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**PROTEOLYTIC PROCESSING OF THE X-LINKED INHIBITOR OF
APOPTOSIS PROTEIN DURING FAS-MEDIATED APOPTOSIS**

A thesis submitted to the School of Graduate Studies at the University of Ottawa in partial fulfillment of the requirements for the degree of Master of Science, Department of Biochemistry, Microbiology and Immunology, Faculty of Medicine

By Hilary D.F. Gibson

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ABSTRACT

Apoptosis is a natural cellular function which is tightly controlled along its multiple pathways by positive and negative regulator proteins. Among the negative regulators are the gene family called the IAPs (inhibitor of apoptosis proteins) which were initially identified in baculoviruses. To date, five vertebrate members of this family have been identified (NAIP, HIAP1, HIAP2, XIAP and survivin). The X-linked inhibitor of apoptosis protein (XIAP), is proteolytically processed during the course of apoptosis in Jurkat lymphoma cells when triggered with anti-Fas monoclonal antibodies and cycloheximide (CHX). Native 55 kDa XIAP undergoes proteolytic processing producing a single 30 kDa fragment containing the carboxy terminus of the protein. *In vitro* cell-free studies, the type I calpain isoform is capable of cleaving XIAP. Calpains are calcium-dependent cysteine proteases, which are activated during apoptosis. Calpain and 26S proteasome specific inhibitors are ineffective in blocking both XIAP cleavage and cell death in Fas/CHX-stimulated Jurkat cells when used individually. However, when used together these two classes were able to block death, but not XIAP cleavage. Caspase specific inhibitors block both cell death and XIAP cleavage. In this study, I have shown that XIAP cleavage is likely induced by a member of the caspase family and that the event occurs upstream of calpain and 26S proteasome activation during the apoptotic cascade. The proteolysis of XIAP during Fas/CHX mediated apoptosis may represent an important step in the propagation of the apoptotic cascade.

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LIST OF ABBREVIATIONS

| | |
|------------------|--|
| PCD | programmed cell death |
| XIAP | X-linked inhibitor of apoptosis protein |
| DNA | deoxyribonucleic acid |
| TNF- α R1 | tumour necrosis factor-alpha receptor 1 |
| NK cells | natural killer cells |
| <i>lpr</i> | lymphoproliferative |
| <i>gld</i> | generalized lymphoproliferative disease |
| DD | death domain |
| DED | death effector domain |
| kDa | kilodaltons |
| DISC | death inducing signal complex |
| FADD | Fas-associated death domain containing protein |
| FLICE | Fas-like ICE protease |
| ICE | interleukin-1 β converting enzyme |
| ATP | adenine triphosphate |
| Apaf-1 | apoptotic protease activating factor-1 |
| CTL | cytotoxic T lymphocyte |
| HIV | human immunodeficiency virus |
| CARD | caspase recruitment domain |
| PARP | poly-(adenine diphosphate ribose) polymerase |
| NF κ B | nuclear factor κ B |

| | |
|-------|--|
| UBCs | ubiquitin conjugating enzymes |
| Ced | <i>C. elegans</i> death gene |
| Egl-1 | egg laying-1 |
| CrmA | cytokine response modifier A |
| IAP | inhibitor of apoptosis protein |
| MIHA | mammalian inhibitor of apoptosis protein homologue-A |
| mRNA | messenger RNA |
| kb | kilobase |
| TRAF | tumour necrosis factor receptor associating factor |
| RING | really interesting new gene |
| IRES | internal ribosome entry site |
| CHX | cycloheximide |
| Fas | anti-Fas monoclonal antibodies |
| GST | gluathione S-transferase |
| BIR | baculoviral inhibitor of apoptosis repeat |

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Introduction

Apoptosis/Programmed Cell Death

Initially characterized by Kerr *et al* (Kerr, 1972), apoptosis or genetically programmed cell death (PCD) has only recently been recognized as an important cellular function. Apoptosis is an integral natural cellular process in organisms of such diverse evolutionary standing as single cell yeast and mammals. Evolutionary conservation of invertebrate and lower vertebrate apoptosis-related genes with those of higher organisms suggests that the common mechanisms of cell death pathways exist in other metazoan organisms. Conservation of the signaling proteins involved in the numerous apoptotic pathways is also maintained between lower organisms and humans. The following review will detail the events which take place during a robust and thoroughly characterized mechanism of death, namely Fas/CHX-mediated apoptosis of the human cultured Jurkat T cell line. In addition to signaling events associated with the Fas-mediated pathway of apoptosis, mechanisms of apoptotic control will be discussed. Finally, X-linked Inhibitor of Apoptosis Protein (XIAP) will be the focus of the study to which this review is related.

Apoptosis is an important process during development and the elimination of self-reactive cells during the maturation of the mammalian immune system. From a pathological perspective, excessive apoptotic cell death characterizes acute neural insults such as stroke and head trauma. Conversely, too little apoptotic death appears to play a major role in proliferative disorders such as autoimmune disease and cancer (Ng, 2000).

Physical manifestations of apoptosis (reviewed in (Allen, 1997)) are oligonucleosomal DNA fragmentation, cellular organelle shrinkage, mitochondrial membrane depolarization, membrane blebbing and the immunologically “silent”

engulfing of discreet apoptotic bodies by surrounding cells. In Greek, the word *apoptosis* means falling leaves, implying a silent, almost peaceful process of death. Importantly, the contents of these dying cells are not exposed to the environment, and are therefore do not become targets for peripheral immune reactions. This form of cell death may be distinguished from necrotic cell death by the fact that the former process does not evoke an inflammatory response. On the other hand, necrotic cells undergo cell swelling, eventually lyse, spreading their contents into the tissue environment thereby evoking an inflammatory response.

The apoptotic cell undergoes dramatic changes as it dies. Dying cells are dismantled in a specific temporal and spatial manner by specific proteases and nucleases. Each of the organelles within an apoptosing cell is broken down and their contents are degraded. The mitochondria are an important example of this disassembly process (Wakabayashi, 1999). The increased permeability of the mitochondrial membrane allows the release of specific proteins, which thereby act in one of the central control mechanisms of some apoptotic cascades. Proteolysis is also an integral part of the apoptotic cascade, now believed to serve as a propagation step in the transduction of apoptotic stimuli within the cell. Selective and stage specific proteolysis of cellular proteins has also provided more precise indicators of the progression of cell death. For example, indications of the early points in the apoptotic cascade include activation of specific proteolytic enzymes and rearrangement of membrane phosphoproteins, whereas cleavage of structural proteins and DNA repair proteins are hallmarks of the latter stages of death (Allen, 1997).

The number of apoptosis-inducing agents is plentiful and they have been shown to act through different pathways. PCD may be stimulated by chemotherapeutic drugs such as etoposide and adriamycin, by viral infection or by receptor-mediated events, such as signals acting through the tumour necrosis factor receptor family. In addition to the complexity of PCD mechanisms according to the inducing stimulus, death occurs with different characteristics in a cell-type specific manner. Although the actual mechanisms of apoptosis are only beginning to be understood, multiple complex signal transduction pathways have been postulated in the progression of apoptosis. The events involved in the Fas pathway of death will be the focus of the next section because I have chosen to use this system of cell death in my study.

Fas/APO-1/CD95 Mediated Apoptosis

The tumor necrosis factor receptor superfamily, including the tumor necrosis factor receptor-1 (TNF- α R1) and APO-1/Fas/CD95R receptors are the most well characterized and robust apoptotic initiation factors (reviewed in (Cleveland, 1995; Rathmel, 1999; Scaffidi, 1999)). The first evidence for the apoptotic effects of the Fas receptor was elucidated in 1991 (Itoh, 1991). In this study, Itoh and colleagues found that anti-Fas receptor monoclonal antibodies were capable of stimulating apoptosis in receptor-bearing cells (Itoh, 1991). Cell surface expression of the Fas receptor makes cells susceptible to binding of the Fas ligand or anti-Fas monoclonal antibodies thus inducing apoptotic death. Fas receptors are expressed on the surface of many lymphoid tissue-derived cells including T and B cells, neutrophils and some peripheral blood mononuclear cells. Within the immune system, Fas signaling is involved in CD8⁺ T cell-

mediated cytotoxicity and is also involved in the process of clearing potentially autoreactive T cells by negative selection (Rathmel, 1999). The Fas ligand may be found on a more limited range of immune related cells, including neutrophils, T cells and NK cells (Cleveland, 1995).

The dramatic phenotypes found in the naturally occurring Fas and Fas ligand mutant mice illustrate the importance of the Fas receptor and its ligand within the apoptotic cascade. Genetic deficiency of the Fas receptor and its ligand cause severe phenotypes in the *lpr* (lymphoproliferation) and *gld* (generalized lymphoproliferative disease) mutant mice, respectively (Hahne, 1995; Takahashi, 1994). The *lpr* mutation blocks Fas mediated apoptosis in these mice, which causes a lymphoproliferative disorder similar to the human autoimmune disease systemic lupus erythematosus (Watanabe-Fukunaga, 1992). The *lpr* and *gld* animal models have provided evidence supporting the importance of the Fas receptor in relation to the maintenance of negative selection in the thymus (Watanabe-Fukunaga, 1992). Negative selection is a process which deletes autoreactive T cells in the thymus and is carried out by apoptotic death. On the other hand, the *gld* mice express a non-functional mutant form of Fas ligand, at similar levels as the wild type protein in normal mice (Hahne, 1995). The *gld* mice also demonstrate a similar lymphoproliferative phenotype as the *lpr* mice, but also have defects in B cell function (Gillete-Ferguson, 1994; Nagata, 1995). These animal models have provided information regarding the importance of the Fas pathway of death and some of the integral events within the cascade.

Fas Signaling Pathway

Jurkat lymphoma cells are an excellent system with which to study specific events in the Fas-mediated apoptotic cascade. The Jurkat cell line expresses Fas-receptors abundantly on their surface making them very susceptible to Fas killing (Morimoto, 1994). Much of the information the subject of Fas-mediated apoptosis was gathered using these cells, stimulated with anti-Fas monoclonal antibodies (Trauth, 1989; Yonehara, 1989). For a schematic representation, refer to Figure I, which outlines a simplified signaling pathway of Fas-mediated apoptosis. The process of Fas-mediated death is initiated by the binding of Fas receptor specific monoclonal antibodies to the Fas receptor (shown in Figure I).

The Fas receptor is a 45 kDa type I transmembrane glycoprotein, characterized by the presence of a cysteine rich death domain (DD) in the carboxy terminus of the protein (see below for details) (Itoh, 1991). Fas specific ligands are type II transmembrane glycoproteins with a molecular weight of 40 kDa (Nagata, 1995). Binding of the specific cell-surface ligands (TNF- α and Fas ligand) to their respective receptors initially triggers the recruitment and trimerization of receptors at the cell surface. Receptor binding and trimerization may also be induced with anti-Fas monoclonal antibodies (Itoh, 1991). Trimerization leads to the recruitment of a protein complex to the membrane, known as the death-inducing signaling complex (DISC) when stimulated with Fas ligand *in vivo* or monoclonal antibodies *in vitro* (reviewed in (Yuan, 1997)).

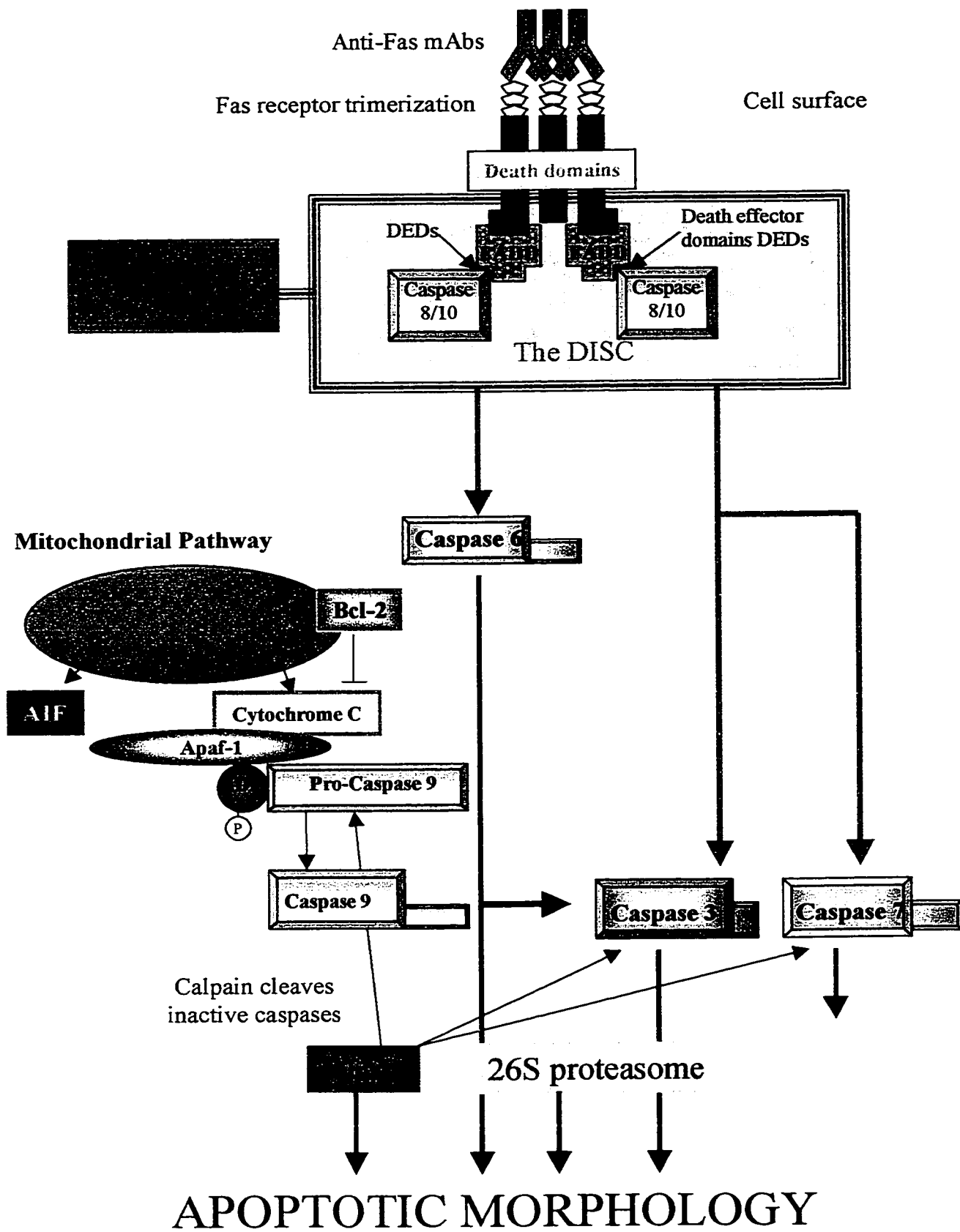
The DISC protein complex is formed by the interaction of proteins through the DDs, and the death effector domains (DEDs-see below). DDs and DEDs are composed of approximately 80 amino acids, which form six amphipathic α -helices (Wallack, 1999).

