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The Candidate Tumour Suppressor, XIAP Associated Factor 1 (XAF1), Directly Inhibits XIAP Activity and Induces G1 Phase Cell Cycle Arrest
The Candidate Tumour Suppressor, XIAP Associated Factor 1 (XAF1), Directly Inhibits XIAP Activity and Induces G1 Phase Cell Cycle Arrest.

A Thesis Submitted to the School of Graduate Studies
University of Ottawa

In Partial Fulfilment of the Requirement for the Degree of
Doctor of Philosophy
Department of Biochemistry, Microbiology and Immunology
Faculty of Medicine

By

Wai Gin Fong

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Abstract

XIAP associated factor 1 (XAF1) was initially isolated as novel 34 kDa protein which bound XIAP in a two-hybrid screening. The XAF1A protein consists of 301 a.a. and contains seven potential zinc finger domains. Two alternatively splice variants of XAF1 were later isolated. One isoform (XAF1B) was formed by the removal of a 57 bp exon, which leads to an in-frame deletion of the third zinc finger and the creation of a shorter 32.5 kDa protein. The other splice variant (XAF1C) contains a 154 bp exon insertion, which truncates the sixth and seventh zinc fingers to produce an 18.7 kDa protein. XAF1A and XAF1B, but not XAF1C, bound XIAP in \textit{in vitro} pull down assays. Northern blot analysis showed at least four distinct sizes of xaf1 mRNA ranging between 3.9 and 7.0 kb, which may indicate other XAF1 isoforms yet to be discovered.

Though the possible role of these zinc fingers on the XAF1/XIAP interaction has yet to be determined, recent experiments indicate that XAF1A can block the ability of XIAP to inhibit caspase-3 \textit{in vitro}. Furthermore, overexpression of XAF1A in HEL299 cells triggered a G1 cell cycle arrest. This G1 arrest coincides with an increase in p21, but not p53. The ability of XAF1 to block XIAP function and induce cell cycle arrest suggests a role for XAF1 in the control of both apoptosis and cell growth.

The coding regions of XAF1A, B and C are encoded on a total of 9 exons within a span of 20 kb. The single copy xaf1 gene has been mapped, using FISH analysis, distal to the \textit{TP53} gene on 17p13.2. Southern blot analysis of YACS within this region further localizes the \textit{xaf1} gene on YAC 746 C 10, which contains
the markers D17S1831, D17S796, and D17S1881. These markers are located approximately 3 cM telomeric to the TP53 gene. Since the xaf1 gene is located in a region commonly deleted in numerous types of cancers, this may suggest a tumour suppressor role for XAF1 in cancer. To test this theory, a 60 cell line panel from the NCI was analyzed for xaf1 RNA expression by Taqman and heterozygosity status of markers proximal to xaf1. Taqman analysis indicated that the majority of cell lines expressed little or no xaf1 RNA while xiap levels were relatively high. A PCR study of markers near xaf1 showed significant loss of heterozygosity (LOH) in this region. The loss of xaf1 expression and significant LOH near the xaf1 gene indicate that the down-regulation of XAF1 may be important in the development of the transformed phenotype.
ACKNOWLEDGEMENTS

I would like to thank my friend and mentor, Dr. Robert G. Korneluk, for his scientific guidance and financial support throughout the duration of my studies. Many thanks to my advisory committee, Dr. Barbara Vanderhyden and Dr. Kathryn Wright, for their direction and helpful discussions. I would also like to thank Edward Sausville and Dominic Scudiero of the National Cancer Institute for providing reagents used in these studies. A special thanks to Dr. Martin Holcik and Dr. Peter Liston for their expertise and encouragement that made this work possible.

Finally, I am extremely grateful to all my colleagues in the Korneluk/MacKenzie group, past and present, who have made my tenure here as happy and enjoyable as I could possibly imagine. Who said science couldn't be fun....
DEDICATION

To Paulina, for her patience, support and love
To Alison and Stephanie, who continue to remind me
how lucky I am...
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<th>Description</th>
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<tbody>
<tr>
<td>a.a.</td>
<td>Amino Acid</td>
</tr>
<tr>
<td>Apaf</td>
<td>Apoptosis Protease Activating Factor</td>
</tr>
<tr>
<td>Bcl</td>
<td>B cell lymphoma</td>
</tr>
<tr>
<td>BIR</td>
<td>Baculoviral IAP Repeat</td>
</tr>
<tr>
<td>BIRP or BIRC</td>
<td>BIR Containing Protein</td>
</tr>
<tr>
<td>bp</td>
<td>Base Pair</td>
</tr>
<tr>
<td>BRUCE</td>
<td>BIR Repeat Containing Ubiquitin Conjugating Enzyme</td>
</tr>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>Caspase</td>
<td>Cysteine Aspartase</td>
</tr>
<tr>
<td>CARD</td>
<td>Caspase Recruitment Domain</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin Dependent Kinase</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CRD</td>
<td>Cysteine Rich Domain</td>
</tr>
<tr>
<td>CrmA</td>
<td>Cytokine Response Modifier A</td>
</tr>
<tr>
<td>dATP</td>
<td>Deoxyadenosine Triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>Deoxycytidine Triphosphate</td>
</tr>
<tr>
<td>DD</td>
<td>Death Domain</td>
</tr>
<tr>
<td>DED</td>
<td>Death Effector Domain</td>
</tr>
<tr>
<td>DISC</td>
<td>Death Inducing Signaling Complex</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas Associated Death Domain</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescent In situ Hybridization</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence Activated Cell Sorting</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S Transferase</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>HIAP</td>
<td>Human Inhibitor of Apoptosis</td>
</tr>
<tr>
<td>IAP</td>
<td>Inhibitor of Apoptosis</td>
</tr>
<tr>
<td>ICE</td>
<td>Interleukin-1β Converting Enzyme</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal Ribosomal Entry Site</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobase pairs</td>
</tr>
<tr>
<td>KIAP</td>
<td>Kidney Inhibitor of Apoptosis</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani Medium</td>
</tr>
<tr>
<td>LOH</td>
<td>Loss of Heterozygosity</td>
</tr>
<tr>
<td>MALT</td>
<td>Mucosa Associated Lymphoid Tissue</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>MIAP</td>
<td>Mouse Inhibitor of Apoptosis</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitre</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<td>---------</td>
<td>----------------------------------------</td>
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<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of Infection</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MTN</td>
<td>Multiple Tissue Northern</td>
</tr>
<tr>
<td>MXAF</td>
<td>Mouse XIAP Associated Factor</td>
</tr>
<tr>
<td>NAIP</td>
<td>Neuronal Apoptosis Inhibitory Protein</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor κB</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve Growth Factor</td>
</tr>
<tr>
<td>ORF</td>
<td>Open Reading Frame</td>
</tr>
<tr>
<td>PBL</td>
<td>Peripheral Blood Leukocytes</td>
</tr>
<tr>
<td>PCD</td>
<td>Programmed Cell Death</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PFU</td>
<td>Plaque Forming Unit</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethysulfonyl Fluoride</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td>RFP</td>
<td>Red Fluorescent Protein</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RPM</td>
<td>Rotations Per Minute</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcription PCR</td>
</tr>
<tr>
<td>RZF</td>
<td>RING Zinc Finger</td>
</tr>
<tr>
<td>SMA</td>
<td>Spinal Muscular Atrophy</td>
</tr>
<tr>
<td>Smac</td>
<td>Second Mitochondrial-Derived Activator of Caspases</td>
</tr>
</tbody>
</table>
CHAPTER 1: INTRODUCTION

Life is pleasant. Death is peaceful. It's the transition that's troublesome

- Isaac Asimov (1920-1992 AD)

Apoptosis is a vital mechanism for the growth and maintenance of higher organisms. The system which controls this process of cellular suicide preserves the balance between anti- and pro-apoptotic signals within every cell. For instance, caspases, the central machinery for executing programmed cell death, are regulated at several steps, including direct inhibition by the inhibitor of apoptosis (IAP) proteins. We describe here the isolation and characterization of a novel IAP antagonist, XIAP associated factor 1 (XAF1), which specifically binds and inhibits the X-linked IAP (XIAP). XAF1 plays a critical role in maintaining the apoptotic threshold by regulating the IAP/caspase balance. Deregulation of this balance is proposed to be necessary for the development of transformed cells. Indeed, loss of XAF1 expression was observed to occur in the majority of transformed cells, likely due to genetic alterations at the xaf1 locus, and re-introduction of XAF1 causes both a cell cycle arrest and sensitization to apoptotic triggers. Therefore, XAF1 appears to meet virtually all requirements for genetic classification as a tumour suppressor gene.

Programmed Cell Death

1. Initial Description of Apoptosis
The physiological regulation of a multicellular organism requires not only the control of cellular proliferation, but also cellular loss. This regulatory mechanism must strictly limit when certain cells will die, as well as prevent inappropriate cell death. Directed cellular destruction is necessary for morphological changes during development, immunological removal of infected cells, and the destruction of damaged or mutated cells. Cellular destruction must also be coupled to the safe removal of cellular debris, preventing inflammation and possible autoimmune responses. This organized method of cell death and removal distinguishes itself from the undirected necrotic cell death triggered by physical or chemical damage which leads to the uncontrolled and harmful release of cellular debris.

The morphological features of cell suicide were described as early as the 19th century in the developing toad embryo (Vogt 1842). Embryonic neuronal cells were known to die in a predictable manner during the formation of the toad nervous system. These dead cells were initially described to have condensed nuclei and chromatin (pyknosis) and fragmented nuclei (karyorrhexis). This form of cellular death was termed ‘programmed cell death’ (PCD) due to the highly directed nature of the dying cells during insect morphogenesis (Lockshin and Williams 1965). Kerr et al. (Kerr et al. 1972) later introduced the term ‘apoptosis’ to distinguish developmental programmed cell death from injury induced necrotic cell death.

Apoptosis is a dynamic and biochemically active process. Blocking RNA or protein synthesis prevents apoptosis during amphibian and insect
development (Tata 1966; Lockshin 1969). Morphologically, apoptotic cells are characterized by nuclear condensation and fragmentation, compacted cytoplasmic organelles, decreased cell volume, and plasma membrane blebbing. In apoptotic cells, fragments of the nuclei are found in small, spherical cytoplasmic fragments termed apoptotic bodies. The fragmented nuclei and cytoplasm are neatly contained within these cellular remnants where they can be efficiently disposed of through endocytosis by neighbouring cells or professional macrophages. The containment of cellular debris prevents the inflammation normally associated with necrotic cell death (Kerr et al. 1972). Another hallmark and early assay for apoptosis was the fragmentation of genomic DNA. During the apoptotic process, the genomic DNA is internucleosomally cleaved by an endogenous DNase into approximately 200 bp fragments, representing histone protected DNA (Riley 1980). When resolved on a conventional agarose gel, these protected fragments can be seen as a ‘DNA ladder’. This method was used by early apoptosis researchers to measure the level of apoptosis, but current knowledge on PCD suggests 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).

Pioneering work on apoptosis began in the field of developmental biology due to the predictable nature of cell death found in embryo formation. Apoptotic cell death was found to be central to embryo structural growth and formation, such as in the regression of interdigital tissue in the hand and feet, and neural development in both the embryo and adults. Beyond these developmental
paradigms, PCD is now found to be critical in immune function and regulation, bone remodeling and general cell homeostasis throughout adult life. It is interesting that not all directed cell death necessarily proceeds through apoptosis. Cytotoxic T lymphocytes can use perforin to directly lyse infected target cells (Russell and Dobos 1980). Apoptotic cells may also indirectly kill neighboring cells due to loss of nutrients or cell surface support, as seen in leaf loss in trees and antler loss in deer. Both of these processes can induce apoptosis and/or necrosis in the target cells. This suggests that some organisms can use necrotic cell death to specifically destroy unwanted or dangerous cells, despite the risk of inflammatory responses.

2. Developmental Apoptosis

Programmed cell death is integral to the development of higher organisms. Directed cell destruction is used by many organisms to form structures and remove superfluous cells. For example, during the formation of the mammalian hand, the fingers are separated through apoptosis of the interdigital webbing (Ganan et al. 1996; Zou and Niswander 1996). Blocking apoptosis through peptide inhibitors prevents proper formation of the digits (Milligan et al. 1995; Jacobsen et al. 1996). Furthermore, apoptosis aids in the formation of the amniotic cavity through the removal of the ectodermal cells within the core of the embryo, as was shown in early mouse development (Coucouvanis and Martin 1995).

It is also evident that evolutionary changes in higher vertebrates require apoptosis to effect modifications. Pronephric tubules, which are important for fish
and amphibian kidney development, are removed by apoptosis during mammalian development. Müllerian ducts, necessary for the formation of the female sexual organs, are eliminated during male sexual development by apoptosis. Similarly, the male equivalent Wolffian ducts, which are necessary for the development of the vas deferens, epididymis and seminal vesicles, are removed by PCD in the female. In these cases, the loss of particular tissues, rendered superfluous during evolution, is through apoptotic regression instead of a lack of cellular development (reviewed in Jacobson et al. 1997).

Apoptosis is especially important during neuronal development. Mature neurons require trophic factors to survive, either through cell-cell contact, neurotrophins, or axonal stimulation. In the development of the vertebrate nervous system immature neurons and oligodendrocytes are produced in excess. During the maturation process, neurons which make proper connections and become innervated receive positive trophic support from axonal signals, soluble neurotrophins such as nerve growth factor (NGF) and cell surface binding to myelinating cells. Neurons which fail to make proper connections and do not receive the proper trophic signals die by apoptosis. In this manner, the organism can produce sufficient cells to properly develop its nervous system and remove the cells which have not achieved their proper role (Barde 1989; Oppenheim et al. 1991; Barres et al. 1992; Mahadevan et al. 1995). This also demonstrates that apoptosis can be induced through either a loss of a positive signal or receipt of a negative signal.
3. Immunological Apoptosis

Programmed cell death plays a pivotal role in the destruction of infected and damaged cells as well as the development of immune cells. This selective killing inhibits the spread of viruses, avoids inflammatory responses and prevents the production of autoimmune lymphocytes. For an immune system to function properly, lymphocytes must recognize foreign antigens but not self antigens. Recognition of self antigen may induce an autoimmune response whereby the cells of the organism are destroyed by its own immune system. To prevent this, the immune system employs a method of screening out T lymphocytes which react to self antigens (reviewed in Sebzda et al. 1999; Yang and Ashwell 1999). Pre-T cells (TCR\(^{+}\), CD4\(^{+}\), CD8\(^{+}\)) destined for the \(\alpha\beta\)TCR lineage migrate from the bone marrow to the thymus where they begin TCR \(\beta\) rearrangement. If the TCR \(\beta\) rearrangement is unsuccessful or the completed \(\alpha\beta\)TCR fails to bind MHC, the pre-T cells receive no positive signals from TCR expression and die by apoptosis. Following positive selection for \(\alpha\beta\)TCR\(^{+}\) T cells with low or moderate avidity to MHC, T cells undergo negative selection whereby T cells which bind MHC with high affinity are forced to self destruct by apoptosis. This mechanism utilizes apoptosis to prevent maturation of both improperly formed and potentially dangerous anti-self reacting T cells. Programmed cell death also occurs during the deletion of anti-self T cells in the periphery, thus maintaining a constant vigilance against self reactive T cells.

Viral infection and replication within host cells are potentially hidden from the immune system. Complex multicellular organisms, such as mammals, have
evolved many defensive mechanisms including the internal processing and presentation of viral antigens to the immune system and the triggering of infected cell death before the virus is able to replicate and spread (reviewed in Alcamí and Koszinowski 2000; Xu et al. 2001). This requires the host cell to internally recognize viral proteins and/or genetic material or react to viral attempts to control cellular functions, especially cell cycle. Many viruses have developed ways to circumvent the host cell apoptotic response, thereby aiding their own survival and proliferation. For example, the baculovirus *Autographa californica* multicapsid nuclear polyhedrosis virus (AcMNPV) expresses the anti-apoptotic IAP protein, which has known eukaryotic homologues, and p35 (reviewed in Stennicke et al. 2002). Both proteins inhibit apoptosis directly through inhibition of caspase activity (described in Section 2). Alternatively, the Kaposi’s sarcoma-associated herpesvirus (KSHV) expresses vFLIP, a viral homologue of the eukaryotic FLIP protein, which blocks Fas mediated cell death by preventing the recruitment of caspase-8 to the death inducing signaling complex (DISC) (Thome and Tschopp 2001). The *nef* gene product of human immunodeficiency virus 1 (HIV-1) is able to block apoptosis through direct inhibition of apoptosis signal-regulating kinase 1 (ASK1), a MAPK3 family protein which activates the c-Jun NH2-terminal kinase pathway and induces apoptosis (Geleziunas et al. 2001). In a dual role, HIV-1 *nef* can also phosphorylate and inactivate the pro-apoptotic Bad protein, thereby hindering the mitochondrial apoptosis pathway (Wolf et al. 2001). By inhibiting apoptosis, viruses are able to minimize detection by the host immune system, as well as increase viral production within each host cell.
The Apoptotic Process

1. Apoptotic Triggers

Beyond genetically activated programmed cell death, numerous apoptotic triggers have been described. These include a variety of cell signaling receptors, noxious chemical and radioactive agents and molecules which interfere with vital cellular functions. Wertz and Hanley (Wertz and Hanley 1996) grouped these diverse stimuli into five general categories based on primary site of perturbation: cell surface, cytosol, cytoskeleton, nucleus and intracellular signaling pathways. Induction of apoptosis through the cell surface may occur by interfering with vital plasma membrane proteins or cell signaling receptors. Disruption of plasma membrane Ca\(^{2+}\) ion regulation via Ca\(^{2+}\) ionophores or inhibition of Na\(^+\)/H\(^+\) ion channels with chemicals such as amiloride disturbs pH balance and solute concentration (Dowd 1995), which in turn induces programmed cell death. Numerous cell surface receptors have been implicated in triggering cell death in certain cells under specific conditions. Several of these well studied receptor pathways, including tumour necrosis factor (TNF) and Fas, show distinct links to the cell death machinery through direct activation of caspases. In the cytoplasm, production of reactive oxygen species through quinone compounds like menadione or introduction of metal chelators such as TPEN induce apoptosis by lipid, protein or DNA damage (Arslan et al. 1985; Hockenbery et al. 1993). Inhibitors of RNA or protein synthesis such as actinomycin-D and cycloheximide also trigger apoptosis in certain cell lines, possibly due to the loss of essential proteins or apoptotic inhibitors. The chemotherapeutic drug Taxol is able to
arrest cells in mitosis by enhancing tubulin polymerization and inhibits isoprenylation of cytoskeletal elements necessary for membrane localization (Liu et al. 1994; Danesi et al. 1995). DNA damaging agents, including 5-fluorouracil (5FU) and UV irradiation, can trigger DNA damage sensors and induce p53 which leads to cell cycle arrest and/or apoptosis. Finally, alterations in internal cell signaling pathways, through inhibition of protein phosphorylation by staurosporine, a serine/threonine kinase inhibitor, or introduction of secondary messenger analogues such as 8-bromo-cAMP, can induce apoptosis by inhibiting positive anti-apoptotic signals or triggering pro-apoptotic pathways. Ultimately, these triggers activate exogenous and/or endogenous apoptotic pathways, which culminate in activation of specific proteases and, eventually, cell death.

2. Caspases – Protease Mediators of Cell Death

Early apoptotic studies utilized model systems with highly predictable and reproducible cell death patterns. During the embryonic development of the nematode Caenorhabditis elegans, 1090 somatic cells are generated to form the adult hermaphrodite, and precisely 131 of these cells are removed by apoptosis (Ellis et al. 1991). Genetic analysis of radiation mutants defective for this process identified many critical genes, including two genes, ced-3 and ced-4, that promote apoptosis and one gene, ced-9, which blocks programmed cell death (Hengartner and Horvitz 1994). CED-9 was identified as a homologue of the anti-apoptotic protein Bcl-2 (Hengartner and Horvitz 1994) and CED-4 was later found to share homology with Apaf-1, a central adapter protein in the
apoptosome (Zou et al. 1997). The identification of CED-3 as a homologue of the interleukin-1β converting enzyme (ICE) (Yuan et al. 1993) was the first hint of the importance of proteases in programmed cell death.

ICE is the founding member of a growing family of cysteine aspartases (caspases); enzymes which play critical roles in a variety of cellular processes including apoptosis. Caspases are functionally defined by their cleavage specificity after aspartate residues. Of the 13 known mammalian caspases, 11 have human homologues, all of which share 29 identical amino acids, including Cys285, His237 and Gly238 of the protease catalytic domain and Arg179, Gln283 and Arg341 (caspase-1 numbering) of the aspartate binding site (see Figure 1-1, reviewed in Earnshaw et al. 1999). All caspases initially exist as zymogens which are cleaved to create a large and small subunit that combine to form a heterodimer core composed of a six-stranded β-sheet flanked by α-helices. The procaspase zymogen, which has low endogenous protease activity, can be cleaved either by autolysis, through the induced proximity of two or more procaspases, or by other active caspases to form the fully functional enzyme. The complete active enzyme is a complex composed of two heterodimers exposing the two active sites at opposite ends of the complex (Walker et al. 1994; Wang et al. 1994; Rotonda et al. 1996; Mittl et al. 1997). The conversion of the caspase zymogen to active enzyme provides a vital control point in the regulation of the apoptotic process.

The caspase family, which are numbered according to date of discovery, can be further subdivided into three groups depending on consensus specificity
Figure 1-1. The Caspase Family.

The human caspase proteins are organized into a phylogenetic tree based on structural similarities and substrate specificity (Cohen 1997). Caspases are numbered according to date of discovery. The primary activating cleavage site is denoted by large arrows. Small arrows indicate secondary cleavage sites. Cleavage sites within each caspase are listed with the primary site listed first. L1-L4 indicates important residues necessary for the formation of the catalytic dyad. Death effector domains (DED) are represented by red boxes (■) and caspase recruitment domains (CARD) are represented by dark blue boxes (■). Substrate specificity and chromosomal location for each caspase are also listed. Caspases and protein domains were drawn to scale.
and, roughly, function (See Figure 1-1). Group I caspases, which include caspase-1 (ICE), caspase-4 (ICE<sub>rel</sub>-II, TX, ICH-2) and caspase-5 (ICE<sub>rel</sub>-III, TY), have the consensus recognition sequence (W/L)EHD and cleaves the target proteins after the aspartate residue. These family members appear mainly to function in the maturation of several pro-inflammatory cytokines. The DExD recognition sequence defines group II caspases, which include caspase-2 (ICH-1) and the effector caspases -3 (CPP32, apopain, Yama) and -7 (Mch3, ICE-LAP3, CMH-1). The role of group II caspases appears to be the cleavage of apoptotic substrates, including crucial proteins that directly lead to the apoptotic phenotype. Finally, group III caspases, which have a less specific consensus sequence (IVL)ExD, appear to function as activators of group II and other group III caspases, as well as cleavage of non-DxxD proteins during apoptosis. This group includes the downstream effector caspase-6 (Mch-2), the initiator caspases -8 (MACH, FLICE, Mch5) and -10 (Mch4, FLICE-2), and the apoptosome-associated caspase-9 (ICE-LAP6, Mch6) (reviewed in Nicholson and Thornberry 1997; Earnshaw et al. 1999).

Caspases play critical roles in the initiation and execution of the apoptotic program. Since caspases are able to self activate, as well as activate other susceptible downstream effector caspases, a single apoptotic trigger may initiate a caspase cascade that leads to apoptosis. Initiator caspases transduce both extracellular and intracellular apoptotic signals to the effector caspases, which function to cleave vital apoptotic substrates. The initiator caspases -8 and -10 contain N-terminal death effector domains, which allow them to be recruited to
several receptor complexes, including FasR and TNFR1. Oligomerization of partially active procaspase molecules occur within the receptor complex, leading to autolytic cleavage and caspase activation. Active initiator caspases then cleave specific target proteins, which includes the pro-apoptotic protein Bid and the effector caspases -3 and -7 (Fernandes-Alnemri et al. 1996; Srinivasula et al. 1996; Stennicke et al. 1998; Chaudhary et al. 2000). Caspase-2 may also associate with the TNFR1 complex through caspase recruitment domain (CARD) mediated RIP/RAIDD binding (Duan and Dixit 1997), but the importance of this interaction remains unclear. Effector caspases, such as caspase-3, may also function to some extent as an initiator caspase by crossactivating the downstream effector caspase-6 (Srinivasula et al. 1998).

The effector caspases -3, -6 and -7 are key executioners of the apoptotic program. Activation of these three caspases by initiator caspases leads to the cleavage of crucial substrates that directly cause the classic features of programmed cell death including plasma membrane blebbing (actin, Gas2 and α-fodrin) (Brancolini et al. 1995; Cryns et al. 1996; Kayalar et al. 1996), nuclear membrane breakdown (lamins A and B) (Rao et al. 1996) and DNA fragmentation (DFF45) (Enari et al. 1998; Liu et al. 1998). Effector caspase activation may disrupt DNA repair (PARP) (Lazebnik et al. 1994), cell signaling (Stat 1, NF-κB) (King and Goodbourn 1998; Ravi et al. 1998), cell cycle (p21waf1, p27kip1, Rb) (Janicke et al. 1996; Levkau et al. 1998), and cell survival (Bcl-2, Bid) (Li et al. 1998; Hsu and Hsueh 2000) resulting in the destruction of cellular functions and death. The ability of effector caspases to
cleave and activate themselves and other caspases creates a positive feedback loop, which, if left unchecked, unleashes an overwhelming wave of protease destruction, culminating in cell death. Cellular survival necessitates both the strict monitoring of apoptotic signaling pathways which lead to caspase initiation, as well as the control of protease activity after the activation of effector caspases.

3. Regulation of Programmed Cell Death

Several important pathways provide links between the apoptotic trigger and the activation of effector caspases. These pathways transduce the apoptotic signal, as well as offer opportunities to block or augment these messages. The means by which the numerous apoptotic triggers activate apoptosis can be divided into two general categories: the exogenous and endogenous pathways (see Figure 1-2). External cellular signaling molecules, such as tumour necrosis factor (TNF) and Fas, directly activate initiator caspases through the exogenous pathway, whereas internal signals, including DNA damage and various drug treatments, trigger apoptosis through a novel endogenous mechanism involving the mitochondrial release of cytochrome c and the formation of the apoptosome. Although these two pathways appear to operate independently, crosstalk does occur which may augment apoptotic signals through the activation of dual pathways.

As mentioned previously, two of the most well studied exogenous apoptotic triggers are the Fas receptor (FasR/CD95/Apo-1) and tumour necrosis factor receptors (TNFR), both members of the TNF receptor superfamily (reviewed in Wang et al. 1998; Fesik 2000; Budd 2002; Chen and Goeddel
2002). Fas has long been recognized as an apoptosis signaling molecule in T lymphocytes and tissues involved in peripheral deletion and immune privilege (Suda et al. 1993). The structure of the Fas receptor closely resembles TNFR1, consisting of an extracellular cysteine-rich domain (CRD), a transmembrane motif and a cytoplasmic conserved 80 amino acid protein-protein binding motif dubbed the death domain (DD). Oligomerization of Fas, through CRD interaction with cell bound or soluble Fas ligand, triggers recruitment of the Fas associated DD (FADD) protein and formation of the death inducing signaling complex (DISC). The FADD adapter protein contains both a DD and a death effector domain (DED) which can bind the DED of procaspase-8. The close proximity between procaspase-8 molecules, resulting from the oligomerization of FasR/FADD complexes, stimulates the cross activating cleavage of the minimally functional procaspase-8 into highly active caspase-8. In turn, the fully active caspase-8 enzyme directly cleaves and activates the effector caspases -3 and -7, leading to apoptosis. Caspase-8 can also cleave the pro-apoptotic Bcl-2 family member Bid into active truncated Bid (tBid), which can interact with Bak and mediate the mitochondrial release of cytochrome c (Lutter et al. 2000). Therefore, caspase-8 can trigger apoptosis via both the exogenous and endogenous pathways.

The formation of the DISC, a critical step in the exogenous pathway, can be impaired by the inhibitor FLIP (FLICE inhibitory protein), an enzymatically inactive homologue of caspase-8. FLIP can not only disrupt formation of the DISC through DD interactions with FADD, but can also stimulate survival signals
Figure 1-2. The Exogenous and Endogenous Apoptotic Pathways.

Triggering of the Fas and TNF receptors leads to initiation of the exogenous pathway through cleavage and activation of caspase-8. Active caspase-8 cleaves and activates effector caspase-3 and -7, which leads directly to cleavage of both cytoplasmic and nuclear substrates. The exogenous pathway is triggered by diverse apoptotic stimuli, which leads to the release of cytochrome c and the formation of the apoptosome. The apoptosome also cleaves and activates the effector caspase-3 and -7, leading to the apoptotic phenotype. The IAP family of proteins interfere with caspase function at various points along both pathways. The numbers represent the IAPs according to BIRC nomenclature. 1-NAIP, 2-HIAP2, 3-HIAP1, 4-XIAP, 5-Survivin, 6-Livin, 7-Bruce/Apollon, 8-Ts-IAP (see text for details). Smac/Diablo and Omi/HtrA2 are released from the mitochondria coincidentally with cytochrome c and are known inhibitors of XIAP function.
by directly stimulating ERK and NF-κB signaling pathways. FLIP directly associates with, and initiates Raf-1, which then activates MEK1 causing the phosphorylation of ERK. FLIP also interacts with TRAF1 and TRAF2, which stimulates IκB degradation and NF-κB activation through stimulation of NIK (Kataoka et al. 2000). The ability of FLIP to both block apoptosis and promote cell survival signals from the same receptor suggests that FLIP may operate as a molecular switch that diverts death signals to survival and proliferation (Budd 2002). This may partially account for the pleiotrophic effects of FasL, which can either cause cell proliferation or apoptosis, depending on the cell type.

