of XIAP, with 80% identity (90% similarity). By contrast the region upstream of the putative translational start sight of TsIAP, while still homologous to XIAP shows a lower level of sequence conservation (73-75%) suggesting that there has been stronger evolutionary pressure to maintain the functional portion of the gene.

Examination of the genomic structure of the *TsIAP* locus reveals the absence of introns. Most gene families are thought to have arisen during evolution by duplication of an ancestral gene, often followed by sequence divergence and ultimately distinct biological functions. In this case, the general intron-exon structure of the gene family should be conserved. This conservation of genomic structure can be seen very clearly with the *HIAP1* and *HIAP2* genes (Young *et al.*, 1999), which are nearly identical in their intron placement. Similarly, the *XIAP* gene locus maintains an overal structure that closely resembles that of *HIAP1* and 2. On the other hand, the lack of introns in the

TsIAP locus strongly suggests that it was generated by the integration into the genome of a fully spliced XIAP cDNA generated by reverse transcription. For the most part retrotransposons generated in this manner are nonfunctional, due to the lack of transcriptional promoter sequences or chromosome position effects, and thus accumulate deleterious mutations throughout the open reading frame as they evolve. However, in some cases retrotransposons have been found to encode functional and essential proteins. One example of this is the phosphoglycerate kinase (PGK) gene family, *pgk-1* and *pgk-2*. PGK is an essential enzyme in the glycolytic pathway. Like *XIAP*, the *pgk-1* gene is located on the X chromosome, which is inactivated during the meiotic phase of male germ cell development. However, the germ cell specific *pgk-2* gene is located on an autosome and activated soon thereafter. Similar to *TsIAP*, the *pgk-2* gene lacks introns and was hypothesized to have evolved as a functional retrotransposon of the X-linked *pgk-1* gene (McCarrey, 1990).

The control of apoptosis plays a crucial role in the development and maturation of spermatozoa (Sinha Hikim & Swerdloff, 1999). It is attractive to postulate that the anti-apoptotic function of an IAP is essential to the developing germ cells and thus, similar to PGK, evolution has provided an alternative gene to fill a necessary role following the inactivation of *XIAP*. It is also possible that the *XIAP* gene is able to escape inactivation on the X-chromosome and that TsIAP plays a unique role in the testis, distinct and complementary to that

performed by XIAP. The latter suggestion seems more likely given our inability to find a murine homologue to the *TsIAP* gene. If XIAP had a critical role in the human Y-bearing sperm one would expect that the murine homologue MIAP-3 would also be necessary and thus would have evolved an autosomal counterpart. *TsIAP*, on the other hand, appears to be limited to humans and to their very close evolutionary counterparts, the great apes. *TsIAP* was detected in human, chimpanzee, and gorilla but could not be isolated from old world monkeys (baboon, cynomolgus monkey, and rhesus monkey) by PCR (Richter *et al.*, 2001). Furthermore, the suggestion that XIAP is dispensable for germ cell development is supported by observation of the lack of gross abnormalities in XIAP-deficient mice (Harlin *et al.*, 2001). On the other hand, there is the possibility that other IAPs are able to compensate for the lack of XIAP. In various tissues in the XIAP knockout mouse, the level of expression of HIAP1 and 2 homologues were elevated. More specific to the testicular role, on a northern blot of rat mRNA, several unique bands appear in the testis when probed with RIAP2 (the rat HIAP2 homologue) coding region (Holcik *et al.*, 2002). Thus, it is conceivable that humans have evolved a unique IAP in the form of TsIAP, while in other mammals other IAPs are able to perform a similar role.

Assuming the role of TsIAP is to substitute for XIAP in the spermatocytes and spermatids following inactivation of the X-chromosome, then clearly it could influence a wide variety of normal and disease effects. Spermatogenesis is a

process that results in the generation of mature sperm cells from primordiary germ cells. As such, regulation of apoptosis plays a prominent role during all stages of sperm development, both to eliminate unwanted cells and to prevent the death of those cells destined to become functional sperm. As of yet, little is known about the regulation of apoptosis during spermatogenesis. Studies with mice transgenic for Bcl-2 and Bcl-X_L under the control of testis specific promoters showed a clear role for the Bcl-2 family in the development of a mature testis, but failed to show significant abnormalities in sperm production or levels of apoptosis in the adult testis (Meehan *et al.*, 2001; Rodriguez *et al.*, 1997). This would suggest genes other than the Bcl-2 family are responsible for the normal apoptotic process that occurs in the adult testis.

These studies increase the family of bacculoviral IAP repeat containing proteins by one to a total of eight human members. In addition to the potential biological significance of its testis-restricted expression, the unique structure of the TsIAP may be useful in better understanding the apoptotic machinery, and in particular the mechanism of action of the original XIAP protein. Finally, TsIAP may represent a novel therapeutic target, both in the case of testicular cancers, and the broader issue of male fertility.

Chapter 5

FUNCTIONAL ANALYSIS OF THE TESTIS-SPECIFIC IAP

Introduction

XIAP functional domains:

The XIAP protein is made up of several distinct functional domains, including a tandem repeat of three BIR domains at the amino terminus, and a carboxy-terminal RING finger domain (see Figure 1-5). Currently, there is no known function for BIR1 (the first BIR domain of XIAP); however, regions encompassing BIR2 specifically target caspases 3 and 7 (Riedl *et al.*, 2001a; Sun *et al.*, 1999), and regions encompassing BIR3 specifically target caspase 9 (Shiozaki *et al.*, 2003). The RING zinc finger domain of XIAP has ubiquitin protein ligase activity and has been shown to promote auto-ubiquitination (Yang *et al.*, 2000), as well as ubiquitination of target proteins including caspase-3 (Suzuki *et al.*, 2001b) and SMAC (MacFarlane *et al.*, 2002).

The XIAP protein, beyond its abilities to function as a multi-caspase inhibitor and ubiquitin ligase, has also been shown to be involved in intracellular signaling. It appears to function as a linker molecule, bridging the bone morphogenic protein receptor with TAB1, an activator of TAK1 and one of the MAP kinase kinase kinase family of signaling molecules (Yamaguchi *et al.*, 1999). This is consistent with the observation that XIAP activates c-Jun N-terminal

kinase 1 (JNK1) (Sanna *et al.*, 1998). Mutated forms of XIAP lacking the linker region between the BIR domains and the RZF domain were unable to activate JNK1 suggesting that the linker region may play an important role.

XIAP – caspase interaction:

The molecular basis for the interaction of XIAP-BIR2 with caspase-3/7 and of XIAP-BIR3 with caspase-9 has been elucidated and is surprisingly distinct. The fundamental mechanism of inhibition of caspases-3 and -7 by BIR2 is the result of restricted substrate access produced by interactions between a region of XIAP N-terminal to the BIR2 domain and the substrate binding cleft of the protease (Huang *et al.*, 2001; Riedl *et al.*, 2001a). The primary interaction between XIAP and caspase 3 involves residues 138-150 of XIAP that bind and block the substrate binding site of caspase 3. The BIR2 domain appears to stabilize this interaction.

The mechanism by which XIAP binds to caspase-9 is entirely different to the manner in which it binds caspase-3 and -7. A pocket in the BIR3 domain of XIAP binds to the small subunit of caspase-9 through a conserved four amino acid sequence at the N-terminus of the cleaved caspase-9 enzyme. Caspase-9 functions in a dimer as part of the 'apoptosome' complex that includes the molecules Apaf-1 and cytochrome-C and it is thought that by binding caspase-9, XIAP is able to sequester caspase-9 in a monomeric form and prevent the formation of the active apoptosome complex (Shiozaki *et al.*, 2003).

XIAP interacting proteins:

Recent studies have led to the identification of several IAP-interacting proteins. SMAC ('second mitochondrial activator of caspases') is a protein that, in non-apoptotic cells, is localized to the inter-membrane space of mitochondria (Du *et al.*, 2000). It is produced as a precursor protein with an amino-terminal 55 residue mitochondrial targeting signal that is proteolytically removed during translocation to the mitochondria. Removal of this leader sequence exposes a short N-terminal peptide sequence remarkably similar to that of the cleaved caspase-9 molecule. Mature SMAC has been found to bind with high affinity to the same pocket in the BIR3 domain of XIAP as caspase-9 and thereby displace caspase-9 from the complex (Liu *et al.*, 2000; Wu *et al.*, 2000). Similar in structure and function to SMAC is another IAP-binding protein, Omi/HTRA2. Omi/HTRA2 is a serine protease that, like SMAC, contains an amino-terminal mitochondrial targeting sequence that is removed to expose a caspase-9-like XIAP binding motif (Hegde *et al.*, 2002; Suzuki *et al.*, 2001a).

Two other XIAP binding proteins that modulate the anti-apoptotic function of XIAP have been identified to date. XIAP-associated factor 1 (XAF1) was identified through a 2-hybrid screening process and has been demonstrated to antagonize XIAP function, possibly through sequestering of the XIAP protein in the nucleus (Fong *et al.*, 2000; Liston *et al.*, 2001). In contrast, ILPIP, also

identified through two-hybrid screening, appears to enhance XIAP mediated protection against Fas and caspase-1 induced apoptosis (Sanna *et al.*, 2002b).

Given the similarities between the sequence of TsIAP and XIAP, we chose to examine the pro- or anti-apoptotic properties of the TsIAP protein. Here we show that TsIAP is capable of inhibiting apoptosis, but only against a more limited subset of apoptotic triggers compared to the full XIAP protein. TsIAP is unable to protect cells from death caused by the chemotoxic drugs etoposide or adriamycin, however it was a potent inhibitor of Bax-induced apoptosis. This most closely mimics the function of the BIR3/RZF portion of XIAP (to which it is homologous), however, significant differences exist with respect to its ability to bind and be affected by SMAC. XIAP, both full length and just the BIR3 fragment are able to bind to SMAC. TsIAP, however, has significant amino-acid substitutions in the conserved binding pocket and we show it is unable to interact with mature SMAC protein. This has a functional significance, as SMAC is able to reverse the protective effects of XIAP on Bax-induced apoptosis, however, it is completely unable to reverse the protection afforded by TsIAP.