Among the proteins which bind to the cytoplasmic tail of the Fas receptor is FADD/MORT-1 (Fas associated death domain-containing protein). FADD is a 23 kDa DD-containing protein that binds to the DD within the Fas receptor. The importance of this protein within the Fas pathway of death is suggested by the fact that overexpression of FADD in Fas-sensitive cells is sufficient to cause apoptosis (Chinnaiyan, 1995). Two proteases, FLICE/caspase 8/Mch5 and caspase 10/Mch4 are also recruited to the DISC protein complex. Caspases 8 and 10 belong to an important protease family that will be the subject of a following section. Caspase 8 and 10 also contain a protein motif similar to the DD, called the death effector domain (DED), which binds to a DED within FADD (Srinivasula, 1996). Binding of these two enzymes within the DISC complex is essential for Fas signaling (Medema, 1997). The binding of the Fas receptor, FADD and the caspases form the apex of the Fas cascade of death (shown in Figure I, below the trimerization of the Fas receptor).

Aside from the core DISC proteins, a number of additional Fas-associated interactive proteins have been identified. A 220 kDa protein, termed FLASH also binds to caspase 8 and FADD and is required for Fas-mediated apoptosis. Containing two DED-like domains, FLASH is also able to self-associate and may act as a catalyst for caspase 8 activation (Imai, 1999). In addition to FLASH, a proapoptotic kinase called RIP (receptor-interacting protein) is necessary for Fas mediated death and binds to the Fas receptor (Stanger, 1995). The list of Fas-interacting proteins is long and diverse. Several members will be discussed in the context of apoptotic control in a later section.

Scaffidi and colleagues (Scaffidi, 1998) have suggested the possibility that at least two distinct Fas/CD95 pathways operate within several different Fas-expressing cell

Figure (I): Schematic illustration of Fas-mediated apoptosis. Simplified representation of the Fas pathway of death was derived from (Cleveland, 1995; Enari, 1996; Scaffidi, 1998; Scaffidi, 1999; Srinivasula, 1996; Weis, 1995). Refer to text for complete explanations. DISC proteins (containing DD and DED domains) are recruited to the cytoplasmic membrane upon Fas receptor binding. Caspase proteases and the mitochondria are largely responsible for the remainder of the signal transduction pathway of Fas-mediated apoptosis.



types. The two death pathways termed types I and II are differentiated according to the elapsed time before the DISC protein complex is recruited to the membrane and subsequent activation of caspases during the course of apoptosis. Differences in mitochondria-related events between the Fas/CD95 mediated death pathways also seem to define the two pathways. The type I pathway in Fas-sensitive cells involves rapid activation of the effector caspases, caspase 3 and 7. However, mitochondrial events such as the formation of the apoptosome are not important in this Fas pathway of death (see below). Conversely, cells that undergo the type II pathway of Fas-mediated death, which include Jurkat T lymphoblasts, undergo an apoptotic cascade which initially is reliant on mitochondrial events (Scaffidi, 1998), followed with slight delay by effector caspase activation. Figure (I) illustrates the simplified version of the Fas-pathway of cell death described here.

Involvement of Mitochondria in Apoptosis

A central facet of many apoptotic cascades is the activity of the mitochondria. When cells become apoptotic, ATP levels drop significantly, probably due to a reduction in the membrane potential of the mitochondria and the formation of permeability transition pores (Wakabayashi, 1999). Mitochondrial membrane proteins, such as cytochrome c are also released from the mitochondria through these pores and act as secondary messengers in the apoptotic cascade. Cytochrome c release stimulates the recruitment of a protein complex called the apoptosome (Rodriguez, 1999). The formation of the apoptosome, which is composed of three proteins: cytochrome c, Apaf-1 and caspase 9, is energy dependent (requires ATP or dATP). Caspase 9, another member

of the central apoptotic protease family, is proteolytically activated only upon binding within the apoptosome complex, specifically to its allosteric regulator Apaf-1 (Rodriguez, 1999). Caspase 9 and Apaf-1 share similar protein motifs called CARDs (caspase recruitment domains) which mediate their binding. It is believed that the binding of CARDs may facilitate in the removal of this domain upon the activation of caspase 9 (Moriishi, 1999). Many cell specific Fas cascades are dependent on the events surrounding the mitochondria.

The mechanisms described above, namely those referring to the receptor and mitochondrial related events, may be categorized as either the initiation or the effector/propagation stages of the apoptotic cascade. The initiation events include, Fas receptor-ligand/mAb binding and recruitment of the DISC protein complex, whereas the initial effector/propagation stage is characterized by the activation of caspase 8 at the DISC and caspase 9, by the formation of the apoptosome. The following sections will outline the third and final phase of apoptosis, the degradation stage, which is catalyzed and propagated by proteases, including the caspases, calpains and the 26S proteasome. The pattern of protein breakdown, or degradation within an apoptotic cell is described by Cryns and colleagues as the “apoptostat”. This pattern provides important indications of the events that are taking place during the apoptotic cascade (Cryns, 1998).

Proteases in Apoptosis

Caspases

The caspase proteases, renamed in 1992 (Alnemri, 1996) according to their cysteiny aspartase specificity, have been described as the most important and potent

proteases in the apoptosis process (reviewed in (Cohen, 1997; Cryns, 1998; Martin, 1995; Patel, 1996; Thornberry, 1997; Zhivotzky, 1997)). The first member of this enzyme family was named according to its function in the proteolytic processing and activation of the immune system related family of proteins, the interleukin 1-converting enzyme (ICE) (Cerretti, 1992). Nomenclature change has designated the ICE protease as caspase 1.

The caspases are synthesized in a proform, as single polypeptides that must be proteolytically processed in a multi-stage process before becoming active. Figure (II) illustrates the primary structure of a generic caspase, both in its proform and the active form (Cohen, 1997). Procaspases contain at least three main domains: a prodomain, small subunit domain and a large subunit domain. These fragments are processed either by autoproteolysis or by another protease, which is often another caspase family member.

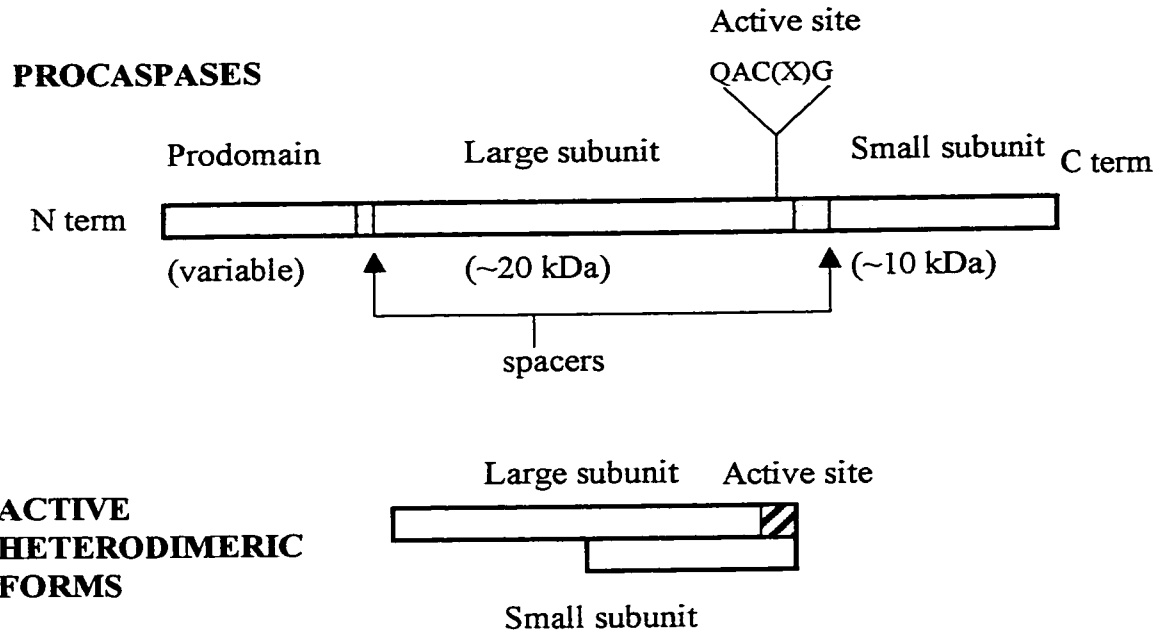
There are notable exceptions to this rule, for instance, it has been shown that granzyme B (another protease involved in CTL killing) is capable of catalyzing the first cleavage event in the caspase 3 enzyme (Medema, 1997), and the HIV protease is also capable of processing and activating caspase 3 (Vocero-Akbani, 1999). Activation of the caspases is a multi-step process, which is initiated by separation of the small, large and prodomains of the proenzyme. The next stage of caspase activation involves the interaction of two of each of the small and large subunits to form an active tetrameric enzyme (reviewed in (Cohen, 1997)).

At present, thirteen caspase members have been identified. The caspase family has been divided into three subfamilies according to substrate specificity and structure. Table 1 lists the members of each subfamily and their substrate specificity. Caspases may also be grouped according to their involvement in the apoptotic cascade. Those that exert

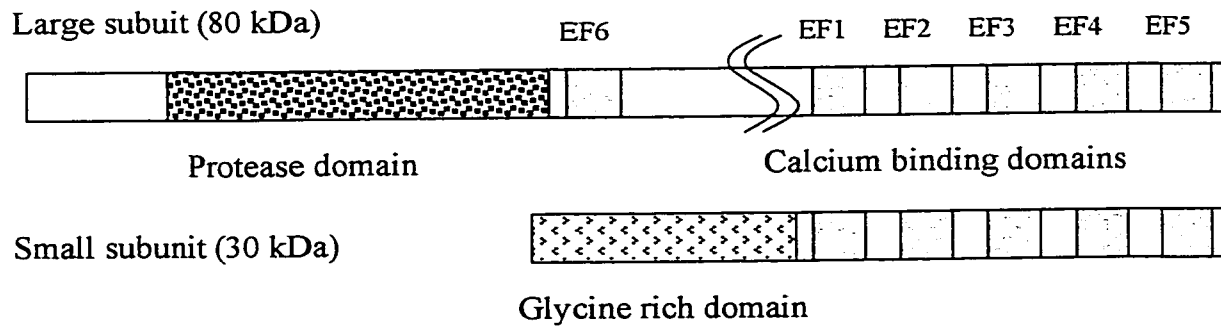
Figure (II) Cartoon representations of Caspase and Calpain Structures: Figure II (i) illustrates the proform and active forms of a generic caspase. Heterodimeric caspase enzymes form tetramers in their active form (Cohen, 1997). Figure II (ii) shows the two subunits that make up the ubiquitous forms of the calpain enzymes. The small (30 kDa) subunit is common between the two isoforms, the large is unique for each isotype. Limited proteolysis is probably required of the calpains (Sorimachi, 1997).

Structure of Proteases

i: The Caspases



ii: The Calpains



their activity in the apex of the cascade tend to bear long prodomains, useful for protein-protein interactions, whereas the effector caspases tend to contain shorter prodomains. Caspases that contain long prodomains act in early apoptotic events, whereas, the effector caspases 3, 6, 7 and 11 contain very short prodomains (reviewed in (Cryns, 1998)). As well, several of the long prodomain-containing caspase family members contain a CARD domain, including caspases 1, 2, 4, 9 (Cryns, 1998). As well, each of the caspase family members contain a conserved active site, the QAC(R/G/Q)G pentapeptide (Cohen, 1997). Caspase-protein interactions are of integral importance in the Fas pathway of death.

The activity of caspases can be blocked specifically by a number of recently developed synthetic peptide inhibitors, designed according to their substrate sequence specificity. Aldehyde derivatives of the tetrapeptide caspase inhibitors are competitive and reversible inhibitors, whereas if the inhibitor contains halogen substitutions it will act in an irreversible manner. Many of these tetrapeptide inhibitors also block cell death pathways. An example of the most potent inhibitors of Fas mediated death is Z-valanyl-alaninyl-aspartate-(OMe)-fluoromethyl ketone (Z-VAD-fmk) an irreversible pan-caspase inhibitor.