Unlike Fas, several TNF receptors exist (TNFR1, TNFR2 and TNFR3) which bind multiple ligands (TNFα, TNFβ and LTβ) and produce both pro- and anti-apoptotic signals. The structure of TNFR1 and TNFR2 consists of a CRD, a transmembrane region and, in the case of TNFR1, a cytoplasmic DD. Soluble TNFα and TNFβ form trimeric aggregates in solution (Eck and Sprang 1989; Eck et al. 1992) which recruit three molecules of TNFR1 or TNFR2 via the CRD. The death domains on the cytoplasmic end of the trimerized TNFR1 can interact with other DD containing proteins, including the adaptor molecule TNF receptor associated DD (TRADD). The interaction between TRADD and the TNFR1 complex can transduce a pro-apoptotic signal by recruitment of FADD and formation of the DISC. On the other hand, an anti-apoptotic signal can be generated through association with TNF receptor associated factor (TRAF) 1 and 2. The FADD adapter protein interacts with TRADD through its DD and recruits procaspase-8 to the DISC through DED-DED binding. As with the Fas receptor,
the formation of the DISC brings several molecules of procaspase-8 within close proximity, which triggers the cleavage of procaspase-8 into fully active caspase-8. Subsequently, caspase-8 directly cleaves and activates effector caspases, eventually leading to apoptosis. FLIP can competitively inhibit caspase-8 activation through its DED by interfering with the induced proximity cleavage of procaspase-8. TNFR1 apoptotic signals can also be produced through the DD mediated recruitment of the protein kinase RIP (receptor interaction protein), which interacts with the adapter protein RAIDD (RIP associated ICH-1 homologue with DD). RAIDD, in turn, induces apoptosis by binding and activating caspase-2.

TNFR1 anti-apoptotic signals can also be generated through the alternative interaction of TRADD with TRAF1 and TRAF2. TRAF2/TRADD can activate NF-κB mediated survival signals through the recruitment of the NF-κB inducing kinase NIK, triggering phosphorylation and degradation of the NF-κB inhibitor Iκb. The TRAF/TRADD interaction has also been shown to stimulate the anti-apoptotic JNK pathway by activating MEKK1. It therefore appears that triggering the TNFR1 pathway provides a balance of survival and death signals that can induce a variety of effects depending on the cellular context. On the other hand, signaling through the DD-less TNFR2, which is unable to form the DISC, seems to transmit primarily anti-apoptotic signals via TRAF1 and TRAF2 induced NF-κB activation.
4. The Apoptosome – Hub of Death

Long known as the cellular engine, the mitochondrion is emerging as a central regulator of apoptosis induced by diverse cellular stresses, including DNA damage, heat shock, and oxidative stress. Mitochondria are dual membrane organelles whose inner membrane contains the electron transfer apparatus used to generate ATP via oxidative phosphorylation. Cytochrome c is an essential electron transfer protein located on the inner membrane and the intermembrane space. Early studies by Liu et al. (Liu et al. 1996) indicated that addition of mitochondrial cytochrome c to cytosolic HeLa extracts could provoke caspase activation. Cytosolic cytochrome c interacts with the C-terminal WD-40 motifs within the adapter molecule apoptosis protease activating factor 1 (Apaf-1), the human homologue of CED-4 (Benedict et al. 2000). This interaction promotes the binding of ATP or dATP to the Walker's consensus nucleotide binding site of Apaf-1 and exposes the CED-4 homology region which supports Apaf-1 oligomerization. Formation of the cytochrome c/Apaf-1 oligomer is accompanied by the simultaneous recruitment of procaspase-9 through its N-terminal CARD motif. Similar to other caspases, the oligomerization of the recruited procaspase-9 can trigger autolytic cleavage of the enzyme, producing active caspase-9 molecules. However, unlike other caspases, the binding to Apaf-1 also results in a conformational change in procaspase-9, fully activating the enzyme without cleavage (Srinivasula et al. 1998; Adrain et al. 1999; Hu et al. 1999; Jiang and Wang 2000). Crystallographic analysis indicated that the apoptosome consists of seven multimers oriented in a circular hub pattern (Acehan et al. 2002). The
active proteinase complex is then able to cleave and activate the effector caspase-3, leading to apoptosis (see Figure 1-2).

The control of apoptosome formation depends on the regulation of cytochrome c release from the mitochondria. The Bcl-2 family of proteins encompasses anti-apoptotic members, including Bcl-2 and Bcl-xL, which blocks release of cytochrome c, and pro-apoptotic members, such as Bax and Bid, which promote release of cytochrome c and apoptosis (reviewed in Wang et al. 2001). The anti-apoptotic subgroup contains several Bcl-2 homology (BH) domains necessary for protein-protein interaction with other BH containing proteins (BH1, BH2, BH3, and BH4). The pro-apoptotic Bcl-2 family members can be divided into two groups: those which contain only a BH3 domain, including Bid, Bad, and Bim, and those which contain more than one BH domain, such as Bax, Bak and Bok (Kelekar and Thompson 1998). The BH3 region is necessary for both the promotion of cell death and interaction with the anti-apoptotic Bcl-2 family members (Chittenden et al. 1995). It appears that the BH3 containing pro-apoptotic proteins help relay pro-apoptotic messages from various triggers, such as growth factor deprivation (Bad), death receptors (Bid, Bkl) and nuclear damage (Bax, Noxa, Rad-9), by promoting the release of cytochrome c or blocking the anti-apoptotic members of the same family (Adrain and Martin 2001). For example, cytosolic Bax appears to translocate to the mitochondrial membrane during the initial stages of apoptosis and form pores on the outer membrane, though there is some debate whether these pores are large enough for the release of the 12 kDa cytochrome c (Saito et al. 2000). Bax can also
interact with voltage dependent anion channel (VDAC) or Bak to form an outmembrane channel capable of cytochrome c passage (Shimizu et al. 1999; Nechushtan et al. 2001). The anti-apoptotic proteins Bcl-2 and Bcl-xL may prevent cytochrome c release by binding to Bax and preventing the creation of mitochondrial pores (Gross et al. 1999). The function of Bcl-2 is inhibited by another BH3 member, Bad. However, Bad is in turn regulated by phosphorylation, which blocks this interaction (Zha et al. 1997).

Bid is another cytoplasmic pro-apoptotic BH3 family member which is activated by caspase-8 cleavage, thereby allowing cross talk between the exogenous cell surface receptor pathway and the endogenous mitochondrial apoptotic pathway (Li and Yuan 1999). Active truncated Bid (tBid) translocates to the mitochondrial outer membrane through association with cardiolipin (Lutter et al. 2000) and may function as a chaperone for Bak. The interaction between tBid and Bak on the mitochondrial cell surface induces the formation of a transmembrane pore that may mediate the release of cytochrome c into the cytoplasm (Luo et al. 1998; Wei et al. 2000).

In addition to the release of cytochrome c, disruption of the mitochondrial membrane may also release other important mitochondrial proteins which have profound effects on apoptosis. Apoptosis inducing factor (AIF) is a 57 kDa flavoprotein which resides in the mitochondrial intermembrane space. When released from the mitochondria, AIF translocates into the nucleus where it triggers chromatin condensation and DNA fragmentation, despite the fact that AIF has no measurable DNase activity (Susin et al. 1999). In a parallel story,
endonuclease G (EndoG) is a known 30 kDa mitochondrial nuclease which is release upon mitochondrial disruption by activated tBid (Li et al. 2001). The release of EndoG from the mitochondria induces nucleosomal DNA fragmentation in the absence of activated caspases or the caspase activated nuclease CAD (Liu et al. 1998; Li et al. 2001), suggesting a caspase-independent method for inducing DNA fragmentation. Finally, mitochondria also harbor the pro-apoptotic proteins Smac/Diablo and Omi/HtrA2, which, when released from the mitochondria, directly inhibit members of the caspase blocking IAP family (see below). All of these proteins are bigger than cytochrome c, and presumably require a much larger channel or even rupture of the outmembrane to mediate their release.

**Direct Caspase Inhibitors**

Several caspase inhibitors have been found in both viruses and higher eukaryotes, including p35, CrmA and the IAP family. Functionally, these proteins share a common goal in directly attaching to the catalytic substrate binding cleft of caspases, preventing the interaction of other caspase substrates. Originally isolated from viruses, only the IAPs have known human homologues that directly inhibit caspases *in vivo.*

1. **CrmA**

   The cytokine response modifier A (CrmA) is a Cowpox virus serpin protein, a member of a family of serine protease inhibitors that were found to inhibit granzyme B, as well as caspases-1, -4 and -8 (reviewed in Zhou and
Salvesen 2000). Blocking caspase-1 and -4 functions can suppress the host inflammatory response, whereas caspase-8 inhibition confers resistance to death receptor signaling by the host cell (Zhou et al. 1997). CrmA acts initially as a substrate for caspase cleavage by binding to the caspase catalytic domain through its LVAD motif. However, upon cleavage, the CrmA protein undergoes a rapid conformational change, irreversibly stabilizing the attachment to the target caspase and preventing further substrate hydrolysis (Komiyama et al. 1994; Zhou et al. 1997).

2. p35

During the analysis of insect baculoviruses, the p35 protein was found to be an irreversible inhibitor of almost all caspases (Fisher et al. 1999). Although p35 is able to inhibit apoptosis in various species such as insects, nematode, flies and mammals, there are no mammalian cellular or viral homologues yet identified. The crystal structure of the p35/caspase-8 interaction shows that p35 uses its DQMD motif to directly attach to the catalytic domain of the target caspase and undergoes normal substrate cleavage. After cleavage, the thioester linkage between C360 of caspase-8 and D87 of p35, which is normally hydrolyzed by water, is sequestered by an N-terminal conformational change which prevents water accessibility and thereby stabilizing the covalent thioester bond. The flexibility of the released N-terminal region allows adaptation to various caspase catalytic sites and promotes broad caspase reactivity (Xu et al. 2001).
3. Inhibitors of Apoptosis Proteins

The inhibitors of apoptosis (IAP) proteins are a family of evolutionarily conserved regulators of programmed cell death. The IAPs themselves are members of a larger group of baculoviral IAP repeat (BIR) domain containing proteins (BIRP or BIRC proteins), which are found in several viruses and many eukaryotic species, ranging from the single celled yeast *Saccharomyces cerevisiae*, the nematode *Caenorhabditis elegans*, the insect *Drosophila melanogaster*, and numerous mammals such as mice, rats, pigs and humans. Named for the initially discovered baculoviral IAP gene, BIR domains are approximately 70 amino acid sequences demonstrating conservatively spaced cysteine and histidine residues that structurally produce a novel zinc binding fold (Cx₂Cx₆Wx₃Dx₅Hx₅C). Though it has yet to be shown whether all BIRPs are able to block caspases, BIRPs have been identified in organisms like yeast, which do not undergo apoptosis. This suggests a role for BIRPs beyond strictly apoptosis. (Verhagen et al. 2001) Indeed, BIRPs can be divided into two subfamilies; those which contain one or more BIR domains and inhibit apoptosis (IAPs) and those which have only one BIR domain and function in cytokinesis and chromatin segregation (reviewed in Miller 1999). This maybe an oversimplification, since at least one IAP, Survivin, has been suggested to play a role in both caspase inhibition and cytokinesis.

3.1 Viral IAPs

The first IAP protein was discovered in a rescue assay looking for novel apoptotic proteins that could functionally replace the known viral apoptotic
inhibitor, p35. Viruses such as the Cowpox virus and the baculovirus *Autographa californica* multicapsid nuclear polyhedrosis virus (AcMNPV) have been shown to encode proteins that inhibit the apoptotic process. In the case of AcMNPV, mutations in its apoptotic inhibitor p35 created strains that rapidly kill its host SF-21 insect cell line, thereby producing a low titre virus. These 'annihilator' strains were co-transfected with fragments of DNA from other baculoviruses, such as *Orgyia pseudosugata* nuclear polyhedrosis virus (OpMNPV) and *Cydia pomonella* granulosis virus (CpGV), and viruses which restored the slow growth/high titre wild-type replication cycle were isolated. Using this method, Crook et al. (Crook et al. 1993) isolated Cp-IAP from CpGV. This 31 kDa protein contained a carboxy terminal C$_3$H$_4$C$_4$ RING zinc finger and two novel 70 amino acid cys/his zinc binding motifs (Cx$_2$Cx$_6$Wx$_3$Dx$_3$Hx$_8$C) at its amino terminus termed the baculovirus IAP repeat (BIR). Similarly, analysis of the OpMNP virus yielded Op-IAP, which has 58% amino acid identity and is functionally identical to Cp-IAP (Birnbaum et al. 1994; Clem and Miller 1994). These genes closely resembled cellular IAP genes from several insect hosts, suggesting that these IAP genes may have been hijacked from their host through evolution (Huang et al. 2000).

The baculoviral IAP genes have a significant impact on the survival of the host insect cell. Both Cp-IAP and Op-IAP have been shown to bind the pro-apoptotic proteins HID, REAPER and GRIM in *Drosophila*; proteins which are known to bind the *Drosophila* cellular IAP equivalent, DIAP. Op-IAP binds HID through BIR2 and this single BIR domain is sufficient to inhibit HID is pro-
apoptotic function, though with far less potency than the full length Op-IAP protein (Vucic et al. 1998). Cp-IAP, but not Op-IAP, has also been shown to inhibit mammalian caspase-9, suggesting a direct role for some viral IAPs in blocking insect caspases (Huang et al. 2000). It has been suggested (Clem 2001) that pro-apoptotic proteins such as HIN sequester cellular IAPs, preventing their ability to block downstream caspases. Viral IAPs can bind HIN and help release cellular IAPs, thereby promoting cell survival.

3.2 Mammalian IAPs

The first mammalian IAP, Neuronal Apoptosis Inhibitory Protein (NAIP/BIRC1), was discovered through efforts to identify candidate genes which cause the human genetic disease Spinal Muscular Atrophy (SMA) (Roy et al. 1995). Since then, seven more BIRC proteins have been identified in humans: XIAP/MIHA/iILP/BIRC4, HIAP1/cIAP2/MIHC/BIRC3, HIAP2/cIAP1/MIHB/BIRC2 (Rothe et al. 1995; Duckett et al. 1996; Liston et al. 1996; Uren et al. 1996), Survivin/BIRC5 (Ambrosini et al. 1997), Bruce/Apollon/BIRC6 (Hauser et al. 1998; Chen et al. 1999), Livin/ML-IAP/BIRC7 (Lin et al. 2000; Vucic et al. 2000; Kasof and Gomes 2001) and ILP-2/Ts-IAP/BIRC8 (Lagace et al. 2001; Richter et al. 2001)(see Figure 1-3). All of these BIRC proteins, with the exception of BRUCE/Apollon, have been implicated in apoptotic regulation and several also play distinct roles in cell division and protein degradation.

3.2.1 Neuronal Apoptosis Inhibitory Protein (NAIP)

Spinal Muscular Atrophy (SMA) is an autosomal recessive degenerative
Figure 1-3. The BIRC Family

The human BIR containing (BIRC) proteins are listed according BIRC nomenclature based on date of discovery. The various domains are represented as follows: ■ - BIR, □ - RING finger, △ - CARD, ▶ - Nucleotide binding loop, ▲ - Leucine rich repeat, ◇ - Coiled-coil, ◆ - Ubiquitin conjugating domain. Amino acid sizes are noted and proteins were drawn to scale, except for Bruce/Apollon. Published molecular weights and chromosome location are also listed.
<table>
<thead>
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<th>Birc1, NAIP</th>
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<th>Chromosome Location</th>
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neuromuscular disease afflicting the population at a frequency of approximately 1:10000 (Emery 1991). SMA patients can be differentiated into three phenotypic variants (types I, II, and III) based on symptom severity, clinical progression and age of onset (Morrison 1996). The disease is characterized by the apoptotic degeneration of α motor neurons in the anterior horn of the spinal cord, leading to progressive voluntary muscle wasting. Linkage analysis of the SMA locus implicated a single genetic locus for all forms of the disease at chromosome 5q13. Analysis of the 500 kb SMA critical region yielded two candidate SMA genes; Survival Motor Neuron (SMN) and Neuronal Apoptosis Inhibitory Protein (NAIP) (Lefebvre et al. 1995; Roy et al. 1995; Rajcan-Separovic et al. 1996). Recent studies suggest that mutations in the SMN gene actually cause SMA by disrupting mRNA splicing (Pellizzoni et al. 1998) while NAIP deletion has been proposed to play a modifying role in the severity of the disease (Gendron and MacKenzie 1999).

The human naip gene is composed of 17 exons spanning approximately 56 kb. One full length copy of naip exists at the telomeric end of 5q13.1, with as many as six other tandem truncated/deleted copies at the SMA locus. The naip mRNA consists of 4212 nt, which encodes a 1403 amino acid protein with a molecular weight of 156 kDa. Structurally, NAIP consists of three N-terminal BIR domains, a central nucleotide binding loop and a C-terminal leucine rich repeat (see Figure 1-3). NAIP shows significant homology to both Cp-IAP and Op-IAP (33% over 189 a.a. and 33% over 180 a.a. respectively) but contains an
additional BIR domain and lacks the RING finger motif found in the baculovirus IAPs (Roy et al. 1995).

Expression of NAIP in the rodent CNS, as measured immunohistochemically, appears to overlap specific neuronal populations lost during the human SMA disease progression (Xu et al. 1997). Interestingly, motor neurons from the anterior horn, which degenerate by apoptosis during the progression of SMA, also express relatively high levels of NAIP (Liston et al. 1997; Pari et al. 2000). This further reinforces the importance of NAIP loss in the development of SMA. Studies also indicate that certain neuronal structures which are resistant to excitotoxic and hypoxic damage have high NAIP levels. For example, mesencephalic trigeminal neurons, which express high levels of NAIP, are resistant to kainic acid induced apoptosis. This is also true for cholinergic interneurons of the striatum which express NAIP and are able to survive transient forebrain ischemia. Not only do these cells survive, but they also appear to elevate their levels of NAIP in response to an ischemic event (Xu et al. 1997). Furthermore, K252a, a bacterial alkaloid which has been shown to be protective in various neurodegenerative models (Knusel and Hefti 1992), can upregulate NAIP in specific cholinergic neurons in the thalamus, striatum, hippocampus and spinal cord. 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 (50% less death) and had almost twice the number of NAIP expressing neurons (Xu et
al. 1997). This indicates that neurons can be saved through stimulation of NAIP expression.

To further illustrate NAIPs neuroprotective abilities in vivo, NAIP was cloned into an adenoviral vector and microinjected into the hippocampus of the rat brain. Transient global ischemia was then induced through a four vessel occlusion (4VO) model whereby blood flow through the vertebral and carotid arteries was blocked for 10 min and then allowed to reperfuse. After fourteen days post ischemia, viable cell counts in the CA1 region of the adeno-NAIP treated hemisphere indicated significant neuronal protection compared to the hemisphere infected with adeno-LacZ (Xu et al. 1997). Adeno-NAIP infection is also able to protect rat motoneurons following sciatic axotomy (Perrelet et al. 2000). This shows that virally transduced NAIP overexpressed can also block apoptosis in a classical gene therapy approach.

Similar to the baculovirus IAPs, NAIP is able to block apoptosis when overexpressed in tissue culture cell lines. Overexpression of full length NAIP through adenoviral vectors or transfection can inhibit apoptosis induced by serum withdrawal, menadione camptothecin or TNFα in various cell lines (Liston et al. 1996; Maier et al. 2002). Transfection of HeLa cells with a construct containing only the three N-terminal BIR domains of NAIP, can prevent apoptosis induced by the topoisomerase II inhibitor etoposide (Maier et al. 2002) suggesting that the NAIP BIR domains alone are sufficient to inhibit apoptosis. Purified NAIP protein containing only the three BIR domains of NAIP, directly inhibits class II caspases 3 and 7, but not the class I caspase 1 or the class III caspase 8. Further
deletion studies showed that constructs containing BIR1 or BIR1+2 could not inhibit caspase-3, but constructs containing BIR2, BIR3 or BIR2+3 blocked caspase 3 \textit{in vitro} and \textit{in vivo} (Maier et al. 2002). Of these, only BIR2 alone could inhibit caspase 3 as effectively as full length NAIP, which may indicate that BIR2 is the effector BIR domain of NAIP. Analysis of additional deletion mutants and/or the NAIP/caspase-3 crystal structure would be helpful to elucidate the sites of interaction.

3.2.2 X-linked Inhibitor of Apoptosis (XIAP)

After the discovery of NAIP, a novel human IAP protein was identified through a database search using the Cp-IAP sequence. The X-linked Inhibitor of Apoptosis (XIAP) protein has a molecular weight of 55 kDa and is composed of three N-terminal BIR domains, similar to NAIP, and a C-terminal RING finger motif (see Figure 1-3, (Liston et al. 1996)). When compared to other human IAPs, XIAP showed 44% and 42% amino acid conservation with HIAP1 and HIAP2 respectively, but appeared more distantly related to NAIP (25-30%), though this may just be a reflection of the highly divergent carboxy terminal ends. As the name implies, the \textit{xiap} gene is located on the X-chromosome at Xq25 (Rajcan-Separovic \textit{et al.} 1996). Structurally, the \textit{xiap} gene consists of 7 exons spanning 34 kb that encodes a 10 kb \textit{xiap} transcript (Lagace \textit{et al.} 2001).

Expression of human \textit{xiap} mRNA is fairly ubiquitous among all adult and fetal tissues tested in Northern blots (Liston \textit{et al.} 1996). The 10 kb \textit{xiap} transcript encodes a 1500 nt coding region, a 6000 nt 3' untranslated region (UTR) and an unusually large 2500 nt 5' UTR (Lagace \textit{et al.} 2001). Long 5'
UTRs are rarely found in eukaryotic mRNAs and usually interfere with efficient translation (Kozak 1989). The relatively high expression of XIAP in most tissues suggests that the translation of XIAP is fairly unaffected by the long 5' UTR. A growing number of eukaryotic genes with long 5' UTRs have been found to translate their proteins through a cap-independent internal ribosomal entry site (IRES, reviewed in Holcik et al. 2000). These IRES elements were initially discovered in picornavirus RNA where they promote the cap-independent translation of viral RNA. Expression of the viral protease 2A blocks host cap-dependent translation by cleaving cap binding proteins, yet IRES initiated translation is unaffected. Thus, the virus is able to favor its own protein production at the expense of the host cell. Cellular IRES elements allow translation of specific mRNAs under stress conditions that shut down translation initiation, including viral infection, serum withdrawal, and growth factor deprivation. These IRES containing mRNAs include survival factors (VEGF, PDGF), oncogenes (c-myc and c-jun) and apoptosis related genes (Apaf-1, XIAP). Expression of XIAP under stressful conditions may promote cell survival while the cell recovers. Indeed, cells under physiological stress, such as low dose γ-irradiation, have been shown to upregulate XIAP translation through an IRES mediated pathway (Holcik et al. 2000) indicating that cells can promote their own survival in the absence of normal protein initiation.

XIAP is one of the most studied and well characterized apoptotic inhibitors. It is well known that full length XIAP can inhibit programmed cell death induced by numerous triggers including serum withdrawal, Fas receptor binding,
the free radical inducer menadione and the topoisomerase II inhibitor etoposide (Liston et al. 1996; Deveraux et al. 1998; Takahashi et al. 1998), to name but a few. The core of XIAPs ability to block cell death are the N-terminal BIR domains which have been shown to directly inhibit both initiator and effector caspases. In vitro studies indicate that XIAP can specifically inhibit the upstream initiator caspase -9 as well as the downstream effector caspases -3 and -7, though not caspases -1, -6, -8 or -10 (see Figure 1-4). The inhibitory constant (Ki) of XIAP against caspases -3 and -7 range from 0.2 to 10 nM, indicating a greater potency than other IAPs and a comparable strength to synthetic caspase inhibitors (Deveraux and Reed 1999).

Initial studies indicated that the N-terminal BIR domains were sufficient for caspase inhibition (Deveraux et al. 1997; Deveraux et al. 1998). Later deletion analysis isolated BIR2 as necessary and sufficient for the inhibition of caspases -3 and -7 (Takahashi et al. 1998). This BIR2 region (XIAP residues 124-240) alone attained a similar Ki (< 2 nM) against caspases -3 and -7 as full length XIAP suggesting that other domains may have only minimal effects on the inhibition of caspases -3 and -7 (Takahashi et al. 1998).

Surprisingly, the caspase -3 and -7 inhibition which was attributed to the BIR2 domain may not be entirely valid. Mutation analysis of acidic amino acids within XIAP indicates that only residues between BIR1 and BIR2 disabled the ability of XIAP to block caspase-3 (Sun et al. 1999). Crystal structure analysis of the XIAP BIR2/caspase-7 complex indicates that the caspase-7 catalytic groove binds specific amino acids (Gly144, Val146, Val147 and Asp148) found within the
Figure 1-4. The Multifunctional XIAP Protein

The published functions of the XIAP protein are indicated relative to known interacting domains (see text for details). The XIAP IRES element in the 5' UTR and protease cleavage site between BIR2 and BIR3 are also noted.
linker region between BIR1 and BIR2 of XIAP. This region is also vital in the formation of the XIAP BIR2/caspase-3 complex. The catalytic domain of caspase-7 is composed of four binding sites (S4-S1) upon which the four residues of the caspase-7 recognition sequence, Asp-Glu-Xaa-Asp (P4-P1), are bound. Computer models generated using data from the crystal structure of XIAP BIR2/caspase-7 complex suggest that the Gly144, Val146, Val147 and Asp148 residues of XIAP binds the S1, S2, S3 and S4 regions respectively, either through Van der Waals or hydrophobic interactions. This interaction is in the reverse orientation of the recognition sequence DEXD (S1-S4 vs S4-S1), indicating that the linker sequence is not a pseudo-substrate but an overlapping competitive inhibitor which binds tightly to the catalytic domain, prohibiting the access of substrate (Chai et al. 2001; Huang et al. 2001). Although BIR2 may not be the main caspase-7 inhibitory domain on XIAP, BIR2 does weakly binds caspase-7, which may help initiate and strengthen the XIAP/caspase-7 interaction. XIAP BIR2 may also function as a regulatory domain by which Smac blocks the XIAP/caspase-7 interaction (Huang et al. 2001).

The catalytic domain of caspase-3 is similarly blocked by the same linker region in XIAP, which is not surprising given the 54% sequence homology and striking structural similarities between caspase-3 and -7 (Chai et al. 2001). However, this binding reaction differs slightly in the interaction between the XIAP linker and the caspase-3 catalytic domain. Residues L140, V146 and L141 engages the hydrophobic pocket created by caspase-3 residues Lys290, Y338 and F381 while XIAP residues T143, G144 and V146 help stabilize the binding
through hydrogen bonds. The V147 residue, which is pivotal for the XIAP/caspase-7 interface, also binds the caspase-3 catalytic domain, blocking substrate access. A novel interaction between XIAP residues 148-156, which includes a portion of BIR2, and several caspase-3 domains (F381b+d, R341, S343, W348, F381b and R233) lock the BIR domain tightly against the side of caspase-3 and stabilizes the entire interaction (Riedl et al. 2001). It therefore appears that the presence of BIR2, though not directly blocking the catalytic center of caspase-3, may be important in enhancing the strength of the binding reaction.

It is interesting to note that BIR1 and 3, though very similar to BIR2 at the protein level (42% and 32% identity respectively) are unable to block caspase-3 or -7, demonstrating that, though closely related, BIR domains may have different functions within the same protein (Deveraux and Reed 1999). This was later found to be true when it was shown that the BIR3-RING finger fragment of XIAP, produced during Fas (CD95) induced apoptosis in 293 fibroblast cells, could bind active caspase-9, a vital component of the apoptosome (Deveraux et al. 1999). The inability of the BIR3-RING fragment to bind procaspase-9 implied that the proteolytic cleavage of procaspase-9 was necessary to permit XIAP BIR3 binding. Autolytic cleavage of caspase-9 at amino acid D315 releases a p35 and p12 fragment and leaves an N-terminal four amino acid sequence (Ala-Thr-Pro-Phe) on p12 which specifically binds XIAP BIR3 (Srinivasula et al. 2001). The association of XIAP with caspase-9 is inhibited by cleavage at the caspase-3 specific D330 site, mutations blocking caspase-9 cleavage at D315 or mutations
within the four amino acid binding domain. The binding of XIAP to caspase-9 can be directly attributed to the BIR3 domain in crystal structure analysis. The free Ala residue at the N-terminus is tightly bound to Glu314 within the BIR3 domain of XIAP. Mutation of Glu314 or Trp310 of XIAP abrogates caspase-9 binding (Srinivasula et al. 2001). It is theorized that upon the cleavage of pro-caspase-9, the p12 fragment, with the tetrapeptide recognition sequence exposed, is bound by XIAP BIR3, preventing substrate access and inhibiting further caspase cascade activation. However, further processing by caspase-3 removes the tetrapeptide sequence from p12, generating a XIAP-resistant form of active caspase-9. The released tetrapeptide sequence may in turn block XIAP from inhibiting caspase-9 through the binding of XIAP BIR3 (Holcik and Korneluk 2001; Srinivasula et al. 2001).