Results

Etoposide/Adriamycin Cell Death Assays:

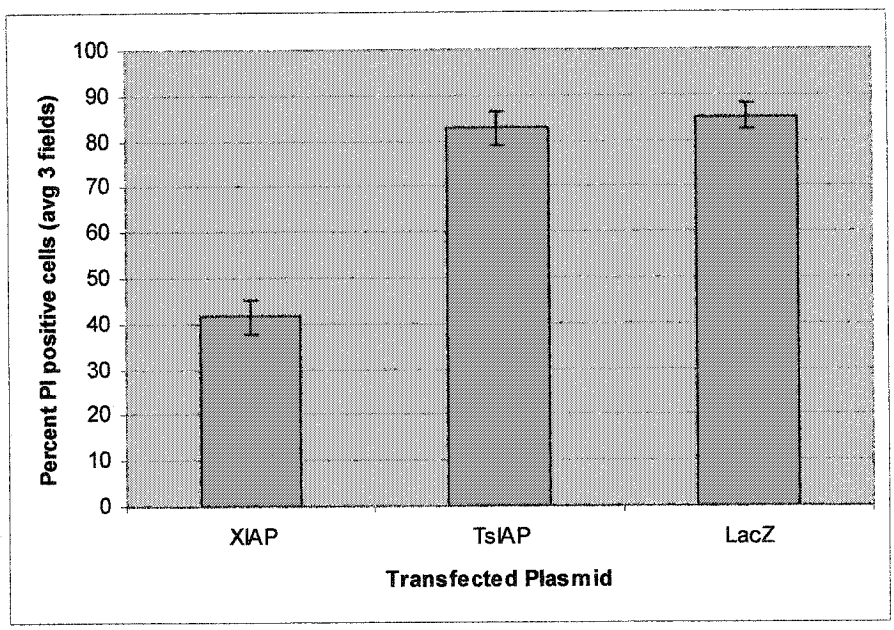
To test the function of the *TsIAP*, we cloned the putative open reading frame by PCR into the pCI vector, a dicistronic GFP vector, and a modified pcDNA3 vector that contains an amino-terminal 6-myc tag. Western blotting of cell lysates of transfected HeLa and probed with anti-*RIAP3* polyclonal antibody showed a non-tagged protein size of approximately 26 kDa, and a myc-tagged size of approximately 39kDa. Two different death assays were performed to test for anti-apoptotic function. The first involved transfection of HeLa cells with pGFP-*TsIAP*, followed by treatment with the drug etoposide. We used pGFP-*XIAP* transfected cells as a positive control for protection and pEGFP-C1 transfected cells as a positive control for cell death. Cell death was visualized by propidium iodide staining and the percentage of surviving cells was determined by counting under an inverted fluorescent microscope. pGFP-*XIAP* showed a significant protective effect versus the pGFP transfected control. No significant protection was observed with pGFP-*TsIAP* (Fig 5-1).

Cell counting can be a cumbersome method when testing multiple samples, so we chose to measure cell proliferation in an assay using the tetrazolium salt WST-1 (2 - (4 - Iodophenyl) – 3 - (4 - nitrophenyl) – 5 - (2,4 - disulfophenyl) - 2H - tetrazolium, monosodium salt). Tetrazolium salts are cleaved by cellular enzymes to form formazan dye, which can then be quantified by measuring the

Figure 5-1. Etoposide cell death assay.

Hela cells were transfected with plasmids expressing XIAP, TsIAP or LacZ (negative control) and exposed to 100 μM etoposide. Cells were stained with propidium iodide and PI positive versus PI negative cells were counted under an inverted fluorescent microscope. Only dying cells take up the PI stain, thus a low percentage of PI positive cells is indicative of protection against apoptosis. The results shown represent the average percentage of PI positive cells counted in 3 fields of view (from separate cell-culture wells) with a minimum of 50 cells per field.

Figure 5-1. Etoposide cell death assay.



absorbance of the dye solution at the appropriate wavelength (Berridge & Tan, 1993). We chose to test out adriamycin (doxorubicin) as a cell death inducing drug for a couple of reasons. First, XIAP had been shown to demonstrate strong protection against cell death induced by adriamycin and secondly, adriamycin, unlike etoposide, is water soluble. Since WST-1 is an indirect method of measuring cell viability, we felt it better to use a cell death trigger that did not require solubilization in DMSO, which could affect cell metabolism independently of drug induced apoptosis. By using adriamycin, we were able to test out a wide range of concentrations without the need for multiple controls to account for varying DMSO concentrations had we used etoposide. Cells were transfected with *XIAP*, *TsIAP*, or *LacZ* plasmid and then treated with various concentrations of adriamycin for 4 hours. WST-1 hydrolysis was measured and normalized against untreated, transfected cells. *XIAP* shows significant protective effects against adriamycin, however, *TsIAP* again showed little to no protection (Fig 5-2).

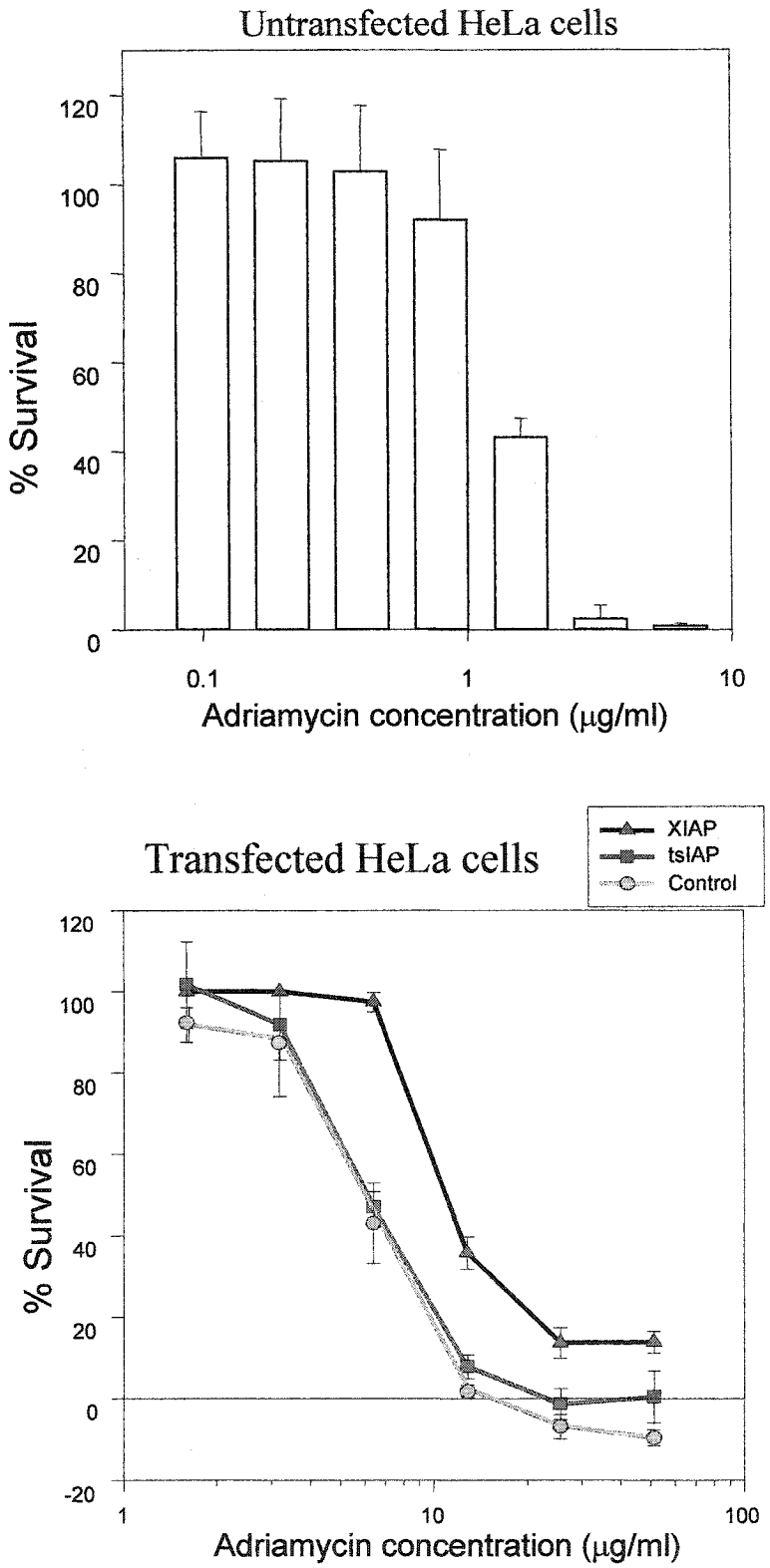
BAX induced cell death assay:

Since the mechanism by which adriamycin induces apoptosis is not fully characterized, we undertook to test out a more defined cell death trigger. Deveraux and colleagues (Deveraux *et al.*, 1999) have shown that the *XIAP* protein is cleaved during the apoptotic process, and that the two fragments produced have distinct anti-apoptotic characteristics. The N-terminal fragment

Figure 5-2. Adriamycin death assays.

Hela cells were treated with varying concentrations of adriamycin (doxorubicin). The upper panel shows a survival curve of untransfected cells. The lower panel shows the survival curves of cells transfected with XIAP, TsIAP, or LacZ (control). Cell death was measured by WST-1 hydrolysis and the death curves were normalized to transfected but untreated (no adriamycin) cells.

Figure 5-2. Adriamycin death assays.



containing the first two BIR domains is a direct and potent caspase3/7 inhibitor. By contrast the C-terminal fragment containing the third BIR domain and the RING zinc finger was not effective at blocking caspase3/7; however, it was shown to be a potent inhibitor of Bax induced apoptosis. Considering the similarities between the BIR3-RZF portion of XIAP and the TsIAP protein we decided to test the anti-apoptotic properties of TsIAP in a bax induced apoptosis system. We co-transfected pGFP tagged BAX into 293 cells with either pcDNA3-6-myc-TsIAP (myc-TsIAP) or a pcDNA3 construct of the BIR3+RZF fragment of *XIAP* (XIAP DB1+2). The myc-TsIAP and the XIAPDB1+2 clones showed significant protection against Bax induced cell death when compared to the negative control (Fig. 5-3).