Table 1: Caspase Subfamilies and Substrate Specificity

| Subfamily | Members | Substrate Specificity | Reference: |
|----------------------|------------------|---|---------------------------------------|
| Type I: ICE-like | 1, 4, 5 11-13 | Bulky hydrophobic residues at P4 (WEXD) | (Thornberry, 1992) (Rathmel, 1999) |
| Type II: CPP-32-like | 2, 3, 7 | Asp at P4 (DEXD) | (Tewari, 1995; Wang, 1994) |
| Type III | 6, 8, 9,10 | Less discriminating ([L/V]EXD) | (Rathmel, 1999) |

There are a large number of caspase substrates identified in association with apoptosis. It is interesting to note that many of the caspase substrate proteins are susceptible to cleavage by caspase 3, the central executioner caspase. The DNA repair protein, poly-(ADP ribose) polymerase (PARP) and the cytoskeletal protein, α -fodrin, are among the caspase 3 substrates (Greidinger, 1996). Alpha-fodrin is also a substrate of the cysteine protease calpain, which is the subject of the next section.

Calpains

Calpain proteases were named due to their strict calcium requirements and their similarity to the papain protease family (Murachi, 1989). In addition to a number of tissue-specific calpain isoforms, two ubiquitous members have been identified and thoroughly studied, namely, type I or mu (μ) and type II or m forms (reviewed in (Sorimachi, 1997)).

First identified two decades ago (Mellgren, 1980), calpains have been associated with the pathology of Alzheimer disease, limb-girdle muscular dystrophy (Baghdiguan,

1999) and ischemia (Sorimachi, 1997). The importance of calpain during thymocyte apoptosis was first recognized by Squire et al (1997), where it was shown that calpains may play a role upstream of the central effector stage (Squire, 1997). In addition to their importance in thymocyte apoptosis, calpains are activated in hippocampal neurons and hepatocytes during apoptosis. Calpain activation is also important in platelet activation, where this protease is centrally responsible for morphological changes.

Calpains are primarily localized in the cytoplasm (Yoshimura, 1984). However, the activation of calpain is believed to take place at the membrane (Baki, 1996; Saito, 1993). Translocation of the calpain isoforms to the plasma membrane and points of attachment must therefore occur before they are autolytically activated (Rock, 1997). Limited autolysis is necessary to activate the 80 kDa large catalytic calpain subunit, inducing the association of a large and 30 kDa small subunit (see below). Alone, the large subunit is unstable and has very little proteolytic activity (Sorimachi, 1997).

Calpain cysteine specific proteinases are ~110 kDa heterodimers (Murakami, 1981). All calpain isoforms share a single regulatory small subunit, which contain calcium-binding regions and a glycine-rich hydrophobic domain. The glycine-clustering domain is involved in the association of calpain with the cytoplasmic membrane (Imajoh, 1986). The large subunit is specific to each isoform and contains the active, catalytic domain. Figure (III) illustrates the structure of a generic calpain isoform, pointing out the various important protein motifs, including the calcium binding domains and protease sites, and hydrophobic glycine-clustering domain (Sorimachi, 1997).

Calpain activation can be inhibited by an endogenous protein called calpastatin. Calpastatin, which binds to the ubiquitous calpain isoforms and regulates their activities,

contains three subdomains, each of which binds reversibly to calcium-binding domain in the active site of the calpain large subunit (Sorimachi, 1997). Proteolytic inactivation of calpastatin leads directly to the activation of calpain isoforms (Wang, 1998).

The first synthetic molecules used in an attempt to inhibit the calpains were leucine peptide derivatives (called E-64 and derivatives thereof) which had varying potencies and poor enzyme specificity (Sorimachi, 1997). Calpain inhibitors I and II (peptide-derivative inhibitors) are used commonly in calpain activation assays. However, these two inhibitors also suppress the activity of a large protease complex called the 26S proteasome however, making these inhibitors ineffective in directly assessing calpain specific events. More recently, an inhibitor has been developed which exhibits slightly greater enzyme specificity called calpeptin (Tsujinaka, 1988). Each of these inhibitors binds to the active site in the large subunit of calpain.

Calpain catalyses the proteolysis of a number of substrates during apoptosis. The canonical calpain substrate is α -fodrin, a cytoskeletal protein, which is cleaved in the latter stages of apoptosis. Calpain isoforms are also responsible for cleavage of a large number of structural proteins, cell signaling molecules and transcription factors, including protein tyrosine phosphatase 1 β , MAP2 (Baki, 1996), c-jun, c-fos and pp60^{src} (Rock, 1997). Recently, Chua *et al* (2000) have shown that calpain can also induce the cleavage of several members of the caspase family (Chua, 2000). This is an important result because it exemplifies the complex system of cross-talk between the protease families involved in apoptotic cascades.

The importance of non-caspase proteases in the process of apoptosis has become more evident recently. Another such protease that is activated during apoptosis is the 26S

proteasome. Knepper-Nicolai and colleagues (1998) have shown that calpains and the proteasome act synergistically in neutrophils during constitutive apoptosis at a point downstream of caspase activation (Knepper-Nicolai, 1998). Neutrophils are induced to apoptose constitutively when aged outside of the body through a mechanism that may involve the Fas signaling pathway (Klein, 2000).

Ubiquitin + 26S Proteasome

The importance of the 26S proteasome during the process of thymocyte apoptosis was recognized in 1996 by Grimm *et al* (Grimm, 1996). They demonstrated that proteasome activity is essential for primary thymocyte cell death induced by glucocorticoids, low-level irradiation and phorbol esters. There are a number of 26S proteasome substrates of the apoptotic pathway that have been well characterized. Most degradation of denatured and improperly folded proteins in the cell is carried out by the 26S proteasome, during the process of apoptosis as well as during the normal cell existence. For example, p53, a protein fundamental to the process of cell growth and apoptosis, is a substrate for degradation by the 26S proteasome (Maki, 1996). In addition, 26S proteasome-mediated processing of the nuclear factor NF κ B, from its native 105 kDa to 50 kDa form, showed that the proteasome could catalyse specific proteolytic processing as well as non-specific degradation (Orion, 1995). These results suggest that proteins relating to the proteasome may have a role in the regulation of the Fas pathway of death.

The process of degradation is quite complicated in that it requires the activity of a number of accessory/chaperone proteins and the activity of an enormous proteolytic

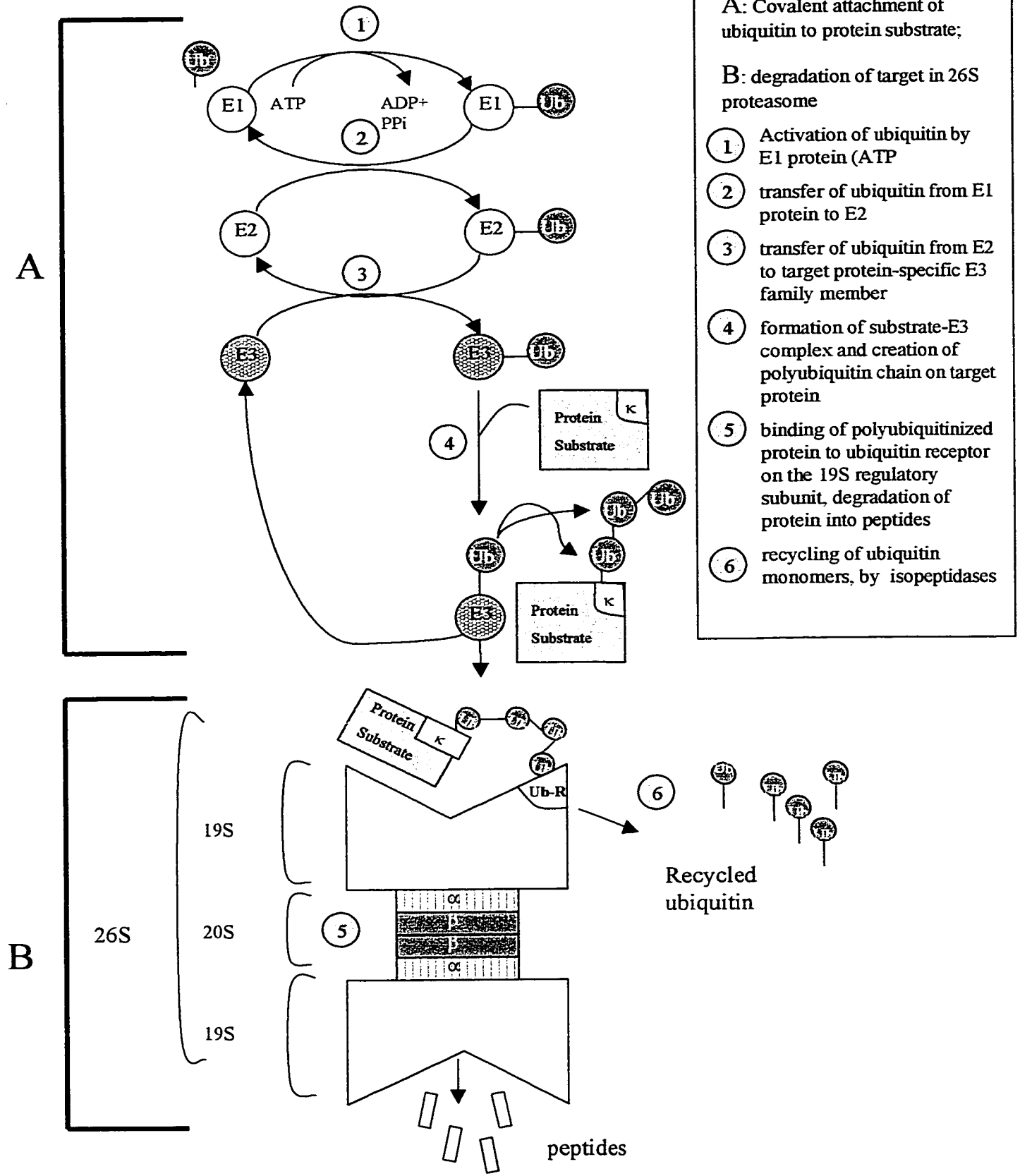
protein complex (~1500 kDa). Small protein molecules called ubiquitin are covalently attached to the target as the first of the two-stage process associated with 26S proteasome-mediated protein degradation. Ubiquitin is an 8.6 kDa peptide marker that is covalently attached to the protein target in an ATP-dependent manner (Ciechanover, 1998). The ubiquitin-mediated process of protein degradation takes place in the nucleus as well as the cytoplasm and is outlined in Figure (III).

The first stage of the degradation process is carried out with the participation of three types of protein chaperones, the E1, E2 and E3 families. This is a hierarchical process, in that there are relatively few E1 molecules, which are ATP-dependent ubiquitin activating enzymes that form high-energy thiol-ester bonds with the target proteins. The more common E2 family members, also called UBCs (ubiquitin carrier/conjugating enzymes) transfer the ubiquitin moiety from E1 to the relatively abundant target specific E3 (ubiquitin ligase) enzymes, which in turn catalyze polyubiquitination of the target substrate (Driscoll, 1990).

This stage is followed by the specific recognition of the poly-ubiquitin chain by the regulatory subunits of the 26S proteasome whereupon the target is passed through the catalytic subunits of the proteasomes. The 26S proteasome is composed of two 19S regulatory complexes and a 20S catalytic complex. Recognition of the poly-ubiquitinated substrate by the 19S regulatory complex initiates the degradation of the protein. The substrate is then passed through the 20S "barrel-like" protease complex. Eight to twelve 20-23 kDa subunits make up the 19S "prosome" or regulatory portion, whereas the 20S is composed of four multisubunit rings, two α s and two β , each made up of 7

Figure (III) Schematic representation of ubiquitination and the 26S Proteasome activity: The first stage of protein degradation by the 26S proteasome is the covalent attachment of multiple ubiquitin monomers to the target protein (A). Following this attachment phase, the protein is degraded in the multi-subunit 26S proteasome enzyme complex (see text and review in (Ciechanover, 1998) for more details).

III: Ubiquitination and the 26S Proteasome



subunits (Driscoll, 1990). Interestingly, as a proteolytic complex the 26S proteasome has trypsin-like, chymotrypsin-like and caspase-like peptidase activities.

For experimental purposes, 26S proteasome activity can be blocked by specific synthetic inhibitors. The irreversible inhibitor, lactacystin has provided the greatest enzyme specificity and effective blockage of the proteasome pathway to date (Fenteany, 1995) (Jenson, 1995). Lactacystin is a bacterial product, from the *Streptomyces* family, which inhibits cell proliferation but has no promiscuous effects on serine or chymotrypsin proteases, or trypsin (Fenteany, 1998).

Regulatory Mechanisms of Apoptosis

The signal transduction pathway associated with Fas-mediated cell death, which include the interaction between receptors, adapter proteins and proteases is tightly regulated by a number of control proteins.

At each level of the apoptotic cascade, regulatory proteins maintain a strict balance of death versus life signals. Balance of these opposing signals seems to be central theme in the control of apoptosis. Interestingly, in a morphological sense, there are a number of similarities between cellular events in mitosis (signals for life) and those in apoptosis (signals for death), including chromatin condensation, loss of substrate adhesion and rapid membrane blebbing (King, 1998). For instance, the oncogene c-myc is capable of transducing signals of death as well as for life. The decision about whether or not to die is also dependent on the environmental growth factors and cellular context (King, 1998).

PCD has been studied extensively in the nematode species *C. elegans*, which has a very defined pattern of apoptosis during development and therefore has become a valuable tool for studying this process. Much of what is currently known about the process of apoptosis was initially recognized in the nematode *C. elegans*. Four main gene products have been isolated in conjunction with this process, termed Ced-3 (*C. elegans* death), Ced-4, Ced-9 and Egl-1 (egg laying) [Hodgkin, 1999 #6; Dragovich, 199? #3]. The mammalian homologs to ced-3 and ced-4 are caspase 3 and Apaf-1 respectively. The mammalian homolog of Egl-1 has not been identified, but is believed to be an inhibitor of Ced-9. The Ced-9 protein is believed to be a homologue of the negative apoptosis regulator protein known as Bcl-2 (Hodgkin, 1999). In mammals, however there also seems to be a larger number of proteins that tightly regulate the process of apoptosis than in the nematode *C. elegans*. In addition, there is a significant amount of functional redundancy in higher vertebrates within the various gene families that control apoptosis.