Beyond the caspase inhibitory capacity of the XIAP BIR domains, XIAP has emerged as a regulator of protein degradation (see Figure 1-4). The C-terminal RING zinc finger of XIAP has E3 ubiquitin ligase activity which specifically recognizes proteins and selectively tags them for proteosome degradation through ubiquitination. For example, during thymocyte apoptosis induced by dexamethasone, proteosome inhibitors prevent cell death. This suggests that the loss of an anti-apoptotic protein is triggered by dexamethasone treatment. Indeed, XIAP was found to be downregulated in dexamethasone treated thymocytes and XIAP can target itself for ubiquitination. Mutation of important Zn binding amino acids within the RING finger eliminates the E3 ligase activity (Yang et al. 2000; Yang and Li 2000). This negative feedback produced
by self-ubiquitination downregulates XIAP expression, which may be necessary for certain cell death paradigms. Furthermore, degradation of caspase-3, which binds XIAP through the N-terminal BIR domains, is greatly increased in the presence of wild type XIAP but not in XIAP RING finger or caspase-3 binding mutants (Suzuki et al. 2001). Caspase-3 protein breakdown, in the presence of XIAP, is coupled with increased ubiquitination and is susceptible to proteosome inhibitors, which prevents ubiquitin mediated protein degradation (Suzuki et al. 2001). It appears that the binding of caspase-3 and the inhibition of its function by full length XIAP may be a prelude to the eventual destruction of the caspase. It remains to be seen if other XIAP bound proteins, such as caspase-7 and -9, are also degraded through XIAP induced ubiquitination.

The multifunctional XIAP protein may also play a role in receptor mediated cell signaling. Yamaguchi et al. (Yamaguchi et al. 1999) used a yeast two-hybrid screen to search for TAB1 interacting proteins and found that XIAP bound both bone morphogenic protein (BMP) type 1 and its signaling molecule TAB1. BMP is a member of the transforming growth factor β (TGFβ) superfamily and is implicated in many cell proliferation and differentiation processes (Massague et al. 2000). The modular XIAP protein appears to function as an adapter protein by connecting the cell surface receptor molecule BMP, which binds the RING zinc finger, with the BIR domain bound TAB1 signaling molecule. This XIAP to TAB1 binding can activate downstream TAK1, a MAP kinase kinase kinase. Overexpression of XIAP, but not a RING deletion mutant of XIAP, within the embryo of Xenopus can induce ventralization in a TAB1-TAK1 dependent
manner (Yamaguchi et al. 1999). The overexpression of XIAP has also been reported to activate the downstream MAP kinase c-jun N-terminal kinase 1 (JNK1), though XIAP did not appear to directly associate with this kinase (Sanna et al. 1998). Interestingly, activation of the BMP receptor also stimulates a rapid rise in downstream JNK1 activity, suggesting a possible mechanism for the observed XIAP mediated activation of JNK1 (Yamaguchi et al. 1999). Blocking JNK1 through the overexpression of a catalytically inactive mutant inhibits apoptosis triggered by caspase-1 or TNFα (Sanna et al. 2002). Expression of inactive TAK1 mutants also inhibits caspase-1 and TNFα induced apoptosis, but did not affect the inhibition of caspases by XIAP (Sanna et al. 2002). It therefore appears that XIAP is able to suppress apoptosis through both caspase inhibition and stimulation of survival signals such as JNK1.

3.2.3 Human Inhibitor of Apoptosis Proteins 1 and 2 (HIAP1 and HIAP2)

Using the newly discovered XIAP sequence, a short 410 bp sequence with moderate homology to XIAP was identified in the GenBank EST database. This sequence was RT-PCR cloned and used to screen a human liver cDNA library, which yielded two novel IAPs termed human IAP1 and 2 (HIAP1 and 2, (Liston et al. 1996). Simultaneously, research into the tumor necrosis factor receptor 2 (TNFR2) signal transduction pathway also isolated two human IAPs dubbed cellular IAP1 and 2 (c-IAP1 and 2, (Rothe et al. 1995). HIAP1 and 2 are identical to c-IAP2 and 1 respectively. While HIAP1 and 2 exhibited 44 and 42% amino acid conservation to XIAP respectively, a high degree of conservation (72% a.a. identity) was found between HIAP1 and 2 (Liston et al. 1996). The hiap1 and
hiap2 genes are arranged in tandem head to tail fashion within 7 kb of each other on chromosome 11q22-23 (Young et al. 1999). The high level of protein identity and the tandem organization of the genes suggest that the hiap1 and 2 genes originate from a gene duplication event. Intriguingly, the chromosome 11q22-23 region is implicated in the development of several leukemias and lymphomas, linking hiap1 and/or 2 to these types of cancers (see Chapter 5).

The hiap1 gene encodes a 8.7 kb transcript on 10 exons whereas the hiap2 gene encodes a 4.5 kb transcript on 9 exons (Young et al. 1999). The 1812 nt coding region of the hiap1 mRNA translates to a 68 kDa protein while the 1854 nt hiap2 coding region translates to a 70 kDa protein. Northern blot analysis of hiap1 reveals high expression in lymphoid tissues, such as thymus, peripheral blood and spleen, as well as fetal lung and kidney. Several hiap1 specific transcripts greater than 8.7 kb were observed in Northern blots, indicating alternatively spliced or poly(A) readthrough transcripts. Expression of hiap2 mRNA is particularly high in adult skeletal muscle, pancreas, thymus and testes, but absent in lung and kidney. Human fetal tissues such as brain, lung, liver and kidney express uniformly high levels of hiap2 (Liston et al. 1996).

Structurally, both HIAP1 and 2 are similar to XIAP in that they consist of three N-terminal BIR domains and a C-terminal RING zinc finger. In contrast to XIAP, HIAP1 and 2 contain a caspase recruitment domain (CARD) located between the BIR and RING finger domains. CARD motifs, a protein structure consisting of six α-helical folds, are members of the death domain superfamily along with death domains (DD) and death effector domains (DED). Although
originally identified as facilitators of caspase aggregation and activation, CARD domains have now been found in non-caspase molecules, such as HIAP1 and 2, and are thought to promote the assembly of protein complexes, including caspases, most of which are involved in the regulation of apoptosis or NFκB activation (Weber and Vincenz 2001). Though a function has yet to be ascribed to the CARD domains of HIAP1 or 2, the interaction of HIAP1 and 2 within the tumour necrosis factor receptor 2 (TNFR2) complex may aid in the recruitment of CARD carrying proteins to this complex. In addition, HIAP2 has been shown to bind CARDIAK/RIP2/RICK, a CARD containing serine/threonine kinase which is known to activate caspase1, but whether HIAP2 interferes with the CARDIAK/caspase1 interaction remains to be determined (McCarthy et al. 1998; Thome et al. 1998).

As IAPs, both HIAP1 and 2 demonstrate anti-apoptotic capability. Overexpression of HIAP1 or HIAP2 blocks apoptosis induced by serum withdrawal, menadione, or etoposide (Liston et al. 1996; Roy et al. 1997). Similar to XIAP, caspase-3, -7, and -9, but not caspase-1, -6, -8 or -10, are inhibited by either HIAP1 or HIAP2 (Ki <= 0.1 μM) and this inhibition is linked to the BIR domains through deletion analysis (Roy et al. 1997; Deveraux et al. 1998). In T cells, apoptotic stimuli, such as glucocorticoids or etoposide, induce a rapid degradation of transfected HIAP2 through the ubiquitin mediated pathway, although RING zinc finger mutants did not degrade (Yang et al. 2000). The presence of E3 ubiquitin ligase activity attributed to the RING zinc finger of XIAP suggests that the ubiquitination of HIAP2 may be self directed and
mediated through the RING finger domain. Recent studies show that HIAP2 also
directs the ubiquitination of TRAF2, a member of the TNF complex which was
previously shown to bind HIAP2, and is dependent on the RING zinc finger for its
E3 function (Li et al. 2002). It has also been reported that HIAP1 mediates the
ubiquitination of caspase-3 and -7, which couples the inhibition and degradation
of caspase-3 and -7 on the same protein (Huang et al. 2000). It is interesting to
note that caspase-3 has been reported to cleave HIAP2 between the BIR
domains and the RING finger and that the RING zinc finger alone has pro-
apoptotic properties (Clem et al. 2001). It has been proposed that caspase
cleavage of HIAP2 transforms an anti-apoptotic protein into a pro-apoptotic
protein, augmenting the apoptotic potency of caspase-3 and further complicates
the analysis of apoptotic pathways influenced by HIAP2. If confirmed, this would
provide another striking parallel with members of the Bcl-2 family where cleavage
of anti-apoptotic Bcl-2 family members has been proposed to generate pro-
apoptotic derivatives (Basanez et al. 2001) and may be a significant event in
amplifying apoptotic signals.

As seen with other IAPs, both HIAP1 and HIAP2 are integral components
of specific cell signaling pathways, in this instance, the TNF receptor 1 and 2
complex. The recruitment of HIAP1 and HIAP2 to the TNFR2 complex requires
the presence of both TRAF1 and TRAF2 (Rothe et al. 1995) and is mediated by
the N-terminal BIR domains (Roy et al. 1997). The interaction is highly specific
since other TRAF family members (TRAF3-6) do not bind either HIAP1 or HIAP2
and other IAPs fail to bind TRAFs (Roy et al. 1997). Furthermore, the TNF
receptor 1 complex (TNFR1) recruits TRAF2 and HIAP2 through binding to another adapter molecule, TRADD. Activation of TNFR1 can stimulate anti-apoptotic NFκB signals through association with TRAF2 and HIAP2 or apoptosis by activating caspases-8 through TRADD/FADD interactions (Shu et al. 1996). Paradoxically, HIAP2 has been shown to bind and degrade TRAF2 through its E3 ubiquitin ligase activity, which logically should suppress NFκB activation and sensitize cells to TNF mediated killing (Li et al. 2002). The exact role for HIAP1 and 2 in TNF signaling remains to be determined.

How does HIAP1 or HIAP2 function within the TNF signaling pathway? It has been shown that overexpression of HIAP1 triggers activation of NFκB and blocks TNFα mediated cytotoxicity. Introduction of a dominant negative IκB, which is resistant to TNF stimulated degradation, blocks the NFκB activation induced by HIAP1 expression, but, as well, enhances the cytotoxicity of TNFα. HIAP1 mutants lacking the RING zinc finger also inhibit TNF mediated activation of NFκB, which in turn augments TNFα induced killing (Chu et al. 1997). The necessity for the RING zinc finger of HIAP1 in NFκB activation suggests a role for the E3 ubiquitin ligase activity of HIAP1 in the TNF pathway. Interestingly, TNFα stimulation also leads the transcriptional upregulation of HIAP1 via NFκB suggesting a positive regulatory loop within the TNF pathway. Therefore, HIAP1 appears to be necessary for the anti-apoptotic triggering of NFκB in the TNF pathway, which is then reinforced by the NFκB upregulation of HIAP1. The NFκB response elements in the HIAP1 promoter, which are responsible for the TNFα inducibility of HIAP1, can also be stimulated by low dose X-irradiation
(Ueda et al. 2001). Consequently, NFκB activation leads to increased HIAP1 expression regardless of the originating trigger. The continual upregulation of HIAP1 through this pathway has a significant impact on cell survival, since HIAP1 plays a dual role in stimulating NFκB, thereby inducing the expression of anti-apoptotic genes such as A20, and directly inhibiting effector caspases. The relative impact of these two anti-apoptotic mechanisms may depend on the cell type and cellular context.

3.2.4 Survivin

The Survivin gene was initially identified as a complimentary coding region within the effector cell protease receptor 1 (EPR-1) gene (Ambrosini et al. 1997). The Survivin/EPR-1 gene is located at chromosome 17q25, where the 1.9 kb human Survivin cDNA is encoded on 4 exons (Ambrosini et al. 1998). The expression pattern of Survivin in human tissues seems strictly embryonic, displaying little or no expression in adult tissues, yet appearing in all embryonic tissues tested. Most interesting is the almost universal expression of Survivin in cancer cell lines and in common cancers such as lung, colon, pancreas, prostate and breast in vivo (Ambrosini et al. 1998). These findings associate the expression of an inhibitor of apoptosis with the transformed phenotype.

The 142 amino acid, 16.5 kDa Survivin protein consists of a single N-terminal BIR domain and a C-terminal coiled-coil domain (Ambrosini et al. 1997). Purified Survivin dimerizes through interactions between the BIR domains as well as conserved residues between the BIR and the alpha helix. Although there are controversies concerning the interpretation of the crystallography data (Reed and
Bischoff 2000), all studies agree that the Survivin dimer peripherally displays the coiled-coil domains whose hydrophobic clusters have the potential for protein-protein interactions (Chantalat et al. 2000; Muchmore et al. 2000; Verdecia et al. 2000). Loss of the coiled-coil domain abrogates spindle microtubule association, but the exact protein which interacts with this domain remains unknown (Li et al. 1999; Reed and Bischoff 2000).

Like other IAPs, overexpressed Survivin can inhibit apoptosis induced by interleukin-3 withdrawal from B-cells (Ambrosini et al. 1997), Fas (CD95) binding, overexpression of Bax, etoposide exposure (Tamm et al. 1998) and treatment with the microtubule stabilizing agent Taxol (Li et al. 1998). Survivin is also able to block programmed cell death triggered by the overexpression of caspase-3 or caspase-7 and prevent the cleavage and activation of the procaspase forms. Immunoprecipitation of Survivin pulled down active caspase-3 and -7, but not the procaspase forms demonstrating that Survivin can inhibit apoptosis through direct binding to caspases (Tamm et al. 1998). Survivin binds tightly to active caspase-3 and -7, with reported dissociation constants ($K_D$) of 20.9 and 11.5 nM respectively. Survivin shows inhibition constants ($K_I$) of 36.0 and 16.5 nM for caspase-3 and -7, respectively, in DEVD tetrapeptide cleavage assays in vitro, which ranks closely with other IAPs (Shin et al. 2001).

Suppression of Survivin, through antisense or introduction of dominant negative mutants of Survivin, not only induces apoptosis, but also results in mitotic disregulation leading to polyploidy, multinucleated cells and abnormal mitotic spindle formation (Li et al. 1999). Immunohistochemistry co-localizes
Survivin with mitotic spindle microtubules, centrosome microtubules and the cytokinetic remnant; interactions that require the C-terminal portion of Survivin (Li et al. 1999). When the localization of Survivin was followed through the various mitotic phases, Survivin was found to associate with the kinetochores, the centromere associate multiprotein complexes that bind the ends of spindle microtubules during mitosis. During anaphase, Survivin translocates to the spindle midzone and later to the midbody during cell cleavage (Skoufias et al. 2000). The close association of Survivin with the mitotic spindle apparatus suggests a role in monitoring spindle integrity and mitotic success. It has also been proposed that the default pathway for proliferating cells is apoptosis unless proper segregation and Survivin mediated caspase inhibition occurs (Reed and Bischoff 2000). Survivin may play a role in sensing problems with the mitotic machinery, as well as directly inhibiting the default apoptotic program.

The control of Survivin expression is closely tied to mitosis and, in turn, Survivin also influences various mitotic proteins. Survivin expression is upregulated during G₂/M phase and is rapidly degraded during G₁ phase of the cell cycle (Li et al. 1998). Several transcriptional control elements, including a pair of cycle dependent elements (CDE) downstream of the transcriptional start site and a cyclin homology region (CHR) within the promoter, may help regulate the cell cycle dependent upregulation of Survivin at G₂/S phase (Li and Altieri 1999). The timely degradation of Survivin at G₁ phase is mediated by the ubiquitin proteosome pathway, but the E3 ubiquitin ligase, which regulates specificity, has yet to be identified (Zhao et al. 2000). Recent studies by Hoffman
et al. (Hoffman et al. 2002) implicate p53 in the repression of Survivin, as well as several important cell cycle gene products such as cyclin B1, cdc2 and cdc25c. Furthermore, the p53 mediated apoptosis caused by UV irradiation is blocked by overexpression of Survivin, suggesting a possible role for Survivin in suppressing p53 mediated apoptosis (Hoffman et al. 2002). Therefore, the suppression of Survivin expression at the key G2/M checkpoint may aid the progression of p53 induced apoptosis.

Survivin is active at several points in the cell cycle. Phosphorylation of Survivin at Thr(34) by cdc2/cyclin B1 is seen at G2/M phase prior to association with the mitotic spindles (O'Connor et al. 2000). Overexpression of Survivin caused an accelerated S phase shift and activation of the cdk2/cyclin E complex, which leads to Rb phosphorylation at G1 (Suzuki et al. 2000). At the G1/S transition, Survivin has been shown to translocate into the nucleus and bind cdk4, possibly inhibiting the association between cdk4 and p16(INK4a) (Suzuki et al. 2000). The sequestration of cdk4 by Survivin also releases p21, which is then allowed to interact with procaspase-3 and prevent cell death caused by Fas (Suzuki et al. 2000). These studies demonstrate that Survivin is a vital link between apoptosis and the cell cycle and that Survivin may play a role in an internal damage sensor which triggers apoptosis when faced with unrecoverable errors during cell proliferation.

3.2.5 Livin

Several independent groups simultaneously isolated a novel BIR containing protein using an 840 nucleotide fragment discovered through a
BLAST database homology search. Lin et al. (Lin et al. 2000) isolated a cDNA clone with an 894 bp coding region encoding a 298 amino acid protein dubbed kidney IAP (KIAP). Similarly, Kasof and Gomes (Kasof and Gomes 2001) discovered a slightly smaller 840 bp coding region, which encodes the 280 a.a. Livin protein. Later analysis shows that Kiap and Livin are splice isoforms of the same gene, which were renamed Livinα and Livinβ respectively (Ashhab et al. 2001).

Three transcripts of Livinα mRNA, with estimated sizes of 1.4, 2.2, and 4 kb, appear in Northern blots and expression is found strictly in the placenta, lymph node and fetal kidney (Lin et al. 2000). Northern blot analysis of Livinβ indicate the same size transcripts, likely due to cross reactivity with Livinα mRNA, and shows a similar expression pattern in human tissues, except it appears in fetal brain instead of fetal kidney. Livinβ expression in human cancer cell lines appears particularly high in the melanoma G361 and SK-Mel29, with minor expression in HeLa S3 cells (Kasof and Gomes 2001). More sensitive analysis using semi-quantitative RT-PCR indicated the presence of Livinα mRNA in heart, brain, placenta, lung, kidney, spleen, ovary and PBLs and Livinβ mRNA in heart, placenta, lung, kidney, spleen and ovary. Only the Livinβ transcripts were found in fetal tissues, especially kidney, heart and spleen. Both Livinα and Livinβ mRNA are detectable in high levels in various cancer cell lines, particularly in melanoma, colon and prostate carcinomas and several different leukemic cell lines (Ashhab et al. 2001). The subcellular localization of Livinβ appears to be
mainly nuclear with microtubule association, similar to Survivin, which suggests a possible role in mitosis (Kasof and Gomes 2001).

The *livin* gene is found on chromosome 20q13.3 and consists of 7 exons. Both livin\(\alpha\) and livin\(\beta\) mRNAs are composed of all 7 exons, but livin\(\beta\) uses an alternative splice acceptor site 54 bp downstream from the livin\(\alpha\) acceptor site on exon 6, which produces an in-frame 18 amino acid deletion (Lin et al. 2000; Ashhab et al. 2001). Livin consists of a single N-terminal BIR domain, similar to Survivin and Bruce/Apollon, and a C-terminal RING domain. The deletion in Livin\(\beta\) does not affect either the BIR or RING finger. The Livin BIR domain shows highest homology to NAIP BIR2 (58.8% identity), as well as to BIR3 of NAIP, XIAP, HIAP1 and HIAP2 (50, 52.9, 52.5 and 53.7% identity respectively). The RING zinc finger also shows high homology with the RING fingers of other human IAPs (XIAP-60%, HIAP1-72%, HIAP2-74% identity)(Lin et al. 2000).

Overexpression of either Livin isoform is very effective in blocking apoptosis triggered through the TNF or Fas pathway or by expression of pro-apoptotic proteins such as Bax (Lin et al. 2000; Ashhab et al. 2001; Kasof and Gomes 2001). However, the two Livin isoforms do demonstrate differential effectiveness against staurosporine and etoposide induced apoptosis. Livin\(\alpha\), but not Livin\(\beta\), was protective against the kinase inhibitor staurosporine, whereas Livin\(\beta\), and not Livin\(\alpha\), was effective against the DNA damaging anticancer drug etoposide. This disparity between the function of the Livin splice variants point to the importance of the 18 amino acid difference, which structurally forms an alpha helix in Livin\(\alpha\) (Ashhab et al. 2001).
The anti-apoptotic capacity of Livin, like most IAPs, is linked to its ability to block caspases. Studies by Kasof and Gomes (Kasof and Gomes 2001) discovered that the shorter Livinβ binds active caspase-3 and -7, directly inhibiting their function through the N-terminal BIR domain. Livinβ also bound unprocessed and active caspase-9, and prevented caspase-9 activation induced by Apaf-1, cytochrome c and dATP. Antisense Livinβ expression leads to the downregulation of Livinβ and triggers apoptosis in Livinβ expressing cell lines with a concurrent increase in DEVD-specific caspase activity (Kasof and Gomes 2001). Aside from caspase inhibition, Livin is able to block apoptosis through activation of the TAK1/JNK1 pathway. Overexpression of catalytically inactive TAK1 or JNK1 blocks the anti-apoptotic ability of Livin upon caspase-1 or TNFα induced apoptosis, but does not inhibit the anti-caspase activity of Livin. This suggests that, like XIAP, Livin is able to block apoptosis by, not only directly inhibiting caspase, but also by activating the anti-apoptotic portion of the TNF pathway (Kasof and Gomes 2001; Sanna et al. 2002).

3.2.6 Other IAPs

Several novel IAPs have been recently discovered, but have yet to be fully characterized. The unusually large BIR repeat containing ubiquitin conjugating enzyme (BRUCE) was initially discovered in mice and later isolated in humans and renamed Apollon (Hauser et al. 1998; Chen et al. 1999). The Bruce/Apollon gene was mapped to chromosome 2p21-22 and Bruce/Apollon mRNA was found in most adult tissues with particularly high expression in brain and kidney. Following immunostaining, the punctate expression of Bruce/Apollon co-localized
with the marker TGN38, indicating expression within the golgi and the vesicular system (Hauser et al. 1998). The 530 kDa human Bruce/Apollon protein consists of a single N-terminal BIR domain and an ubiquitin conjugating (UBC) domain at the C-terminus (Chen et al. 1999). Although no function has been currently ascribed to mammalian Bruce/Apollon, the Drosophila homologue, dBruce, has been shown to suppress apoptosis in insect cells (Vernooy et al. 2002). The similarity between the BIR domains of Bruce/Apollon and BIR containing proteins important in mitosis, such as Survivin, also suggests a role for Bruce/Apollon in cell cycle regulation.

In Northern blots of human tissues probed for XIAP, a second 2.2 kb crossreactive band was found expressed only in the testis. When cloned, this transcript proved to encode a novel IAP with high homology to XIAP (80% a.a. identity, 90% a.a. similarity) termed IAP like protein 2 (ILP-2) or testes specific IAP (Ts-IAP) (Lagace et al. 2001; Richter et al. 2001). The 711 bp coding region encodes a 26 kDa protein that consists of a single N-terminal BIR domain and a C-terminal RING zinc finger motif. The ts-iap gene, located on chromosome 19q13.3-4, is intronless and contains remnants of the xiap BIR1-2 coding sequence within its 5' UTR, suggesting that the ts-iap gene originated from a reverse transcription/genomic reintegration event (Lagace et al. 2001). Overexpression of Ts-IAP blocked cell death induced by BAX expression or coexpression of caspase-9 and Apaf-1, but was unable to affect TNF or Fas mediated cell death. Active caspase-9 can be immunoprecipitated with Ts-IAP indicating that Ts-IAP may be a direct inhibitor of caspase-9, similar to XIAP
BIR3 (Richter et al. 2001). Due to the similarity to XIAP, the RING finger of Ts-IAP may have ubiquitin ligase activity, but this has yet to be proven.

**IAP Antagonists**

As guardians against apoptosis, IAPs must themselves be closely regulated to prevent IAP hyperactivity and allow organized cell death when appropriate. It is therefore not surprising that several antagonists of IAP function have been recently characterized. The Smac/Diablo protein was simultaneously isolated from cell lines by two separate groups. Du et al. (Du et al. 2000) used a six step purification process to isolate a caspase-3 activation enhancing factor from the mitochondrial membrane fraction of HeLa cells. Peptide sequencing of the approximately 25 kDa protein identified a previously uncharacterized protein which they named the second mitochondrial-derived activator of caspase or Smac (Du et al. 2000). Using a different approach, Verhagen et al. (Verhagen et al. 2000) immunoprecipitated several proteins which bound Flag tagged mouse XIAP and identified one of them as a direct IAP binding protein with low pH (Diablo). The human homologue of Diablo was isolated through database searches and found to encode a 26.8 kDa protein with the identical sequence as Smac (Verhagen et al. 2000). Analysis by both groups indicated that the Smac/Diablo protein showed no sequence similarity to any known proteins or motifs. Overexpression of Smac/Diablo, which did not induce apoptosis alone, potentiates apoptosis induced by UV irradiation and enhances caspase-9 and caspase-3 activity through XIAP inhibition (Chai et al. 2000; Du et al. 2000; Verhagen et al. 2000; Ekert et al. 2001). Similar results were seen with Survivin,
whereby Smac/Diablo expression stimulated adriamycin induced apoptosis and blocked Survivin interactions with caspase-9 (Vucic et al. 2002).

The 1.4 kb Smac mRNA is moderately expressed in most human tissues and highly expressed in the heart, liver, kidney, pancreas, spleen, prostate, testes, and ovary (Du et al. 2000; Verhagen et al. 2000). Full length 26.8 kDa Smac is found within the mitochondrial intermembrane space and co-localized with cytochrome c, whereas the processed 25 kDa Smac co-localized with XIAP in the cytoplasmic fraction (Du et al. 2000; Verhagen et al. 2000). The immature pro-form of Smac has a 55 a.a. mitochondrial localization signal located at its N-terminus which is cleaved to produce the mature protein. Loss of the localization signal is induced by several apoptotic triggers, including UV or gamma irradiation, chemical insult, and Fas binding, which results in the efflux of mature Smac into the cytoplasm with kinetics similar to cytochrome c. Cleavage of immature Smac appears downstream of caspase activation, since it is blocked by broad spectrum caspase inhibitors or expression of the anti-apoptotic protein Bcl-2 (Du et al. 2000; Verhagen et al. 2000; Adrain et al. 2001)

The removal of the amino terminal end is not only necessary for the release of Smac from the mitochondria, but is also vital for the activation of its IAP binding activity. The new N-terminus exposes a novel tetrapeptide (Ala-Val-Pro-Ile) that is crucial for the interaction between Smac and several IAPs, including XIAP, HIAP1, HIAP2, OplAP and Survivin (Du et al. 2000; Verhagen et al. 2000; Vucic et al. 2002). This tetrapeptide sequence shows high homology to the IAP interacting domain at the N-terminus of the caspase-9 fragment p12 (Ala-
Thr-Pro-Phe) (Chai et al. 2000; Srinivasula et al. 2000). Similar sequences are found in several *Drosophila* pro-apoptotic proteins: Hid (Ala-Val-Pro-Phe), Reaper (Ala-Val-Ala-Phe) and Grim (Ala-Ile-Ala-Tyr) (Chai et al. 2000; Srinivasula et al. 2000). Though these proteins do not share significant sequence homology with Smac, they are thought to be functional homologues by promoting cell death by inhibiting IAPs.

Smac exists as a dimer through interactions between its extensive hydrophobic interfaces formed by four antiparallel \(\alpha\)-helical bundles (Chai et al. 2000).

Smac binds to either BIR2 or BIR3, but not to BIR1. Homodimerization appears to be critical for BIR2 binding, yet is dispensable for the interaction with BIR3 (Chai et al. 2000). Smac mutations which prevent formation of the homodimer are unable to bind XIAP BIR2 and potentiate caspase-3 activity, whereas deletion or mutation of the Smac N-terminal tetramer abrogates binding to XIAP BIR3, but is still able to bind XIAP BIR2 and augment caspase-3/7 activity, though at a far lower potential (Chai et al. 2000; Srinivasula et al. 2001). Therefore, it appears that homodimerization of Smac, and not the tetrapeptide sequence, is largely responsible for the Smac/XIAP BIR2 interaction and the resulting increase in caspase-3/7 activity.