Caspase 9 inhibition assay:

BAX induces cell death through the release of cytochrome C from the mitochondria, which leads to the formation of the apoptosome and activation of caspase-9 (Adams & Cory, 2002). Since it has been shown previously that the C-terminal portion of XIAP is capable of inhibiting caspase-9 (Deveraux *et al.*, 1999) we decided to test whether TsIAP can function in the same fashion. Pure recombinant caspase-9 activity was very low, presumably due to the need for the formation of the apoptosome for proper activation, thus we chose to use S-100 fractions of transfected cells to compare the inhibitory properties of TsIAP and XIAP. Figure 5-4 shows the results of these experiments. Over the course of

Figure 5-3. TsIAP protects against bax induced apoptosis.

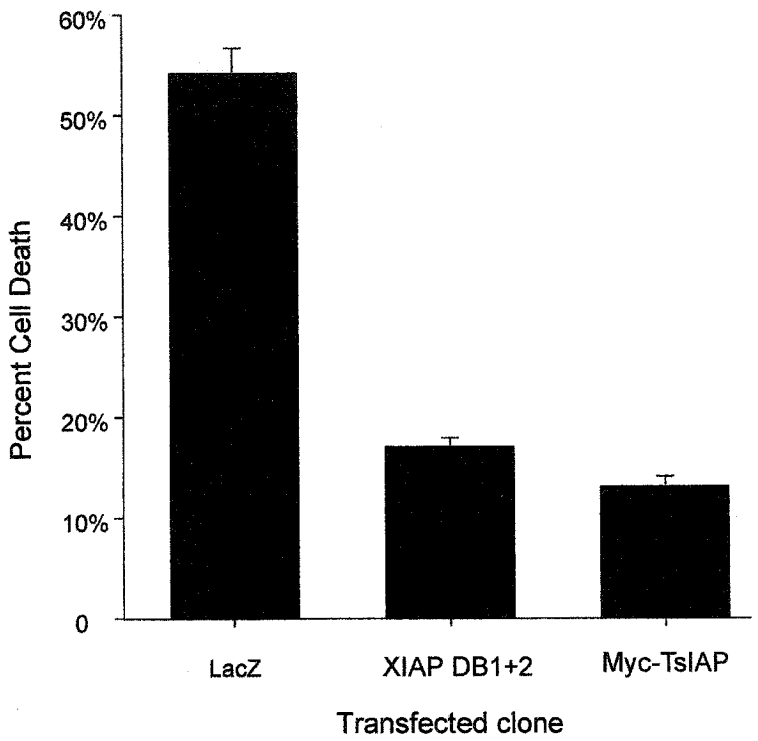
Survival of 293T cells transfected with BAX. 293T cells were transfected with GFP-BAX and co-transfected with either XIAP lacking the first two BIR domains (XIAP DB1+2), or with TsIAP.

(A) GFP-positive cells were examined by flow cytometry and the percentage of live versus apoptotic cells was determined by PI exclusion. The error bars represent standard error of the mean of three samples. TsIAP clearly protects against BAX-induced apoptosis.

(B) Anti-RIAP3 antibody was used to detect the expression of the transfected constructs in a western blot analysis. Clearly detectable levels of both myc-tagged TsIAP (myc-TsIAP) and the XIAP fragment (XIAP DB1+2) was observed.

Figure 5-3. TsIAP protects against bax induced apoptosis.

A.



B.

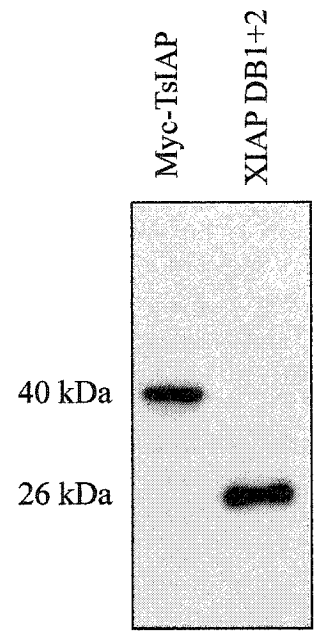
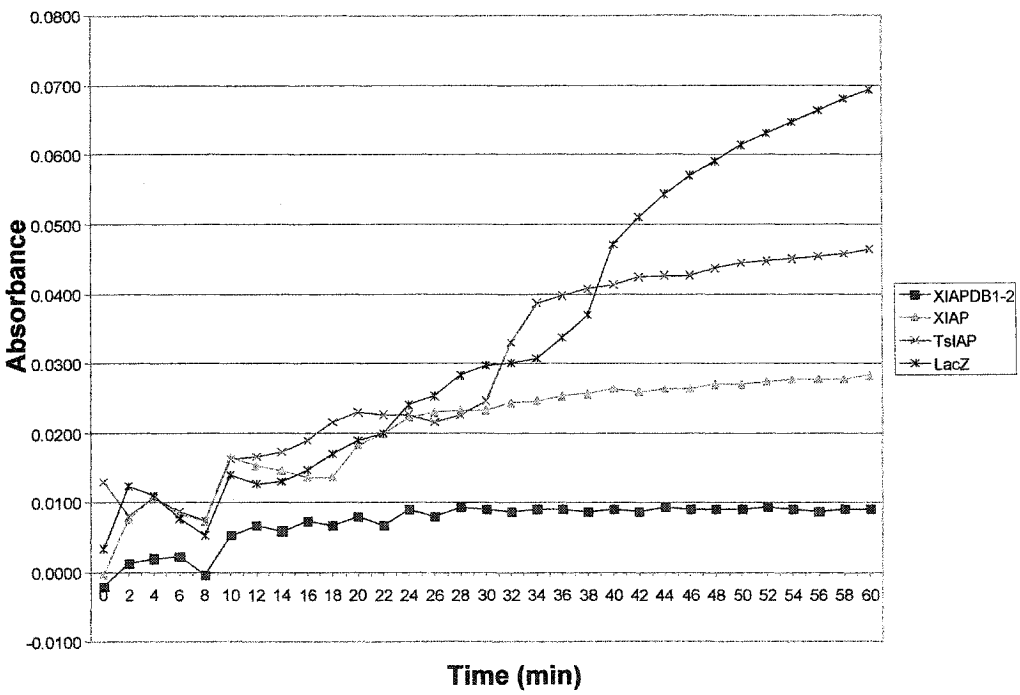


Figure 5-4. Caspase-9 inhibition by XIAP and TsIAP.

Since it has been shown previously that the C-terminal portion of XIAP is capable of inhibiting caspase-9 we decided to test whether TsIAP can function in the same fashion. Caspase activity was assayed at 37 °C in 50 μl of caspase buffer. 30U of recombinant caspase-9 was pre-incubated with varying amounts of the S-100 fractions from transfected and untransfected cells. The reaction was initiated by the addition of 10mM ATP along with 4 mM colorimetric substrate Ac-LEHD-pNA in caspase buffer. The absorbance at 405nm was monitored continuously. Over the course of this assay, XIAPDB1-2 was the most potent inhibitor, however, both TsIAP and full length XIAP also showed an inhibitory effect when compared to the LacZ control

Figure 5-4. Caspase-9 inhibition by XIAP and TsIAP.



this assay, XIAPDB1-2 was the most potent inhibitor, however, both TsIAP and full length XIAP also showed a significant inhibitory effect when compared to the LacZ control ($p < 0.05$).

SMAC interaction:

SMAC (Second Mitochondrial Activator of Caspase) is a recently discovered protein that is released from the mitochondria under apoptotic conditions (Du *et al.*, 2000). It is able to bind to XIAP through interaction between a short 4 amino acid sequence at the amino terminus of processed SMAC that binds tightly into a pocket on the 3rd BIR domain of XIAP (Srinivasula *et al.*, 2001; Wu *et al.*, 2000). This interaction displaces XIAP from caspase-9, permitting activation of caspase-9 and the progression of the apoptotic cascade. To test whether SMAC is able to bind to TsIAP, we performed pull-down experiments using bead-bound IAPs and overexpressed SMAC. SMAC is normally produced in a cell with a mitochondrial targeting sequence at the amino terminus, which gets cleaved off to produce the active SMAC protein. Since the N-terminal amino acids of the processed SMAC are critical to the interaction with XIAP, we used a Ubiquitin-SMAC construct developed by Dr. P. Liston (Hunter *et al.*, 2003) to produce active SMAC protein. Despite repeated attempts, TsIAP was unable to pull-down the active SMAC protein in our experiments (Fig 5-5A). XIAP, HIAP2, and to a lesser extent HIAP1, in contrast show clear binding to mature SMAC protein. On the other hand, TsIAP was capable of binding to the

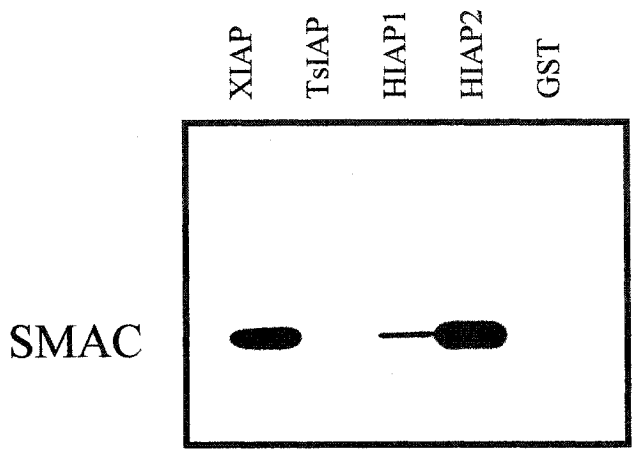
Figure 5-5. Effects of SMAC on TsIAP function.

(A) To test whether SMAC is able to bind to TsIAP, we performed pull-down experiments using bead-bound IAPs and overexpressed SMAC. XIAP and HIAP2 were clearly able to pull down SMAC, but no interaction with TsIAP was detectable.