There are three major levels under which the mechanisms of apoptotic control can be described: transcriptional control, translational control and post-translational modification control. For the purpose of this thesis, I will focus on the latter mechanism. Apoptosis is a multi-stage process, in which several types of post-translational modifications may exert their control in the various signaling pathways. At present, it has been shown that these post-translational modifications may be categorized into four subsets. Firstly, post-translational modifications can be catalysed by protein kinases, which propagate signals between accessory proteins in cell cycle control by phosphorylation of target molecules (King, 1998). For instance, the protein kinase

B/Akt pathway, positively regulates expression of anti-apoptotic gene products such as anti-apoptotic members of the Bcl-2 family of proteins (see below) [Dragovich, 199? #3].

The second type of post-translation control lies in the interactions or dimerization between control proteins. An example of this is found with the Bcl-2 family members, where the ratio of negative and positive regulators has been referred to as the “rheostat” [Dragovich, 199? #3; Korsmeyer, 1993 #41]. Bcl-2 is a potent inhibitor of apoptosis, which acts in the mitochondrial pore complex. This protein regulates the flow of cytochrome c, which is a downstream mediator of the late stages of apoptosis (66)(Kluck, 1997). There are several other proteins related to Bcl-2, which together comprise a family including both negative and positive regulators of the apoptosis process (reviewed in (Korsmeyer, 1993)). Regulating ratios of pro- and anti-apoptotic signals seems to be the common theme in a cell’s commitment to apoptosis. For example, Bax, another member of the Bcl-2 family of control proteins has pro-apoptotic characteristics (Rosse, 1998). The Bcl-2/Bax example is a perfect example where dimerization acts as a mechanism of apoptotic control (Korsmeyer, 1993). Saturation of the apoptotic agonist (Bax) by the antagonist (Bcl-2) with the formation of dimers is sufficient to block apoptosis (Diaz, 1997; Zha, 1996).

A third and central theme in the control of the apoptotic process is the localization of the various control proteins. In order to propagate the apoptotic signal, interacting proteins must be in the same locale. There may be cross talk between post-translational control mechanisms where signals such as phosphorylation and processing may regulate the translocation of these proteins. For instance, the presence of the death agonist, Bax in the mitochondria is necessary for apoptosis (Diaz, 1997),(Fadeel, 1999). The integration

of Bax into the mitochondrial membrane induces the release of apoptotic regulatory proteins, which initiates an increase in mitochondrial membrane permeability (Korsmeyer, 1993).

In addition to phosphorylation, dimerization and localization, a fourth mechanism of apoptotic control is the endolytic processing of proteins. This mechanism will be the central theme of this discussion, with respect to both positive and negative regulation of the apoptotic event. For example, proteolytic processing brings about the activation of caspases, a central protein family in the course of most, if not all apoptotic cascades. . When the anti-apoptotic Bcl-2 is cleaved, it is converted into a proapoptotic protein, which could be a “feed-forward” mechanism for propagating the apoptotic signal (Fadeel, 1999). In these two cases, proteolysis serves to shift the balance in the direction of the apoptotic event. Conversely, proteolysis can result in the activation or gain of function of some proteins. An example of this is the anti-apoptotic viral gene product p35, which is a direct inhibitor of the caspases upon cleavage by the enzymes that it inhibits (Fischer, 1999). Other viral and mammalian inhibitors of the caspases, are also potent inhibitors of apoptosis.

Recently, another such family of apoptosis inhibitory proteins has been identified, the IAPs (inhibitor of apoptosis proteins) which appear to have potent protective effects against a number of apoptotic stimuli.

Inhibitor of Apoptosis Proteins (IAPs)

Viral IAPs were first identified in the baculovirus species *Orgyia pseudotsugata* and *Cydia pomonella* (reviewed in (Clem, 1995)). These proteins are important

propagation factors which function by ensuring survival of host cells by blocking the activation of ICE-like caspases. IAP homologues have been identified from vertebrate and invertebrate species, including *Drosophila*, *C. elegans* and yeast (reviewed in (Uren, 1998)). The IAPs represent the first evidence of an endogenous (cellular) caspase inhibitor (Uren, 1998), and alluding to their importance, they appear to be highly evolutionarily conserved. Much like the IAPs, the baculoviral proteins, p35 and CrmA (cytokine response modifier A) are promiscuous caspase inhibitors (Bump, 1995). IAPs share no sequence homology with these baculoviral inhibitors of apoptosis, but are able to functionally complement p35 and CrmA proteins (Tamm, 1998).

The first mammalian IAP homologue was isolated in 1995, in a positional cloning effort to find the gene responsible for spinal muscular atrophy (SMA), an inherited rare progressive motor neuron loss disease (Roy, 1995). The encoded protein was named the neuronal apoptosis inhibitory protein (NAIP) based upon homology with baculoviral IAPs. Three other members of this gene family which were subsequently identified using database and genome analysis were termed HIAP 1 and 2 (human IAPs), and XIAP (X-linked IAP) (Liston, 1996). At the same time, other laboratories had identified the same IAP family members. The XIAP, HIAP 2 and HIAP1 genes were also called MIHA, MIHB and MIHC, respectively by Uren *et al* (Rothe, 1996). XIAP was also cloned as hILP (Duckett, 1996) by Duckett *et al*, and HIAPs 1 and 2 were designated as c-IAP2 and c-IAP1, respectively, by Rothe *et al* (Uren, 1996).

The members of the cellular IAP family, HIAP1 and HIAP2, are highly homologous proteins, and are found in tandem on chromosome 11q22-q23 (Young, 1999). These genes are expressed in a tissue specific manner (Liston, 1996; Rothe, 1996;

Young, 1999). Northern blot analysis has shown that HIAP1 mRNA is expressed at high levels in lymphoid tissues, including the thymus, spleen and peripheral lymphocytes; HIAP2 is expressed in the thymus, testes, skeletal muscle, pancreas and in many fetal tissues (Liston, 1996; Uren, 1996). HIAP1 and 2 mRNAs, 5 kb and 4.5 kb respectively, are elevated in certain cancer cell lines, including the Burkitt's lymphoma cell line, Raji, and others (Liston, 1997). Transcription of HIAP1 is controlled by the nuclear replication factor NF κ B, a protein which is known to be associated with the transcriptional regulation of other anti-apoptotic proteins (Chu, 1997). Both HIAP-1 and HIAP-2 are capable of binding to several members of the TRAF (tumor necrosis factor receptor associating factor) family of proteins, which functions within the TNF α signaling pathway (Rothe, 1996).

More recently an additional member of the IAP family has been identified, called survivin (Ambrosini, 1997). Survivin mRNA is expressed in the narrowest range of adult mouse and human tissues of all of the IAPs, however its importance is exemplified in the fact that it is upregulated in a number of cancer cell lines (Ambrosini, 1997).

Upregulation of a gene product in a cancer cell line suggests the protein's importance in the dysregulation in cell proliferation. The expression of survivin may be negatively regulated by the epr-1 gene, which shares the same coding region, though transcribed in the opposite direction (Adida, 1998; Ambrosini, 1997). Survivin expression appears to be cell cycle regulated in that it is expressed at the G2/M interphase at which time the survivin protein binds to microtubules (Tamm, 1998).

Structure of the IAPs

The IAPs are characterized by zinc-finger repeat domains called BIRs (baculovirus IAP repeats). The five cellular IAP proteins, NAIP, XIAP, survivin, HIAP1 and 2 share extensive sequence homology, especially within the BIR domains. BIR domains are approximately 70 amino acids that contain histidine and cysteine residues spaced in a way that accommodates a novel metal binding pocket. Unlike the other mammalian IAP homologs that contain three BIR domains, survivin contains only a single BIR region, though retains its apoptotic inhibitory function. The three members XIAP, HIAP1 and 2 also contain an additional motif at the carboxyl terminus called the RING (really interesting new gene), a zinc-finger like domain. RING zinc finger domains have been isolated in at least 100 proteins to date. These domains are generally approximately 80 amino acids in length and are usually located at the amino terminus. IAPs are an exception in that the RING domains are located at the C terminal and are more than likely involved in protein-protein interactions rather than DNA-binding (Deveraux, 1998). Figure (IV) outlines the general structure of five mammalian IAP proteins mentioned as well as several of the baculoviral and drosophila IAP homologues.

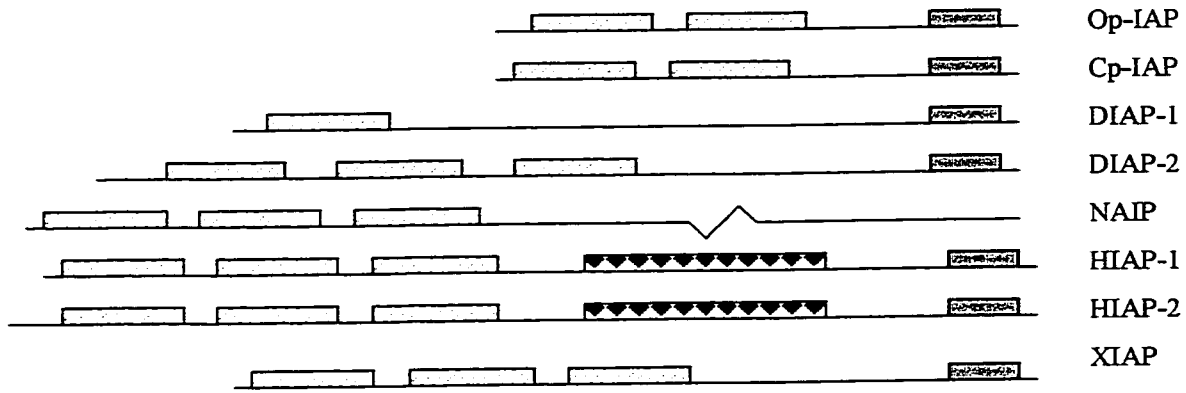
X-linked inhibitor of apoptosis protein (XIAP)

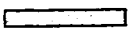


Genetics and Expression

The gene that encodes XIAP is located on the human X chromosome at position q25 and in the mouse (designated miap-3 (Farahani, 1997)) at position A3-A5 (Rajcan-Separovic, 1996). The human XIAP mRNA is approximately 9 kb in length, of which only 1.5 kb contains the XIAP coding region (Legacé unpub). XIAP is a 497 amino acid

Figure (IV) Cartoon representation of Inhibitor of Apoptosis Protein (IAP)
structure: The family of IAPs contains three important protein motifs, the BIR (baculoviral inhibitor of apoptosis protein repeat) zinc finger, RING zinc finger, and possibly the CARD (caspase recruitment domain. This representation shows baculoviruses (Op-IAP, Cp-IAP), Drosophila (DIAP-1, DIAP-2) and human IAPs (NAIP, HIAP-1, HIAP-2, XIAP and survivin).

Inhibitor of Apoptosis Protein Family



 BIR domains $X_3RX_{20-23}GX_{11}CX_2CX_{16}HX_5CX_3$
 RING Zinc Finger
 CARD domain

protein with a molecular weight of approximately 55 kDa. XIAP protein is expressed ubiquitously throughout mammalian tissues and cells (Liston, 1996) implying that this protein may serve as the “house-keeping” member of the IAP family. This gene contains an unusually long 5' untranslated region that has recently been found to contain an IRES (internal ribosome entry site) (Holcik, 1999). IRES elements were first identified in viruses and in this case are responsible for the translational control of this gene product. In viruses, IRES sequences provide an efficient mechanism to over-ride the cellular control of the host's translation machinery during the propagation phase of infection. During virus propagation, cap-dependent translation is turned off by certain viral gene products and as a consequence, protein translation within the infected cell is mostly driven by the IRES element. Cellular genes, whose expression are regulated by IRES elements are upregulated in periods of physiological stress (Holcik, 1999).

XIAP Function

XIAP has been shown to be the most potent of the IAP family, protecting cells against a wide variety of apoptosis-inducing agents (Deveraux, 1997; Deveraux, 1998; Duckett, 1998; Liston, 1996; Takahashi, 1998; Uren, 1996; Yamaguchi, 1999). Specifically, exogenous overexpression of XIAP protects numerous cell types (Rat-1, CHO, 293, 293T) from apoptotic death using a large number of apoptotic triggers: including anti-Fas mAbs, TNF α , dexamethasone, etoposide, serum deprivation, cisplatin, menadione, γ - and UV-irradiation, Bax overexpression, caspase overexpression and viral infection (reviewed in (Liston, 1997)).

The mechanism by which XIAP inhibits apoptosis is probably based on its ability to directly inhibit the activity of specific caspases during the receptor-mediated pathway of death. In 1997, it was shown that XIAP, survivin, HIAP1 and 2 are able to reversibly bind to and thereby inhibit active caspases 3 and 7 *in vitro* (Deveraux, 1997; Roy, 1997; Tamm, 1998). Figure (I) shows the position where the effector caspase 3 and 7 exert their degradation effect in the common downstream event of the Fas apoptotic cascade. XIAP binds and inhibits these caspases with the greatest potency of all tested IAPs (Deveraux, 1997). When overexpressed ectopically, XIAP is also able to partially block caspase 8-mediated activation of caspase 3. XIAP blocks the initial autocatalytic cleavage event of caspase 3 and not the proteolytic activity of caspase 8 (Deveraux, 1997). The second BIR unit seems to be necessary and sufficient for caspase 3 and 7 inhibition under both *in vitro* conditions and in transient transfection studies (Deveraux, 1997; Takahashi, 1998; Tamm, 1998; Yamaguchi, 1999). The activation of caspases 3 and 7 is essential for most apoptotic cascades, illustrated by the fact that caspase 3 specific inhibitors are able to block death in many cell types (reviewed in (Thornberry, 1997)). As mentioned before, caspases 3 and 7 are activated rapidly in the type I Fas pathway (see also Scaffidi *et al* (Scaffidi, 1998)).