The Smac tetrapeptide motif, though not vital for XIAP BIR2 binding, is absolutely vital for XIAP BIR3 and Survivin BIR interactions. Deletion or mutation of important residues within the Smac tetrapeptide sequence abrogated XIAP BIR3 and Survivin BIR binding and permitted XIAP or Survivin inhibition of
caspase-9 (Chai et al. 2000; Srinivasula et al. 2001; Vucic et al. 2002). A novel Smac splice variant, which excludes the first two exons, is also unable to bind and block XIAP BIR3 (Srinivasula et al. 2001). Crystallographic studies of the Smac/ XIAP BIR3 complex indicate that the N-terminal Ala of the Smac tetrapeptide sequence is bound in a hydrophobic pocket within XIAP BIR3, forming up to five hydrogen bonds with neighbouring BIR3 residues. The other three residues of the tetrapeptide sequence are hydrophobic and may provide alignment and stabilization of the Ala bond through binding to other hydrophobic residues lining the BIR3 surface groove. The proline at position P3 is especially important for alignment with the surface groove of BIR3 by introducing a kink within the peptide (Liu et al. 2000; Wu et al. 2000). Similarly, the active caspase-9 tetrapeptide also utilizes its N-terminal Ala residue to anchor the protein into the same BIR3 hydrophobic pocket, along with hydrophobic associations which overlaps the Smac binding domain (Srinivasula et al. 2001). Adherence to XIAP BIR3 by caspase-9 and Smac is mutually exclusive, with Smac functioning as a competitive inhibitor of XIAP function.

Another mitochondrial protein, Omi/HtrA2, has been implicated in IAP interference. Utilizing full length XIAP or XIAP BIR3 as bait, a 37-38 kDa protein was isolated from cell lysates, protein sequenced and identified as a truncated version of Omi/HtrA2 (Suzuki et al. 2001; Hegde et al. 2002; Martins 2002; Verhagen et al. 2002). Originally identified as a homologue of the E. coli heat shock inducible serine protease HtrA (Faccio et al. 2000; Gray et al. 2000) the Omi protein is a 458 amino acid protein with a molecular weight of 49 kDa.
Structurally, the full length Omi protein consists of an N-terminal mitochondrial targeting sequence, a conserved serine protease domain and a single PDZ domain at the C-terminus. The Omi protein is primarily localized to the mitochondria, but is released into the cytosol upon introduction of an apoptotic trigger, such as UV irradiation, tBid expression, Fas and TNF activation (Hegde et al. 2002; van Loo et al. 2002; Verhagen et al. 2002). Mitochondrial release of Omi coincides with release of Smac and cytochrome c. As with Smac, mature cytosolic Omi loses the mitochondrial targeting sequence through protein cleavage and Bcl-2 overexpression prevents mitochondrial release (Hegde et al. 2002; Verhagen et al. 2002).

In addition to XIAP, Omi is also able to bind HIAP1, HIAP2 and OplAP, but not Survivin (Hegde et al. 2002; Verhagen et al. 2002). The removal of the mitochondrial signal peptide of Omi exposes an N-terminal tetrapeptide sequence (Ala-Val-Pro-Ser) similar to Smac and caspase-9. This tetrapeptide domain is necessary and sufficient to bind both XIAP BIR2 and BIR3. Unlike Smac, Omi interacted more strongly with XIAP BIR2 than with BIR3, suggesting a preferred role in promoting caspase-3 activity. Mutation of the Omi tetrapeptide or XIAP BIR3 amino acids important for Smac binding abolished the interaction between Omi and XIAP BIR3, which indicates that a similar binding domain exists on XIAP BIR3 for both Omi and Smac (Hegde et al. 2002; Verhagen et al. 2002). Moreover, the Omi tetrapeptide interaction with IAPs potentiates apoptosis by permitting caspase-3 and -9 activities (Hegde et al. 2002; Verhagen et al. 2002). In contrast to Smac, overexpression of Omi alone can induce
apoptosis. Tetrapeptide deletion mutants of Omi still induced apoptosis, but mutants which have lost vital residues within its serine protease domain did not cause cell death. The binding of XIAP to Omi did not affect its serine protease activity nor its ability to induce apoptosis, indicating that the XIAP inhibition function of Omi is separate from its ability to induce apoptosis (Hegde et al. 2002; Verhagen et al. 2002). Omi aggregation is vital for its serine protease activity and its apoptotic abilities, since disruption of the Omi trimer structure by mutagenesis destroys these functions (Li et al. 2002). Overall, Omi appears to both induce and promote the apoptotic process.

Smac and Omi both represent a novel class of IAP antagonist based on the AxPxA consensus tetrapeptide sequence. These mitochondrial proteins likely work within the endogenous apoptotic pathway, concurrently released from the mitochondria with cytochrome c. Other proteins, such as XAF1, embody a new class of IAP antagonists which block IAP function in the absence of an AxPxA tetrapeptide sequence.

Thesis Outline

We isolated the novel XIAP interacting protein, XIAP associated factor 1 (XAF1) in a yeast two-hybrid system utilizing full length XIAP protein as bait. The open reading frame encodes a unique 35 kDa protein, which potentially contains seven zinc fingers. This thesis documents the characterization of XAF1 genomic and mRNA structure, as well as detailing XAF1 function and its importance in cancer. Our initial studies cover the characterization of the xaf1 gene, the isolation of new xaf1 mRNA isoforms, as well as the tissue and subcellular
localization of XAF1 protein. Due to the inhibitory nature of other XIAP interacting proteins, the functional consequences of the XAF1/XIAP interaction on caspase activity and etoposide induced apoptosis were also examined. These studies showed that XAF1A inhibits the caspase-3/XIAP interaction and, consequently, is able to reverse the anti-apoptotic abilities of XIAP in vivo. Next, we describe the tissue expression of the murine IAP homologues and the sequence analysis of the mouse xaf1 gene and mRNA isoforms. These studies lead to the construction of the xaf1 targeting vector, which is currently being used to develop the xaf1 null mouse. Finally, we report a common loss of XAF1 expression in the majority of cancer cells, and the possible role of XAF1 in cell cycle regulation. The decrease in xaf1 mRNA expression, coupled with the general loss of heterozygosity with the xaf1 locus suggests that the removal of XAF1 may be an important factor in the development of the malignant phenotype. Indeed, XAF1A expression can cause cell cycle arrest at G1 through the p53-independent induction of p21. Therefore, the loss of XAF1 expression relieves both the inhibition of XIAP function and cell cycle repression, thereby promoting cancer growth. We propose that xaf1 is a tumour suppressor gene which functions as a novel antagonist of XIAP function and triggers G1 cell cycle arrest through the upregulation of p21.
CHAPTER 2: GENERAL MATERIALS AND METHODS

1. DNA Preparation and Analysis

1.1 Vector Construction

XAF1 isoforms were amplified using primers beginning at the known ATG (AGATCTATGGAAGGAGACTTCTCGGTG) and ending at the XAF1A stop codon (ATCTAGCTGAAATTTCCTCATTGA). These primers were used to amplify a Marathon ready cDNA library (Clontech) according to manufacturers' instruction using PFU polymerase (New England Biolabs). PCR products were cloned into the PCR2.1 vector using the TOPO TA cloning kit (Invitrogen) and sequenced on a 373A DNA sequencer (Applied Biosystems). The cDNA clones were subcloned into pGex-KG (Pharmacia) for GST protein production through a simple EcoRI (Gibco/BRL) ligation. The XAF1 isoforms were also cloned into the pEGFP-C1 (Invitrogen) vector through a BglII/Smal ligation.

1.2 Recombinant Adenovirus

The recombinant adeno-xf1 was constructed through a Clal/Swal blunt end ligation with the pAdex1CA cosmid. The cosmid was co-transfected with Ad5dlX-TPC complex into 293 fibroblast cells using the calcium phosphate transfection method (Current Protocols in Molecular Biology, Ch. 9.1). Recombinant viruses were isolated, plaque purified, and amplified as previously described (Miyake et al. 1996).
1.3 Genomic DNA Isolation

Total genomic DNA was isolated from normal human peripheral blood and cancer cell lines by standard phenol/chloroform extraction as described (Current Protocols in Molecular Biology, Ch. 2.2). Briefly, cell were lysed in 300 µl 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 49%pheno/49%chloroform/2% isooamyl alcohol, again with 1 volume chloroform and precipitated with ½ volume 7.5 M ammonium acetate and 2 volumes 95% ethanol. DNA pellets were washed with 70% ethanol, dried and resuspended in TE (10 mM Tris-HCl, pH 8.0, 25 mM EDTA). The DNA concentration was determined on a Genequant spectrophotometer (Pharmacia).

1.4 Plasmid DNA Isolation

Mini-prep plasmid DNA was isolated by standard alkaline lysis method as described (Current Protocols in Molecular Biology, Ch. 1.6). Briefly, clones were grown in 5 ml aliquots of Luria Broth (LB, 1% tryptone, 0.5% yeast extract, 1% NaCl) containing the appropriate antibiotics (50 µg/ml ampicillin or kanamycin) overnight at 37°C with vigorous shaking. Cells were pelleted and sequentially treated with 100 µl GTE (25 mM Tris-HCl, pH 8.0, 10 mM EDTA, 50 mM glucose), 200 µL 1% SDS/0.2 N NaOH, and 150 µL 5M KAc at 5 min intervals on ice. The supernatant was precipitated in 90% ethanol, washed in 70% ethanol and resuspended in 50 µL TE. Maxi-prep plasmid DNA was prepared
using a Qiagen column maxi-prep kit according to manufacturer’s instructions (Qiagen).

1.5 Southern Blotting

Isolated genomic or YAC DNA (5 μg) or plasmid DNA (1 μg) was digested with the appropriate restriction enzyme overnight at 37°C and separated on a 1% agarose gel in 1X TAE overnight at 45 V. The DNA was partially depurinated for 30 min. with 0.25 M HCl 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, Gaithersburg, MD). Blots were baked for 40 min. at 80°C and pre-hybridized overnight at 65°C in hybridization buffer (1.5 X SSPE, 7% SDS, 10% PEG). Southern blots were probed with the 32P labeled 750 bp fragment of XAF1A at 1 X 10^6 CPM/ml overnight at 65°C. After appropriate washings of the membranes at 65°C with buffers of increasing stringency (2-0.01 X SSC, 0.1% SDS), the blots were exposed to film (Kodak X-OMAT) overnight at -80°C.

1.6 Transposon Sequencing

Genomic clones from human and mouse library screenings were cloned into the pKO1 vector and transposon sequenced using the TN1000 transposon sequencing kit (Gold Biotechnology Inc., St. Louis, MO). In brief, the pKO1 genomic clone was transformed into the DPWC bacterial host strain, which contains a transposon on the F factor. The transposon randomly integrates into the target plasmid, which fuses the F factor with the pKO1 vector. The ampicillin resistant F factor/pKO1 fusion plasmid is transferred from DPWC to the
kanamycin resistant BW26 by mating (100 μL overnight culture of each in 500 μL LB), where the co-integrate is resolved back into the original F factor and a pKO1 recombinant containing a randomly integrated transposon sequence. Samples were taken at 1, 2, 4 h post-inoculation and plated on kanamycin/ampicillin (50 μg/mL each) LB agar plates. Double antibiotic resistant clones were isolated and analyzed by Sall digests for a properly integrated transposon within the sequence of interest. Positive clone plasmids were isolated and sequenced using the G186 (ATATAACAAACGAATTATCTCC) and G187 (GTATTATAATCAATAAGTTATACC) primers found within the transposon sequence. Sequence files were organized into contigs using the GCG sequence analysis program.

2. RNA Isolation and Analysis

2.1 Isolation of Total RNA

Total RNA was isolated from 100 mg of murine tissues using Trizol reagent (Gibco/BRL). Mouse tissues were placed in 1 ml Trizol reagent and homogenized using a Polytron tissue homogenizer (Brinkmann). 200 μL chloroform was added, vortexed and incubated at room temperature for 3 min. The sample was centrifuged for 15 min at 12000 rpm and the aqueous phase transferred to a new tube. RNA was precipitated through the addition of 500 μL isopropanol and again centrifuged for 15 min at 12000 rpm. Pellets were washed with 1 ml 70% ethanol, dried for 5 min and resuspended in 50 μL RNase free H2O. Purified RNA was incubated at 60°C for 10 min to facilitate complete
resuspension and the RNA concentration measured on a Genequant spectrophotometer (Pharmacia). RNeasy mini spin columns were used to isolate total RNA from cancer cell line pellets provided by the National Cancer Institute. Total RNA was isolated from ~1 x 10^7 cells using RNeasy mini spin columns combined with DNase treatment (Qiagen) according to the manufacturer’s instruction.

3. Protein Isolation and Analysis

3.1 Western Blot Analysis

Proteins samples were analyzed by Western blot as described (Current Protocols in Molecular Biology, Ch. 10.2). 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 (15% acrylamide/0.4% 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 transferred onto PVDF membranes (Immobilon) using a semi-dry gel transfer apparatus (Hoefer Scientific). Blots were probed with a polyclonal anti-XAF1, a polyclonal anti-XIAP, a monoclonal anti-p53 or various monoclonal antibodies from the cell cycle sampler kit (Calbiochem) at a 1/1000 dilution in blotting buffer (5% skim milk/0.1% Tween20/1X PBS) for 1 h. The blots were washed with 0.1% Tween20/1X PBS 4 times for 15 min. Secondary anti-rabbit or anti-mouse antibodies fused to horse radish peroxide (HRP, Amersham) were used at a
concentration of 1/2500 in blotting buffer and incubated for 1 h with shaking. Blots were washed 4 times with 0.1% Tween20/1X PBS for 15 min each before chemiluminescent processing (ECL, Amersham) and exposure to X-ray film.

3.2 Preparation of Antibodies

Polyclonal anti-XAF1 and anti-XIAP antibodies were generated by immunizing rabbits with GST-XAF1A or GST-XIAP fusion proteins in RIBI adjuvant (Sigma). Polyclonal anti-XAF1A serum was cleared of anti-GST antibody on a GST-agarose column (Pharmacia) then positively selected on a GST-XAF1-agarose columns, generated using purified GST-XAF1 protein and glutathione agarose beads (Pharmacia).

3.3 Protein Purification

GST fusion proteins were grown in the BL21 E. coli strain overnight in a 20 ml LB culture, supplemented with 50 µg/ml ampicillin, at 37°C. The 20 ml culture was then used to inoculate 180 ml LB, supplemented with ampicillin and 10 µM zinc acetate and grown at 30-37°C until OD_{600}=0.7-1.0. IPTG was added to a concentration of 0.1 mM and grown for an additional 4 h. The bacteria was spun down at 13000 rpm for 5 min and resuspended in 20 ml of STE (10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 1 mM EDTA), 5 mM DTT, 0.1 mg/ml lysozyme and 1 mM PMSF (Sigma). After 20 min on ice, 4 ml of 10% taurocholic acid was added and the bacterial cells were lysed by sonication (1 min at 35% amplitude, Vibra Cell, Sonics and Materials). Cellular debris was removed by centrifugation for 10 min at 10000 rpm and filtered through cheesecloth. Triton X-100 (BDH)
was added to a final concentration of 2% and 500 µl of glutathione sepharose beads (Pharmacia) was added. The mixture was incubated for >1 h at 4°C. The glutathione beads were spun down and washed 3 times with STE. Proteins were eluted with 50 mM Tris-HCl, pH 8.0 supplemented with 10 mM free glutathione (Sigma). Purified proteins were stored in aliquots at -80°C.

pET-28a(+) His-tagged caspase-3 (Novagen) expressed in BL21 E. coli was grown in 10 ml LB cultures, supplemented with 50 µg/ml ampicillin and 20 µg/ml chloramphenicol, overnight at 37°C with shaking. 1 ml of culture was used to inoculate 100 ml LB/amp/chloramphenicol, which was grown at 37°C until the OD<sub>600</sub>=0.5-1.0. IPTG was then added to a final concentration of 1 mM and the culture incubated for an addition 3 h. Cells were pelleted by centrifugation at 5000 rpm for 10 min and lysed by sonication in 5 ml MCAC buffer (20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 10% glycerol, 0.1% Triton X-100 and 1 mM PMSF). MgCl<sub>2</sub> and DNase I were added to a final concentration of 10 mM and 10 µg/ml respectively and incubated 10 min at room temperature. Cell extracts were cleared by centrifugation at 15000 rpm for 15 min and applied to a Ni<sup>2+</sup>-NTA agarose column (Qiagen). The column was washed once with MCAC buffer, and then eluted with MCAC buffer containing increasing concentrations of imidazole (0.01-1 M). Collected fractions were analyzed for protein content and high protein fractions were combined and analyzed by SDS-PAGE gel.
CHAPTER 3: XIAP ASSOCIATED FACTOR 1 – GENOMIC STRUCTURE, SPLICE ISOFORMS AND FUNCTIONAL CHARACTERIZATION

We all labor against our own cure, for death is the cure of all diseases.

- Unknown

1. Introduction

The IAPs are evolving into a group of multifunctional proteins with pleiotrophic effects in eukaryotic cells. Although the majority of IAP proteins have been identified as direct caspase inhibitors, many IAPs have been ascribed additional roles in diverse cellular functions. These alternative functions include directed degradation of specific proteins, cell cycle regulation and association with certain cell signaling pathways (see Figure 3-1). The caspase inhibitory function of certain IAPs may be a secondary function relative to other roles they may play within the cell.

Several RING zinc finger (RZF) containing IAPs, including XIAP and HIAP2, have been described as E3 ubiquitin ligases (Yang et al. 2000; Li et al. 2002). These IAPs appear to target specific proteins by BIR domain binding and promote their ubiquitination and degradation through the E3 ubiquitin ligase activity of the RZF. Interestingly, these highly potent caspase inhibitors also promote caspase degradation, thereby inhibiting apoptosis by both the functional and physical removal of caspases. In the case of XIAP, self-ubiquitination is also seen, which may provide a negative feedback mechanism by which XIAP regulates its own function (Yang et al. 2000). Other XIAP binding partners, such
Figure 3-1. IAP Roles Beyond Caspase Inhibition

Numerous diverse roles have been demonstrated for many IAP family members, including caspase inhibition, protein ubiquitination, cell surface signaling and cell cycle regulation (see text for details). Several BIRCs, including Bruce/Apollon and Ts-IAP, do not yet have well defined caspase inhibitory properties and, therefore, were not listed.
as Smac, are also ubiquitinated through their interaction with XIAP (MacFarlane et al. 2002). The removal of Smac may play a significant role in the anti-apoptotic function of XIAP, since the elimination of the Smac binding site abrogates the ability of XIAP to inhibit apoptosis in UV irradiated cells, while removal of caspase-3 and/or -9 binding sites has little effect (Silke et al. 2002). Although Smac has been established as an inhibitor of the caspase/XIAP interaction, it is possible that Smac and caspases are actually ubiquitin targets of XIAP. In this model, the inhibition of caspases by XIAP, and the subsequent reversal by Smac overexpression, reflect competition for XIAP binding. The inhibition of apoptosis linked to the removal of Smac suggests an alternative pro-apoptotic function for Smac, which has yet to be discovered.

IAPs have also been implicated in the regulation of cell cycle and cell receptor signaling. Survivin expression is cell cycle regulated and can associate with mitotic spindles. It has been suggested that the expression of Survivin during G2/S phase of the cell cycle provides an internal sensor to gauge mitotic success and prevents the execution of the default apoptotic pathway during mitosis (Reed and Bischoff 2000). Loss of these controls through downregulation of Survivin produces polyploidy and cell death (Li et al. 1999). Thus, nuclear IAPs such as Survivin play dual roles both in the direct inhibition of caspases and as a vital regulator of the cell cycle. On the other hand, HIAP1 and HIAP2 are found associated with the TNF receptors and appear to tip the balance of TNF signals toward the anti-apoptotic NF-kB signaling pathway (Shu et al. 1996). Similarly, XIAP has been shown to associate with the BMP receptor.
and trigger pro-survival JNK signals through TAB1/TAK1 activation (Yamaguchi et al. 1999). These IAPs appear to augment their anti-apoptotic potency through the stimulation of cell survival signals. Clearly, IAP proteins are not strictly caspase inhibitors and participate in many different cellular functions.

The IAP family originally distinguished themselves as potent inhibitors of caspase function. With so many IAPs targeting similar caspases within an organism, the question of functional redundancy arises. This may be partially explained by the localization of IAPs within specific tissues. XIAP appears to be a widespread caspase inhibitor, being expressed in almost all human tissues and potently suppresses many different cell death triggers. HIAP1, on the other hand, has a more restricted expression pattern, being found primarily in lymphoid tissues and suppresses TNFα induced killing more potently than XIAP (Wright et al. 2000). The expression of HIAP1 within certain tissues provides necessary apoptotic resistance within specific cells, resistance not offered by other IAPs. The localization of Survivin within the nucleus suggests directed caspase inhibition at a subcellular level, thereby granting specific nuclear caspase protection. It appears that different IAPs can provide specialized protection to particular cells or cellular compartments. In addition, IAPs are emerging as multifunctional protein with other tasks beyond caspase inhibition. Therefore, though IAPs share the ability to inhibit caspases, each IAP member has developed their own specialized role within an organism, thus necessitating their place within the genome.
In an effort to identify novel XIAP interacting proteins, the yeast two-hybrid system was employed. Full length XIAP protein, cloned into the bait plasmid pAS2, was expressed in yeast and used to screen a human placental cDNA library. Of the 24 clones isolated, 7 non-identical clones encoding a novel zinc finger protein, termed XIAP associated factor 1 (XAF1), were obtained. Using the cloned cDNA, a human genomic and additional cDNA libraries were screened, which yielded the xaf1 gene structure and two novel XAF1 splice isoforms (XAF1B and XAF1C). Studies also showed that XAF1A is able to inhibit the caspase-3/XIAP interaction, similar to Smac, and that overexpressed XAF1A can reverse the anti-apoptotic function of XIAP in transduced cell lines.

2. Methodology

2.1 Human Genomic and cDNA Library Screening

A λ FIX II human male placenta genomic library (Stratagene) and a Uni-ZAP XR human liver cDNA library (Stratagene) were both probed with the first 750 bp of the XAF1A cDNA according to the manufacturer’s instructions. Selected human genomic clones were subcloned into the pKO1 vector (Holck and Korneluk, unpublished data) and sequenced utilizing the TN1000 transposon sequencing kit (Gold Biotechnology Inc., St. Louis, MO). Human cDNA clones were sequenced directly from in vivo excised Bluescript SK-plasmids. Full-length xaf1 cDNAs were isolated from human liver and placenta by RT-PCR using PFU polymerase and the following primers:
ATGGAAGGAGACTTCTCGGTG and TTAAAGCCCAACGAATAAAA. All sequencing was performed on a 373A DNA sequencer (Applied Biosystems).

2.2 Mutiple Tissue Northern Blot Analysis

Human multiple tissue northern blots (Clontech MTN blot I, blot II, and cancer cell line blot I), containing 2 μg poly(A)^+ mRNA, were probed with a [32P]CTP (Amersham) random-primed labeled (Rediprime, Amersham) DNA probe spanning the first 750 nucleotides of the xaf1 ORF. Blots were hybridized in ExpressHyb hybridization solution (Clontech) overnight at 65°C with constant shaking. Hybridized blots were washed several times with SSC/0.1% SDS wash buffers at increasing stringency (1-0.1X SSC) and exposed to X-ray film at -80°C.

2.3 Fluorescence Imaging

To examine the subcellular localization of the various XAF1 isoforms, GFP-XAF1 fusion constructs (see above) were transfected into subconfluent 293 fibroblasts using the Lipofectamine 2000 reagent (Invitrogen) according to manufacturers instructions. At 48 h post-transfection, cells were washed twice with 1X PBS and fixed with 2% paraformaldehyde for 15 min at 4°C. Slides were washed five times with 1X PBS and imaging was performed on a Zeiss Axiohot fluorescence microscope using a Sony 3CCD PowerHAD camera and Northern Eclipse 5.0 software.

2.4 GST Pulldown Assays

To test binding affinities, purified recombinant GST proteins were used to pulldown [35S] labeled proteins. In vitro translated proteins were generated by
the TNT quick coupled transcription/translation system (Promega) and labeled through the addition of [\textsuperscript{35}S]methionine (Amersham). Proteins were combined in binding buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, and 1 mM PMSF) + 50 µl glutathione sepharose beads and incubated >1 h at 4°C. Bound proteins were spun down for 30 sec at 4°C and washed seven times with wash buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100 and 10 mM EDTA). Beads were resuspended in protein loading buffer and run on a 15% SDS-PAGE gel. The gel was dried flat on a gel dryer (GD-3, Owl Scientific Plastics) and exposed to X-ray film at room temperature for 24 h.

2.5 Caspase-3 Inhibition Assays

Purified GST, GST-XIAP and GST-XAF1 were combined with purified His-caspase-3 (50 pM) in 200 µL caspase reaction buffer (10 mM Hepes, pH 7.5, 1 mM EDTA, 5 mM DTT and 10% glycerol) and caspase-3 activity was measured using 10 µM p-nitroanilide (pNA) conjugated DEVD peptide substrate (ApoAlert, Clontech). DEVD-pNA hydrolysis was monitored at 30 second intervals using a spectrophotometer at OD\textsubscript{405} nm. To measure endogenous caspase activity, adenovirus transduced SF-295 cells were collected 8 h post-infection, lysed and the protein concentration measured using Bradford reagent (BioRad). Caspase activity was tested at various protein concentrations and the pNA-DEVD hydrolysis/mg total protein extract/hr calculated for each protein sample.

2.6 Survival Assays

Etoposide death assays were performed in 96 well plates containing 3 x
10^3 cells per well in 100 μL MEM + 10% heat inactivated fetal calf serum (FCS). Cells were infected with various recombinant adenoviruses at the stated MOI. At 24 h post-infection, various dilutions of etoposide were added for 4 h and the media replaced. Cell viability was determined 18 h later by adding 10 μL WST-1 reagent (Boehringer-Mannheim) and incubating the cell at 37°C in 5% CO₂ for 4 h. WST-1 hydrolysis was measured in a colorimetric plate reader (SpectraMAX 340, Molecular Devices) at 405 nm and the percent survival calculated relative to untransfected cells.

3. Results

3.1 Genomic Organization of the xaf1 Gene

The original xaf1 cDNA was isolated as a 1325 bp (ATG to poly(A)) DNA sequence. Unfortunately, a frequent, repetitive Line1 element makes the 3' UTR of the cDNA useless as a probe. Therefore, from this cDNA, a 750 bp coding region probe fragment was generated using BamHI, which cut the multiple cloning site just upstream of the ATG and within the xaf1 cDNA. Southern blot analysis of normal human DNA using this probe reveals a single xaf1 locus with the human genome (see Figure 5-3). To characterize the xaf1 gene, the 750 bp fragment was used to probe a human placental genomic λFixII library. From this genomic library screening, 50 xaf1 clones were isolated and mapped with various restriction enzymes (see Figure 3-2A). Clones ranged in sized from 9 to 20 kb. Six clones, which were determined to span the entire xaf1 gene, were subcloned into pKO1 and sequenced by transposon sequencing. Sequence data revealed
that the original xaf1 cDNA was encoded by seven exons (Exons 1-4 and 6-8) spanning approximately 20 kb (see Figure 3-2A and B). These intron-exon splice boundaries follow the standard GT/AG consensus at the intron ends (see Table 1). A 14mer CA repeat located between exons 2 and 3 (see Table 1) was identified, which would be useful for heterozygosity analysis if found to be polymorphic in the normal population. To examine this possibility, primers were designed at either side of the CA repeat, which produced an approximately 68 bp product. Analysis of 20 random human genomic DNA samples by PCR found only a single size band (data not shown), which indicated that the CA repeat is not sufficiently polymorphic, and therefore was not useful for the heterozygosity analysis employed in Chapter 5.

3.2 Isolation of XAF1 Isoforms

The original 903 bp xaf1 open reading frame was found to encode a 301 amino acid protein with a predicted molecular weight of 34 kDa. Structurally, the XAF1 protein has the potential to form seven zinc fingers, five of which are clustered close to the amino terminus (see Figure 3-3B). No complex zinc binding motifs, nor other known domain structures, were deduced from the protein sequence. Searching the NIH protein database using the first five amino terminal zinc fingers found a 41% and 22% amino acid identity with similar domains of FLN 29 and INFα receptor associated factor 6 (TRAF6) respectively. In an effort to isolate other XAF1 isoforms, as suggested by the Northern blot analysis, the amino terminal 750 bp BamHI fragment was used as a probe on a human liver cDNA uni-ZAP XR library. Human liver was used because it was
Figure 3-2. Organization of the xaf1 Gene and Splicing Isoforms

(A) The genomic structure of the xaf1 locus. Known exons are numbered. Certain restriction sites are labeled as follows: B – BamHI, E – EcoRI, H – HindIII. (B) Splicing pattern of xaf1 isoforms. Three cDNAs are formed by alternative splicing of the xaf1 gene. XAF1A represents the originally isolated xaf1 cDNA. XAF1B does not contain exon3 which causes an in-frame deletion of the third zinc finger. The addition of exon5 into XAF1C causes the truncation of the protein after the fifth zinc finger. White blocks represent zinc fingers. Proteins were drawn to scale.
Table 3-1. Intron-Exon Boundaries of the *xafl* Gene.