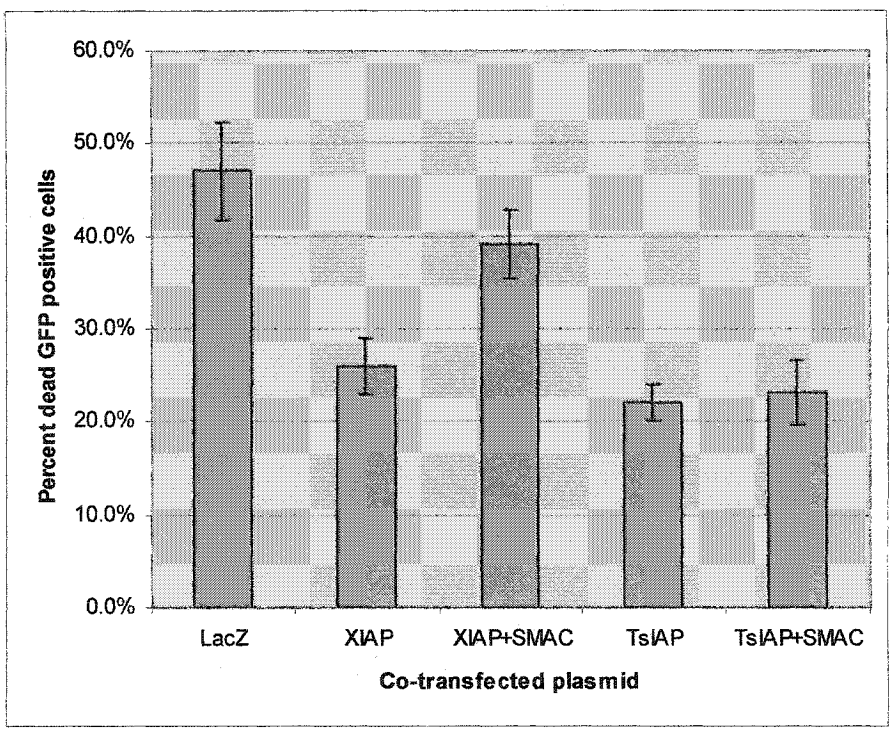
(B) To determine the physiological relevance TsIAP's inability to bind SMAC, we performed Bax cell death assays in the presence and absence of SMAC. XIAP and TsIAP were co-transfected with GFP-Bax alone, or GFP-Bax and SMAC. Cells were stained for cell death using propidium iodide and were analysed using a Coulter EpicsXL flow cytometer. Only GFP-positive (i.e Bax transfected) cells were counted, and the percentage of PI positive (dying) versus PI negative (live) cells is shown.

Figure 5-5. Interaction of TsIAP and SMAC.

(A) SMAC does not bind TsIAP



(B) SMAC does not reverse TsIAP protective effects



XAF1A and XAF1B isoforms of the XIAP inhibitor XAF1 (W.G. Fong, unpublished observations).

To test whether there was a functional significance to the lack of interaction between TsIAP and SMAC, we decided to repeat our earlier BAX-induced cell death experiments, however, this time we co-transfected Ubiquitin-SMAC with the IAPs to determine it affected their anti-apoptotic properties. Interestingly, SMAC was able to reverse the protective effects of XIAPΔB1-2 but had no significant effect on TsIAP protection (Fig 5-5B) suggesting that the mechanism by which TsIAP is able to interefere with BAX induced cell death is distinct from that used by XIAP.

Discussion

XIAP is a potent inhibitor of apoptosis which exerts its effects, at least in part, through the direct binding and inhibition of specific caspases. For example, XIAP has been shown to bind and inhibit the function of active caspase-3, caspase-7 and caspase-9. Caspase-3 and -7 are described as 'effector' caspases, responsible for many of the downstream events associated with apoptosis. The ability to block these enzymes permits XIAP to suppress a wide variety of apoptotic signals, including death receptors such as Fas and DNA-damaging agents such as irradiation and chemotoxic drugs. In this chapter, we describe the characterization of a novel IAP protein, TsIAP. Despite its similarity to XIAP, TsIAP was found to function in a much more restricted manner. Overexpression of TsIAP was unable to protect cells from apoptosis induced by the chemotoxic drugs etoposide and adriamycin (Fig. 5-1). In contrast, it proved to be a potent inhibitor of Bax-induced apoptosis (Fig 5-2).

These results are not entirely unexpected when considered in the context of the different functional domains of XIAP. XIAP consists of three tandem BIR domains, followed by a short spacer region and then a RING zinc finger domain (RZF). One report has suggested that during apoptosis, the XIAP protein is cleaved between the second and third BIR domain resulting in two fragments with distinct anti-apoptotic properties (Deveraux *et al.*, 1999). The N-terminal fragment is a potent inhibitor of caspase-3 induced apoptosis. The crystal structure of the interaction between BIR2 and caspase-3 has been determined (Riedl *et al.*, 2001a), confirming the mechanism by which

that N-terminal fragment functions. The C-terminal fragment interacts directly with caspase-9 (Shiozaki *et al.*, 2003) and is thus able to block cell death triggers that rely on the formation of the apoptosome and activation of caspase-9. Given the similarity in overall structure between the TsIAP protein and the BIR3-RZF fragment of XIAP, it is not surprising to see that the TsIAP protein behaves in a manner very similar to that observed for the XIAP fragment. Similar experiments by another group have confirmed TsIAP's ability to protect against Bax induced apoptosis, and have also shown that it is protective against caspase-9 over expression, while being completely non-protective against Fas induced cell death (Richter *et al.*, 2001). All of these protective effects can likely be attributed to TsIAP's ability to inhibit caspase-9 activity, since we see in a cell-free system that TsIAP overexpression is able to block the activation of caspase-9 (Fig. 5-3). Richter and colleagues have also gone on to show that TsIAP is able to bind to caspase-9, although whether the interaction is direct, or through an intermediary molecule is not known (Richter *et al.*, 2001).

Recent studies have identified an IAP-interacting protein known as SMAC (Du *et al.*, 2000) in humans and DIABLO (Verhagen *et al.*, 2000) in mice. SMAC can be released from the mitochondria, along with cytochrome C in response to apoptotic stimuli. On its release, mature SMAC can bind to XIAP in a manner that displaces caspase-9 and thus enhances apoptosis. Examining SMAC in the context of Bax induced apoptosis shows that overexpressed mature SMAC is able to partially reverse the protective effects of XIAP. In contrast, TsIAP protection is largely unaffected (Fig

5-4). These results suggest a profound difference in the mechanisms by which the two proteins inhibit caspase-9. The structural basis for the interaction between SMAC and BIR3 of XIAP has been determined (Liu *et al.*, 2000; Wu *et al.*, 2000). In brief, the binding of SMAC to XIAP is mediated by the interaction between the N-terminal amino acids of SMAC (AVPI) with a binding groove formed on the surface of the BIR3 domain of XIAP. This short peptide sequence (AVPI) is very similar to that present in human caspase-9 (ATPF), mouse caspase-9 (AVPY), and the drosophila proteins Reaper (AVAF), and HID (AVPF). It is postulated that SMAC functions by displacing caspase-9 from that binding pocket, thus allowing activation of the caspase.

A comparison between the primary sequence of XIAP and TsIAP, however, shows some significant changes in the amino acids responsible for forming the binding pocket on BIR3. Of the 8 amino acids in closest proximity to the binding pocket, 5 are identical between XIAP and TsIAP; lysine 299 is changed to glutamine; threonine 308 is changed to alanine; and aspartic acid 309 is changed to asparagine. In contrast, all of the critical amino acids that define a BIR domain ($RX_{20-23}GX_{11}CX_2CX_{16}HX_6C$) are perfectly conserved. Clearly the changes in the SMAC binding groove on the BIR domain of TsIAP abolish any interaction between TsIAP and SMAC (Fig 5-4), however, this does not appear to affect the ability of TsIAP to inhibit caspase-9 activity (Fig 5-3). Since caspase-9 uses the same 4-amino acid motif to interact with the binding pocket of XIAP it begs the question: How does TsIAP inhibit caspase-9 without apparently having a functional binding pocket?

Crystallographic analysis of the catalytic domain of caspase-9 (residues 139-416) in an inhibitory complex with XIAP-BIR3 provides some insight (Shiozaki *et al.*, 2003). The XIAP-BIR3 domain forms a heterodimer with one caspase-9 monomer. In the complex the BIR domain forms a large continuous interface with caspase-9, resulting in the burial of 2200 $Å^2$ of exposed surface area. Thus, a large portion of the BIR3 domain of XIAP is involved in the interaction with caspase-9 and it is conceivable that the ATPF interaction with the binding pocket is but a small fraction of the forces that allow binding. In contrast to the manner in which BIR2 inhibits caspase-3, the binding of BIR3 to caspase-9 does not block the substrate binding cleft of caspase-9, but rather holds it in an inactive conformation. With BIR2, the 'hook-line-sinker' model requires that key amino acids are present to bind in an anti-sense orientation through the active site of caspase-3 (Huang *et al.*, 2001; Riedl *et al.*, 2001a). The BIR domain in this case merely provides additional structural support. In the caspase-9/BIR3 interaction, there are no key amino acids that block the binding groove, but rather a large surface interaction that prevents homodimerization of caspase-9 and thus maintains the enzyme in an inactive conformation. In light of the TsIAP data, it seems likely that the binding pocket in the BIR3 domain of XIAP is, in fact, more critical to the interaction of SMAC with XIAP than it is to the proper blocking of caspase-9 function.

Chapter 6

GENERAL DISCUSSION AND CONCLUSIONS

The regulation of cell number is a crucial property of complex multicellular eukaryotes, and elegant mechanisms to modulate the rates of both cell division and cell death have evolved. Programmed cell death can be seen throughout the life of an organism and is required for diverse roles in both early development and adult life. Disruption of the balance between cell growth and cell death components results in numerous pathological consequences, including cancers, neurodegenerative disorders, and pathology associated with various infectious organisms (Thompson, 1995).

The biochemical basis upon which cells make their life and death decisions has been the subject of much scrutiny. Initial characterization of genes that regulate cell numbers focused primarily on those that control cell growth and differentiation, however, the process of cell death is certainly not a new phenomenon. The concept of "apoptosis" was first put forth in the early 1970s, along with a careful description of the morphology associated with apoptosis versus trauma-induced cell death known as necrosis (Kerr *et al.*, 1972). It was not until the late 1980s, however, with the discovery of the *B-cell lymphoma-2 (bcl-2)* gene that the idea of impaired apoptosis as a crucial step in tumorigenesis was truly embraced (Cory *et al.*, 1999).