XIAP also binds to inactive procaspase 9, an important protease in the type II Fas pathway (Scaffidi, 1998) thereby preventing its proteolytic activation as well as binding and inhibiting directly the active form. Caspase 9 is able to catalyze the proteolysis and activation of caspase 3 thereby beginning the amplification of further caspase activation (Deveraux, 1999b). Caspase 9 is also an important protease in the Bax pathway of apoptosis that is involved in the release of cytochrome c from mitochondria and the

activity of the Ced-9 homologue, Apaf-1 (Rodriguez, 1999). By inhibiting the activity of caspase 9, XIAP is able to block cytochrome c-mediated activation of downstream caspases. Binding of the caspases has been shown to cause the cleavage of certain apoptotic regulatory proteins.

Processing of Anti-Apoptotic Proteins

Anti-apoptotic viral gene products have provided the scientific community with new avenues of investigation in the field of apoptosis. Viruses utilize cellular machinery for their vital processes, and therefore require a mechanism for maintaining cell viability. One of the first proteins to be discovered was the cowpox virus gene product called CrmA. This gene product is an inhibitor of caspase 1 and 8, and to a lesser extent caspase 6 and acts to diminish the host's inflammatory response (Rathmel, 1999). P35, a gene product of the *Autobiographica californica* mononuclear polyhedrosis virus is another example of a promiscuous caspase inhibitor (Bump, 1995). P35 is a suicide substrate, that is, its anti-apoptotic activity is dependent on its ability to be cleaved by the enzymes that it inhibits (Zhou, 1998). A cleaved protein fragment, where its protease-binding site lies, remains bound to the active site within caspase 3 (Fischer, 1999).

Apoptosis is an evolutionarily conserved process and occurs in an orderly fashion through numerous different pathways. The mechanisms of receptor mediated apoptosis, such as Fas killing of Jurkat T cells, are well defined, and represent an ideal system for studying the events which regulate the apoptotic cascade. Within this pathway, there are a number of inhibitory proteins, including the IAPs, which exert their potent effects. An important protease family, the caspases are activated during the receptor-mediated

pathway of death and are directly inhibited by the IAPs. However, death does occur in spite of the presence of these potent intrinsic apoptosis inhibitors. This fact strongly argues that the apoptotic inhibitor(s) itself must be removed or inactivated for death to progress.

THESIS OVERVIEW

There are numerous examples where inhibitory proteins are cleaved during the Fas-mediated apoptosis pathway. Examples of these proteins are the baculoviral proteins, p35 and CrmA (Zhou, 1998). An important protease family, the caspases are activated during the receptor-mediated pathway of death and are directly inhibited by the IAPs. In this study, we have shown that the negative regulator of apoptosis, endogenous XIAP is proteolytically processed during Fas-mediated apoptosis in Jurkat T cells. A single 30 kDa proteolytic product is formed during apoptosis. Cell viability was also investigated in the Fas-mediated apoptosis assay. I examined the activity of three cytoplasmic protease groups induced during Fas-mediated apoptosis, the caspases, the calpains and the 26S proteasome in relation to XIAP proteolysis. The conclusions of this study are that the protease responsible for XIAP cleavage may be a member of the caspase family of proteases.

Materials and Methods

Plasmids, Reagents and Antibodies

Anti-Fas monoclonal antibody (clone DX2), calpain inhibitors calpeptin and calpastatin peptide and all caspase inhibitors were purchased from Calbiochem and/or Novabiochem. Anti-Fas monoclonal antibody (clone CH11) was purchased from Molecular and Biological Laboratories. Anti-XIAP monoclonal antibody was from BD Transduction Laboratories (clone H62120). Mouse anti-actin monoclonal antibodies were purchased from Sigma. Alexa™ 488 Signal Amplification kit (Molecular Probes) fluorescence-conjugated secondary antibodies were used to detect the XIAP and calpain-specific primary antibodies. All other chemicals and reagents were purchased from Sigma, including poly-L-lysine solutions and cycloheximide.

Death and Inhibitor Assays

Jurkat T lymphoma cells (ATCC- TIB-152) were grown in RPMI media (Gibco-BRL) supplemented with 10% heat-inactivated fetal calf serum (FCS) and 5% penicillin and streptomycin at 37°C humidified in 5% CO₂. Apoptosis was induced with anti-Fas mAbs DX2 or CH11 (1 µg/mL) and cycloheximide (20 µg/mL). Time course experiments were performed in 6 or 12 well plates with cells that had been split and fed with fresh RPMI media (+ FCS) overnight.

Calpain, proteasome and caspase inhibitors were added one hour prior to the addition of apoptotic stimuli. Calpeptin was used at concentrations ranging from 5 to 40 µM on Jurkat T cells. The endogenous inhibitor peptide, calpastatin was used at a concentration of 0.5 µg/mL for all experiments. Lactacystin was used at a concentration

of 5, 10 or 20 μM . Caspase inhibitors were used at a standard concentration of 100 μM with the exception of DEVD-fmk and LEHD-fmk, which were used at concentrations of 25, 50 and 100 μM .

Cells death was measured by trypan blue exclusion. The criteria for identifying apoptotic cells under light microscopy (Nikon TMS-F model microscope) was used by Weis *et al* (Weis, 1995) by morphology and trypan blue dye exclusion. Each determination was made in quadruplicate and the results were averaged. Error bars represent one standard deviation from a single representative experiment. Each experiment was repeated at least two times. Nuclear morphology was assessed using Hoescht 33258 DNA specific dye at a concentration of 2.5 $\mu\text{g}/\text{mL}$, in 1 X PBS.

SDS-PAGE & Western Blots

Jurkat cells were washed with 1 X PBS and lysed in 1 X PBS containing 1% Triton X100, 0.1% SDS, 1mM EDTA, 1 mM EGTA and 0.1 M sodium deoxycholate and 1 μM PMSF. Whole Jurkat cell lysates were quantitated by the Bradford method, using reagents purchased from Biorad, according to the manufacturer's instructions. Samples were analysed by SDS-polyacrylamide gel electrophoresis (PAGE) on 10 or 12% resolving gels. Gels were transferred to PVDF membranes (Immobilon-P Millipore) using the Hoefer Semiphor semidry transfer apparatus (Amersham Pharmacia biotech). Membranes were incubated with primary and secondary antibody solutions in 2% skim milk with 0.5 % Tween 20 in 1X PBS. Horseradish peroxidase conjugated secondary antibodies were detected using enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Kodak X-OMAT X-

ray film was used in all exposures. Densitometric analyses were performed using the Molecular Analyst™/PC Image Analysis software (version 1.1.1) for the Model GS-670 densitometer from Biorad.

Production of Recombinant Proteins and Calpain Assays

For the expression of glutathione S-transferase (GST)-XIAP fusion protein, the XIAP gene was subcloned in-frame between the *Bam*HI and *Xho*I restriction sites in pGEX-KG-XIAP and pGEX-KG-BIR23 (Pharmacia). Cloning of the XIAP cDNA into the vector in frame was verified by sequence analysis. The pGEX-KG-XIAP and pGEX-KG-BIR23 transformed *Eschericia coli* DH5 α were grown overnight in Luria Bertania (LB) broth with shaking at 23 °C. The culture was diluted 100X in LB media and grown to an approximate optical density of 1.0. GST-XIAP expression was induced with 0.1-0.2 mM isopropyl- β D-galactoside (IPTG) at 37°C for 5 hours. Bacteria were pelleted by centrifugation at 10 000 rpm at 4°C (Beckman J2-21 M/E centrifuge, JA-10 rotor). Pellets were resuspended in 20mM phosphate buffer (1 M NaH₂PO₄ and 1 M Na₂HPO₄ pH 7.25, containing 2 mM EDTA, 150 mM NaCl and 0.1% CHAPS detergent), frozen in liquid nitrogen and stored at -80°C for protein extraction. Pellets were thawed, resuspended in 20 mM phosphate buffer, 5 M dithiothreitol (DTT) and 0.3 mg/mL lysozyme then incubated on ice for 30 minutes. Samples were adjusted to 2mM PMSF and 10 ug/mL leupeptin and 10% Sarkosyl, sonicated using a Vibracell Ultrasonic processor VCX 400 at 25% amplitude for 2 minutes with 1 second on and 4 seconds off, then adjusted to 10% Triton X100. Samples were centrifuged at 15 000 rpm for one hour at 4 ° C. Glutathione Sepharose 4B™ beads (Amersham Pharmacia Biotech) were added

to the cleared supernatant and incubated at 4° C overnight under slow agitation. Beads were collected and washed with 3-5 volumes of 1 X PBS (phosphate buffered saline- 0.01 M Na₂HPO₄, 0.01 M KH₂PO₄, 0.15 M NaCl and 0.15 M KCl pH 7.4) + 10 % Triton X100 and 5 mM DTT. Fusion protein was eluted three or four times with 50 mM Tris, 50mM glutathione at room temperature for 15 minutes. Combined eluates were dialysed overnight in 20 mM phosphate buffer with 5 mM DTT.

In vitro XIAP cleavage assays were performed in HEPES buffer (containing 10 mM HEPES, 140 mM NaCl, 5 mM KCl (pH 7.3)). GST-XIAP samples were incubated with 50 ug/mL calpains I or II in the presence of excess calcium (20 mM). Cleavage was assessed using Western immunoblotting techniques with XIAP specific antibodies.

Immunofluorescent Staining of Jurkat Cells

Jurkat T lymphoma cells were treated with anti-Fas monoclonal antibodies and cycloheximide as described. Cells were washed in cold 1 X PBS, cells were adhered to poly-L-lysine (Sigma Diagnostics) coated coverslips according to manufacturer's instructions and fixed in fresh 4% paraformaldehyde. The fixation solution was incubated on the coverslips for 30 minutes at room temperature and washed 3 times with approximately 1 mL 1X PBS. Cells now fixed to the coverslips were then treated with 0.5% Triton X100 to permeabilize the cells to antibody detection and blocking solution (1X PBS and 10% goat serum) was added. These coverslips were then subjected to immunofluorescent staining by previously reported methods (Rosenthal, 1997). XIAP serum antibodies (of unknown concentration) were diluted to 1:100 and secondary antibodies were used at 1:200. Fluorecence microscopic visualization of the cells was

performed using a Zeiss Axiophot microscope at a wavelength of 488 nm. Photographs were taken with a standard 35 mm camera.

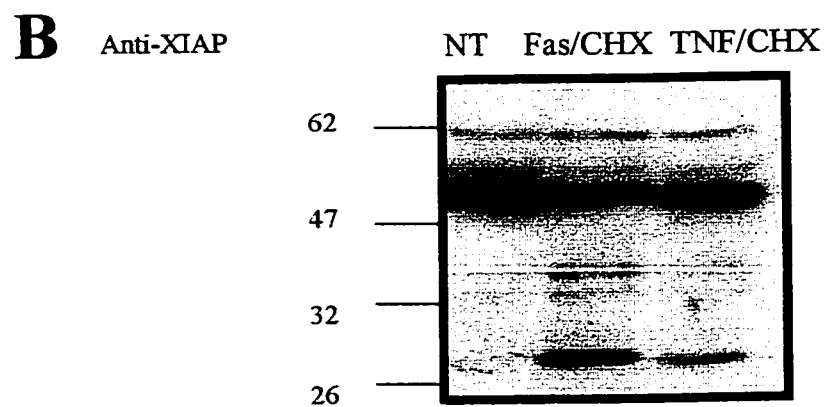
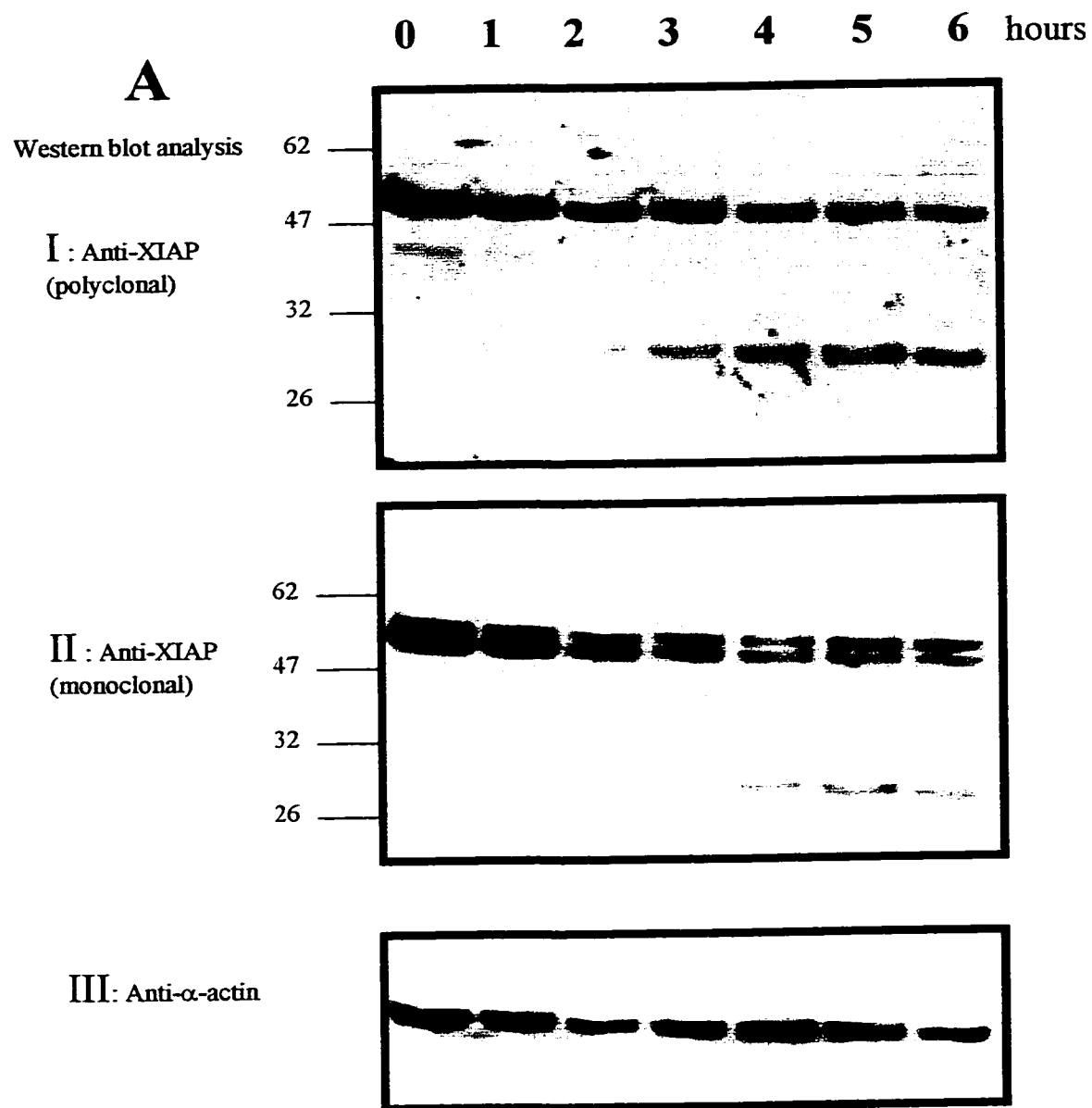
Results