Nucleotides in bold represent splice donor and acceptor sites. Underlined nucleotides show non-consensus splice acceptor. Intron and exon sizes are also listed.
<table>
<thead>
<tr>
<th>Intron</th>
<th>Exon (Size)</th>
<th>Intron</th>
<th>Exon (Size)</th>
</tr>
</thead>
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<tr>
<td>N/A</td>
<td>1</td>
<td>GCAGGAACCTGGtAaagaaagtgctttct−ccagggcagacccgggtgga</td>
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</tr>
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<td>GCACCAGCAGGgtgaggagcgagggaggtggtcagagttcaag</td>
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<tr>
<td>tctggtgtgtgtgtgtgtgtggtgtgtttagGTGTTGGTGA</td>
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<td>689 bp</td>
</tr>
<tr>
<td>ggccagtgatcatgcctttcctctgtgcctccacagGCCAAATGAGT</td>
<td>4 (197 bp)</td>
<td>GTCGGGAAGGtaagacacaaactgggtggaagagagagcttcaag</td>
<td>1.22 kb</td>
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<tr>
<td>tgccatccggtgtctacattcctctctctactagAAACAGTCC</td>
<td>5 (153 bp)</td>
<td>TCAAGCCAGGCagttccggggtgggtctctctacgtgacagcagcc</td>
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</tr>
<tr>
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</tr>
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<td>8 (290 bp)</td>
<td>CAAACCGGCAGtcaagaaaaaatccccccccccccccccccccccaccag</td>
<td>N/A</td>
</tr>
</tbody>
</table>
demonstrated to have relatively average levels of xaf1 mRNA and at least four
different sized splice variants on Northern blots (Figure 3-3A). Screening of the
human liver cDNA library yielded seven clones, which revealed at least two novel
alternatively spliced xaf1 cDNAs, in addition to the original xaf1A. One of these
isolates (xaf1B, see Figure 3-3A) had spliced out the 57 bp exon 3 which resulted
in an in-frame 19 amino acid deletion and a total loss of the third zinc finger (see
Figure 3-3B). A second novel isoform spliced in a new exon, exon 5 (see Figure
3-3A), a 153 bp exon which added 24 novel amino acids but truncated the
protein before the sixth zinc finger (see Figure 3-3B). The 5’ splice site between
exons 5 and 6 is an unusual GC instead of the GT consensus, suggesting a
possible regulatory mechanism for the expression of this isoform (Table 1). All
isoforms were verified by RT-PCR on human liver RNA, TA cloned and
sequenced (Data not shown).

3.3 Expression of xaf1 mRNA in Human Tissues and Cell Lines

Human poly(A)$^+$ mRNA multiple tissue Northern blots (Clontech) were
probed using a 750 bp DNA fragment from the amino terminus of xaf1. Analysis
revealed widespread expression of xaf1 in most tissues, with the highest levels
found in heart, spleen, thymus and ovary (see Figure 3-4A). Interestingly, at
least four distinct xaf1 transcripts were discovered, with approximate sizes of 3.9,
4.5, 6.0 and 7.0 kb. These transcript variants suggests the presence of
alternative splicing mechanisms or poly(A) readthrough. Analysis of poly(A)$^+$
mRNA from eight cancer cell lines indicted little or no expression of xaf1 mRNA
in any of the samples (see Figure 3-4B). Those cell lines which did express
Figure 3-3. xaf1 Isoform cDNA and Protein Sequence

(A) Alignment of the xaf1 cDNA sequence. (B) Comparison of the amino acid sequences between the XAF1 isoforms (A, B and C). Lower case amino acids represent residues not found in original XAF1 sequence. Shaded and bold characters illustrate the zinc finger consensus sequence and important cysteine and histidine spacing (in bold).
some xaf1 mRNA, such as Molt4 and Raji, were restricted to certain mRNA isoforms, suggesting the loss of certain mRNA variants in cancer cells. As will be revealed in Chapter 5, the loss of xaf1 expression is a common feature of cancer cell lines.

3.4 Expression of XAF1 Proteins in Human Tissues

The expression of XAF1 in human tissues was further examined at the protein level by Western blots. Polyclonal and monoclonal antibodies, developed against purified GST-XAF1, were both used to probe Western blots prepared with human tissue protein medley extracts (Clontech). The blots probed with the polyclonal or monoclonal anti-XAF1 antibodies showed a similar pattern of bands in the majority of tissues tested, which reduced the possibility of the bands being non-specific (see Figure 3-5). Of the ten tissues tested, only ovary demonstrated high expression of an approximately 32.5 kDa band. Unfortunately, the band may either be XAF1A or XAF1B, since their molecular weights of 34 and 32 kDa respectively are very similar. Other tissues, such as brain, skeletal muscle and testes, have bands of similar size, but at much lower expression levels. Most tissues, with the exception of skeletal muscle, express a 16 kDa band, which corresponds with the size of XAF1C. Several strong smaller bands (<16 kDa) and an approximately 20 kDa band highly expressed only in the human brain are seen with both the polyclonal and monoclonal antibodies (see Figure 3-5). These bands may represent XAF1 cleavage products or other yet undiscovered isoforms.
3.5 Subcellular Localization of XAF1 Isoforms

The subcellular localization of the various XAF1 isoforms was examined using green fluorescent protein (GFP)-XAF1 fusion proteins expressed in the fibroblast cell line 293. GFP-XAF1A, B, and C were present in both the cytoplasm and the nucleus, while GFP and GFP-LacZ were mainly cytoplasmic. GFP-XAF1B transfected cells also showed peculiar punctate concentrations of GFP-XAF1B proteins within the cytoplasm, but mainly in cells that expressed high levels of protein (see Figure 3-6).

3.6 XIAP Interaction with XAF1 isoforms

To examine the interaction between the various XAF1 isoforms and XIAP, RT-PCR amplified XAF1A, B and C were individually cloned into pGEX KG. GST-XAF1 fusion proteins were produced in the BL21 bacterial strain and purified using glutathione agarose beads. These proteins were added to in vitro translated and radioactively labeled XIAP and pulled down with glutathione sepharose beads. GST-XAF1A and B, and to a lesser degree GST-XAF1C, were able to bind and pull down XIAP (see Figure 3-7A). The reciprocal assay using GST-XIAP bound and pulled down XAF1A and B, but not XAF1C (see Figure 3-7B). GST alone did not bind XIAP or any of the XAF1 isoforms. Apparently, the loss of the third zinc finger had little effect on XIAP binding, but the loss of zinc fingers 6 and 7 impaired XIAP binding.

3.7 Reversal of XIAP mediated anti-apoptotic protection

Due to its ability to bind to XIAP, we proposed that this interaction
Figure 3-4. Expression of xaf1 mRNA in Normal Human Tissues and Cancer Cell Lines

Multiple normal human tissue (A) and cancer cell line (B) Northern blots were probed with a 750bp fragment of xaf1A coding region and exposed for 24 h. Each lane contains 2 μg of poly(A) RNA. Sizes were estimated from the RNA markers on the blot.
Figure 3-5. XAF1A Protein Expression in Human Tissues

(A) Western blot analysis of human tissues using the polyclonal anti-GST-XAF1A antibody. (B) Western blot analysis of the same human tissues using a monoclonal anti-XAF1A antibody. Bands representing XAF1A+B and XAF1C are indicated by arrows. Gels were run simultaneously with 20 μg of protein in each lane and both gels were exposed on the same film for 30 sec. Band sizes were estimated using prestained protein markers on the transfer membrane.
Figure 3-6. Subcellular Localization of xaf1 Isoforms in 293 Cells.

XAF1 isoforms were fused to the C terminal end of GFP and transfected into the 293 fibroblast cell line. GFP localization was visualized using UV microscopy. Empty GFP vector and a GFP-LacZ construct were used as controls.
may inhibit the ability of XIAP to block caspases -3 and -7, similar to Smac or Omi. To this end, an in vitro colorimetric caspase-3 assay was utilized which employs His-tagged caspase-3 and the colorimetric substrate DEVD-pNA. The addition of purified GST-XIAP protein blocks the caspase-3 mediated cleavage of the DEVD-pNA substrate, as measured by a spectrophotometer at 405 nm (see Figure 3-8). The addition of purified GST-XAF1A at an increasing ratio relative to XIAP reverses the XIAP mediated inhibition of caspase-3 function, and restores almost full caspase activity at a 1:3 ratio of XIAP:XAF1A (see Figure 3-8). The addition of GST neither affected caspase function, nor reversed the XIAP mediated inhibition of caspase function. Therefore, the ability of XAF1A to bind XIAP has a direct negative impact on XIAP function.

To further determine the extent of XAF1A function in a cellular context, adenoviral XAF1A expression was tested against XIAP mediated anti-apoptotic protection. It has been previously established that XIAP expression protected cells from serum withdrawal and anticancer drugs such as etoposide. We examined the effect of XAF1A on the survival of the SF-295 human glioblastoma cell line after etoposide treatment. SF-295 cells were infected on 96 well plates with different combinations of recombinant adenoviruses. After 24 h, infected cells were treated in triplicate with various dilutions of etoposide ranging from 1 mM to 2 μM for 4 h. The etoposide was removed and the cells were incubated in normal media for an additional 18 hrs. Viability was measured by hydrolysis of a water soluble tetrazolium dye (WST-1). As expected, adeno-XIAP expression protected SF-295 cells from etoposide treatment, whereas adeno-LacZ had no
Figure 3-7. XAF1A and XAF1B Protein Isoforms Bind XIAP.

The ability of XAF1 isoforms to bind XIAP was tested in *in vitro* pull down assays. (A) Purified GST-XAF1A, B and C proteins were incubated with S\textsuperscript{35} labeled *in vitro* translated XIAP proteins and pull down using glutathione sepharose. Proteins were run on 15% acrylamide gels and exposed for 24 h. GST protein and glutathione beads alone were used as negative controls. (B) Purified XIAP was used to precipitate S\textsuperscript{35} labeled *in vitro* translated XAF1A, B and C proteins. GST proteins and glutathione beads alone were used as negative controls on XAF1A only.
Figure 3-8. XAF1A Interferes with XIAP Mediated Caspase-3 Inhibition

_in vitro_ caspase-3 reactions were monitored at 30 sec intervals using the DEVD-pNA colorimetric substrate. The addition of excess GST-XIAP inhibits >90% of this activity, but is reversed by increasing ratios of GST-XAF1A. Purified GST was used to maintain protein concentrations at 500 nM. ‘XIAP’ = 125 nM GST-XIAP protein + 375 nM GST. ‘3:1 XIAP/XAF1A’ = 125 nM GST-XIAP protein + 40 nM GST-XAF1A + 335 nM GST. ‘1:1 XIAP/XAF1A’ = 125 nM GST-XIAP protein + 125 nM GST-XAF1A + 250 nM GST. ‘1:3 XIAP/XAF1A’ = 125 nM GST-XIAP protein + 375 nM GST-XAF1A. ‘Caspase-3’ = control reaction containing 500 nM GST. XAF1A antagonizes the XIAP mediated inhibition of caspase-3 in a dose dependent manner.
effect on survival. On the other hand, adeno-XAF1A infection appears to potentiate the cell death induced by etoposide in SF-295 cells (see Figure 3-9A). When adeno-XIAP is co-infected with an increasing ratio of adeno-XAF1A, the expression of XAF1A protein again appeared to reverse the anti-apoptotic effects of XIAP in a dose dependent manner (see Figure 3-10A). The expression of XAF1A and XIAP protein was verified by Western blot, which demonstrated a steady level of XIAP concomitant with increased XAF1A expression roughly correlating with the adenoviral infection ratio (see Figure 3-9B and 10B). SF-295 cells expressed no endogenous XAF1A, which is a common feature in cancer cell lines (See Chapter 5), and adenoviral infection did not induce the endogenous expression of either XIAP or XAF1A.

The inhibition of XIAP function was predicted to have a direct positive effect on caspase-3 function. This was tested using cellular extracts from adenovirus infected SF-295 cell lines in an in vitro colorimetric caspase-3 assay. Treatment of SF-295 cells with 100 µM etoposide significantly increases the level of DEVD-pNA cleavage, indicating a large increase in caspase-3-like activity (see Figure 3-11). Infection of SF-295 cells with adeno-XIAP blocked production of this caspase activity by approximately 70% relative to adeno-LacZ transfected cells, whereas adeno-XAF1A individually had no effect. On the other hand, the co-infection of adeno-XAF1A at an increasing ratio to adeno-XIAP (1:3, 1:1 and 3:1 XAF:XIAP) restored caspase-3-like activity in a dose dependent manner (approximately 90% of adeno-LacZ levels), which correlated with the increased apoptosis seen previously (see Figure 3-11). Although the expression of XAF1A
Figure 3-9. Adeno-XIAP Protects SF-295 Cells from Etoposide Mediated Cell Death.

The effect of Adeno-XIAP or Adeno-XAF1A on etoposide induced death was examined in SF-295 cells. (A) Cells were infected with adeno-XIAP + adeno-LacZ (Multiplicity of Infection (MOI) = 5 pfu/cell each) or adeno-XAF1A + adeno-LacZ (MOI = 5 each). Adeno-LacZ (MOI = 10) and uninfected cells were used as controls. After 24 h post infection, cells were exposed to etoposide for 4 h, and viability was from etoposide mediated cell death, whereas LacZ overexpression had no effect. XAF1A overexpression appears to potentiate the etoposide mediated cell death. (B) Transduction of the target gene was verified using Western blots on infected cells from control wells. determined 18 hrs later by WST-1 assay. XIAP expression protects SF-295 cells
Figure 3-10. Adeno-XAF1A Increases Sensitivity of SF-295 Cells to Etoposide Triggered Apoptosis

Co-infection with adeno-XAF1 reverses adeno-XIAP mediated protection from etoposide induced apoptosis. SF-295 cells were infected with recombinant adenoviruses as follows: ‘XIAP + XAF1(1:3)’ = infection with adeno-XIAP (MOI of 5) + adeno-xaf1 (MOI of 1.5). ‘XIAP + XAF1(1:1)’ = infection with adeno-XIAP (MOI of 5) + adeno-xaf1 (MOI of 5). ‘XIAP + XAF1(3:1)’ = infection with adeno-XIAP (MOI of 5) + adeno-xaf1 (MOI of 15).
A

![Graph showing % survival vs. etoposide dose (μM)]

- XIAP + LacZ
- XIAP + XAF1A (3:1)
- XIAP + XAF1A (1:1)
- XIAP + XAF1A (1:3)

B

<table>
<thead>
<tr>
<th>XIAP + LacZ</th>
<th>XIAP + XAF1A</th>
</tr>
</thead>
<tbody>
<tr>
<td>3:1</td>
<td>1:1</td>
</tr>
</tbody>
</table>

- anti-XIAP
- anti-XAF1A
Figure 3-11. Etoposide Sensitivity Induced by Adeno-XAF1A Transduction Correlates with Increase Caspase-3-like Activity

Cell extracts were prepared from SF-295 cells prepared as in Figure 3-10. Extracts were prepared 8 h post-etoposide exposure (100 μM). The overexpression of XIAP significantly reduced caspase-3-like activity in SF-295 cell extracts, an effect which is reversed by the co-infection of increasing concentrations of adeno-XAF1A. Uninfected and untreated cell extracts were used as a control for caspase-3 activity.
clearly had a pro-apoptotic effect on cells treated with etoposide, the infection of adeno-XAF1A alone did not directly kill infected cells. The overexpression of XAF1A promoted apoptosis through inhibition of XIAP function, which thereby promoted caspase activity, but did not appear to induce apoptosis itself.

4. Discussion

In addition to targeting caspases, the anti-apoptotic XIAP protein interacts with a growing number of inhibitors, including Smac and Omi. A novel protein, XIAP associated factor 1 (XAF1), was initially isolated as a XIAP interacting protein and appears to operate, at least in part, by interfering with XIAP function. Our first objective was to characterize the genomic organization of xaf1. The xaf1 gene was found to consists of eight exons spanning approximately 18 kb. The number of exons, as well as the size of exon 1, will likely increase when the entire 5' UTR of the xaf1 mRNA is fully defined. Only one intron-exon splice junction, at the 3' end of exon 5, contained a non-consensus splice acceptor (GC instead of GT, see Table I). This may partially explain the alternative splicing of exon 5 in XAF1c. The CA repeat found just 5' of exon 3 was shown to be non-polymorphic by PCR (data not shown). Analysis of the DNA sequence in and around the xaf1 gene using the GRAIL analysis program did not yield any obvious promoter regions or alternative start sites, though complete sequencing of the 5' UTR may aid in their discovery.

Studies presented here indicated that the original xaf1 isolate was just one of at least three alternatively spliced isoforms of the xaf1 gene. Northern blot analysis of xaf1 mRNA indicated that multiple size isoforms exist in normal
human tissue. Though the splicing variants presented here are not large enough to entirely account for the size variation seen in the poly(A) Northern, it is clear that alternative splicing occurs in xaf1 and that splicing is likely to take place outside of the coding region as well. The RNA size variations may also be caused by other transcriptional controls such as poly(A) read through or alternative initiation sites. The nucleotide sequence changes in the two novel xaf1 isoforms had a profound effect on the overall structure of these proteins. The in-frame deletion of exon 3 in xaf1b, not only removes the third zinc finger entirely, but also moved the second and fourth zinc fingers closer together. The juxtaposition of zinc fingers 2 and 4 did not appear to result in a more complex zinc binding motif (Saurin et al. 1996). The splicing of xaf1c added an exon just after the fifth zinc finger. Though the rest of the RNA is identical to xaf1a and b, there were 3 sequential stop codons beginning 72 bp into the exon, which truncated the protein. The 24 amino acids added to the C-terminus had no obvious motifs. Zinc fingers have been implicated in both protein-protein and DNA binding (Saurin et al. 1996). Therefore, though the interaction between XIAP and XAF1 has not been mapped to a specific region of XAF1, it is likely that these new isoforms will have different effects on XIAP function. Indeed, recent studies indicated that, while expression of full length XAF1A in the A375 cell line increased IFN-β dependent sensitization to TRAIL induced cell death, a truncated form of XAF1A that contained the first six zinc fingers made these cells more resistant to the same apoptotic trigger (Leaman et al. 2002). This data
indicated that the full length XAF1A and the truncated XAF1C isoforms may play opposite roles in apoptotic regulation, at least under certain cell death triggers.

Expression of xaf1 mRNA appeared fairly ubiquitous among human tissues, with the highest level observed in the heart, spleen, thymus and ovary. The presence of four size variants also appeared in all the tissues tested, indicating little tissue specific expression. Sequence data of xaf1A, B and C, isolated from both library screening and PCR amplification of human liver mRNA, showed highly variable 3' UTRs that support both alternative splicing and poly(A) read through as possible mechanisms for the variation seen (Data not shown). Northern blot analysis using isoform specific probes may help sort out which bands are associated with which coding region isoforms, although it may be possible that two or more isoforms are represented by a single band on the Northern blot due to the very small size differences between each isoform. Contrary to mRNA levels, XAF1 protein expression within these tissues, especially XAF1A and B, was surprisingly low. With the exception of ovary, there was little or no XAF1A or XAF1B protein expression in all the tissues tested. On the other hand, the 16 kDa XAF1C appeared in the majority of tissues tested, suggesting that XAF1C may be the predominant expressed isoform in humans. Several smaller bands (<16 kDa) were found in most tissues, as well as a novel 20 kDa species in the brain. It remains to be determined whether these bands represent novel, uncharacterized isoforms, cleavage products of existing XAF1 isoforms or non-specific cross-reactivity. The fact that these bands were present
in Western blots probed with both a polyclonal and a monoclonal anti-XAF1A antibody suggested that non-specific antibody binding was unlikely.

The intracellular localization of the XAF1 was assessed using a GFP fusion construct designed for each XAF1 isoform and transfected into the 293 fibroblast cell line. XAF1A-GFP expression, as denoted by GFP fluorescence, appeared cytoplasmic, but may be nuclear in certain rounded cells. This expression pattern differed from what was previously reported for the XAF1A-RFP (Liston et al. 2001), but consistent with cell fractionation studies (Leaman et al. 2002), suggesting localization influence from the XAF1A fluorescent fusion partner or cell line specific effects (HeLa vs 293). In contrast, XAF1B-GFP was mainly cytoplasmic with many of the cells also containing punctate concentrations of GFP fluorescence in the cytoplasm. Such unusual concentrations of XAF1B-GFP have yet to be localized to a specific subcellular structure. Finally, the XAF1C-GFP demonstrated both cytoplasmic and nuclear fluorescence. None of the XAF1 isoforms demonstrated the mitochondrial staining found with other XIAP inhibitors such as Smac and Omi. This suggests that regulation of XIAP/XAF1 interaction is fundamentally different than the partitioning/regulation of the Smac/XIAP and Omi/XIAP interaction.

The XIAP/XAF1 isoform interaction was tested in an in vitro binding assay, using purified GST proteins to pull down $^{35}$S labeled in vitro translated proteins. Using this assay, GST-XAF1A and B were shown to bind in vitro translated XIAP protein, whereas GST-XAF1C and GST control protein did not. In the converse assay, GST-XIAP bound and pulled down XAF1A and XAF1B, but not XAF1C.
Clearly, the loss of the third zinc finger of XAF1 did not affect XIAP binding, but the loss of the carboxy-terminal end after the fifth zinc finger abrogated the XAF1/XIAP interaction. These results have been verified by similar GST binding assays using cellular extracts overexpressing the XAF1 isoforms. Once again, GST-XIAP was able to bind XAF1A and B, but not XAF1C, as shown in Western blots (Liston and Ho, unpublished data). The inability of XAF1C to interact with XIAP, and its expression in many different tissues, suggest a role for XAF1C beyond XIAP inhibition. The loss of the sixth and seventh zinc finger in XAF1C implicates these putative protein-protein binding domains in the interaction with XIAP, although it is possible that the intervening amino acids between the last two zinc fingers may have a yet unknown XIAP binding motif.

The XIAP interacting capability of XAF1A and B suggested that XAF1A and B may function to inhibit XIAP function, similar to other XIAP binding proteins such as Smac and Omi. To test this theory, an in vitro colorimetric caspase-3 assay was employed using purified GST-XIAP as the caspase-3 inhibitor and varying concentrations of GST-XAF1A. This assay showed that the XIAP inhibition of capase-3 can be reversed by the addition of increasing concentrations of XAF1A. The inhibition of XIAP function was unaffected by the presence of excess GST protein or general protein concentration, since purified GST was used to equalize protein concentrations in all test samples. Although the method by which XAF1A binds and inhibits XIAP is unknown, XAF1A does not contain an AVPI-like amino acid motif that is central to the function of other XIAP inhibitors such as Smac and Omi. It is likely, though yet to be tested, that
XAF1B can also inhibit XIAP function, due to its ability to directly bind XIAP. On the other hand, XAF1C is likely unable to block XIAP function because of its inability to bind XIAP. Interestingly, the data presented in Leaman et al. (Leaman et al. 2002) suggested that deletion of the carboxy terminal zinc finger of XAF1 resulted in a dominant-negative protein isoform. XAF1C is therefore predicted to function analogous to this artificial construct and antagonize the effects of XAF1A and B. Although the mechanism of action of XAF1C remains to be determined, it does not appear to function by competing with XAF1A for XIAP interaction. We are currently determining whether XAF1 proteins oligomerize, which may explain the basis of XAF1C function.

*In vitro* biochemical assays do not always correlate with protein function within the complex environment of the cell. In order to move the XAF1/XIAP interaction studies from a purely *in vitro* situation to an *in vivo* assay system, we employed tissue culture models of apoptosis. Adeno-XIAP transduced SF-295 cells were co-infected with adeno-XAF1A in an attempt to reverse the anti-apoptotic function of XIAP. As expected, overexpression of XIAP protects SF-295 cells from apoptosis induced by the topoisomerase II inhibitor etoposide. Co-infection with adeno-XAF1A decreased the viability of XIAP overexpressing cells throughout the range of etoposide treatment. The decreased viability directly correlated with increased XAF1A/XIAP ratio and with increasing XAF1A protein expression as shown by Western blot. The increase in apoptosis was not directly due to XAF1A toxicity, since adeno-XAF1A transduction alone had little effect on cell viability. The augmentation of apoptosis by adeno-XAF1A
transduction correlated with an increase in caspase-3 activity. The simplest interpretation is that the overexpression of XAF1A promoted apoptosis through the suppression of XIAP function, which lead to increased caspase activity and apoptosis. Other studies have shown that transduction of an anti-sense XAF1A adenovirus (adeno-asXAF1A) into the SF-539 cell line, which expressed high levels of endogenous XAF1A, partially protected cells from etoposide induced cell death. In contrast, the related cell line SF-295, which had no endogenous XAF1A expression, demonstrated no such protection from apoptosis upon adeno-asXAF1A infection (Liston et al. 2001). It appeared that downregulation of endogenous XAF1A expression increased the viability of cells treated with etoposide, presumably by relieving the repression on endogenous XIAP function, which in turn lead to suppression of caspase activity.

The studies presented above implicated XAF1A in the repression of XIAP activity, thereby promoting apoptosis. Although several XIAP inhibitors exist, including Smac and Omi, XAF1 differed in that the interaction with XIAP did not rely on an AVPI-like motif, and XAF1 isoforms did not localize to the mitochondria. XAF1A may operate by lowering the apoptotic threshold through XIAP inhibition, which sensitizes cells to apoptotic stimuli without requiring mitochondrial release. On the other hand, XAF1C may function as a dominant negative possibly by interfering with XAF1A function. Other mitochondria based XIAP inhibitors may function to potentiate apoptotic signals after the endogenous apoptotic pathway is triggered, and may have little effect on the mitochondria-independent exogenous pathway. The interaction between IAP and IAP
inhibitors is reminiscent of the Bcl-2 pathway, where apoptosis is mediated by the balance between multiple pro- and anti-apoptotic proteins. Cellular signals which control the function or expression of these proteins have a direct influence over the life or death of the cell.

From a different point of view, the ability of XIAP to function as an E3 ubiquitin ligase suggests an alternative interpretation of the XIAP/XAF1 interaction. XIAP has been shown to aid in the ubiquitination of several interacting proteins including caspase-3 and Smac (Suzuki et al. 2001; MacFarlane et al. 2002). The Smacβ splice isoform lacks the mitochondrial targeting sequence and the AVPI tetrapeptide. Despite the inability of Smacβ to interact with XIAP, Smacβ still displayed proapoptotic characteristics, suggesting a function for Smac beyond IAP inhibition (Roberts et al. 2001). XIAP may prevent apoptosis through the removal of pro-apoptotic proteins such as Smac and caspase-3 by ubiquitination, and not strictly through the inhibition of caspase function. As with Smac, XAF1A could also have cellular functions other than XIAP inhibition and the XAF1/XIAP interaction may be directed at the inhibition of XAF1A function, instead of XIAP function. As Chapter 5 will show, XAF1A expression blocks cell cycle at G1, indicating an alternative function for XAF1A which would be disrupted by the destruction of XAF1A through XIAP mediated ubiquitination. Further study is under way to determine whether other XAF1 isoforms inhibit XIAP function and whether XIAP mediates the destruction of XAF1A.
CHAPTER 4: MOUSE INHIBITORS OF APOPTOSIS TISSUE EXPRESSION
AND CHARACTERIZATION OF THE MOUSE xaf1 HOMOLOGUE

If we don't know life, how can we know death?
- Confucius (551-479 BC)

1. Introduction

1.1 Murine IAPs

The IAP family of proteins plays important roles in the regulation of both cell signaling and apoptosis. Therefore, it was not surprising that IAPs are highly conserved amongst higher eukaryotes from insects to mammals. Murine homologues were found for almost all human IAPs, including NAIP (Yaraghi et al. 1998), XIAP (Farahani et al. 1997), HIAP1 and HIAP2 (Liston et al. 1997), Survivin (Conway et al. 2000) and Bruce (Hauser et al. 1998). In addition, rat homologues of NAIP (Simons et al. 1999), XIAP, HIAP1 and HIAP2 (Holcik et al. 2002) were also been recently characterized.

Multiple copies of the murine naip homologue (naip1-6) exist on mouse chromosome 13. As with humans, the mouse naip loci, which number at least 6 in the 129/SvJ mouse strain, are clustered telomeric to the SMN gene (DiDonato et al. 1997; Yaraghi et al. 1998). Of the six mouse naip loci, three (naip1-3) contain the proper promoter elements for neuronal expression, unlike the situation in humans, with only one functional naip copy and one or more pseudogenes. The three mouse naip loci show significant differences in size, intronic sequence and tissue expression, suggesting a family of functional mouse
naip copies. NAIP1, which is expressed in mouse brain, but not the spleen, demonstrates 77% nucleotide homology to human NAIP and 68% identity at the amino acid level (Yaraghi et al. 1998). On the other hand, NAIP2 protein was found in the mouse spleen, lung and liver, but not the brain (Diez et al. 2000). Although the naip2 mRNA shows 90.4% nucleotide homology with naip1 and 77% homology with human naip, the genomic structure more closely resembles human naip than mouse naip1 (Yaraghi et al. 1999). The mouse naip2 and 5 genetic regions have been implicated in the resistance of most mouse strains to Legionella pneumophila, the causative agent of Legionnaires’ disease (Growney and Dietrich 2000). It was postulated that NAIP2 expression prevented primary macrophage apoptosis, allowing them to phagocytose the Legionella bacteria, whereas susceptible mouse strains, such as A/J, expressed little NAIP2, making their macrophages more vulnerable to apoptosis induction (Yaraghi et al. 1999). This was supported by studies which showed high expression of NAIP2 in primary macrophages of the L. pneumophila resistant C57BL/6J strain, but significantly less expression in the permissive strain A/J (Diez et al. 2000).