Following the discovery of Bcl-2, numerous inhibitors of apoptosis were described - primarily of viral origin, having evolved as mechanisms to evade the host immune response to infection. The initial member of the Inhibitor of Apoptosis (IAP) family of proteins was discovered by Crook and colleagues based on their ability to restore wild-type replication to a mutant strain of AcMNPV (Crook *et al.*, 1993). The first mammalian IAP, NAIP, was discovered in a positional cloning effort to isolate the causative mutation in spinal muscular atrophy (Roy *et al.*, 1995). Following the discovery of NAIP we isolated several other members of the mammalian IAP family of genes, including Human IAP 1 (HIAP1), Human IAP 2 (HIAP2), and the X-linked IAP. This thesis documents the genetic characterization of the X-linked IAP gene and the subsequent discovery and characterization of a previously unknown member of the human IAP gene family.

At the time this project was started, knowledge of the genetic structure of the *X-Linked IAP* was limited to a single cDNA clone spanning the coding region and extending less than 500 base pairs into the 3' UTR (Liston *et al.*, 1996). Given the importance of apoptosis to normal and diseased cell processes we undertook the task of cloning and sequencing the complete *XIAP* gene in an effort to better understand its genetic regulation and gene structure. Initial Southern blot analysis showed multiple bands present in human genomic DNA digested with *EcoRI* and probed with XIAP coding region DNA (Figure 3-1). Only two of those bands, however, displayed any difference in intensity between male (1X) and female (2X) DNA. This suggested that

there are several regions of the human genome that are cross-reactive with the XIAP cDNA probe used. This is not unprecedented in the IAP family, as the NAIP locus is also complex with up to 6 truncated copies of NAIP present (Roy *et al.*, 1995; Yaraghi *et al.*, 1998). Genomic library screening was complicated by the presence of the cross-reactive regions, however, after multiple rounds of screening an overlapping contiguous array of phage clones was obtained that covered the entire known coding region of the gene (Figure 3-3). The phage were subcloned and sequenced using a transposon-facilitated sequencing technique (Strathmann *et al.*, 1991) that permitted the large scale sequencing required to cover the entire genomic region.

Since the completion of our sequencing efforts, the human genome project (Lander *et al.*, 2001) as well as the private human genome sequencing effort by Celera Genomics (Venter *et al.*, 2001) both released sequence covering the XIAP genomic region. There were no differences over the entire 33 kb region of DNA we sequenced and that released by the Human Genome Project.

Northern blot analysis of XIAP revealed the presence of a 10 kb transcript in virtually all tissues tested, with the exception of peripheral blood leukocytes (PBLs) (Figure 3-2). It is unclear why PBLs would lack XIAP expression by northern blot. It is possible that the cells of the immune system are more sensitive to, or primed to undergo apoptosis due to the lack of XIAP expression. On the other hand, it is more likely that other IAPs are able to compensate, as high levels of HIAP1 and HIAP2

expression are seen in the cells of immune origin (Young *et al.*, 1999). Another possibility is that *XIAP* is expressed at levels too low to detect by northern blot. Indeed, by western blot analysis, the mouse expresses detectable levels of XIAP in T and B cells (Conte *et al.*, 2001) and thymocytes (Yang *et al.*, 2000).

The region upstream of the transcription start site in *XIAP* provides few clues to the transcriptional control of the *XIAP* gene. Other IAPs are subject to tight transcriptional control, such as the cell-cycle dependant transcription of *survivin* (Ambrosini *et al.*, 1997; Li *et al.*, 1998a), or the tissue specific expression pattern of *HIAP1* and *2* (Young *et al.*, 1999). The region upstream of the transcriptional start site of *XIAP* shows generic low-level promoter activity (Figure 3-4), but no obvious transcription factor binding sites were detected when the region was searched using the TRANSFAC database of transcription factor binding sites (Matys *et al.*, 2003; Wingender *et al.*, 2000). It is likely that elements further upstream from the region we tested can modulate XIAP expression levels, as XIAP levels have been seen to be upregulated by NF-κB (Stehlik *et al.*, 1998; Tang *et al.*, 2001). It is also possible that regions within the first intron have some regulatory role, as it is common for 5' introns to facilitate transcription (Kozak, 1991). It is also clear now that post-transcriptional features regulate differential expression of XIAP in those tissues that require more or less XIAP protein. The extensive 5' untranslated region (UTRs) present in the mRNA has been shown to possess an internal ribosome entry site (IRES) in the 5' UTR that is

able to enhance protein expression under cellular stress conditions (Holcik *et al.*, 1999; Holcik *et al.*, 2000).

The *XIAP* cross reactive regions of the genome all appear to code intronless copies of the *XIAP* gene with varying degrees of sequence conservation. The presence of these *XIAP* homologous regions would be consistent with a reverse transcription event occurring with *XIAP* message and re-integration into the chromosome. Of particular interest is that one of these intronless genes appears to have maintained an open reading frame and is expressed at the mRNA level in the testis (Figure 3-2, 4-5). This phenomenon appears to be limited to the human *XIAP* gene, as there is little evidence of intronless *XIAP*-like retrotransposons in the mouse (Farahani *et al.*, 1997).

The presence of a significant open reading frame in one of the *XIAP* 'pseudogenes' was investigated further. Using a probe derived entirely from unique, non-XIAP related sequence we probed a multiple tissue northern blot and saw a unique band of roughly 2kb in size was picked up in the testis lane. This band corresponded to the smaller transcript seen in the testis lane of the multiple tissue Northern blot that had been probed with *XIAP* coding region sequence and thus confirmed that it is the product of a unique gene rather than a splice variant of *XIAP*. We called this gene the *Testis-specific IAP (TsIAP)* for its unique expression pattern.

Given the unusual 5' UTR present in the *TsIAP* message, there was the distinct possibility that *TsIAP* could not be translated into a protein. The 5' UTR of *TsIAP*

maintains some homology with the 2nd BIR domain of XIAP, as well as possessing multiple upstream AUG codons prior to the TsIAP open reading frame, suggesting that translation may be impaired or completely abrogated. Western blot analyses of both transfected and endogenous TsIAP however, have confirmed that the mRNA is able to produce a protein.

The control of apoptosis plays a crucial role in the development and maturation of spermatozoa (Sinha Hikim & Swerdloff, 1999). It is attractive to postulate that XIAP's anti-apoptotic function is essential to the developing germ cells and thus, similar to other essential genes like PGK (McCarrey, 1990), evolution has provided an alternative gene to fill a necessary role following the inactivation of *XIAP* on the X-chromosome. It is also possible that the *XIAP* gene is able to escape inactivation on the X-chromosome and that TsIAP plays a unique role in the testis, distinct and complementary to that performed by XIAP. The latter suggestion seems more likely given our inability to find a murine homologue to the *TsIAP* gene. If XIAP had a critical role in the human Y-bearing sperm one would expect that the murine homologue MIAP-3 would also be necessary and thus would have evolved an autosomal counterpart. *TsIAP*, on the other hand, appears to be limited to humans and their very close evolutionary counterparts the great apes. *TsIAP* was detected in human, chimpanzee, and gorilla but could not be isolated from old world monkeys (baboon, cynomolgus monkey, and rhesus monkey) by PCR (Richter *et al.*, 2001). Further suggesting that XIAP is dispensable for germ cell development is the lack of

gross abnormalities in XIAP-deficient mice (Harlin *et al.*, 2001). On the other hand, there is the possibility that other IAPs are able to compensate for the lack of XIAP. In various tissues in the *XIAP* knockout mouse, the level of expression of *HIAP1* and *2* homologues were elevated. More specific to the testicular role, on a northern blot of rat mRNA, several unique bands appear in the testis when probed with RIAP2 coding region (Holcik *et al.*, 2002). Thus, it is conceivable that humans have evolved a unique IAP in the form of TsIAP, while in other mammals other IAPs are able to perform a similar role.

Further suggesting the unique role for TsIAP are the results of the functional assays. Despite its similarity to XIAP, TsIAP was found to function in a much more restricted manner. Overexpression of TsIAP was unable to protect cells from apoptosis induced by the chemotoxic drugs etoposide and adriamycin (Fig. 5-1). Likewise, Richter and colleagues showed that TsIAP was unable to protect against Fas-mediated apoptosis (Richter *et al.*, 2001). In contrast, it proved to be a potent inhibitor of Bax-induced apoptosis (Fig 5-2). Given the similarity in overall structure between the TsIAP protein and the BIR3-RZF fragment of XIAP, it is not surprising to see that the TsIAP protein behaves in a manner very similar to that observed for the BIR3/RZF portion of XIAP. XIAP BIR3/RZF is able to interact with and directly inhibit caspase-9. Both XIAP, and TsIAP were able to inhibit caspase-9 activity as measured in an in-vitro caspase assay system. Thus, since Bax-induces cell death through the mitochondrial pathway leading to release of cytochrome C and activation

of caspase-9, it is not surprising to see both XIAP and TsIAP able to inhibit cell death caused by Bax overexpression.

On the other hand, XIAP is able to bind caspase-9 through the interaction between the N-terminal 4 amino acids of the small subunit of caspase-9 with a pocket formed in the BIR3 domain of XIAP (Shiozaki *et al.*, 2003). This 'binding pocket' in the BIR3 domain of XIAP is also responsible for XIAP's interaction with the inhibitor molecules SMAC and Omi (Chai *et al.*, 2000; Verhagen & Vaux, 2002; Wu *et al.*, 2000). Smac and Omi are thought to function by displacing caspase-9 from XIAP, thus sequestering XIAP and permitting formation of the apoptosome and activation of the caspase molecule. In pull-down experiments, however, we were unable to show an interaction between the TsIAP protein and the mature SMAC protein (Fig 5-5), suggesting that binding does not occur. Indeed, while the amino acids crucial to the formation of the BIR and RZF domains in TsIAP are perfectly conserved, there are significant changes in the amino acids responsible for forming the binding pocket when compared to those in the BIR3 domain of XIAP. Functionally, where SMAC is able to reverse the protective effects of XIAP, it is unable to do so for TsIAP (Fig 5-5), further confirming that SMAC and TsIAP do not associate. Thus, the mechanism by which TsIAP is able to inhibit apoptosis poses a paradox. If it is given that XIAP (and by homology TsIAP) inhibit Bax-induced apoptosis through the interaction with caspase-9, and that SMAC uses the same mechanism as caspase-9 to bind to XIAP, then how does TsIAP inhibit apoptosis without the ability to bind mature SMAC? The answer to

this will likely have to wait until detailed structural analysis can be performed on the interactions between TsIAP and members of the cellular apoptosis machinery, but it does pose some intriguing questions as to the mechanisms by which XIAP is able to block apoptosis.