XIAP is proteolytically processed during Fas/CHX-mediated apoptosis in Jurkat lymphoma cells

Treatment of cells with α -Fas monoclonal antibody and cycloheximide (CHX), a general protein synthesis inhibitor, is a well-characterized system for the study of apoptotic events *in vitro* (Weis, 1995). Jurkat T cells typically have been used in Fas-apoptosis studies because they express high levels of Fas antigen, and are therefore extremely susceptible to Fas-mediated cell death (Yonehara, 1989). In these cells, the apoptotic effects of Fas- and other TNF superfamily receptor mediated-apoptotic pathways are intensified with CHX treatment. CHX-mediated apoptosis may follow a similar pathway to Fas-mediated apoptosis including the involvement of FADD, thus the two triggers together amplify the apoptotic stimulus. (Tang, 1999). The purpose of my initial experiment was to investigate a possible cell-type specific proteolysis of the apoptosis inhibitor protein, XIAP within the defined process of Fas/CHX-apoptosis. Other apoptotic cells, including HeLa cells, stimulated with anti-Fas mAbs and CHX, were tested, but little evidence of XIAP cleavage was seen (results not shown). Accordingly, Jurkat lymphoblastoid cells were treated with anti-Fas monoclonal antibodies and CHX in a time course assay over six hours, and then samples were subjected to Western blot analysis. Anti-XIAP Western blots (Figure 1AI and 1AII) were performed using 50 μ g of Jurkat whole cell lysate at each time point. Figure 1A illustrates the incidence of an \sim 30 kDa XIAP cleavage product in samples at various times following the initiation of apoptosis (Figure 1AI and 1AII, lanes 2-6) using two anti-XIAP antibodies (polyclonal [I] and commercial monoclonal [II]).

Figure 1A: Endogenous native 55 kDa XIAP is proteolytically processed into a single product of approximately 30 kDa during Fas/CHX mediated apoptosis. Approximately 50 ug of whole Jurkat T cells lysate were run on 12% SDS-PAGE following treatment with Fas/CHX. Cells were treated in a time course assay with 1 ug/mL anti-Fas mAb (MBL) and 20 ug/mL cycloheximide. Samples were taken hourly for 6 hours (lanes 0-6 hours) post treatment. Western blots were conducted using (I) rabbit anti-XIAP polyclonal antibody, (1:1500 dilution). (II) Mouse anti-XIAP monoclonal antibody (1:2000); (III) mouse anti-actin monoclonal antibody as a loading control (1:5000 dilution). Anti-rabbit and anti-mouse horseradish peroxidase conjugated secondary antibodies (1:2000) were detected on PVDF membranes with ECL. A distinct 30 kDa cleavage product is seen over the time course with both antibodies. At least three independent experiments were conducted.

Figure 1B: XIAP Cleavage in Fas/CHX and TNF- α /CHX treated Jurkat Cells XIAP cleavage is common between the two TNFR superfamily receptor mediated apoptotic events, Fas and TNFR1. Anti-XIAP polyclonal antibody (1:1500) was used in this Western blot to show XIAP cleavage during both Fas and TNF-mediated apoptosis. Contents of the lanes are whole Jurkat cell lysates treated with: lane 1: no treatment; lane 2 Fas/CHX; lane 3 TNF- α /CHX for 6 hours; (Fas/CHX refers to anti-Fas monoclonal antibodies and cycloheximide; TNF/CHX refers to TNF α and cycloheximide treated Jurkat cells



In this experiment, cleavage of XIAP was investigated using two XIAP specific antibodies in order to assess which fragment of XIAP corresponds with the 30 kDa cleavage product (Figure 1A). The monoclonal antibody used in Figure 1A(II) was directed against the carboxyl terminus of XIAP (BD Transduction Laboratories, clone H62120-see Appendix I). The immunoblot analysis detected with the polyclonal antibody (Figure 1AI) showed the same pattern of XIAP cleavage as the immunoblot using the monoclonal antibody (Figure 1AII) but with a minor exception. There was a pair of bands in the 55-60 kDa range only detected using the anti-XIAP monoclonal antibody (compare Figure 1AI with Figure 1AII). However, the top band within this doublet was shown previously to be due to non-specific antibody binding (Deveraux, 1999). The results from this experiment showed that the cleavage product in lanes 2-6, Figure 1AI & II contain the carboxyl terminus of the XIAP protein and these two antibodies were both effective tools for assessing the cleavage of XIAP in this system.

Interestingly, no additional band was observed in Figure 1AI that could correspond to the amino terminus of the XIAP protein. The amino terminus cleavage product may have been selectively degraded after the cleavage event during Fas-mediated apoptosis in Jurkat cells (Deveraux, 1999). It was also interesting to note that the amount of full-length protein seems to decrease slightly but it did not seem to decrease in proportion to the amount of newly formed 30-kDa XIAP cleavage product.

The Fas receptor is not the only receptor within the TNFR superfamily that can mediate apoptosis. The founding member of this superfamily, TNFR1 is also capable of mediating programmed cell death (Robaye, 1991). In order to assess the universality of XIAP cleavage within similar apoptotic systems the TNFR1 specific ligand, TNF α , was

used with CHX as an apoptotic stimulus (Figure 1B). The anti-XIAP Western blot shown in Figure 1B contains 50 ug of whole cell lysates after treatment with either Fas/CHX (lane2) or TNF α /CHX (lane 3) trigger for 6 hours. Interestingly, TNF α plus CHX treatment also induces a XIAP cleavage product which appears to be the same as that seen in Fas/CHX-treatment in this cell type (Figure 1B lane 3). This 30-kDa fragment was assumed to be a proteolytic product of the full-length protein because protein synthesis was inhibited selectively by cycloheximide, as was also the case in the previous experiment (Figure 1A). Together, these results showed that the X-linked inhibitor of apoptosis protein was proteolytically processed in anti-Fas monoclonal antibodies/CHX- or TNF α /CHX-treated Jurkat cultured T cells (Figure 1A). It seemed likely that the proteolytic events involved in both TNFR1 and FasR apoptosis pathways converge on the cleavage of XIAP. Cleavage of XIAP shown in this experiment was more striking in the sample containing Fas/CHX treated cells (lane 2) than in the cells treated with TNF α /CHX (lane 3) after 6 hours of treatment. Therefore Fas/CHX was chosen as the apoptotic trigger for the further experiments, particularly since the death pathway used herein was a well-established and robust system where many of the molecular events have been determined.

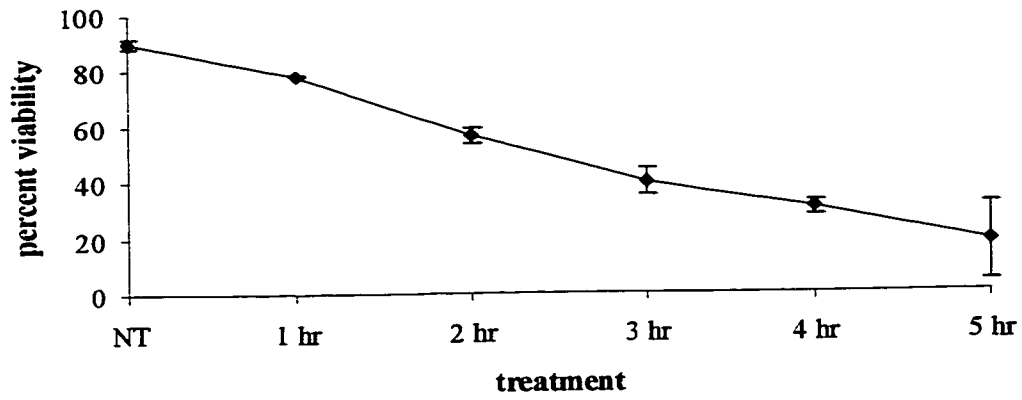
Viability of Jurkat Cells during Fas/CHX-mediated Apoptosis

Determination of the apoptotic status of the Jurkat cells treated with Fas/CHX was important in order to correlate this with the incidence of the XIAP ~30 kDa cleavage product. Figure 2A shows the viability of Jurkat T lymphocytes as measured by both vital staining with trypan blue and by apoptotic morphology with Hoescht 33258 fluorescence

Figure 2: Viability of Jurkat Cells during Fas/CHX mediated apoptosis

Jurkat T cells were treated with 1 ug/mL anti-Fas mAb and 20 ug/mL CHX over a six hour time course assay; Viability progressively decreases with duration of exposure to the Fas and CHX apoptotic triggers. **A:** Samples were taken hourly for the duration of the six hour experiment, and counted using trypan blue exclusion staining. Four fields of cells were counted for each sample, where each field contained at least 100 cells. This figure is representative of data collected from at least three assays using either monoclonal anti-Fas antibody (Calbiochem or MBL). **Figure 2B/C/D:** Hoescht staining (2.5 ug/mL) was conducted to assess apoptotic status of Jurkat cells during the time course assay described in Figure 1A. **B:** untreated Jurkat cells stained with Hoescht, **C:** Fas/CHX treated Jurkat cells stained with Hoescht (2 hour); **D:** Fas/CHX treated Jurkat cells stained with Hoescht (3 hour); Jurkat T cells were treated with 1 ug/mL anti-Fas mAb and 20ug/mL CHX over a five hour time course assay. Viability progressively decreases with duration of exposure to the Fas and CHX apoptotic triggers. Error bars represent standard deviation within a single experiment.

A

Viability of Jurkats with α -Fas mAb and Cycloheximide Treatment

Hoescht stained Jurkat cells

B



Untreated Cells

C



2 hours Fas/CHX treatment

D



3 hours Fas/CHX treatment

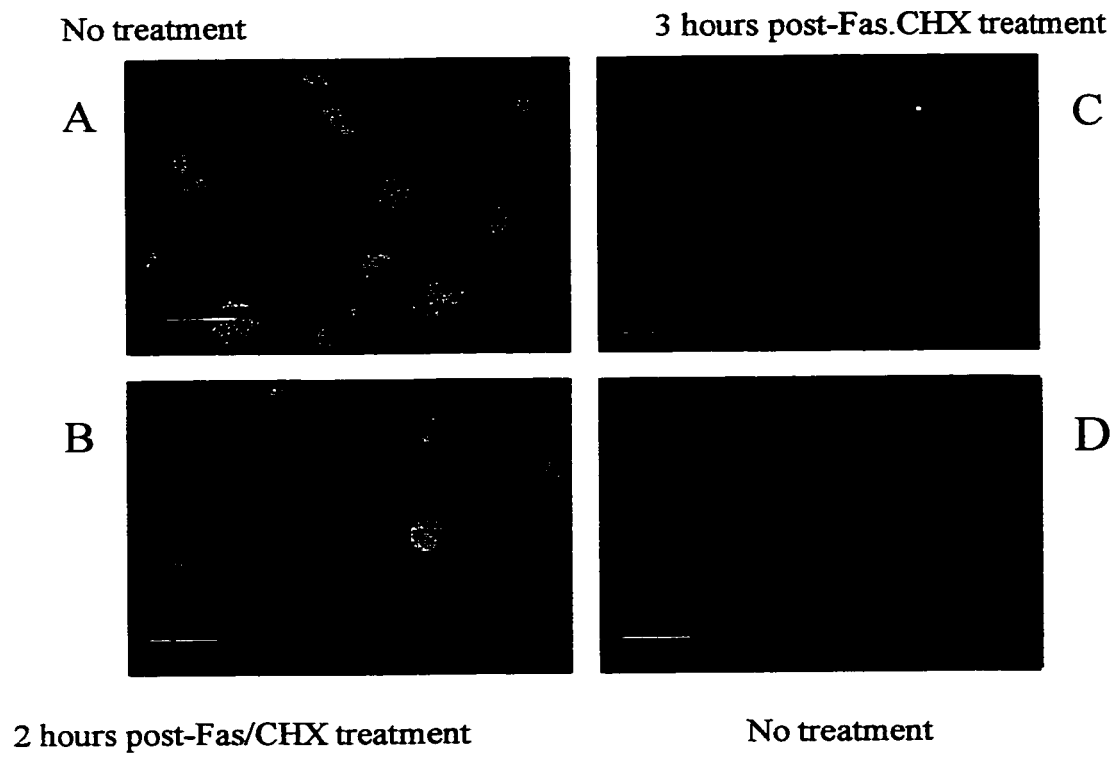
staining of cellular DNA. The data shown in Figure 2A demonstrates that Jurkat cells die rapidly following anti-Fas/CHX treatment. Approximately 20% of cells survived by 5 hours post-treatment; in contrast, 80-95% viability was observed in the untreated negative control. As well, in order to show that the death occurring in these cells was indeed apoptotic, cells were stained with the DNA stain Hoescht 33258. This stain may be employed to distinguish nuclear morphology associated with healthy cells (round whole nuclei) from apoptotic cells (segmented nuclei). The results from the Hoescht 33258 staining of Jurkat cells show segmentation of nuclei in apoptosing cells two (Figure 2B) and three hours (Figure 2C) post-treatment. In summary, the Fas/CHX treatment of Jurkat cells was a reproducible apoptotic system that can be used to study XIAP proteolysis more fully. In the time frame when cells demonstrate greater than 40% cell death, which appears clearly apoptotic, XIAP cleavage has occurred in these cells (see Figure 1A hours 3-6).