The murine equivalents of HIAP1, HIAP2 and XIAP have also been identified as mouse IAP (MIAP) 1, 2 and 3 respectively (Farahani et al. 1997; Liston et al. 1997). MIAP1 is a 602 amino acid protein with a predicted molecular weight of 67.3 kDa and showed a 69% and 73% amino acid identity with MIAP2 and HIAP1 respectively. The slightly larger MIAP2 protein (612 a.a., 69.7 kDa) had 82% a.a. identity with HIAP2 and an unusually large 1574 nucleotide 5' UTR which may contain an internal ribosomal entry site. The genomic organization of
miap1 and miap2 was found to be similar to their human homologues, located in a tandem head-to-tail arrangement on chromosome 9A2. As with mouse naip, both miap1 and miap2 have pseudogene copies, a situation that was not duplicated in the human genome (Liston et al. 1997). The murine xiap gene, or miap3, was localized within the A3-A5 region of the mouse X chromosome and consisted of six exons spanning approximately 20 kb. The 1491 bp miap3 coding region encoded a 496 amino acid, 55 kDa protein similar in size to its human homologue. MIAP3 protein demonstrated significant evolutionary conservation, with 94% amino acid identity with its human homologue (Farahani et al. 1997).

The mouse survivin gene, like its human homologue, was found to be composed of four exons spanning >50 kb, and was localized to chromosome 11E2. The primary transcripts were spliced to yield a 850 bp mRNA which encoded a 140 amino acid, 16.2 kDa protein with 84% a.a identity to its human homologue (Li and Altieri 1999; Conway et al. 2000). Mouse Survivin mRNA synthesis was initiated at three transcriptional start sites located -32, -36, and -40 from the original ATG start codon (Li and Altieri 1999). In addition, studies indicated two splice variants of mouse Survivin; one isoform retained intron 3, which created a truncated 121 amino acid protein with no C-terminal coiled-coil domain (Survivin121), while the other removed exon 2, generating a short 40 amino acid protein which contained neither the BIR or coiled-coil domains (Survivin40). As predicted, Survivin40 was unable to block caspase-3 activity, unlike its BIR containing cousins. All three splice isoforms were variably
expressed in the developing mouse fetus and have known human counterparts. In adult tissues, thymus and testis accumulated high levels of Survivin$_{140}$, while intermediate levels of Survivin$_{121}$, but no Survivin$_{40}$, were found in all tissues tested (Conway et al. 2000). The differential expression of these isoforms suggests diverse biochemical functions within fetal and adult tissues.

1.2 Apoptotic Inhibitors in Transgenic Mice

The creation of a transgenic animal is a powerful tool for analyzing the in vivo effect of overexpressing a target gene within specific tissues. Transgenic mice are created by microinjecting a cDNA copy, under the control of a tissue specific promoter, into fertilized murine oocytes. In the case of apoptotic inhibitors, overexpression may help evaluate the affect of these protective molecules on programmed cell death triggered by various stimuli under specific physiological conditions. Cell survival in transgenic animals can be assessed for long term function, in a specific tissue and injury context. Transgenic mice expressing apoptotic inhibitors can also be assessed for developmental problems arising from the inappropriate disruption of apoptosis.

Transgenic mice have been developed which overexpress Bcl-2 in various blood lineages, liver, heart, and neurons in the brain and optic nerve (reviewed in Martinou et al. 1994; Bonfanti et al. 1996; Strasser et al. 1996; Chen et al. 2001; Selzner et al. 2002). Overexpression of Bcl-2 had a significant impact on distinct populations within the transgenic mouse. Transgenic overexpression of Bcl-2 in B cells, which mimics the t(14;18) translocation induced overexpression seen in follicular B cell lymphomas, promoted clonal B cell expansion by providing a
survival advantage to these cells (McDonnell et al. 1989). The negative selection of thymic pre-T cells was also impaired by the expression of high levels of Bcl-2, though maturation due to positive selection was unaffected (Strasser et al. 1994). Interestingly, although female Bcl-2 transgenic mice demonstrated decreased ovarian follicle apoptosis and enhanced folliculogenesis, the adult males exhibited impaired sperm production, suggesting that Bcl-2 inhibitable apoptotic pathways may be necessary during spermatogenesis (Yamamoto et al. 2001).

Aside from developmental alterations, Bcl-2 transgenic mice displayed cellular protection from apoptosis induced by various triggers. For example, Bcl-2 transgenic mice are resistant to apoptotic liver damage triggered by ischemic/reperfusion injury (Selzner et al. 2002). Similar protection from ischemic damage was found in the brain and heart of Bcl-2 transgenic mice (Martinou et al. 1994; Chen et al. 2001), which demonstrated the broad protective capability of Bcl-2 in different tissues. Moreover, retinal ganglion cells, which normally die in response to transection of the optic nerve, were also rescued by the transgenic expression of Bcl-2 (Chen et al. 2001).

The use of transgenic animals in the study of IAP function had yet to be fully exploited. This was likely due to the advent of adenoviral vectors whose ability to transduce various cell types both in vitro and in vivo allowed expression of the gene of interest without the slow and expensive creation of transgenic animals. Interestingly, the majority of in vivo studies utilizing adenoviral IAP vectors involved murine models of brain injury, which reflects the importance of apoptosis in the induction and severity of CNS damage (reviewed in Robertson
et al. 2000). Employing an adenovirus expressing human XIAP or NAIP, studies by Xu et al. (Xu et al. 1997; Xu et al. 1999) found that stereotaxic injection of either XIAP or NAIP adenovirus into the rat brain protected the sensitive CA1 neurons of the hippocampus from cell death induced by ischemia. Not only were neurons saved from apoptosis, but higher order memory function within the hippocampus was also preserved by XIAP transduction, as assessed in the Morris water maze test (Xu et al. 1999). In contrast, direct inhibition of caspases through the use of the peptide inhibitors preserved neuronal cell bodies, but not the dendritic connections within the hippocampus following an ischemic event, and thus failed to preserve the function of this region (Gillardon et al. 1999). This suggests that, though the XIAP mediated inhibition of caspases protects neurons from apoptosis, XIAP may also have other functions which help maintain neuronal connections. Adenoviral vectors expressing IAPs have also been employed in several other murine models of neuronal cell death, including motor neuron axotomy (Perrelet et al. 2000) and treatment with the free radical inducing drug 6-hydroxy dopamine (6-OHDA) (Crocker et al. 2001)). These studies demonstrated the neuroprotective abilities of certain IAPs in vivo, and, in the case of the global ischemia and 6-OHDA model, preservation of neuronal function (Xu et al. 1999; Crocker et al. 2001).

One transgenic mouse model was developed to express XIAP specifically within the T lymphocyte lineage (Conte et al. 2001). Driven by the p56lck promoter, expression of the human XIAP protein was verified in the thymus and lymphoid tissues, such as the spleen. As expected, T cells isolated from XIAP
transgenic animals were resistant to apoptosis induced by non-specific triggers, such as C2 ceramide, UV irradiation and Fas/cycloheximide treatment, as well as T cell specific agents, including dexamethasone and anti-CD3 antibody. Moreover, the perturbation of apoptosis through XIAP expression also affected T cell maturation, a process which requires the apoptotic mechanism to eliminate unwanted cells. This leads to the accumulation of double negative thymocytes (CD4−, CD8−), which normally die in great numbers during negative selection, and skewed the mature T cell populations towards a CD4−CD8+ phenotype; a phenomenon also seen in Bcl-2 transgenic mice (Conte et al. 2001). Clearly, XIAP overexpression in murine T cells had a profound effect on both T cell survival and development in vivo.

1.3 Apoptotic Inhibitor Knockout Mice

The ablation of the in vivo cellular expression of apoptotic inhibitors can provide a unique method to study the importance of various genes in the context of the organism. The creation of these ‘knockout’ animals requires a recombination event between the gene of interest within the embryonic stem cell line and a knockout recombination vector. The vector was designed with a copy of the target gene which had large deletions of vital exons, and extensive 5’ and 3’ regions immediately flanking the targeted exons. The flanking arms are large to raise the probability of a recombination event occurring within this region, and the deletion inside the target gene effectively destroys expression. The deletions in the gene of interest may also be replaced by an in frame reporter cDNA, such as LacZ or GFP, whose ‘knock in’ expression aids in screening embryonic cells
for proper recombination and tracking the expression pattern of the target gene within an organism.

Knockout mice have been used in the analysis of several antiapoptotic genes including members of the Bcl-2 and IAP families. Bcl-2 knockouts were small yet viable, though about half of the mice died before 6 weeks of age. These deaths correlated with the gradual decrease in lymphocyte numbers, especially in mature T cells, although lymphocyte function appeared unaffected and other hematopoietic lineages appeared normal (Nakayama et al. 1994). Hematopoietic bone marrow progenitors from adult Bcl-2 knockout mice were unable to fully reconstitute lymphocyte development in γ-irradiated mice, a problem not found in fetal progenitors from the same knockout strain (Matsuzaki et al. 1997). Other changes include decreased bone deposition due to osteoclast dysfunction (Boot-Handford et al. 1998), graying hair at 4-5 weeks of age and the development of polycystic-like damage in the renal tubules of the kidney (Nakayama et al. 1994). Mice generated through homozygous deletion of the Bcl-2 homologue Bcl-x were not viable, dying at embryonic day 13. Analyses of the Bcl-x deleted embryos indicated massive apoptosis in the developing brain, spinal cord, and dorsal root ganglia, as well as defects in early lymphocyte maturation (Motoyama et al. 1995). Such data showed both overlapping and separate functions within the antiapoptotic members of the Bcl-2 family. In addition, Bcl-2/Bax double knockouts did not show the lymphocyte defects inherent in Bcl-2 knockouts, suggesting that the systemic loss of Bcl-2 can be offset by the additional removal of Bax (Knudson and Korsmeyer 1997).
The complete removal of an antiapoptotic gene from an organism may have dire consequences in the cellular response to apoptotic stimuli. Primary cultured cerebellar granule neurons from Bcl-2 knockouts, which normally express high levels of Bcl-2, were more susceptible to apoptotic triggers, such as potassium loss, serum deprivation and etoposide treatment, than cells derived from their wild-type littermates (Tanabe et al. 1997). The loss of Bcl-2 in knockout mice exacerbated apoptosis related to focal ischemic brain injury, underlining the importance of Bcl-2 in the resistance to hypoxic damage (Hata et al. 1999). UV irradiation triggered higher levels of apoptosis in the epidermal cells of Bcl-2 knockout animals relative to their wild-type littermates, linking Bcl-2 deficiency with susceptibility to UV induced damage (Gillardon et al. 1999).

Several IAP knockouts have been created, each having significantly different effects on the mouse. Construction of the naip knockout was complicated by the presence of three functional copies at the mouse chromosome 13 locus (Yaraghi et al. 1998). Therefore, a knockout vector was designed which targeted only the neuronally expressed naip1 gene. Although the naip1-/- mouse develops normally, the pyramidal neurons in the hippocampus are highly vulnerable to cytotoxic kainic acid induced limbic seizures (Holcik et al. 2000). Hence, although naip was not necessary for the normal development of the mouse, it had significant effects on the resistance of neurons to apoptotic triggers. The loss of naip1 may also be compensated for by the other functional copies of naip, thereby creating virtually normal progeny.
The *miap3* deficient mouse also appeared perfectly healthy and was histopathologically indistinguishable from wild-type littermates. The susceptibility of thymocytes and fibroblasts isolated from MIAP3-/- mice to several apoptotic triggers, including anti-CD3 and anti-Fas treatment and UV irradiation, was no greater than cells from wild-type animals. This indicates that the apoptotic resistance within these cells was not diminished by the abrogation of MIAP3 expression. Interestingly, the loss of MIAP3 within these mice correlated with an increase in MIAP1 and MIAP2 expression (Harlin *et al.* 2001). The upregulation of existing IAPs in the absence of others suggests a functional redundancy between IAPs which can maintain the apoptotic resistance in these cells. In contrast, the Survivin knockout was not viable and failed to survive beyond embryonic day 4.5. Survivin null embryos displayed disrupted microtubule formation and polyploidy due to the vital role of Survivin in cell division (Uren *et al.* 2000). The loss of the essential Survivin gene was not functionally replaceable by other IAPs and therefore leads to non-viable embryos.

We endeavored to analyze the tissue expression of the murine IAPs, as well as characterize the murine mXAF1 homologue. Initial studies utilized standard 3' UTR probes to examine the expression of *miap1* and *miap2* mRNA in mouse tissues. Subsequently, riboprobes were produced for *miap1*, *miap2* and *miap3* and tested in mouse total RNA Northern blots. These probes will later be used in *in situ* hybridization experiments of murine tissue. We then turned our attention to the mouse homologue of XAF1, based on a murine cDNA fragment discovered by a Blast search. This cDNA fragment was used to uncover the
mxaf1 gene, several cDNA splice isoforms, and mRNA expression pattern in murine tissues. The characterization of the mxaf1 gene leads directly to the construction of the knockout vector, which will later be used to create a knockout mouse. Deletion of the mxaf1 gene in vivo will lead to a better understanding of mXAF1 function within an organism.

2. Methodology

2.1 Mouse Genomic and cDNA Library Screening

A 129 SVJ mouse liver λ FIX II genomic library (Stratagene) and a C57BL/6 mouse spleen λ FIX II cDNA library were probed with the mxaf1 coding region fragment (GenBank accession # AA059908). Selected genomic clones were subcloned into the pKO1 vector (Holcik and Korneluk, unpublished data) and sequenced utilizing the TN1000 transposon sequencing kit (Gold Biotechnology Inc., see Materials and Methods). Murine cDNA clones were recloned into Bluescript SK- plasmids and sequence using T7 and T3 primers on a 373A DNA sequencer (Applied Biosystems).

2.2 Murine Mutiple Tissue Northern Blot Analysis

The mouse multiple tissue Northern blots, also containing 2 μg poly(A)+ mRNA, were probed with the [32P]CTP (Amersham) random-primed labeled (Rediprime, Amersham) mxaf1 fragment (GenBank accession # AA059908). Blots were hybridized in ExpressHyb hybridization solution (Clontech) overnight at 65°C with constant shaking. Hybridized blots were washed several times with SSC/0.1% SDS wash buffers at increasing stringency (1-0.1X SSC) and exposed
to X-ray film at -80°C.

2.3 Riboprobe Northern Blot Analysis

Isolated total RNA from murine tissues was used for Northern blot analysis of miap1, miap2 and miap3 expression. 20 µg total RNA in RNA sample buffer (1X MOPS running buffer, 2.2 M formaldehyde, 50% formamide final) was run on 1% gels supplemented with 2.2 M formaldehyde in 1X MOPS running buffer (0.2 M MOPS, 0.5 M sodium acetate, 0.01 M EDTA). RNA markers and test RNA were used as controls, stained with ethidium bromide and visualized on UV transilluminator. The unstained samples were washed with RNase free H₂O and cleave by alkaline hydrolysis (0.05 NaOH/1.5 NaCl, 30 min). The gel was neutralized by 0.5 Tris-HCl, pH 7.4/ 1.5 M NaCl for 20 min and washed with 20X SSC. The RNA was blotted onto Pall Biodyne B nylon membranes (Gibco/BRL) through capillary transfer in 20X SSC. Northern blots were baked for 2 h at 80°C and prehybridized in formamide hybridization buffer (5X SSC, 5X Denhardt solution, 50% formamide, 1% SDS, 100 µg/ml denatured salmon sperm DNA).

Miap1, 2 and 3 riboprobes were generated using the Maxiscript T7 kit (Ambion) and labeled through the addition of 5 µL [³²P]-UTP (Amersham). Labeled riboprobes were added to the mouse total RNA Northern blot at a concentration of 1-2 x 10⁶ CPM/ml in fresh hybridization solution and incubated overnight at 65°C with shaking. The blots were then washed with SSC/0.1% SDS at increasing stringency (1-0.1X SSC) at room temperature and exposed to X-OMAT film (Kodak) at -80°C.
2.4 Construction of the mxaf1 Targeting Vector

The mxaf1 knockin vector was constructed using the mxaf1 genomic clone pKO14.4-10 and the β-gal containing vector pG1.8-SA-IRES-βgeo (see Figure 4-7). To prepare the genomic clone, the start codon was removed by PCR amplification of a HindIII/HindII DNA fragment using a DNA thermal cycler (Perkin Elmer), Taq polymerase (Gibco/BRL) and specially designed primers (AAGCTTAGCATCTCAGGAG and GGTACCCTCGAGGTCTAGCTTCCTGCAGT GCTTCTGTG), which destroyed the 3’ HindIII site and the mxaf1 start codon and added 3’ Sall, Xhol and KpnI sites. Amplified PCR products were recloned into the pKO14.4-10 parent vector at the HindIII/KpnI restriction sites, which results in a 1429 bp deletion that including the majority of exon 1. The β-gal/NeoR cDNA fragment was cut out of the pG1.8-SA-IRES-βgeo vector using HindIII and Sall (Gibco/BRL) and ligated to a synthetic oligo linker with a Sall restriction site and HindIII overhang (AGCTAGTCGACT). The β-gal/NeoR cDNA fragment (Sall) was then cloned into the modified pKO14.4-10 vector using the synthetic Xhol site (see Figure 4-7).

3. Results

3.1 Expression of miap1, miap2 and miap3 RNA in Murine Tissues

Miap1 and miap2 mRNA expression was tested in an adult mouse multiple tissue Northern (MTN) blot, which contained 2 μg/lane poly(A)+ RNA (see Figure 4-1). The miap1 and miap2 specific probes, using 315 bp and 560 bp of 3’ UTR respectively, were radiolabeled and used sequentially to probe the same MTN
Figure 4-1. Northern Analysis of miap1 and miap2 mRNA Expression

Murine poly (A)$^+$ multiple tissue Northern blots were analyzed for miap1 (A) and miap2 (B) expression using DNA probes derived from the 3' UTR. $\beta$-actin was used as a control probe (C). Each lane represents 2 $\mu$g of poly (A)$^+$ RNA. Position of the RNA size markers are indicated.
blot. A mouse β-actin cDNA probe was used to confirm equal loading. The tissue distribution of miap1 and miap2 differed slightly from the corresponding human pattern, with the 2.9 kb miap1 transcript being highly expressed in the lung, liver, heart, and spleen and the 3.9 kb miap2 transcript being ubiquitously expressed in all murine tissues examined (see Figure 4-1). The transcript sizes corresponded closely with the known cDNA sequences (Liston et al. 1997). In addition, since larger mRNA species for both miap1 and miap2 were discovered by Northern blot, possibly formed by alternative splicing or poly(A) readthrough, the human and mouse homologues may differ significantly in transcriptional regulation despite their similarities at the protein level (Liston et al. 1997). This was especially true in skeletal muscle and lung, where the large band of approximately 6 kb was prominent (see Figure 4-1A).

*In situ* hybridization is a powerful method to examine the expression of specific RNA within cells and tissues. For this purpose, radiolabeled RNA riboprobes were designed at the 3' UTR of miap1 and miap2 and within the coding region of miap3, between the BIR and RING domains. To test the effectiveness and specificity of these riboprobes, [*32P*-labeled *in vitro* translated RNA was used to probe separate mouse tissue total RNA Northern blots containing 20 μg RNA/lane. As seen with the DNA probe, the predominant miap1 transcript was identified at 2.9 kb, and was particularly abundant in thymus, lung, liver and kidney (see Figure 4-2). The miap1 Northern also demonstrated larger molecular weight species, previously seen with the normal DNA probe (see Figure 4-2A). Northern blots probed with the miap2 riboprobe
Figure 4-2. Riboprobe Analysis of Mouse IAP Expression in Total RNA Northern Blots

Murine multiple tissue Northern blots containing 20 μg of total RNA per lane were probed for (A) miap1, (B) miap2 and (C) miap3 using in vitro transcribed RNA probes labeled with [32P]-ATP. Probe location and size are indicated at the lower left corner of each gel. A β-actin DNA probe was used as a loading control (D). RNA sizes were estimated from RNA size markers on the gel.
showed a fairly ubiquitous single 3.9 kb band. In addition, the miap3 mRNA was ubiquitously expressed in all murine tissues. Its unusually large 8.5 kb mRNA and correspondingly large 5' UTR is suggests the presence of an IRES element, similar to its human xiap homologue.

3.2 Genomic Structure and Partial cDNA Isoform Characterization of Mouse xaf1

After the initial isolation of the human xaf1 cDNA, a Blast search of the murine EST database yielded a partial cDNA clone (GenBank accession number AA059908) with significant homology to human xaf1b (76% identity over 275 bp). This mxaf1 clone encodes 3 zinc finger domains, roughly corresponding to zinc fingers 1, 4 and 5 of human xaf1A (see Figure 4-3). The mxaf1 cDNA fragment was cloned by RT-PCR, sequenced and used as a probe for both a λgt11 mouse liver cDNA library and a 129/Sv liver genomic library. Probing of these libraries produced 10 non-redundant genomic and 7 individual cDNA clones. Genomic clone number 4 was subcloned into pKO1 and transposon sequenced. The mxaf1 genomic sequence data identified 4 exons over 14 kb, which covered the known cDNA fragment (see Figure 4-3A). Each of the 7 cDNA clones yielded only fragments of the mxaf1 cDNA, with clone 4 and 5 containing the largest fragment. Both clones 4 and 5 encompass the known mxaf1 cDNA fragment, but included an additional zinc finger on a novel exon 5 (see Figure 4-4 and Figure 4-5). Subsequent Blast database searches lead to the discovery of several new splice isoforms of mxaf1. The first sequence (GenBank accession number BB645287) did not splice in exon 5, continuing at least 46 codons beyond the intron-exon boundary (see Figure 4-4). Two cDNAs (GenBank accession
A) The genomic structure of the *mxaf1* gene. Known exons are numbered. Certain important restriction sites are labeled as follows: B – BamHI, E – EcoRI, H – HindIII. (B) Splicing pattern of *mxaf1* isoforms. Several partial *mxaf1* isoforms were isolated from both λ cDNA libraries (MXAF5-1) and Blast EST database searches (AA059908, BB645287 and BB645745). The MXAF5-1 is an extension of the originally isolated *mxaf1* fragment (AA059908). The BB645287 EST contains an extended exon 4 and the BB645745 EST excludes exon 3, producing an ORF out of frame with the original ORF, which leads to the truncation of the protein after the first zinc finger. The cDNA fragments are drawn to scale. Open arrows represent incomplete sequence.
number BB645745 and BB329028) splice out exon 3, leading a reading frame shift between exon 2 and exon 4 (see Figure 4-4). The predicted protein would continue for another 19 amino acids before truncating between zinc fingers 1 and 2 (see Figure 4-5). The splice sites followed conserved AG-GT splice donor/acceptor site (see Table 2). No new protein motifs were identified within the novel sequence using the conserved domain architecture retrieval tool (CDART, NCBI).

3.3 Expression of mxaf1 mRNA in Mouse Tissues

The mRNA expression of mxaf1 was tested using a murine MTN blot (Clontech) probed with the original mxaf1 cDNA fragment (see Figure 4-6). These blots showed a single mxaf1 band at approximately 1.3 kb, with the highest expression found in the mouse heart, lung and kidney. The mxaf1 murine Northern did not demonstrate the multiple size isoforms found in the human xaf1 Northern, and the 1.3 kb band is significantly smaller than those found in the human xaf1 Northern blot. The thickness of the 1.3 kb band allows for the possibility of other splice isoforms with relatively small size changes (see Figure 4-6).

3.4 Construction of the mxaf1 Targeting Vector

The mxaf1 knockout mouse would provide an invaluable tool to study the role mXAF1 in apoptosis regulation and cancer development. By cloning the β-gal cDNA into the mxaf1 gene sequence, the tissue expression of mxaf1 can also be traced through β-gal staining. To construct the knockin vector, the β-gal gene,
Figure 4-4. Alignment of mxaf1cDNA Sequences.

Sequence alignments were based on cDNA sequences retrieved from the public databases (GenBank, NCBI).
Table 4-1. Intron-Exon Boundaries of the *mxaf1* Gene.

Nucleotides in bold represent splice donor and acceptor sites. Intron and exon sizes are also listed.
<table>
<thead>
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<th>Intron</th>
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</tr>
</thead>
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Figure 4-5. Protein Sequence Alignment of MXAF1.

The amino acid sequences of the various MXAF1 protein isoforms were compared. Shaded and bold characters illustrate the zinc finger consensus sequence and important cysteine and histidine spacing (in bold).
AA059908 1  MEADFQVRNCNRKRLNVLHFMHEAHLRFIVLCEPEEPIESKMKHEMVELVHQQTKE
MXAFS-1 1  MEADFQVRNCNRKRLNVLHFMHEAHLRFIVLCEPEEPIESKMKHEMVELVHQQTKE
BB645287 1  MEADFQVRNCNRKRLNVLHFMHEAHLRFIVLCEPEEPIESKMKHEMVELVHQQTKE
BI067986 1  MEADFQVRNCNRKRLNVLHFMHEAHLRFIVLCEPEEPIESKMKHEMVELVHQQTKE
BB645745 1  MEADFQVRNCNRKRLNVLHFMHEAHLRFIVLCEPEEPIESKMKHEMVELVHQGREL

AA059908 61  QHPAKCFCLEAVQLSNLDVDHSHGSRTEHCPCONQPITLQLQVLSQNKHAKCLSAKGRP
MXAFS-1 61  QHPAKCFCLEAVQLSNLDVDHSHGSRTEHCPCONQPITLQLQVLSQNKHAKCLSAKGRP
BB645287 61  QHPAKCFCLEAVQLSNLDVDHSHGSRTEHCPCONQPITLQLQVLSQNKHAKCLSAKGRP
BI067986 61  QHPAKCFCLEAVQLSNLDVDHSHGSRTEHCPCONQPITLQLQVLSQAQSHVSECK------
BB645745 56  YHLLEEKPVVIFANK*

AA059908 121  EGKRIVSX-----------------------------------------------
MXAFS-1 121  EGKRIVSSPGRKRCICLCKQMPENTYASHMKSAPNTVTIRIDXE-----
BB645287 121  EGKRIVSSPGRKRCICLCKQMPENTYASHMVSGNDPLSNQARQFQASPTADSRYT
BI067986 116  ----------------------------------------------
BB645745 56  ----------------------------------------------

AA059908 121  ----------------------------------------------
MXAFS-1 167  ----------------------------------------------
BB645287 181  SQTVIKGSDVYFYILX-
BI067986 116  ----------------------------------------------
BB645745 115  ----------------------------------------------
Figure 4-6. Northern Blot Analysis of mxafl mRNA Expression

(A) The normal human tissue expression of mxafl was analyzed on murine poly (A) superscript multiple tissue Northern blots using a DNA probe derived from the original mxafl cDNA fragment (AA059908). Each lane contains 2 μg of mRNA. A β-actin DNA probe (B) from the manufacturer was used as a control. RNA size markers are indicated at the left of the blot.
Figure 4-7. Contraction of the mxaf1 Knockin Vector.

The mouse xaf1 knockin vector was constructed using the mxaf1 genomic clone pKO14.4-10 and the β-gal containing vector pG1.8-SA-IRES-βgeo. The knockin vector was prepared as described in the Methodology (see text for details). Restriction endonucleases are labeled as follows: H - HindIII, K - KpnI, S - Sall, X - Xhol.
along with the neomycin resistance marker, were cut out of the pG1.8-SA-IRES-βgeo vector using HindIII and Sall and ligated to a synthetic oligonucleotide linker designed with Sall and Xhol restriction sites and HindIII overhangs ready for cloning (see Figure 4-7). This DNA fragment was cloned and cut with Sall, producing the β-gal/Neo<sup>R</sup> knockin cassette to be cloned into the mxaf1 gene vector. To prepare the mxaf1 genomic clone pKO14.4-10, which contains the entire known mxaf1 gene sequence, a HindIII/HindII DNA fragment just before the start codon was PCR amplified using specially designed primers which destroyed the start codon and the proximal HindIII site and added sites for the restriction enzymes Sall and KpnI. The resulting PCR products were recloned back into pKO14.4-10 using HindIII and KpnI, which results in a 1429 bp deletion that includes the majority of exon 1. The β-gal/Neo<sup>R</sup> cDNA fragment (Sall ends) was then cloned into the modified pKO14.4-10 vector at the synthetic Xhol site (see Figure 4-7). The final vector has removed most of exon 1, including the start codon, and replaced it with the β-gal cDNA sequence, which is now under the transcriptional control of the mxaf1 promoter.