APPENDIX 1

Here follows the Clustal W (1.82) multiple sequence alignment of *XIAP* cDNA with sequence obtained from 3 of the *XIAP* cross-reactive bands seen on a Southern blot of genomic DNA digested with EcoRI (Fig 3-1A). *XIAP* sequence is numbered from the start of transcription site of the *XIAP* gene. The remaining sequences are numbered from the start of the region of homology with *XIAP*. 3' and 5' regions of XIAP where there is no homologous pseudogene have been cut out to save space. Asterisks underneath the alignment indicate conserved nucleotides.

```
CLUSTAL W (1.82) multiple sequence alignment

4.3kb           ------------------------------------------------------------
1.8kb           ------------------------------------------------------------
XIAP            GAAATCCATCCATGGCAGATTATGAAGCACGGATCTTTACTTTTGGGACATGGATATACT 2520
TsIAP           ------------------------------------------------------------

4.3kb           -------CAAGGAGCAGCTTTCAAGAGCTGGATTTTATGCTTTAG-GTGAAGGTGATAAA 52
1.8kb           ----------------------------TACACTG-GCTTTAG-GTGAAAGTGATAAA 28
XIAP            CAGTTAACAAGGAGCAGCTTGCAAGAGCTGGATTTTATGCTTTAG-GTGAAGGTGATAAA 2579
TsIAP           --------------------CAACTACACACGTGTGTGTGCGCGTGTGTATAAAACACA 39
                                                 *  *     * *** *    * * *

4.3kb           GTAAAGTGCT-TTCACTGTGGAGGGGGGCTAACTGATTGGAAGCCCAGCGAAGACCCTTG 111
1.8kb           GT-----GCT-TTCACTGTGGAGGAGGGCTAACTGATTGGAAGCCCAGTGAAGACCCTTG 82
XIAP            GTAAAGTGCT-TTCACTGTGGAGGAGGGCTAACTGATTGGAAGCCCAGTGAAGACCCTTG 2638
TsIAP           GTGCACTAATACTCAGCCTTTAAAAAAAATG-CCACTTGCAACAACGTAGATGGAGCTGG 98
                **       *  ***   *  *       *  *   *** **   *   ** *   ** *

4.3kb           GGAACAACATGATAAATGGCATCCAGGGTGTAAATATCTGTTAGAACAGAAGACACGAAA 171
1.8kb           GGAACAACATGCTAAATGGTATCCAGGGTGTAAATATCTGTTAGAACAGAAGGGACAAGA 142
XIAP            GGAACAACATGCTAAATGGTATCCAGGGTGCAAATATCTGTTAGAACAGAAGGGACAAGA 2698
TsIAP           ACGATATCATGCTAAA-ATTATGCAAAGTG-AAACAAGCACAAAAAAGAACGAGACACGG 156
                   * * **** ****    ** **  *** *** *      * **   * *  **

4.3kb           ATATA---TAAACAATATTCATTTAT-----CCCATTCACTTGAGGAGTGTCTGGTAAGA 223
1.8kb           ATATA---TAAACAATATTCATTTAA-----CTCATTCACTTGAGGAGTGTCTGCTAAGA 194
XIAP            ATATA---TAAACAATATTCATTTAA-----CTCATTCACTTGAGGAGTGTCTGGTAAGA 2750
TsIAP           GCGTGGGGCACGAGGTGCTCACTGGCAAGCGCCCACTCCACCGCGTGGTTTCCAGCTGGA 216
                   *     *     *  *** *        * ** **    * *  ** **      **

4.3kb           ACTGCTGAAAAAACGCCATCACTAACTAGAAAAATTGAT---ACCATCTTCCATAATCCT 280
```

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1.8kb       ACTACTGAGAAAACACCATCACTAACTAGAAGAATTGATGATACCATCTTCCAAAATCCT 254
XIAP        ACTACTGAGAAAACACCATCACTAACTAGAAGAATTGATGATACCATCTTCCAAAATCCT 2810
TsIAP       G--GCTGGGA--GCGTTGTGGCTTCCTCTTTTC-TTGCT-----GACCCTTCGGAGCTCT 266
                ***  *   *    *  **  **       *** *      * * * *  *   **

4.3kb       ATGGTACAAGAAGCTATATGAATGGGGTTCAGTTTCAAAGACATTAAGAAAATAATGGAG 340
1.8kb       ATGGTACAAGAAGCTATATGAATGGGGTTCAGTTTCAAGGATGTTAAGAAAATAATGGAG 314
XIAP        ATGGTACAAGAAGCTATACGAATGGGGTTCAGTTTCAAGGACATTAAGAAAATAATGGAG 2870
TsIAP       GGG----AAGTGGCTGCACCTTGGCGGCTC--CCCAGAGCGCGCGGTGCTAATCGTGG-G 319
              *    ***  ***  *     * ** **       *         *  ***  *** *

4.3kb       GAAAAAATTCAGACATCTGGGAGCAACTGTAAATCACTTGAGGTTCTGATTGCAGATCCA 400
1.8kb       GAAAAAATGCAGATATCTGGGAGCAACTATAAATAACTTGAGGTTCTGGTTGCAGATCTA 374
XIAP        GAAAAAATTCAGATATCTGGGAGCAACTATAAATCACTTGAGGTTCTGGTTGCAGATCTA 2930
TsIAP       TCGTCAGCCTGGGTGGCTGGGCCCGGCT-TAGGGCA---GGGTTTGGCATTTCCAATG-G 374
                 *     *    *****  *  ** **    *   * * **    ** *  **

4.3kb       GTGAAGGCTCAGAAAGACAGTACACAAGACGAATCAAGTCAGACTTCATTGCAGAAAGAG 460
1.8kb       GTGAATGCTCAGAAAGACAGTACACAAGATGAGTCAAGTCAGACTTCATTACAGAAAGAG 434
XIAP        GTGAATGCTCAGAAAGACAGTATGCAAGATGAGTCAAGTCAGACTTCATTACAGAAAGAG 2990
TsIAP       TAGGGGGCTCGGACCGTCCCTCCGCGGGACCCTCCCGTTGGGACAAGGCCGATCGCCTGG 434
              *   **** **  * *  *   *  **     *   *  ***                *

4.3kb       ATTAGTACTGAAGAGCAGCTAAGACACCTGCAAGAGGAGAAGCTTTGCA---AAATCTGT 517
1.8kb       ATTAGTACTGAAGAGCAGCTAAGGCACCTGCAAGTGGAGAAGCTTTGCA---AAATCTGT 491
XIAP        ATTAGTACTGAAGAGCAGCTAAGGCGCCTGCAAGAGGAGAAGCTTTGCA---AAATCTGT 3047
TsIAP       GCGGTTG--GAGCCGCTATCCTGGCGCGAGACGGTGGACAAGTCCTATATTCAAGAGAAG 492
                 *   **   **      * * *  *   * *** ***   *  *   **

4.3kb       ATGGATAGAAATATTGCTGTCGTTTTTATTCCTTGTGGACATCCAGTCACTCGTAAACAA 577
1.8kb       AGGGATAGAAATATTGCTATCGTTTTTGTTCCTTGTGGACATCTAGTCACTTGTAAACAA 551
XIAP        ATGGATAGAAATATTGCTATCGTTTTTGTTCCTTGTGGACATCTAGTCACTTGTAAACAA 3107
TsIAP       ATAACTTTGAACAGTTTCGAAGGATCTAAAACGTATGTGTCTGCAGACA----TCAATAG 548
            *    *   ** * *      *  * *    * * **    *  ** **    * ** *

4.3kb       TGTGCTGAAGTGGTTGACAAATGTCTCAAGTGGTACGCAGTCATTACTTTCAAGCAAAAA 637
1.8kb       TGTGCTGAAGCAGTTGACAAGCGTCCCATGTGCTACACAGTCATTACTTTGAAGCAAAAA 611
XIAP        TGTGCTGAAGCAGTTGACAAGTGTCCCATGTGCTACACAGTCATTACTTTCAAGCAAAAA 3167
TsIAP       GATGAAGAATTAGTAAAGAGAT-TAATAGATCAAAAACGTTTGCTGGCTTTGCAGGTGGT 607
              **  ***   **  * *    *   *  *   *  *  *   *   **

4.3kb       AATTTTATGTCTTAATCTA-ACGCTATAGTAGGCATATTATGTT---CGTATTATCCTGA 693
1.8kb       ATTTTTATGTCTTAATCTA-ACTCTATAGTAGGCATGTTATGTTGTTCTTATTACCCTGA 670
XIAP        ATTTTTATGTCTTAATCTA-ACTCTATAGTAGGCATGTTATGTTGTTCTTATTACCCTGA 3226
TsIAP       GGGCCTGCCTGGGCATCGGCGCGTTGGAGGAGACGCCCTGGGGGGCCTTAGCTGCCCTGA 667
                 *   *    ***    *  *  ** ** *    *  *          *  *****

4.3kb       TTGAATGTGTGATGTGAACTGACTTTAAGTAATCAGGATTGAATTCCATTAGCATTTGCT 753
1.8kb       TTGAATGTGTGATGTGAACTGACTTTAAGTAATCAAGATTGAATTCCATTAGCATCTGCT 730
XIAP        TTGAATGTGTGATGTGAACTGACTTTAAGTAATCAGGATTGAATTCCATTAGCATTTGCT 3286
TsIAP       ---AGCGGTAGACAGGTGGCAACGTGGGGGCTCAGGAGTTGACAAACACAAGAAAGCAGC 724
              *  *   **   *     ** *   *         ****    **  ** *