Cellular Localization of XIAP during Fas/CHX-mediated Apoptosis

I chose to investigate the protease responsible for the proteolysis of the anti-apoptotic protein XIAP. The proteases involved in the initial phase of the apoptotic process are primarily cytoplasmic (reviewed in (Cryns, 1998; Martin, 1995; Patel, 1996; Thornberry, 1997; Zhivotsky, 1997)). The next experiment was conducted to determine the localization of XIAP during the apoptotic process, which would provide an indication as to which proteases to look at. In this experiment, Jurkat cells were treated with anti-Fas mAbs and CHX in a four-hour time course assay and then immuno-stained for XIAP protein levels using polyclonal anti-XIAP antibodies (Figure 3).

Figure 3: XIAP localization in the cytoplasm during apoptosis shown through Immunofluorescent Staining of Jurkat Cells Treated with anti-Fas mAbs and cycloheximide. Jurkat T cells were treated with the apoptotic stimuli, Fas/CHX, and localization of XIAP was assessed using XIAP polyclonal antibodies (1:150 dilution), detected with Alexa (fluorescence-conjugated) secondary antibodies (1:200) **A:** untreated Jurkat cells, detected with anti-XIAP polyclonal antibody. **B:** Jurkat cells two hours post-Fas/CHX treatment detected with anti-XIAP polyclonal antibody, secondary Alexa fluorescence conjugated antibody. **C:** 3 hour Fas/CHX treated Jurkat cells detected with anti-XIAP polyclonal antibody; **D:** negative control: untreated Jurkat cells stained with preimmune serum and fluorescence-conjugated secondary antibody. XIAP immunostaining, primarily in the cytoplasm, seems to decrease in intensity in cells undergoing apoptosis. Bars represent 15 μ m.

Anti-XIAP primary antibody detected (A-C); pre-immune serum detected (D) Jurkat cells following apoptotic stimuli



The results of this experiment showed that in untreated cells, XIAP was distributed equally throughout the entire cell. Apoptotic as well as healthy Jurkat cells were present two (Figure 3B) and three hours (Figure 3C) following triggering of apoptosis by Fas/CHX. In the healthy cells (Figures 3B and 3C), staining of the XIAP protein was more intense than that of the apoptotic cells. As the cells progressed through apoptosis, XIAP immuno-staining distribution became less prominent suggesting that XIAP protein levels are being depleted. Although it is difficult to assess, potential cytoplasmic distribution in dying Jurkat cells suggested that XIAP cleavage might be propagated by the activation of cytoplasmic proteases.

Induction of the Fas pathway of death results in the activation of one or all three of the protease families involved in apoptosis namely, the caspases (Martin, 1995), calpains (Squire, 1994) and the 26S proteasome complex (Grimm, 1996). The next stage of investigation in this study therefore was centered on determining which class of protease was responsible for XIAP cleavage.

Candidate Proteases for XIAP Cleavage during Fas/CHX-mediated apoptosis

Role of Calpains in XIAP Cleavage

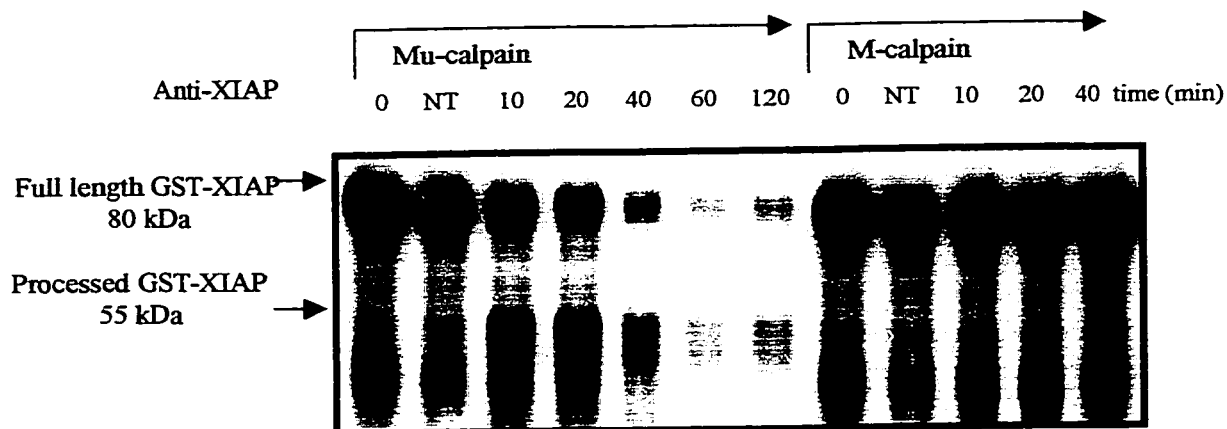
In previous *in vitro* studies, two groups had concluded that caspases are not directly responsible for XIAP cleavage (Deveraux, 1998; Duckett, 1998). My investigations were therefore directed towards identifying other candidate proteases responsible for XIAP cleavage. The first enzymes that I chose to investigate were the calpains, a decision based upon two reasons. Firstly, I identified a potential calpain cleavage site in the region between BIR 2 and 3 of XIAP at position 252 (amino acids -

Proline-Asparagine-Serine-Threonine-). A number of groups have shown that calpains cleave proteins at sites which are rich in proline (P), glutamic acid (E), serine (S) and threonine (T) amino acids, called PEST sequences (Sorimachi, 1997; Squire, 1994; Squire, 1997). There does not seem to be a completely conserved consensus sequence associated with calpain cleavage, instead processing is dependent upon protein secondary structure. However, PEST-like regions have been found in a number calpain substrates (Sorimachi, 1997; Squire, 1994). The products from cleavage at the 252 site would be consistent with the size of the actual XIAP cleavage product seen in my experiments (Figure 1AI and 1AII). Secondly, Vanags *et al* (1996) have shown that the TNF α /CHX pathway of death is calpain-dependent (Vanags, 1996), a trigger which I have shown induces the cleavage of XIAP. Calpains became the focus of my attention as they may also be involved in the Fas pathway of death, and therefore may be responsible for the cleavage of XIAP.

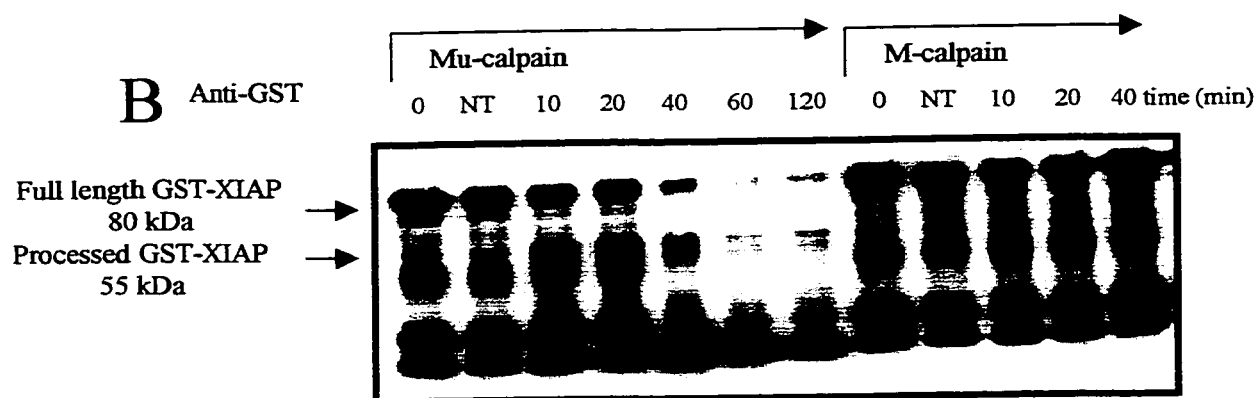
The initial investigation of potential XIAP cleavage by calpain isoforms was conducted using *in vitro* protease studies (Figure 4). For this purpose, an assay was performed using recombinant GST-XIAP protein and one of two calpain isoforms (types I (μ) or II (m)) in the presence of excess calcium. Anti-XIAP polyclonal antibodies reproducibly detect a 55 kDa cleavage product of recombinant GST-tagged XIAP at early time points using the type I isoform, and not the type II isoform (Figure 4A lanes 3-7). Recombinant calpain isoforms were not tested for their activity in this assay however. The size of this cleavage product is consistent with an ~ 30 kDa N terminal XIAP fragment attached to the GST-tag (26 kDa protein). Comparison of the experiment shown in Figure 4A with Figure 4B, in which an anti-GST antibody was used, confirms that the

Figure 4: Type I (μ) calpain cleaves recombinant GST-XIAP under *in vitro* conditions in a time course assay Recombinant GST-XIAP proteins were placed *in vitro* with one of the two calpain isoforms in the presence of excess calcium. Figure 3A shows an immunoblot detected with the affinity purified anti-XIAP polyclonal antibody; arrows represent full-length 80 kDa GST-XIAP and 50 kDa cleavage product. Figure 3B shows the same experiment using an anti-GST monoclonal antibody; Figure 3C shows an immunoblot, detected with anti-XIAP polyclonal antibodies, using a truncated recombinant XIAP protein (containing only BIR2 & 3). Contents of the lanes are as follows: A: (full-length GST-XIAP proteins): lane 1 no calpain (0). lane 2 two hours 37°C no calpain (NT); lane 3-7 μ calpain: lane 3 ten minutes; lane 4 twenty minutes; lane 5 forty minutes; lane 6 one hour; lane 7 two hours; lanes 8-12: m calpain: lane 8 ten minutes; lane 9 twenty minutes; lane 10 forty minutes; lane 11 one hour; lane 12 two hours B: same as above (A); C: (GST-BIR2 and 3 truncation XIAP mutant): lane 1 no calpain, on ice. lane 2, 2 hours at 37°C, no calpain, lane 3 μ -calpain 2 hours 37°C, lane 4 m-calpain 2 hours 37°C. (μ refers to type I (μ) calpain isoform; m refers to the type II (m) calpain isoform). Two independent experiments were conducted with similar results.

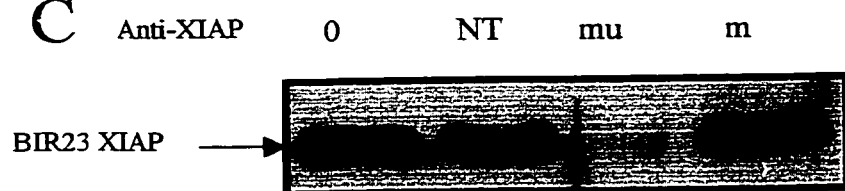
A Western blot analysis



B Anti-GST



C Anti-XIAP



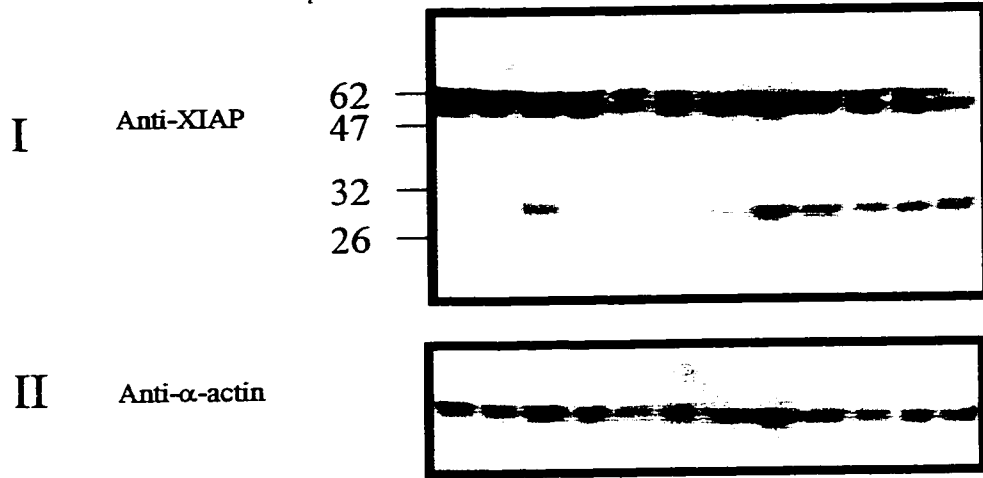
55 kDa proteolytic fragment contains the GST tag (Figure 4B lane 3-7). A truncated form of XIAP that contains only the BIR 2 and 3 motifs with no GST tag was also tested (Figure 4C). In this assay, BIR23-XIAP disappeared following exposure to the type I calpain isoform (Figure 4C lane 3). Using the type II calpain isoform in this assay did not result in the loss of the XIAP fragment (Figure 4C lane 4). The results from this experiment suggest that the type I calpain is capable of processing XIAP under *in vitro* conditions, and that the cleavage site within lies within the BIR23 region of XIAP. The site where the PEST sequence was found (aa 252) was present in this truncated protein.