4. Discussion

The study of murine gene homologues gives us insight into the evolutionary conservation and importance of human genes. Conserved tissue expression also suggests a vital function for certain genes within specific cell types. Our studies examined the mRNA expression of miap1, miap2 and miap3 in mouse tissue Northern blots using both conventional DNA probes and riboprobes. The analysis of miap1 mRNA showed high expression of the 2.9 kb
transcript in spleen, lung and liver, with moderate expression in heart, skeletal muscle and kidney. The human hiap1 mRNA pattern (Liston et al. 1996) differed slightly, with the highest expression found in spleen, thymus and peripheral blood leukocytes. No expression was found in heart and lung, two tissues which demonstrated high expression in the mouse. The miap1 Northern also showed larger bands, especially in lung and skeletal muscle. One particular band, at approximately 6 kb, exhibited a fairly high levels of expression in both the lung and skeletal muscle, suggesting that alternatively splicing or poly(A) readthrough may be prominent mechanisms during the transcription of the miap1 mRNA. On the other hand, miap2 is expressed as a single band at 3.9 kb, which appeared in moderate quantities in all tissues except spleen. The absence of splenic expression differed from the human hiap2 Northern pattern, suggesting that miap2 mRNA production in the mouse may not be as distinctly immune cell related as the human. Unfortunately, fetal mouse MTN blots were unavailable for comparison to the human expression pattern.

The use of anti-sense RNA as a probe for specific RNA within a cell has become a useful tool in histology due to the strength of RNA-RNA hybridization. Radioactively labeled riboprobes are used in in situ hybridization experiments on cell monolayers and tissue sections to accurately pinpoint target RNA expression within specific cells. This is an especially useful tool in the absence of a highly specific antibody and may be used to correlate protein and RNA expression in conjunction with a specific antibody. Riboprobes for the mouse IAPs were constructed in an effort to study the expression of these genes in adult and
embryonic mouse tissues. Our first step following construction of these probes was to test the specificity and accuracy of these probes in Northern blots. Total RNA was isolated from CD1 mouse tissues and standard Northern blots were run using 20 μg RNA per lane. All three probes recognized bands of approximately the correct size and the expression patterns resembled those found on the multiple tissue Northern blots probed with conventional DNA fragments. Of the blotting experiments, only miap1 showed different size bands, including the large isoforms seen previously in the commercial MTN blot. These bands may represent cross-hybridizing RNA species or splice variants which have yet to be identified. Overall, the target specificity and general affinity of these riboprobes are suitable for in situ hybridization experiments, which will be tested in future investigations.

During the initial analysis of the novel human xaf1A cDNA, Blast database searches of the mouse cDNA database yielded a partial cDNA with high homology to xaf1B. This cDNA fragment contained three zinc finger motifs, which corresponded to zinc fingers 1, 4 and 5 of human xaf1. In the human xaf1B isoform, a 57 bp exon was spliced out, which lead to the deletion of the third zinc finger. This exon was also missing in the mouse xaf1 fragment and analysis of the mouse genomic sequence does not identify a corresponding exon, suggesting a complete absence of an mXAF1A protein isoform. The mxaf1 sequence also did not have a complete zinc finger matching the human zinc finger 2. Three of the four zinc coordinating amino acids were still present, which suggested that a point mutation may be responsible for the elimination of
this zinc finger. In addition, the loss of this zinc finger suggested that the usefulness of this domain may have been lost during murine evolution. Clones isolated through λ phage libraries yielded only fragments of mxaf1 cDNA, possibly due to adenine rich sequences which bind the poly(T) primers used to create the mouse cDNA library. Of the isolated clones, several (#4 and 5) encompassed the known section of mxaf1 open reading frame and revealed a fourth zinc finger. This zinc finger corresponded to zinc finger 6 in the human xaf1A sequence, both in spacing and homology at key zinc binding residues. It appears from these cDNA clones that mxaf1 most closely resembles xaf1B in overall amino acid structure, except for the absence of a full second zinc finger.

Later sequence searches of the ever expanding murine EST database yielded several novel mxaf1 splice isoforms. The first EST (BB645287) did not splice in exon 5, but instead continued expanding exon 4 another 46 codons. Although incomplete, this extra sequence did not display any known protein domains when analyzed by CDART. The splicing of two other novel sequences (BB645745 and BB329028) excluded exon 3 and created an out of frame connection between exon 2 and 4. This open reading frame continues 19 codons past the splice site, at which point it truncated with three successive stop codons within 20 amino acids. This created an unusually short ORF encoding a potential protein consisting only of zinc finger 1. Although this may well reflect an infrequent splicing error, two non-identical clones were isolated by database searches, which indicated prominent expression of this isoform in this particular cDNA library. No cDNAs have yet been found to correspond to the human
xaf1A and xaf1C sequence, although this has been far from a comprehensive search. As with the human xaf1 gene, there are indications of many more splice isoforms which have yet to be characterized. Although the functional significance of so many splice forms remains obscure, the absence of a mouse xaf1A homologue is troubling due to the significant advances on the function of this particular isoform. The ability of both human XAF1A and XAF1B isoforms to interact with XIAP hints at a possible functional redundancy that may not be maintained in the mouse. It is equally possible that the human XAF1A isoform developed during evolution to serve a particular function. It remains to be seen if further cDNA analysis will yield a mouse xaf1A homologue.

The mxaf1 mRNA tissue expression pattern demonstrated high levels of mxaf1 in heart, lung and kidney, which is similar to xaf1 mRNA expression in human tissues. Interestingly, mxaf1 mRNA appears as a single diffuse band on Northern blots, and did not demonstrate the multiple bands characteristic of human xaf1 mRNA expression in tissues. This implies that, unlike its human counterpart, variation in UTR sizes does not exist in mxaf1. On the other hand, the diffuse mxaf1 mRNA band left open the possibility of multiple mxaf1 isoforms which varied only slightly in size.

Genetic manipulation of target gene expression within an organism can provide useful information on gene function within an in vivo setting. This is especially true in the case of xaf1 which has been demonstrated to have tumour suppressor properties (see Chapter 5). The direct elimination of xaf1 expression within an organism is expected to inhibit general apoptotic function by relieving
the suppression of XIAP. This increase in the apoptotic threshold may promote
tumour formation by aiding inappropriate cell survival. In addition, human XAF1
has been demonstrated to induce G1 cell cycle arrest and the loss of this
function would also advance tumour development. The mxaf1 knockin mouse
will resolve the importance of mXAF1 expression on the survival and proper
development of the mouse, as well as provide mxaf1 null cells to further the
study of mxaf1 function.
CHAPTER 5: DIMINISHED XAF1 EXPRESSION IN CANCER CELL LINES
AND INVOLVEMENT IN CELL CYCLE REGULATION.

Our life is made by the death of others.

- Leonardo da Vinci (1452-1519 AD)

1. Introduction

1.1 Apoptosis and Cancer

Diseases of uncontrolled cellular proliferation are generally categorized as cancers. Cancer is a pleiotropic disease, with no single cause and manifesting itself in almost any tissue. Cellular malignancy requires several mutational steps, culminating in unrestricted cell growth, which is then detrimental to the organism. Hanahan and Weinberg (Hanahan and Weinberg 2000) summarized the vast array of catalogued genetic alterations within cancer cells into six fundamental categories: self-sufficient in cell growth signals, insensitivity to antigrowth signals, evasion of programmed cell death, unlimited growth potential, sustained angiogenesis and tissue invasion and metastasis. These genetic mutations advance tumour development by promoting cell growth and survival (oncogenes) or by the inactivation of the cellular anti-cancer defense mechanism (tumour suppressors). To prevent the advancement of malignant cell growth, the process of cell proliferation is coupled to programmed cell death. This cellular safety mechanism activates the apoptotic pathway, thereby eliminating cells that are proliferating in an inappropriate fashion or location. Thus, overexpression of
growth promoting oncogenes generally leads to apoptosis instead of cancer (Harrington et al. 1994; Evan and Littlewood 1998).

Examples of the linkage between growth promoting oncogenes and apoptosis include the inappropriate expression of the Myc oncogene, which sensitizes or induces cells to apoptosis, in addition to its transforming capabilities (Harrington et al. 1994). The triggering of the p19ARF/Mdm-2/p53 tumour suppressor pathway may be responsible for the oncogenic induction of apoptosis. The oncogenes Ras, Myc, E1A and E2F all stimulate p19ARF, which blocks the Mdm-2 dependent ubiquitination of p53 (Lowe 1999). This stabilization of the p53 protein promotes both cell cycle arrest and apoptosis. The apoptosis inducing abilities of p53 are mediated through several mechanisms including the transcriptional activation of the pro-apoptotic proteins Bax, Noxa and PUMA, and the upregulation of death receptor expression, such as the Fas and DR5 receptors (Owen-Schaub et al. 1995; Newton and Strasser 2000). The sensitization to death signals by p53 can be counteracted by cellular survival signals and trophic support by neighbouring cells, creating a balance of positive and negative signals within each individual cell. Perturbing this precarious equilibrium through inactivation of p19ARF and p53, or overexpression of Mdm-2, blocks p53 dependent cell death, allowing the uncontrolled proliferation of the damaged cell. This has been shown to be an important and frequent mutation in human cancer, with over 50% of tumours expressing mutated p53 (Hollstein et al. 1991) and 30-40% of sarcomas with wild-type p53 expressing elevated levels of Mdm-2 (Oliner et al. 1992). Clearly, transformation
through oncogene overexpression, such as Myc or Ras, requires inactivation of
one or more apoptotic pathways, including mutations in the p19ARF/Mdm-2/p53
pathway.

The consequences of oncogene activation, in some circumstances, may
be cell cycle arrest rather than apoptosis. Overexpression of the Ras oncogene
can induce p19ARF transcription and stimulate the transcription of Mdm-2 through
the MAP kinase signaling pathway. The balance between p19ARF and Mdm-2
expression regulates p53 expression leading to cell cycle arrest and resistance to
the p53-dependent apoptosis induced by DNA damage. Activation of p53 leads
to the transcriptional upregulation of p21WAF1/CIP1, which inhibits the G1 cyclin-
dependent kinase cyclin-E/cdk2. This stops the phosphorylation of Rb and
prevents cells from entering into S phase (Harper et al. 1993; Xiong et al. 1993).
By blocking cell cycle, cells are able to affect genetic repairs and prevent the
propagation of DNA changes, an important mechanism that confers resistance to
DNA damaging agents. The combination of cell cycle arrest and apoptosis
presents a two pronged attack by p53 tumour suppressor activation on the
development of cancer.

1.2 Apoptosis Inhibitors and Cancer

Blocking programmed cell death, through the expression of direct
apoptosis inhibitors, has obvious selection benefits for the development of the
transformed phenotype, as well as for resistance to chemotherapy drugs.
Overexpression of anti-apoptotic proteins elevates the apoptotic threshold, thus
raising the apoptotic signal strength needed to impel the cell to commit
programmed cell death. As a consequence, cells are allowed to survive when they would otherwise perish. One such example is the anti-apoptotic gene Bcl-2, which was first described as an oncogene overexpressed in human follicular B cell lymphomas. A reciprocal chromosomal translocation t(14;18) placed the Bcl-2 gene under the control of the immunoglobulin heavy chain enhancer, resulting in massive overexpression of Bcl-2 within these B cells. Although Bcl-2 is not an inducer of cell proliferation, B cells which express high levels of Bcl-2 grow under normal B cell stimulation and form slow growing tumours due to the inhibition of the apoptosis pathway (Tsujimoto et al. 1985). The tumorigenic ability of Bcl-2 was verified in transgenic mouse models, which also showed that the accumulation of additional mutations, such as Myc overexpression, creates a far more aggressive tumour (Vaux et al. 1988).

B cell lymphomas are not the only cancer which overexpresses Bcl-2. It has been estimated that increased expression of Bcl-2 is found in up to 50% of all human cancers, suggesting that upregulation of anti-apoptotic genes may be a common, if not universal, factor in cancer development (Jaattela 1999). In addition to hematopoietic malignancies, elevated Bcl-2 is found in cancers of the breast, prostate, ovary, colon, and lungs (McDonnell et al. 1992; Pezzella et al. 1993; Hague et al. 1994; Leek et al. 1994). The correlation between high Bcl-2 expression and disease prognosis is mixed; some groups reported a correlation with poor prognosis in lymphomas, leukemias and prostate cancers, whereas others groups demonstrate good prognosis in many cancers including breast, colon and kidney (Jaattela 1999). The Bax/Bcl-2 ratio is a better prognostic
indicator, with higher ratios correlating with longer survival and a better response to therapy in breast cancers (Krajewski et al. 1995; Binder et al. 1996).

The inhibition of programmed cell death through overexpression of the IAPs would also be predicted to favour the development of cancers. Therefore, it is not surprising that several studies have demonstrated elevated levels of IAPs in cancer cell lines and tumour samples (Ambrosini et al. 1997; LaCasse et al. 1998; Tamm et al. 2000; Vucic et al. 2000). Initial Northern blot studies of cultured cancer cells showed high expression of xiap mRNA in cervical carcinoma, leukemia, adenocarcinoma and melanoma cell types, relative to the ubiquitous and consistent levels observed in normal tissues (Liston et al. 1996). Tamm et al. (Tamm et al. 2000) found that high XIAP levels in biopsy samples from patients with acute myelogenous leukemia (AML) and treated with the chemotherapy drug cytarabine (AraC) correlated with shorter remission duration and shorter overall survival. A closer correlation exists between XIAP expression and resistance to anti-cancer therapies. Downregulation of XIAP expression through anti-sense adenoviral vectors sensitized the non-small cell lung carcinoma cell line H661 to γ-irradiation and ovarian carcinoma cells to cisplatin (Holcik et al. 2000; Sasaki et al. 2000). The expression of XIAP may play a role in both the development and the maintenance of malignant cells.

The most consistent link between IAP overexpression and transformation is found in Survivin expression patterns, which demonstrates high levels in embryonic tissues and many cancer cell lines and biopsy samples, but is entirely absent from normal adult human tissues (Reed 2001). High Survivin expression
levels is proving to be a excellent prognostic indicator of disease severity in many malignancies, including neuroblastoma, colorectal cancer, non-small cell lung cancers, breast carcinoma and acute myeloid leukemias (Adida et al. 1998; Kawasaki et al. 1998; Monzo et al. 1999; Adida et al. 2000; Tanaka et al. 2000). Survivin proteins can be detected in the urine from patients with bladder carcinoma, which may eventually be used as a diagnostic test for this type of cancer (Sharp et al. 2002). As with XIAP, the expression of Survivin in cancer cells may serve to increase the apoptotic threshold, which provides protection from various programmed cell death triggers, including certain chemotherapeutic drugs. Survivin anti-sense oligonucleotide treatment sensitizes the lung adenocarcinoma cell line A549 to the drug etoposide (Olie et al. 2000), suggesting a role for Survivin in the chemotherapeutic resistance of this cell line. Expression of the p53 tumour suppressor inhibits transcription of the anti-apoptotic survivin gene, thereby reinforcing the importance of the p53 dependent pro-apoptotic pathway (Hoffman et al. 2002).

Direct genetic evidence is now lending credence to the idea that the IAPs may function as oncogenes. The HIAP1 and HIAP2 locus on chromosome 11q21-q23 is amplified in various cancers, such as medulloblastosomas, renal cell carcinomas, glioblastomas and gastric carcinomas. Transcriptional analysis of esophageal squamous cell carcinomas displaying the 11q21-q23 amplification indicates that HIAP2 mRNA is the most frequently overexpressed gene within this region, suggesting that HIAP disregulation may be important in the development of certain cancer types (Imoto et al. 2001).
Genetic rearrangements within the HIAP1/HIAP2 locus have also been demonstrated in extranodal marginal zone mucosa-associated lymphoid tissue (MALT) B cell lymphomas. MALT B cells initially hyperproliferate in response to chronic inflammation, such as during persistent Helicobacter pylori infection or certain autoimmune diseases. These inflammatory responses lead to chronic NF-κB activation in B cells, resulting in the upregulation of the anti-apoptotic HIAP1 protein, and slow growing tumours resulting from aberrant B cell survival. Proper treatment of H. pylori infection with antibiotics leads to decreased inflammation, decline in NF-κB activation, downregulation of HIAP1 and tumour cell regression (reviewed in Mullauer et al. 2001; Du and Isaccson 2002). Several genetic changes can arise in MALT lymphomas which produce feedback loops leading to chronic NF-κB activation. These lymphomas continue to grow and survive independent from inflammatory stimulation and are no longer responsive to antibiotic treatment.

MALT lymphomas demonstrate two recurring translocation events involving genes in the TNF/MALT1 signaling pathway: t(11;18)(q21;q21) and t(1;14)(p22;q32) (see Figure 5-1). The MALT1 protein is a purported paracaspase, with a caspase homology region and a CARD domain, but no reported enzymatic activity. MALT1 activates NF-κB through association with the Bcl10 protein via CARD-CARD interactions (Uren et al. 2000; Vega and Meadeiros 2001). Bcl10 is CARD containing protein with a Pyrin domain, a common motif among proteins involved in the inflammatory response. HIAP1 and TRAF2 are also able to bind Bcl10, possibly through their CARD domains
and this interaction is regulated by the phosphorylation of Bcl10. The TNF/MALT pathway may activate anti-apoptotic NF-κB signals by the direct interaction of TRAF2 and MALT1, which in turn binds hypophosphorylated Bcl10. The hyperphosphorylation of Bcl10 triggers the dissociation from TRAF2 and the sequestering of HIAP1; an event which correlates with programmed cell death induction. The Bcl10/HIAP1 interaction may promote apoptosis though the interference of HIAP1 activity (Yoneda et al. 2000; Yui et al. 2001).

Of the two common MALT B cell lymphoma translocations, the t(11;18)(q21;q21) translocation is the more frequent, occurring in greater than 50% of all extranodal MALT lymphomas (Baens et al. 2000). This translocation combines the hiap1 and mlh/malt1 genes and creates an in-frame protein consisting of the HIAP1 BIR domains, with or without the CARD domain, and the carboxy terminus of the MLT/MALT1 protein (see Figure 5-1, (Uren et al. 2000)). The HIAP1/MLT fusion protein triggers the anti-apoptotic NF-κB pathway and upregulate its own transcription though the NF-κB responsive hiap1 promoter (Chu et al. 1997; Erl et al. 1999; Hong et al. 2000). This positive feedback loop continually stimulates NF-κB and may explain why the majority of antibiotic resistant gastric MALT lymphomas display the HIAP1/MLT translocation (Liu et al. 2001). The less frequent t(1;14)(p22;q32) translocation fuses the immunoglobulin heavy chain locus with the bcl10 gene. The resulting gene fusion is also under the control of an NF-κB responsive promoter and can trigger NF-κB, which results in a constitutively high level of NF-κB activation and thus transcriptional activation of the hiap1 gene. Once again, the maintenance of high
Figure 5-1. Recurrent Translocations in MALT Lymphomas.

(A) In B cells, the common t(11;18)(q22;21) translocation occurs between exons 7 and 8 of hiap1 and several positions within the malt1 gene (indicated by arrows). This produces a fusion protein which is both anti-apoptotic and NF-κB activating. Activation of NF-κB leads to upregulation of the fusion protein through the hiap1 promoter region, which produces a positive feedback loop that generates constitutive expression of the HIAP1/MALT1 fusion protein. The anti-apoptotic function of this fusion protein blocks programmed cell death in these B cells, which leads to aberrant B cell survival and the formation of low grade lymphomas. CHD = caspase homology domain (B) In the less common t(1;14)(p22;q32) translocation, the NF-κB stimulating bcl10 gene is fused next to the NF-κB responsive immunoglobulin heavy chain enhancer. The overexpression of the Bcl10 gene can lead to NF-κB activation and cell survival through the recruitment of TRAF2 or apoptosis induction through sequestration of HIAP1, depending on the phosphorylation status of Bcl10. Under the correct phosphorylation conditions, Bcl10 expression can lead to NF-κB activation, Bcl10 stimulation and upregulation of NF-κB responsive anti-apoptotic proteins, such as HIAP1. This combination may also lead to abnormal B cell survival and growth.
NF-κB levels protects cells from apoptosis and renders the tumours resistant to antibiotic therapies.

The observation that xaf1 mRNA expression is high in normal tissues, but very low in cancer cell lines suggests a possible anti-cancer role for XAF1. We expanded our study using the National Cancer Institute (NCI) 60 cell line panel and found that the mRNA expression of xaf1 was low to non-existent in the majority of cell lines tested. Analysis of genetic markers proximal to the xaf1 gene suggested a general decrease in heterozygosity at the xaf1 locus, which was indicative of genetic alterations within this region. Finally, we propose a novel anti-tumour role for xaf1 in cell cycle regulation. The expression of XAF1A triggered G1 cell cycle arrest, likely through the p53-independent activation of p21. This data indicated a new function for XAF1 beyond XIAP inhibition and lent credence to the proposed tumour suppressor role for XAF1.

2. Methodology

2.1 FISH Analysis

Fluorescence in situ hybridization was performed according to previously documented methods (Rajcan-Separovic et al. 1996). Briefly, 1 μg of DNA from the human xaf1 genomic clone #28 was labeled with biotin by nick-translation (Gibco/BRL). Chromosome spreads prepared from peripheral blood cells were denatured in 70% formimide/2X SSC for 2 min and probed with the biotin labeled xaf1 genomic DNA in 2X SSC/50% formimide/10% dextran sulfate solution for 18 h at 37°C. Slides were washed in 2X SSC/50% formimide, followed by washed
in 2X SSC at 42°C. Biotin labeled DNA was detected using a fluorescein isothiocyanate-conjugated avidin (ONCOR detection kit). For chromosome identification, slides were destained, dehydrated and digested with trypsin for 30 s, then stained with 4% Giemsa stain for 2 min. Slides were examined on an Olympus BX60 epifluorescence microscope.

2.2 Real-Time Quantitative RT-PCR

Specific xaf1 RNA from the NCI 60 cell line panel was measured using a real-time quantitative RT-PCR method (Heid et al. 1996; Lo et al. 1999). This method is based on a fluorescent DNA probe designed between two amplification primers. This dual-fluorescent probe contains the reporter dye 6-carboxyfluorescein (6-FAM) whose emission spectrum is quenched by the second dye, 6-carboxy-tetramethylrhodamine (TAMRA). During PCR amplification, the 5' to 3' exonuclease activity of Taq DNA polymerase cleaves 6-FAM from the probe, releasing 6-FAM from the quencher TAMRA. The quantity of free 6-FAM, which directly correlates with DNA amplification, can be continuously measured by spectrophotometric readings at 518 nm. Simultaneously, amplification of the gene GAPDH can be use as an endogenous PCR control by designing probes using JOE (6-carboxy-4,5-dichloro-2,7-dimethoxyfluorescein), a reporter dye with a widely different emission spectrum (554 nm). By continuously monitoring free 6-FAM and JOE, it is possible to normalize the amplification curve of the gene of interest. Using the fractional cycle number at a fixed threshold during log phase (C_T), the quantity of specific
target RNA can be calculated relative to a standard sample using the following formulae:

\[ \Delta C_T = \text{Average } C_T, \text{ sample} - \text{Average } C_T, \text{ GAPDH} \]

Relative amount of target = \[ 2^{-\Delta \Delta C_T} = 2^{-\left(\Delta C_T, \text{ sample} - \Delta C_T, \text{ standard}\right)} \]

In this study, primers and probes were made for both XAF1 and XIAP. The XAF1 primers and probe (see Table 3) were derived from exon 1 and 2 sequences and are found in all isoforms of xaf1. The XIAP primers and probes (see Table 3) were designed to span exons 3 and 4. All probes were designed to overlap an intron-exon boundary to block measurement of any possible genomic DNA contamination. Total RNA was isolated from \( \sim 1 \times 10^7 \) cells using RNeasy mini spin columns combined with DNase treatment (Qiagen). For each cell line sample, 100 and 10 ng of total RNA was used for xaf1 and xiap analysis respectively. The RNA was reverse transcribed and PCR amplified using the Taqman EZ RT-PCR kit (Applied Biosystems) in the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Primers and probes were used at a concentration of 600 nM and 200 nM respectively for both xaf1 and xiap. The thermal cycling conditions for the RT step was 50°C for 2 min, 60°C for 30 min and 95°C for 5 min, which was followed by 45 cycles of PCR (94°C for 20 s and 60°C for 1 min). The concentration of each sample was calculated relative to leukemic cell line HL-60.

2.3 YAC DNA Isolation

YAC clones (746 C 10, 946 B 1, 892 A 1, 769 B 4, and 728 B 11) from the human 17p13.2 region were ordered from Genome Systems (St. Louis, MO).
Table 5-1. PCR Primers Used for Real Time RT-PCR and Heterozygosity Analysis.

(A) Real time RT-PCR requires two primers and an intervening fluorescent probe labeled with TAMRA and FAM. Primers were designed to span an intron/exon boundary to prevent amplification of genomic DNA. (B) Primers for STS genetic markers were designed from the established sequence and observed heterozygosity within the general population have been previously published (Genbank).
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### B

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Yeast genomic and YAC DNA was isolated using established protocols (Current Protocols in Molecular Biology, Ch. 13.11). Yeast cells were grown overnight at 30°C in 10 ml YPD media and collected by centrifugation at 1200 x g. Cells were washed in H$_2$O and disrupted by 3 minutes of vortexing in the presence of 200 μL breaking buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0), 0.3 g glass beads and 200 μL phenol/chloroform/isoamyl alcohol. After 5 min centrifugation, the DNA in the aqueous phase was precipitated in 1 ml of ethanol and resuspended in 0.4 ml TE buffer. The DNA is further treated with 30 μg DNase-free RNA for 5 min at 37°C, precipitated with 10 μL 4 M ammonium acetate and 1 ml ethanol, and resuspended in 100 μl TE.

2.4 Loss of Heterozygosity Analysis

Polymorphic CA repeat markers were amplified by PCR using primers spanning the repeat region (see Table 3). Each 5' primer was end-labeled with γ-[³²P]ATP using T4 polynucleotide kinase according to the manufacturers instructions (Gibco/BRL). Labeled products were partially purified on G50 spin columns and used in the PCR reaction at a concentration of 10 μM. Products were amplified in 25 μl volumes using 1 μg genomic DNA and 1.25 units of Taq DNA polymerase (Gibco/BRL) under standard buffer conditions (20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM MgCl$_2$, 0.2 mM dNTP). After denaturation at 95 °C for 5 minutes, the DNA was amplified for 35 cycles under typical PCR conditions (95 °C, annealing temperature (see Table 3), and 72 °C for 30s each step) in a DNA thermal cycler (Perkin Elmer). An equal volume of formimide loading buffer
was added and the samples heated to 75 °C for 5 minutes. After cooling on ice for 1 min, 5 µl from each sample was loaded onto a pre-run Long Ranger polyacrylamide gel (Ultrapure Bioreagents) containing 40% formimide in 1X TBE running buffer. Gels were run on a S2 sequencing electrophoresis apparatus (Gibco/BRL) at 90 W for 2 h. A 20% ethanol/10% acetic acid solution was used to fix the gel prior to transfer onto Whatman 3mm paper. The gels were dried under vacuum in a GD-3 gel dryer (Owl Scientific Plastics) at 80°C for 2 h and exposed to X-ray film overnight at −80 °C.

3. Results

3.1 Lack of xaf1 Expression in Cancer Cell Lines

Initial Northern blot analysis showed a loss of xaf1 mRNA expression in a small panel of cancer cell lines (see Figure 5-2). The low expression of xaf1 mRNA in most cancer cell lines was further examined using a large panel of 60 cell lines compiled by the National Cancer Institute. A relatively new real-time quantitative fluorogenic PCR method (Heid et al. 1996; Lo et al. 1999) was used to further examine xaf1 RNA levels in these cancer cell lines. Using the fraction of the cycle number at a set fluorescence threshold, while compensating for background and endogenous gene expression, it is possible to calculate the expression levels of the target sequence relative to a particular reference sample (see Materials and Methods). Using this method, total RNA from the NCI 60 cell line panel was analyzed by RT-PCR utilizing primers designed to amplify all known xaf1 mRNA isoforms and a 6-FAM/TAMRA probe spanning
Figure 5-2. Expression of xaf1 RNA is Lost in the Majority of Cancer Cell Lines

Total RNA from the NCI 60 cell line panel was examined for xaf1 and xiap expression using a real-time quantitative PCR method. The data was analyzed using a comparative method with the HL-60 cell line as the baseline (=1). Normal human liver xaf1 expression was beyond the scale of the graph (16.11). The majority of cancer cell lines express little to no xaf1 mRNA, compared to normal human tissues like liver. On the other hand, xiap mRNA is found in significant quantities in all cell lines tested.
intron 1 to prevent genomic DNA amplification (see Materials and Methods). The data shown in Figure 5-2 were calculated relative to HL-60, a leukemia cell line with a moderate level of xaf1 RNA expression. The levels of xaf1 RNA were low to non-existent in the majority of the cell lines when compared to the expression levels in total human liver RNA (16.11 times HL-60, see Figure 5-2). Several notable exceptions include SF-539 (4.38), OVCAR-5 (3.25) and NCI-H226 (2.03), but these values were still significantly below normal human liver. Evidently, despite the high levels of xaf1 mRNA in normal human tissues (Figure 3-4A), cancer cell lines from all tissues showed little to no expression of xaf1 RNA. Given the anti-apoptotic function of XIAP can be blocked by XAF, the NCI 60 cell line panel RNA was also examined for xiap RNA levels. In contrast to xaf1, xiap RNA levels were high, even when compared to normal human liver (see Figure 5-2). Though not all individual cancer cell lines showed a direct inverse correlation between xaf1 and xiap levels, the overall data suggests that the high expression of xiap RNA coupled with the low expression of xaf1 RNA is a very common characteristic of cancer cell lines.