4.3kb       ACCAAGTAGGAAAAAAAAATGTAAATGGCAGTGTTTTAGTTGGCAATATAATCTTTGAAT 813
1.8kb       ACCAAGTAGGAAAAAAAA-TGTACATGGCAGTGTTTTAGTTGGCAATATAATCTTTGAAT 789
XIAP        ACCAAGTAGGAAAAAAAA-TGTACATGGCAGTGTTTTAGTTGGCAATATAATCTTTGAAT 3345
TsIAP       GCCGAATTGCAGGTTTATCCGCAGCTTTTATT-TTGAAGACAGTGCCACGAAACCTGCAA 783
             ** * * * *     *   * *  *   * * **  **   *    *  *    ** *

4.3kb       TTCTGGATTTTTCAGT-TATTAGCTGTCTTATTTATCCAATTTTTTTTTACTGTTATTTAA 872
1.8kb       GTCTGGATTTTTCAGGGTATTAGCCATATTATCCAT------TTTTTTACTGTTAATTAA 843
XIAP        TTCTTGATTTTTCAGGGTATTAGCTGTATTATCCAT-----TTTTTTTACTGTTATTTAA 3400
TsIAP       ATCCTGGTGTCCCAAA-TAGTCAATACCAAGTTGAAA-ACCATCTGGGAGAGGAAAAGCG 841
```

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                 **  * * *  **   ** *          *  *       * *   *  *  *

4.3kb            TTGAAACCCTAGACTAAGAA------GCATCATATT-ATAACTGATCACAATGTGTATTC 925
1.8kb            TTGAAACCATAGACCAAGAA------GCATCATATT-ATAACTGAACGCAACGTGTATTC 896
XIAP             TTGAAACCATAGACTAAGAATAAGAAGCATCATACT-ATAACTGAACACAATGTGTATTC 3459
TsIAP            TTGTG-CTTTAGAC-AGGCCGTATGAGACTCGTGCAGACCGGCTTTTGAGAGCTGGACAG 899
                 ***   *  ***** * *        *  ** *    *            *  ** *

4.3kb            ATAGTATATTGACTTAATTTC-TAAGTGTAAGTGAATTAATCATCTG-AATTTTTTATT- 982
1.8kb            ATAGTATACTGATTTAATTTC-TAAGTGTACATGAATTAGTCATCTG-GATTTTTTATTC 954
XIAP             ATAGTATACTGATTTAATTTC-TAAGTGTAAGTGAATTAATCATCTG-GATTTTTTATTC 3517
TsIAP            GTGGTGGATAGATCAGACTCCATACACCCGAGGAGCCCCGCCATGCATAGTGAAGAAGCT 959
                  * **  *  **    * * * **                   ***      *     *

4.3kb            --TTCAGATAGGCTTAACAAATAGAACATTCTGTATATAAATGTGTAGATTAGAGTTAAT 1040
1.8kb            TTTTCAGATGGGCTTAACAAATGGAGCTTTCTGTATATAAATGTGGAGATTAGAGTTAAT 1014
XIAP             TTTTCAGATAGGCTTAACAAATGGAGCTTTCTGTATATAAATGTGGAGATTAGAGTTAAT 3577
TsIAP            AGATAACAGTCGTTTCACAACTGGC-CAGCCTCTGCCCACTTGACCCCGAGAGAGCTGGC 1018
                    * * *   * ** **** * *  *   ** *    *   *        **** *

4.3kb            CTTTCCAATCACATAATTCGTTTTATGTGAAAAAGGAATGAACTG-TTCCATGCTGGTGG 1099
1.8kb            CTCCCCAATCACATAATTTGTTTTGTGTGAAAAACGAATAAATTG-TTCCACGCTGGTGG 1073
XIAP             CTCCCCAATCACATAATTTGTTTTGTGTGAAAAAGGAATAAATTG-TTCCATGCTGGTGG 3636
TsIAP            CAGTGCTGGGCTGTACTACACAGGCACTGATGACCAAGTGCAGTGCTTCTGTTGTGGCGG 1078
                 *    *       ** *          ***  *   * *  * ** ***     *** **

4.3kb            AAAGATAGAGATTATTTTTAGAGGTTTGTCGTTGTGTTTTGGGATTCTGT-----TTTCT 1154
1.8kb            AAAGATAGAGATTGCTCTTAGAGGTTGGTTATTGTGTTT-AGGATTCTGT-----CCATT 1127
XIAP             AAAGATAGAGATTGTTTTTAGAGGTTGGTTGTTGTGTTTTAGGATTCTGTCC-ATTTTCT 3695
TsIAP            AAAACTGAAAA----ACTGGGAACCTGGTGATCGTGCCTGGTCAGAACACAGGAGACATT 1134
                 ***  *  * *      *  **   * **  * ***  *    *                *

4.3kb            TTTAAAATTGTAAATATGTACTTGTGTGAATGATTTTTTAAAATGATTTTACCATTTTTG 1214
1.8kb            TTC----TTCT---TTTTTTCTTTTTTTTTATGATTATT----ACACTTTAAGTTTTAGGG 1176
XIAP             TTTAAAGTTATAAACACGTACTTGTGCGAATTATTTTTTTAAAGTGATTTGCCATTTTTG 3755
TsIAP            TTCCTAATTGCT--TCTTTATTTTGGGCCACAACGTTAATATTCGAGGTGAATCTGATGT 1192
                 **     **         *  **      *  *   *           *     *

4.3kb            GAAGGGTATTTAATGATAGAATATCATCGAGCCAACATGCACTGACATAGAAAGATGTCA 1274
1.8kb            TATGTGTGCACAACGTGCAGGTTT---------------------------------- 1200
XIAP             AAAGCGTATTTAATGATAGAATACTATCGAGCCAACATGTACTGACATGGAAAGATGTCA 3815
TsIAP            TGCGAGT-TCTGATA--GGAATTTCTCAAATTCAACAAGTTCT--CCAAGGAATCCATCC 1247
                    * **     *        *

4.3kb            AAGATATATTAAGTGTAAAATGCAAGAGGGAAAACACTATGTACAGTCTGAGCCAAATCA 1334
1.8kb            ------------------------------------------------------------
XIAP             AAGATATGTTAAGTGTAAAATGCAAGTGGCAAAACACTATGTATAGTCTGAGCCAGATCA 3875
TsIAP            ATGACGGGTTATGAAGCCCGGCTCATTACTTTTGGGACATGGATGTACTCCGTCAACAAA 1307

4.3kb            AAGCATGTATGTTTTTTATATGTGTACAACAAAAGGTTTGGAAAGATATGCACCAAATTG 1394
1.8kb            ------------------------------------------------------------
XIAP             AAGTATGTATGTTTTTAATATGCATAGAACAAAAGATTTGGAAAGATATACACCAAACTG 3935
TsIAP            GAGCA-GCTTGCAAGAGCTGGATTTTATGCTATAGGTCAAGAGGATAAAGTACAGTGCT- 1365

4.3kb            TTAAATGTGGTTTCACTT-------GAGGGGGTGGGAGGATGGGCCCCAGAGGGGTTTTT 1447
1.8kb            ------------------------------------------------------------
XIAP             TTAAATGTGGTTTCTCTTCGGGGAGGGGGGGATTGGGGGAGGGGCCCCATAGGGGTTTTA 3995
TsIAP            TTCACTGTGGAGGA-----------GGGCTAGCCAACTGGAAGCCCAAGGAAGATCCTTG 1414

4.3kb            ATAGGGGCCTTTCACTTGGTATTTTTTTCATTTTGTTCTGTTTGAAATTTTGTTTTTTCT 1507
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1.8kb          ------------------------------------------------------------
XIAP           TAGGGGCCTTTTCACTTTCTACTTTTTTCATTTTGTTCTGTTCGAATTTTTTATAAGTAT 4055
TsIAP          GGAACAGCATGCTAAATGGTATCCAGGTTGCAAATATCTGCTAGAAGAGAAGGGACATGA 1474

4.3kb          TTTTA-AATGGAGTTTCACTCTTGTCGCCTAGGCTGCAATGTAGTGGCGTGAA---CTCA 1563
1.8kb          ------------------------------------------------------------
XIAP           GTATT-ACT---TTTGTAATCAGAATTTTTAGAAAGTATTTTGCTGATTTAAAGGCTTAG 4111
TsIAP          ATATATAAACAACATTCATTTAACCCGTTCACTTGAGGGAGCTCTGGTACAAACTACCAA 1534

4.3kb          GCTCACTGCAACCTCCGCCTCCCAGGTTCAAGTGATTCTCCTGCCTCAGCCTCCCATGCC 1623
1.8kb          ------------------------------------------------------------
XIAP           GCATGTTCAAACGCCTGCAAAACTACTTATCACTCAGCTTTAGTTTTTCTAATCCAAGAA 4171
TsIAP          GAAAAC-ACCATCACTAACTAAAAGAATCAGTGATACCATCTTCCCTAA--TCCTATGCT 1591

4.3kb          TCCTGTGTAGCTGGGATTACAGGCACCCATCACCATGCCTGGCTAATTTTTGTATTTTCA 1683
1.8kb          ------------------------------------------------------------
XIAP           GGCAGGGCAGTTAACCTTTTTGGTGCCAAT-GTGAAATGTAAATGATTTTATGTTTTTCC 4230
TsIAP          AC--AAGAAGCTATACGAATGGGATTTGATTTCAAGG--------ACGTTAAGAAAATAA 1641

4.3kb          GTAGAGATGGGGTTTCACCATGTTGGCCAGGCTGGTCTGTAATTCCTGACCTCAAGTGAT 1743
1.8kb          ------------------------------------------------------------
XIAP           TGCTTTGTGGATGAAAAATATTTCTGAGTGGTAGTTTTTTGACAGGTAGACC---ATGTC 4287
TsIAP          TGGAGGAAAGAATTCAAACATCTGGG---AGCAACTATAAAACGCTTGAGGT-------- 1690

4.3kb          CCACCCACCTTGGCCTCCCAAAGTGCTGGGATTTCAGGTGTGAGCCACCACGCCCAGCCC 1803
1.8kb          ------------------------------------------------------------
XIAP           TTATCTTGTTTCAAAATAAGTATTTCTGATTTTGTAAA-ATGAAATATAAAATATGTCTC 4346
TsIAP          -------TCTTG--TTGCAGATCTAGTGAGCGCTCAGAAAGACACTACAGAAAATGAATT 1741

4.3kb          TGTTTAAATTTT-TTATAAGTATGTACTACTTTTGTAATCAGAATTATTAGAAAGCATTT 1862
1.8kb          ------------------------------------------------------------
XIAP           AGATCTTCCAAT-TAATTAGTAAGGATTCATCCTTAATCCTTGCTAGTTTAAGCCTGCCT 4405
TsIAP          GAATCAGACTTCATTGCAGAGAGAAATCAGCCCTGAA----GAGCCGCTAAGGCG---TC 1794

4.3kb          TACTGATTTAAAAGCTTAGACATGTTCAAATGCCTGCAAAACTACTTAACACTCAGCTTT 1922
1.8kb          ------------------------------------------------------------
XIAP           AAGTCACTTTA---CTAAAAGATCTTTGTTAACTCAGTATTTTAAACATCTGTCAGCTT- 4461
TsIAP          TGCAAGAGGAGAAGCTTTGTAAAATC----TGCATGGACAGATATATCGCTGTTGTTTTT 1850

4.3kb          AGTTTTTCTAATCCAAAAAGGCCGGGCAGTTAATCTTTTTGGTGCCAATGTGAAATTTAA 1982
1.8kb          ------------------------------------------------------------
XIAP           -ATGTAGGTAAAAGTAGAAGCATGTTTGTACACTGCTTGTAGTTATAGTGACAGCTTTCC 4520
TsIAP          ATTCCTTGTGGACATCTGGTCACTTGTAAACAATG-TGCTGAAGCAGTTGACAGATGTCC 1909

4.3kb          ACGGTTTTATGTTTTTCCTGTGTTGTGAATGAAAAATATTTCTGAGTGG---TGGTTTTT 2039
1.8kb          ------------------------------------------------------------
XIAP           ATGTTGAGATTCTCATATCATCTTGTATCTTAAAGTTTCATGTGAGTT-------TTTAC 4573
TsIAP          CATGTGCAGCGCGGTTATTG-ATTTCAAGCAAAGAGTTTTTATGTCTTAATGTAACTCTA 1968

4.3kb          TGACAGGTAGACCATGTCTTGTCTTGTTTCAAA-ATAAGTATTTCTGATTTTGTAAAATG 2098
1.8kb          ------------------------------------------------------------
XIAP           CGTTAGGATGATTAAGATGTATATAGGACAAAATGTTAAGTCTTTCCTCTACCTACATTT 4633
TsIAP          CAGTGGGTGTGCTATGTTCT-TATTACCCTGATTAAATGTGTGATGTGACTCAAAAAAAA 2027
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4.3kb          AAATATACAATATGTCACAG-ATCTTCCAATTAAGTAGTAAGGGTTTATCCTTAATCCTT 2157
1.8kb          ------------------------------------------------------------
XIAP           GTTTTCTTGGCTAGTAATAGTAGTAGATACTTCTGAAATAAATGTTCTCTCAAGATCCTT 4693
TsIAP          AAAAA------------------------------------------------------- 2032

4.3kb          G---CTAATTTAAGCTTGCATAAGTCACTTTACTAAAAGA----TCTTTGTTAAGCTAGT 2210
1.8kb          ------------------------------------------------------------
XIAP           AAAACCTCTTGGAAATTATAAAAATATTGGCAAGAAAAGAAGAATAGTTGTTTAAATATT 4753
TsIAP          ------------------------------------------------------------

4.3kb          ATTTTAAACATCTGTCAGCTTATGTAGGTAAAA-GTAGAAGCATGTTTGTACACTGTTGT 2269
1.8kb          ------------------------------------------------------------
XIAP           TTTTAAAAAACACTTGAATAAGAATCAGTAGGGTATAAACTAGAAGTTTAAAAATGCCTC 4813
TsIAP          ------------------------------------------------------------

4.3kb          AGTTATAGTGACAGCTTTCCATGTTGAGGTTCTCATATCACCTTGTATCTTGAAGTTTCA 2329
1.8kb          ------------------------------------------------------------
XIAP           ATAGAACGTCCAGGGTTTACATTACAAGATTCTCACAACAAACC-CATTGTAGAGGTGAG 4872
TsIAP          ------------------------------------------------------------

4.3kb          TGTGAGTTTTTACCATTAGGATGATTAAGATGTATATAGGACAAAATATTAAGTCTTTCC 2389
1.8kb          ------------------------------------------------------------
XIAP           TAAGGCATGTTACTACAGAGGAAAGTTTGAGAGTAAAACTGTAAAAAATTATATTTTTGT 4932
TsIAP          ------------------------------------------------------------

4.3kb          TTTACCTAAGTTTGCTTTCTTGACTAGTAATAGTAGTAGATATTTCTGTAATAAATGTTC 2449
1.8kb          ------------------------------------------------------------
XIAP           TGTAC----------TTTCT--------AAGAGAAAGAG-TATTGTT-------ATGTTC 4966
TsIAP          ------------------------------------------------------------

4.3kb          TCTCAAGATCCTTAAAAT-CTCTTGGAAATTATAAAAATATTGGAAAGAGAAGAACAGTT 2508
1.8kb          ------------------------------------------------------------
XIAP           TCCTAACTTCTGTTGATTACTACTTTAAGTGATATTCATTTAAAACATTGCAAATTTATT 5026
TsIAP          ------------------------------------------------------------

4.3kb          TTTAAAATATATATATATATATATATTTTTTTTGAGATGGAGTCTTGCTCTGTCGTCCAG 2568
1.8kb          ------------------------------------------------------------
XIAP           T----------TATTTATTTA-ATTTTCTTTTTGAGATGGAGTCTTGCT-TGTCACCCAG 5074
TsIAP          ------------------------------------------------------------

4.3kb          GCTGGAGTGCAGTGGCGCAAACTTGGTTCACCACAACCTCTGCCTCCCGGGTTCAAGCGA 2628
1.8kb          ------------------------------------------------------------
XIAP           GCTGGAGTGCAGTGGAGTGATCTCTGCTCACTGCAACCTCCGCCTTCTGGGTTCAAGCGA 5134
TsIAP          ------------------------------------------------------------

4.3kb          TTCTTCTGCCTCAGCCTCCTGAGTAGCTGGGACTACAGGCGCCCGCCACCACGCCCAGCT 2688
1.8kb          ------------------------------------------------------------
XIAP           TTCTCGTGCCTCAGCTTCCTGAGTAGCTGGAATTACAGGCAGGTGCCACCATGCCCGACT 5194
TsIAP          ------------------------------------------------------------

4.3kb          AATTTTTGT--ATTTTTAGTAGACACGAGGTTTTACTATGTTGGCTAGGCTGGTCTCAAA 2746
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1.8kb          ------------------------------------------------------------
XIAP           AATTTTTTTTTATTTTTAGTAGAGACGGGGTTTCACCATGTTGGCCAGGCTGGTATCAAA 5254
TsIAP          ------------------------------------------------------------

4.3kb          CTCCTGACCTT--GTGATCTGCCCGCCTTGGCCTCCCAAAGTGCTGGGATTACAGGTGTG 2804
1.8kb          ------------------------------------------------------------
XIAP           CTCCTGACCTCAAGAGATCCACTCGCCTTGCCCTCCCAAAGTGCTGGGATTACAGGCTTG 5314
TsIAP          ------------------------------------------------------------

4.3kb          AGCCACTGCACCTGGCCAGTTTTTTAAATATATTTTTAAAAACACTTGAAT---AAGAGT 2861
1.8kb          ------------------------------------------------------------
XIAP           AGCCACCACGCCCGGCTAAAACATTGCAAATTTAAATGAGAGTTTTAAAAATTAAATAAT 5374
TsIAP          ------------------------------------------------------------

4.3kb          CAGTGTAAACTAGAAGTTTAAAAATGCTT-CACAGAACACCCAGGGTTTACATTACAAGA 2920
1.8kb          ------------------------------------------------------------
XIAP           GACTGCCCTGTTTCTGTTTTAGTATGTAAATCCTCAGTTCTTCACCTTTGCACTGTCTGC 5434
TsIAP          ------------------------------------------------------------

4.3kb          TTCTCACAACAAACCTATTGTAAAGGTGAGTAAGGCATGTTATTACAGAGAAAAGTTTGG 2980
1.8kb          ------------------------------------------------------------
XIAP           CACTTAGTTTGGTTATATAGTCATTA-ACTTGAATTTGGTCTGTATAGTCTAGACTTTAA 5493
TsIAP          ------------------------------------------------------------

4.3kb          GAGCAAAACTGT--AAAAAATTATATTTTTGTTGTATTTTCTAAGAGAAAGAGTATTGTT 3038
1.8kb          ------------------------------------------------------------
XIAP           ATTTAAAGTTTTCTACAAGGGGAGAAAAGTGTTAAAATTTTTAAAATATGTTTTCCAGGA 5553
TsIAP          ------------------------------------------------------------

4.3kb          ATGTTC--TCCTAACCTCTGTTGATTACT-ACTTTAAGTGATGTTCA-----TTTAAAAC 3090
1.8kb          ------------------------------------------------------------
XIAP           CACTTCACTTCCAAGTCAGGTAGGTAGTTCAATCTAGTTGTTAGCCAAGGACTCAAGGAC 5613
TsIAP          ------------------------------------------------------------

4.3kb          ATTGCAAATTTAA-ATAAGAGTTTTAAAAAT---------------------------- 3120
1.8kb          ------------------------------------------------------------
XIAP           TGAATTGTTTTAACATAAGGCTTTTCCTGTTCTGGGAGCCGCACTTCATTAAAATTCTTC 5673
TsIAP          ------------------------------------------------------------
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