3.2 Chromosomal Localization of the xaf1 Gene

The loss of xaf1 mRNA expression was proposed to correlate with loss of heterozygosity, a genetic phenomenon linked to tumor suppressor genes. To examine this hypothesis, further refined localization of the xaf1 gene was necessary. The first step was the chromosomal localization of the xaf1 gene, which was done through fluorescent in situ hybridization (FISH) analysis using an isolated genomic clone (#28) which spans the majority of the xaf1 gene. A single
copy of the xaf1 gene (see Figure 5-3A) is found on the short arm of chromosome 17 at approximately 17p13, as measured by chromosomal banding pattern (see Figure 5-3B). This was verified by dual coloured FISH, using a chromosome 17 centromere specific probe, the TP53 gene and the xaf1 gene (see Figure 5-3C).

To further define the xaf1 locus, eight yeast artificial chromosomes (YAC) spanning 17p13 were analyzed by Southern blot (see Figure 5-4). YACs 769B4 and 892A1, which have been localized near the telomere of chromosome 17, have been previously identified as regions that contain possible tumour suppressors (Merlo et al. 1992; Pietsch et al. 1997; Steichen-Gersdorf et al. 1997; Eiriksdottir et al. 1998; Konishi et al. 1998). The YACs 770F3, 898A10 and 767F9 have been used to map the cystinosis gene on 17p13.2 (McDowell et al. 1996). The three other YACs used (746C10, 728B11 and 946B1) are located between 17p13.2 and 17p13.1. One clone, 728B11, is known to contain the tumor suppressor gene TP53, which encodes the p53 protein (Dib et al. 1996)(see Figure 5-4A). Figure 5-4C shows a Southern blot of YAC containing yeast DNA digested with HindIII and probed with the 750 bp xaf1 cDNA fragment. HindIII digested control DNA from two normal individuals was used for comparison (see Figure 5-4B). Of the eight YACs, only 746C10 showed two prominent bands similar to the control lanes (17 and 8.5 kb, see Figure 5-4C). Comparable results were found with two other restriction enzymes tested (Data not shown). YAC 746C10 is telomeric to the TP53 gene, which is located on the neighboring 728B11 YAC.
Figure 5-3. FISH Localization of the xaf1 Locus.

(A) Metaphase spreads of human peripheral blood leukocytes were hybridized with the xaf1 genomic phage clone#28. Specific fluorescent signals on 17p13.2 are indicated by the arrows. (B) Same metaphase spread after G-banding. 33/37 (86%) metaphase spreads had signals on 17p13.2 in one or both chromosome 17 homologues. (C) Multicoloured interphase FISH analysis of HEY cells. Metaphase spreads of HEY cells were probed with genomic fragments of the TP53 and xaf1, as well as a chromosome 17 centromer specific probe. The three copies of the xaf1 gene (small red fluorescence, large arrows) lie distal to the TP53 locus (blue fluorescence, small arrow heads) on the four copies of chromosome 17 (large red fluorescence) in this cell.
Figure 5-4. Localization of the xaf1 Gene on Chromosome 17.

(A) Position of the various YAC clones tested on chromosome 17p13. Filled circles indicate polymorphic markers and hollow circles represent non-polymorphic markers. Numbers beneath the contigs indicate genetic distance between markers according to RH maps, where available. Figure is not to scale. (B) Southern blot analysis of HindIII digested normal control human DNA probed with xaf1A cDNA. λHindIII size markers are labeled on the left of the gel. (C) Southern analysis of YAC clones in the vicinity of 17p13.2. HindIII digested YAC DNA was probed with the xaf1A cDNA. The xaf1 gene was localized on YAC 746 C 10, a clone proximal to the p53 locus on 728 B 11.
3.3 Decreased Heterozygosity in the xaf1 Region

The loss of xaf1 mRNA expression in cancer cells suggests that xaf1 downregulation may be important in the development of malignancy. One early event frequently associated with loss of gene expression in cancer is the loss of heterozygosity of polymorphic markers in the immediate area surrounding the gene of interest. A decrease in heterozygosity at markers in the region of interest may indicate the loss of one allele due to chromosomal deletion or unequal crossover events. This allelic loss may duplicate the mutations from one allele, thereby totally impairing gene function from both copies. To determine whether there is decreased heterozygosity in the xaf1 region of cancer cells, the three polymorphic markers found on YAC 746C10 were analyzed. All three STS markers have high heterozygosity levels in the general population (>77% heterozygous, see Table 3). Genomic DNA was extracted from frozen cell pellets from the NCI 60 cell line panel. The 5' primer from each STS primer set was end-labeled with [32P]-ATP using T4 polynucleotide kinase, partially purified using spin columns and used in the PCR at a concentration of 10 μM. Approximately 1 μg of genomic DNA was used as template in each reaction. Samples with two distinct bands of different sizes but equal intensity were deemed heterozygous, whereas one single distinct band was judged to be homozygous. Indistinct bands or two bands of different intensities were considered indeterminant. Figure 5-5 summarizes the results of this study, which showed a low level of heterozygosity among the three markers tested (42.3%, 38.9% and 19.6% for D17S1832, D17S796 and D17S1881 respectively). Only 7 cell lines sho
Figure 5-5. Loss of Heterozygosity in the NCI 60 Cell Line Panel.

Cell line genomic DNA was tested for LOH at 3 polymorphic markers located on YAC 746 C 10. Genomic DNA was isolated from the NCI 60 cell line panel and tested for heterozygosity at specific markers by PCR. Cell lines were grouped according to the tissue of origin. Symbols represent ◊ Heterozygosity, ● homozygosity or ○ untested/unknown at a particular marker. % heterozygosity was 42.3%, 38.9% and 19.6% for D17S1832, D17S796 and D17S1881 respectively. Two cell lines were unavailable for analysis.
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heterozygosity across this region whereas 22 were homozygous on all markers tested. Of the 22 homozygous cell lines certain cancer cell types were highly represented, such as non-small cell lung carcinomas (NSCLC, 7/9, 77.7%), breast cancer (5/7, 71.4%) and CNS (3/6, 50%). None of the cell lines tested showed any gross rearrangements of the xaf1 gene by Southern blot (data not shown). Therefore, the loss of xaf1 expression in these cancer cells lines is not due to large deletions of the xaf1 gene.

3.4 XAF1A Induced Cell Cycle Arrest

Tumour suppressor genes, such as p53, have functions detrimental to the development of cancer cells, such as involvement in pro-apoptotic pathways or inhibition of cell cycle. In Chapter 3, XAF1A was shown to promote apoptosis through the obstruction of XIAP function. Here, we examined the effect of XAF1A overexpression, using recombinant adenoviral vectors, on the proliferation of the human embryonic lung fibroblasts HEL 299 and WI-38. Both HEL 299 and WI-38 are relatively normal cell lines with a limited growth potential. Cells were plated at a low density and transduced at an MOI of 10 plaque forming units (pfu)/cell. Transfected cells were collected 48 h post-infection, fixed with methanol and stained with the DNA dye propidium iodide. Fluorescence activated cell sorting (FACS) analysis (see Figure 5-6) indicated that cells infected with the control adeno-p53 vector inhibited cell cycle progression at G1, as measured by the significant loss of S phase cells (6.6 and 4.6% for HEL 299 and WI-38 respectively) when compared to adeno-LacZ infected cells (18.7% for both HEL 299 and WI-38). Adeno-XAF1A infected cells
Figure 5-6. XAF1A Induces G1 Cell Cycle Arrest in HEL299 and WI-38 Cell Lines.

(A) HEL299 cells were transduced with adeno-LacZ, adeno-p53 or adeno-XAF1A at a MOI of 20 pfu/cell. Cells were collected 48 h post-infection and analyzed by fluorescence activated cell sorting (FACS). Untransduced cells were used as a control. XAF1A triggers G1 cell cycle arrest, similar to p53 overexpression. Similar results were found for the WI-38 cell line (data not shown). (B) The percentage S-phase cells were determined for both HEL299 and WI-38 cell lines transduced with adeno-LacZ, adeno-p53 or adeno-XAF1A.
also significantly (p<0.001) suppressed cell cycle progression through G1 phase, yielding 12.5 and 10.2% S phase cells for HEL 299 and WI-38 respectively (see Figure 5-6). This XAF1A induced G1 arrest, though not as potent as p53, may be an important function of the nuclear localized XAF1A, beyond its XIAP inhibiting capabilities. On the other hand, the highly transformed HeLa cell line showed no cell cycle effects when infected with either adeno-p53 or adeno-XAF1A (see Figure 5-7). Loss of downstream G1 phase regulators, such as p21, may be responsible for this lack of responsiveness.

In an attempt to elucidate the mechanism for the observed G1 arrest, protein extracts from adeno-p53 and adeno-XAF1A transduced HEL 299 cells were examined for expression of important cell cycle regulating proteins (see Figure 5-8). As expected, the expression of p53 upregulated p21^{WAF1/CIP1} protein production, which prevented Rb phosphorylation by inhibiting cyclinE/cdk2. Overexpression of p53 also appeared to prevent Rb phosphorylation by downregulating CDK2 expression in these cells. In contrast, adeno-XAF1A transduction of HEL 299 cells leads to the upregulation of p21^{WAF1/CIP1}, without inducing p53 or blocking CDK2 expression (see Figure 5-8). It appeared that XAF1A induced G1 cell cycle arrest in HEL 299 cells, at least in part, through the p53-independent upregulation of p21^{WAF1/CIP1}.

4. Discussion

One of the most intriguing observations from the Northern blot analysis was the lack of xaf1 mRNA expression in cancer cell lines. Of the eight cell lines tested, five had undetectable levels of xaf1 mRNA while the other two expressed
Figure 5-7. XAF1A Does Not Affect the Proliferation of HeLa Cells.

(A) HeLa cells were transduced with adeno-LacZ, adeno-p53 or adeno-XAF1A at a MOI of 20 pfu/cell. 48 h post-infection, HeLa cells were collected and analyzed by FACS. Untransduced HeLa cells were used as a control. (B) The percentage of S-phase cells was determined for HeLa cells transduced with adeno-LacZ, adeno-p53 or adeno-XAF1A.
Figure 5-8. XAF1A Triggers p21\textsuperscript{waf1/cip1} Protein Expression in HEL299 Cells.

HEL299 cells were transduced with adeno-p53 or adeno-XAF1A at an MOI of 20 pfu/cell. Cells were collected 48 h post-infection and the proteins analyzed by Western blot for XAF1, p53, p21\textsuperscript{waf1/cip1} and CDK2 protein expression. An anti-\alpha-tubulin antibody was used as a loading control. XAF1A induces a dramatic increase in p21\textsuperscript{waf1/cip1} protein expression, but did not affect CDK2 levels, as seen in adeno-p53 transduced cells.
low levels of the 3.9 kb RNA species (see Figure 3-4A and B). Since XAF1 is known to block the anti-apoptotic capability of XIAP (See Chapter 3), there are obvious growth advantages in downregulating XAF1 for a cancer cell. To examine the extent of XAF1 suppression in cancer cell lines, a panel of 60 cell lines was obtained from the NCI and the expression of xaf1 RNA was measured using a real time RT-PCR protocol. As predicted, the relative levels of xaf1 RNA was extremely low. Several cell lines, such as SF-539, OVCAR-5 and NCI-H226, expressed relatively high levels of xaf1 (2.03-4.38), but they still had ~4 times less xaf1 RNA than normal human liver (16.11). On the other hand, xiap RNA expression levels appeared high for almost all the cell lines tested relative to normal liver. This alteration in xaf1 and xiap RNA expression levels is proposed to lead to increased apoptotic resistance due to unregulated XIAP function in these cancer cells.

The method by which xaf1 expression is suppressed may lend credence to the importance of XAF1 in the development of certain cancers. The observation that xaf1 is located distal to p53 on 17p13.2 is striking due to the numerous studies showing the importance of loss of heterozygosity at 17p13 in a variety of cancers (Casey et al. 1993; Phillips et al. 1993; Fujii et al. 1998; Konishi et al. 1998). LOH studies examine the heterozygosity level of polymorphic STS markers at various chromosomal locations and searches for differences in heterozygosity between cancer samples and matched normal tissue control. Significantly lower heterozygosity scores in the cancer tissue indicate a loss or duplication of one allelic region, which increases the functional
significance of any mutations on the other allele.

The first step in this study was to sublocalize the xaf1 gene on 17p13. This was accomplished by Southern blots on YAC contigs spanning the entire 17p13 region. The YAC 746C10 was found to encompass the xaf1 gene as well as three polymorphic STS markers (D17S1832, D17S796 and D17S1881). When the heterozygosity of these markers were examined in the NCI 60 cell line panel, it was found that all three markers had significantly lower heterozygosity levels than published data (Dib et al. 1996). Though there were no individual matched controls for these cell lines, the low heterozygosity levels apparent in many of these cancer cell lines suggests a frequent loss or duplication of one allele in the xaf1 region. Southern blot analysis showed no gross deletions of the xaf1 gene in these cell lines, but this does not discount the possible deletion of one entire xaf1 allele or deletions outside of the xaf1 coding region. Of the 58 cell lines tested, 22 (37.9%) were homozygous at all three markers. Within these 22 cell lines, it is interesting to note that several highly represented cell types, like NSCLC, breast cancer and CNS, have previously documented LOH in the 17p13 region (Merlo et al. 1992; Pietsch et al. 1997; Steichen-Gersdorf et al. 1997; Eiriksdottir et al. 1998; Konishi et al. 1998).

The loss of xaf1 RNA expression in cancer cell lines suggests a possible role for XAF1 in the suppression of malignancy, although the method by which xaf1 expression is turned off is still unclear. The decreased heterozygosity within the xaf1 region observed in the NCI 60 cell line panel demonstrates that allelic loss is common to 17p13.2 in cancer, but fails to correlate with the significant
reduction in xaf1 RNA expression. Examination of the remaining xaf1 allele for mutations that prevent RNA expression would be most beneficial. Other possible transcriptional regulatory mechanisms, such as hypermethylation of CpG islands (Laird and Jaenisch 1994; McBurney 1999), should also be investigated. Sequence analysis of the existing xaf1 genomic sequence using the GRAIL analysis program revealed a CpG island at the 3' end of the xaf1 gene locus. Recent studies indicate that treatment of bladder cancer cells with the methylation inhibiting compound 5-aza-deoxycytidine leads to the activation of numerous genes, including xaf1 (Liang et al. 2002). This data suggests that methylation may also be important in the silencing of the xaf1 gene and may be a significant contributor to the general loss of xaf1 expression in cancer cell lines.

Many tumour suppressor proteins, including p53 and p21, have significant effects on both apoptosis and cell cycle. In particular, p53 is a strong inducer of apoptosis and an important cell cycle mediator, through the induction of p21. In Chapter 3, XAF1A was shown to promote apoptosis by inhibiting XIAP, thereby supporting caspase function. We now provide evidence that the overexpression of XAF1A causes G1 cell cycle arrest in human fibroblast cell lines, similar to p53. XAF1A induced inhibition of cell cycle progression correlates with a profound increase in $p21^{WAF1/CIP1}$ protein accumulation, along with the distinct absence of p53 expression. XAF1A overexpression does not lead to the downregulation of the G1 cyclin-dependent kinase CDK2, as seen in p53 transduced cells. This suggests that the XAF1A may block cell cycle at the G1 checkpoint through $p21^{WAF1/CIP1}$ inhibition of cyclin-E/CDK2 dependent
phosphorylation of Rb. The lack of p53 induction by XAF1A indicates that the upregulation of \( p21^{WAF1/CIP1} \) does not occur along the well defined \( p19^{ARF/MDM-2/p53} \) pathway, which suggests alternative mechanisms for \( p21^{WAF1/CIP1} \) stimulation. One such mechanism may be the direct promotion of \( p21^{WAF1/CIP1} \) transcription through XAF1A binding, since XAF1A has been localized to both the cytoplasm and nucleus and zinc fingers may function as protein-DNA binding domains. The ability of XAF1A to bind DNA, as well as the influence of the other XAF1 isoforms on cell cycle, is currently under investigation.

Overall, the loss of XAF1 expression would be highly beneficial to the developing cancer cell. Removal of XAF1A increases the apoptotic threshold by relieving XIAP repression, which permits inappropriate and unfortunate cell survival. The increased apoptotic threshold, due to the loss of XAF1A, is coupled with the loss of cell cycle inhibition, thereby promoting both apoptotic resistance and cell cycle progression. Since an exact cellular function has yet to be attributed to the XAF1B and XAF1C isoforms, the impact of their loss in cancer cells is difficult to gauge. Nonetheless, the evidence supports a tumour suppressor role for XAF1 in human cells and suggests a novel therapeutic target, which may be exploited to sensitize or kill tumour cells.
CHAPTER 6: GENERAL DISCUSSION

The process of cellular suicide is vital for the biological development and maintenance of multicellular organisms. The self directed death of specific cells aids in the removal of superfluous cells during tissue formation, as well as the elimination of certain cells which are dangerous to the health of the organism. Not only are unwanted cells destroyed, but the cellular debris is neatly packaged in apoptotic bodies where they can be safely removed by phagocytosis. Although apoptosis offers great benefits to higher organisms, the process itself must be strictly controlled to avoid the loss of essential cells or the survival of hazardous cells; either of which are detrimental to the health of the organism. The ever expanding field of apoptosis research is just beginning to elucidate the complex mechanisms by which the organism controls the death of its own cells.

The apoptotic process is regulated in multiple layers, beginning with the control of multiple apoptotic triggers and culminating in the restriction of effector caspase function. Between these two control points lie the endogenous and exogenous apoptotic signaling pathways, both of which contain their own regulatory elements. All these control mechanisms form a relative apoptotic threshold, which balances apoptotic signals with cell survival signals. In addition to the plethora of pro-apoptotic proteins found to trigger caspase activation, a growing number of anti-apoptotic proteins have been discovered, which block apoptosis at different levels. This includes Bcl-2 family members, which help control the release of cytochrome c, a key event in the endogenous apoptotic pathway, and the inhibitor of apoptosis family of proteins, many of which have
been shown to directly inhibit effector caspases. These proteins endeavor to increase the apoptotic threshold by interfering at certain points along the apoptotic pathway. A new group of proteins are emerging which are able to block the function of anti-apoptotic proteins, but do not necessarily induce apoptosis. For example, the Bcl-2 family member Bad binds and prevents Bcl-2 from interfering with the Bax-dependent release of cytochrome c (Datta et al. 1997). Although the function of Bad promotes apoptosis, Bad itself does not induce apoptosis alone, but instead lowers the apoptotic threshold through the inhibition of Bcl-2.

As with Bcl-2, several antagonists of IAP function have been discovered, including Smac and Omi. These mitochondrial proteins have been shown to directly bind XIAP BIR2 and BIR3, thereby interfering with the caspase/XIAP interaction. These interactions with the XIAP BIR domains requires an exposed tetrapeptide sequence (AXPX) at the amino terminal end of the Smac or Omi protein, with the exception of the Smac/XIAP BIR2 interface which requires the formation of a Smac dimer. The regulation of Smac and Omi function involves multiple levels of control, including release from the mitochondria, protein cleavage to expose the active tetrapeptide sequence and the degradation of Smac and Omi through ubiquitination by XIAP. This degree of regulation is necessary to maintain a balanced apoptotic threshold, as well as controlling other possible functions of Smac and Omi.

We describe here a novel antagonist of XIAP, XAF1, which also directly binds and inhibits XIAP function. However, unlike Smac and Omi, XAF1 does
not contain an AXPX-like sequence, which suggests another method of XIAP binding. The original XAF1A isoform consist of 7 potential zinc finger domains, but no other known protein binding motifs. Studies of the existing XAF1 isoforms indicate that XIAP binding is abrogated by the loss of the sixth and seventh C-terminal zinc fingers (XAF1C), but is unaffected by the loss of the third zinc finger (XAF1B). It is likely that XIAP interacts with one or both of these C-terminal zinc fingers, since zinc fingers frequently function as protein-protein binding domains. Alternatively, XIAP may interact with the intervening sequence between XAF1 zinc finger domains, analogous to the XIAP BIR1-BIR2 linker region, which plays a critical, if unanticipated, role in XIAP/caspase-3 interactions. There is also a possibility that XAF1 requires oligomerization to interact with XIAP, similar to the interaction between Smac and XIAP BIR2. The loss of the two C-terminal zinc fingers on XAF1C may prevent oligomerization, thereby preventing XIAP binding. Additional mutation analysis is underway to determine whether oligomerization occurs and which domains are essential for the XIAP interaction. X-ray crystallography studies are also underway, in collaboration with Dr. Hao Wu (Cornell University), which will ultimately resolve these issues.

According to Leaman et al. (Leaman et al. 2002), XAF1 is induced by IFN-β and sensitizes IFN-β treated cells to INF related apoptosis inducing ligand (TRAIL) triggered apoptosis. This IFN-β induced upregulation of XAF1A and/or XAF1B likely potentiates TRAIL triggered apoptosis by antagonizing XIAP function, as seen in etoposide mediated apoptosis. Interestingly, a construct consisting of the first six carboxy terminal zinc fingers of XAF1 (ZF-XAF1) acted
as a dominant negative inhibitor of IFN-β/TRAIL induced apoptosis (Leaman et al. 2002). ZF-XAF1 may function to block oligomerization of XAF1 or inhibit the ability of XAF1 to interact with XIAP. Our studies of the XAF1C isoform, consisting of the first five zinc fingers, showed a total inability of XAF1C to bind, and possibly inhibit, XIAP. While the effect of XAF1C overexpression on IFN-β/TRAIL induced apoptosis has yet to be determined, it is highly probable that the XAF1C isoform also functions as a dominant negative inhibitor similar to the ZF-XAF1 deletion mutant. If verified, XAF1C may represent a novel antagonist of the pro-apoptotic function of XAF1A and/or XAF1B, analogous to the Bcl-2/Bax interaction. The production of antagonistic splice isoforms also provides a novel control mechanism by which the cell maintains the apoptotic threshold.

We have demonstrated that XAF1A expression, through adenoviral transduction, antagonizes the ability of XIAP to inhibit the effector caspase-3, thereby potentiating apoptosis induced by etoposide and serum withdrawal. However, these experiments used high levels of exogenously produced proteins, which may not be a true representation of physiological conditions within the cell. To examine the effect of endogenously expressed XAF1, an adenoviral XAF1A antisense (adeno-xafas) vector was used to downregulate XAF1 expression in the high XAF1 expressing SF-539 and the low XAF1 expressing SF-295 cell line (Liston et al. 2001). These studies indicated that adeno-xafas transduction increased the apoptotic resistance of SF-539 to etoposide induced cell death, but had little effect on the XAF1 non-expressing cell line SF-295 (Liston et al. 2001). Therefore, the removal of the endogenous XIAP antagonist XAF1 increases the
apoptotic threshold, thereby inhibiting programmed cell death induced by certain triggers.

Our research also indicated that XAF1 may have other roles beyond the promotion of apoptosis. Adeno-XAF1A transduction of HEL299 and WI-38 human embryonic lung cell lines produced a significant G1 cell cycle arrest, similar to adeno-p53 transduction, which coincided with a dramatic upregulation in p21\textsuperscript{WAF1/CIP1} protein expression. Interestingly, overexpression of XAF1A does not stimulate p53 expression or downregulate the G1 cyclin-dependent kinase CDK2, which suggested that XAF1A works independent of the p19\textsuperscript{ARF}/MDM-2/p53 signaling pathway. XAF1A may induce G1 arrest by blocking the phosphorylation of Rb through the p21\textsuperscript{WAF1/CIP1} inhibition of cyclin-E/cdk2. On the other hand, the cell cycle of the highly transformed HeLa cell line was unaffected by either p53 or XAF1A overexpression. It is possible that mutations within the HeLa genome prevent the induction of G1 cell cycle arrest. Cell lines like HEL299 and WI-39, which are less transformed and survive only a limited number of passages, are highly affected by both p53 and XAF1A expression.

In an interesting twist, it was demonstrated that XIAP functions as an E3 ubiquitin ligase and mediated the ubiquitination of various binding partners including caspase-3, Smac and XIAP itself (Yang et al. 2000; Suzuki et al. 2001; MacFarlane et al. 2002). It is possible that the interaction between XAF1 and XIAP may also lead to the ubiquitination and degradation of XAF1. Moreover, the primary purpose of the XIAP/XAF1 interaction may be the degradation of XAF1 protein, rather than the inhibition of XIAP/caspase binding. The targeted
destruction of XAF1 would prevent the G1 cell cycle arrest observed here, which, along with caspase-3 and Smac degradation, blocks apoptosis and promotes cell growth. Additional pro-apoptotic functions for Smac and XAF1 are likely to be found in the near future.

The two known biological functions of XAF1 provide distinct benefits to the organism. By decreasing the apoptotic threshold and blocking cell cycle progression, XAF1 expression prevents the growth and survival of specific target cells. In contrast to other IAP inhibitors, such as Smac and Omi, which appear to be sequestered in the mitochondria and released upon apoptotic stimulation, XAF1 expression can be induced through pathways such as IFN-β (Leaman et al. 2002), which results in the sensitization of specific cells to other apoptotic triggers. We hypothesize that XAF1 functions as a homeostatic apoptosis regulator whose expression, along with XIAP, helps maintain the apoptotic threshold, whereas Smac and Omi operate as abrupt apoptotic stimulators that potentiate the endogenous apoptotic pathway upon release from the mitochondria. The ubiquitous expression of XAF1 in normal tissues and distribution throughout the cell suggests an unrestricted inhibitor which helps establish basal sensitivity to apoptotic triggers. This may be important in the nucleus, where inhibitors of nuclear IAPs, such as Survivin and Livin, have yet to be discovered. Finally, there is evidence that XAF1A sequesters XIAP in the nucleus following etoposide treatment (Liston et al. 2001), which potentially promotes apoptosis by removing XIAP from the same cellular compartment as caspases. This data suggests that XAF1 may antagonize XIAP function by both
directly inhibiting XIAP function and physically removing XIAP from potential substrates.

While beneficial to the entire organism, XAF1 expression has dire consequences for individual cells. In the case of the developing cancer cell, the ability of XAF1 to promote apoptosis and block cell growth offers serious obstacles toward the progression of the malignant phenotype. Although the expression of xaf1 mRNA in normal human tissue is high, it appears that the majority of human cancer cell lines downregulate the expression of xaf1, with obviously favorable consequences for tumour cell development. This profound loss of xaf1 mRNA expression coincides with significantly upregulated xiap mRNA expression in these cell lines and decreased heterozygosity in polymorphic genetic markers close to the xaf1 locus. These findings suggest that the majority of cancer cells develop an imbalance between XAF1 and XIAP function which results in a significantly elevated apoptotic threshold. This leads directly to increased cell survival and resistance to anti-cancer therapies. The diminished expression of xaf1 mRNA may be partially due to genetic alterations in the vicinity of the xaf1 locus, which leads to the observed decreased heterozygosity within this region. Indeed, the telomeric end of chromosome 17 is known to harbour several important tumour suppressor genes such as TP53 and hic-1 (Wales et al. 1995; Dib et al. 1996) and various cancers have marked loss of heterozygosity within this region (Merlo et al. 1992; Pietsch et al. 1997; Steichen-Gersdorf et al. 1997; Eiriksdottir et al. 1998; Konishi et al. 1998). There is also evidence of methylation induced silencing of the xaf1 gene, since
treatment of bladder cancer cells with the methylase inhibitor 5-aza-deoxycytidine stimulates expression of xaf1 mRNA (Liang et al. 2002). We propose that xaf1 is a tumour suppressor gene, whose loss promotes tumour development through the disregulation of apoptosis and release from cell cycle repression. XAF1 offers insights into the growth and formation of the malignant phenotype and provides a novel target for the development anti-cancer gene therapy approaches.

Concluding Remarks

The novel xaf1 gene fulfills most of the requirements for tumour suppressor gene classification. Firstly, xaf1 operates as an apoptosis promoting gene by antagonizing the anti-apoptotic function of XIAP. XAF1 also plays an added role in triggering G1 cell cycle arrest through the p53 independent upregulation of p21^{WAF1/CIP1}. This dual role of XAF1 prohibits formation of the transformed phenotype through the inhibition of cell growth and the promotion of the apoptotic program. In addition, the loss of xaf1 expression is a common trait among cancer cell lines, which supports both growth and survival of these cells. Finally, genetic alterations near the xaf1 locus, as well as methylation silencing of the xaf1 gene, have been implicated as mechanisms which abrogate xaf1 expression. Additional studies to correlate the loss of xaf1 expression with the development of malignancy, as well additional analysis of XAF1 function, are needed to further establish the importance of xaf1 as a tumour suppressor gene.
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