The purpose of the next experiment was to assess involvement of the calpains in the Fas and/or CHX pathway of cell death and in the context of XIAP cleavage. Several groups have suggested that Fas-mediated apoptosis is calpain-independent, whereas CHX mediated death is dependent on calpain's involvement (Knepper-Nicolai, 1998; Squire, 1999). Therefore participation of the calpains in the cleavage of XIAP may be dependent on one or both of the stimuli used in my study, namely anti-Fas monoclonal antibodies and cycloheximide. To further investigate the possibility that calpain was a candidate protease responsible for XIAP cleavage, Fas- and/or CHX- stimulated Jurkat cells were treated with calpain specific inhibitors [calpeptin and calpastatin] (Figure 5). XIAP cleavage was investigated using Western blotting techniques with whole cell lysates from Jurkat cells, treated with cycloheximide and/or anti-Fas antibodies. Cells were also treated with one of four calpain inhibitors, calpeptin, calpain inhibitor I, calpain inhibitor II or calpastatin. Calpeptin, and calpain inhibitors 1 and 2 are synthetic peptide inhibitors, whereas calpastatin is a truncated, cell permeable form of an endogenous inhibitor of

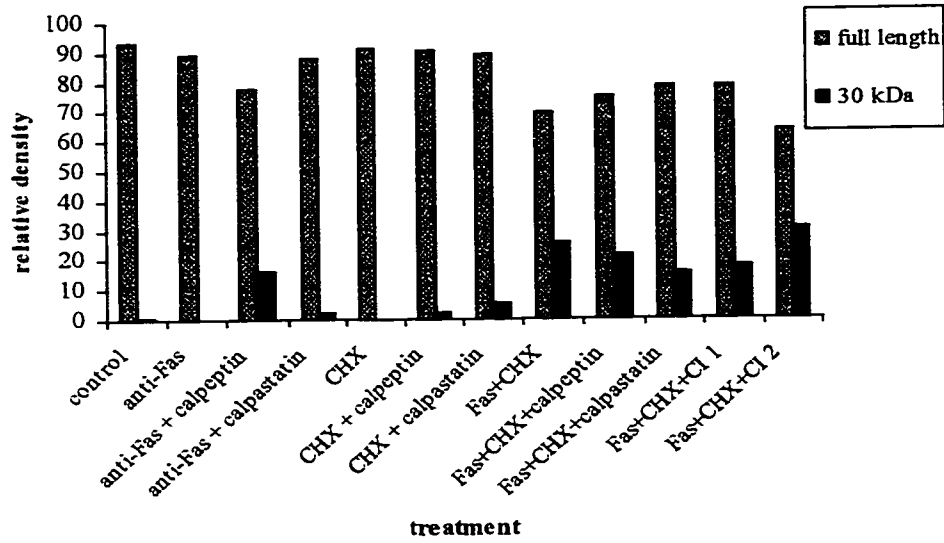
Figure 5: Calpain Inhibitors have little or no effect on XIAP Cleavage or cell viability in Fas and/or CHX treated Jurkat Cells Cleavage of endogenous XIAP proteins was assessed in this assay using Fas/CHX stimulated Jurkat cells treated with calpain specific inhibitors. **Figure 5A** Western blots were conducted using whole cell lysates detected with **I**: anti-XIAP monoclonal antibodies or **II**: anti-actin monoclonal antibodies. Contents of the lanes are as follows: lane 1 untreated Jurkat cells; lanes 2-4 anti-Fas mAb; lane 2 alone, lane 3 calpeptin (20 uM); lane 4 calpastatin (0.5 ug/mL); lanes 5-7 CHX; lane 5 alone; lane 6 calpeptin; lane 7 calpastatin; lanes 8-12 anti-Fas mAb + CHX; lane 8 alone; lane 9 calpeptin; lane 10 calpastatin; lane 11 calpain inhibitor I (50 uM); lane 12 calpain inhibitor II (50 uM); anti-Fas mAb were used at a concentration of 1.0 ug/mL; CHX was used at a concentration of 20 ug/mL. Densitometry data of Western blot in **Figure 5B**, where peak 1 refers to full-length XIAP proteins, peak 2 refers to 30 kDa proteolytic product of XIAP. “Total” refers to the total density shown in each lane, whereas “selected” refers to the density value according to the full-length and 30 kDa protein fragments; viability of this experiment shown in **Figure 5C**. Viability of Jurkat cells was assessed after 5 hours of treatment. Three independent experiments were conducted with similar results.

A Western Analysis

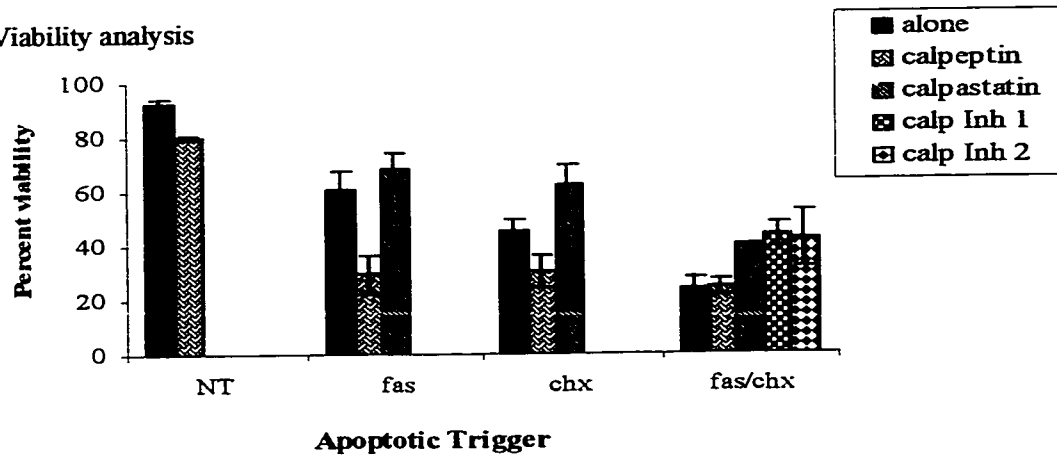
| | | | | | | | | | | | | |
|---------------|---|---|---|---|---|---|---|---|---|---|---|---|
| Anti-Fas | - | + | + | + | - | - | - | + | + | + | + | + |
| CHX | - | - | - | - | + | + | + | + | + | + | + | + |
| Calpeptin | - | - | + | - | - | + | - | - | + | - | - | - |
| Calpastatin | - | - | - | + | - | - | + | - | - | + | - | - |
| Calpain inhbs | - | - | - | - | - | - | - | - | - | - | 1 | 2 |



B Densitometric Analysis



C Viability analysis



calpain (Tsujiyama, 1988). The effectiveness of the four inhibitors in ablating the cleavage event and their effects on cell viability were then determined.

In the first part of this experiment, the anti-Fas and CHX-triggers were used in isolation, in the presence of one of the two inhibitors, calpeptin or calpastatin peptide (Figure 5A lanes 2-7). Surprisingly, anti-Fas antibodies, when used in isolation did not induce cleavage of XIAP or significant death at 5-6 hours post-treatment (Figure 5A lane 2). This result is in direct contrast to the study conducted by Deveraux *et al* (1999), where it was shown that when used alone, anti-Fas antibodies induced XIAP cleavage and cell death in Jurkat T cells (Deveraux, 1999). In my study, the decrease in viability was only about 25% between controls without inhibitors and the Fas monoclonal antibody alone. However, treatment of Fas-stimulated cells with calpeptin did cause the cleavage event and more death in Jurkat cells (Figure 5 lane 3). In this case, according to densitometric analysis (Figure 5B) of the Western blot shown in Figure 5A (lane 3), a decrease in cell viability corresponds directly with an increase in the amount of the XIAP cleavage product. Neither of the apoptotic stimuli (Fas or CHX), when used in isolation resulted in a significant decrease in cell viability (Figure 5C). Likewise, treatment of Jurkat cells with CHX and one of the calpain inhibitors had little effect on cell viability or XIAP cleavage (Figure 5A lanes 5-7). XIAP proteolysis is not evident in Jurkat cells treated with either the Fas or CHX trigger in isolation, or when treated with the calpastatin inhibitor and Fas stimuli. The results from this first part of this experiment (Figure 5A lanes 1-7) suggest that the Fas or CHX triggers, when used in isolation do not induce XIAP cleavage or cell death. However, these results must be considered taking into account that the efficacy of the inhibitors was not assessed.

The next portion of this experiment was conducted in order to assess the effects of the calpain inhibitors on cell viability following Fas and CHX treatment. As previously demonstrated, the combination of Fas and CHX had a significant negative effect on the viability of these cells (Figure 5C). Cells exhibited a decrease in viability of over 75% when both triggers were used. However, even in the presence of calpain inhibitors, there was little to no protection of Jurkat cells treated with anti-Fas mAb and cycloheximide (Figure 5C). There appeared to be a slight protection from cell death in CHX treated cells in the presence of the endogenous inhibitor calpastatin, although the effect did not appear to be dramatic.

The purpose of the last part of this experiment was to investigate XIAP cleavage in Jurkat cells that had undergone Fas and CHX stimulation with the addition of calpain specific inhibitors (Figure 5A lane 8-12). According to densitometric analysis of the immunoblot in Figure 5B (lanes 8-12), the cleavage product comprises between 20% and 35% of total XIAP protein. When Jurkat cells are treated with Fas and CHX together, even in the presence of any of the four inhibitors, the XIAP cleavage product is abundant. In summary, the data shown in Figure 5 suggests that calpain proteases are not centrally responsible for the initial XIAP cleavage event, assuming that the inhibitors were effective. No assessment of the inhibitor's effectiveness was conducted however.

Possible Role of the 26S Proteasome in XIAP Cleavage

Next I chose to investigate the possibility that another cytoplasmic protease complex may be responsible for XIAP cleavage, the 26S proteasome. The possible importance of this protease complex in my study is exemplified by the study conducted

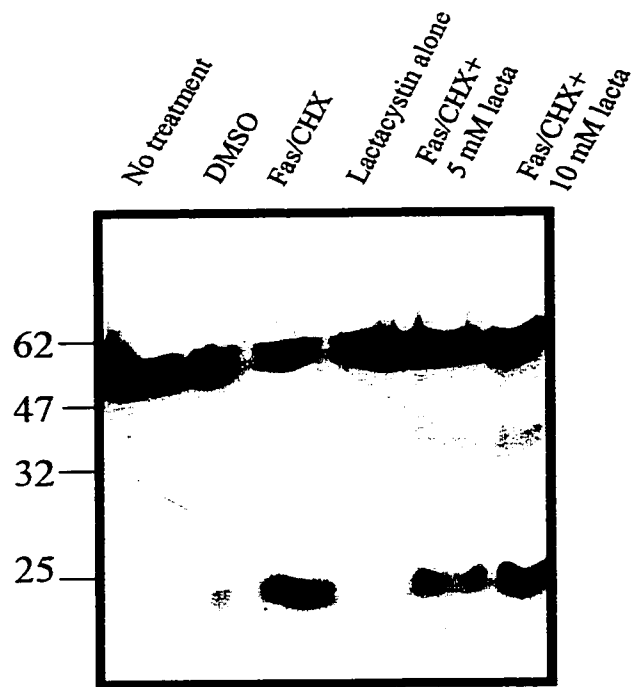
by Grimm *et al* (1996), where it was shown that the 26S proteasome was essential for thymocyte apoptosis. The 26S proteasome is responsible for the majority of general degradation and processing of cellular proteins including those specifically involved in the apoptotic cascade. For example, a substrate of the 26S proteasome is NF- κ B, which is proteolytically processed from its native 110 kDa form to 50 kDa during apoptosis (Orian, 1995). As the 26S proteasome is capable of processing proteins during apoptosis, this protease complex was investigated as a candidate responsible for XIAP cleavage.

The involvement of the 26S proteasome in XIAP processing during Fas-mediated apoptosis in Jurkat cells was investigated in the experiment shown in Figure 6. In this study, Jurkat cells were treated with Fas/CHX, and lactacystin, (an irreversible and specific inhibitor of the 26S proteasome (Fenteany, 1998)) whereupon XIAP cleavage (Figure 6A) and cell viability (Figure 6B) were assessed. In Fas/CHX treated Jurkat cells, lactacystin did not block XIAP cleavage, in any of the three concentrations tested (5, 10 and 20 μ M) (Orian, 1995). The effectiveness of this inhibitor was not assessed however. After 4 hours, Fas and CHX-treated Jurkat cells had undergone approximately 50% apoptotic death. Conversely, cells treated with lactacystin (20 μ M) in isolation experienced a very slight decrease (about 5-10% compared to the negative control) in viability. Of the three concentrations used in this experiment, 10 μ M lactacystin had a slight protective effect on cell viability following Fas/CHX treatment. Specifically, the increase in viability in cells treated with 10 μ M lactacystin and Fas/CHX was only 10%, compared to cells treated with Fas/CHX alone (Figure 6B). In this experiment, much like the previous experiment (Figure 5), the incidence of death correlates directly with the

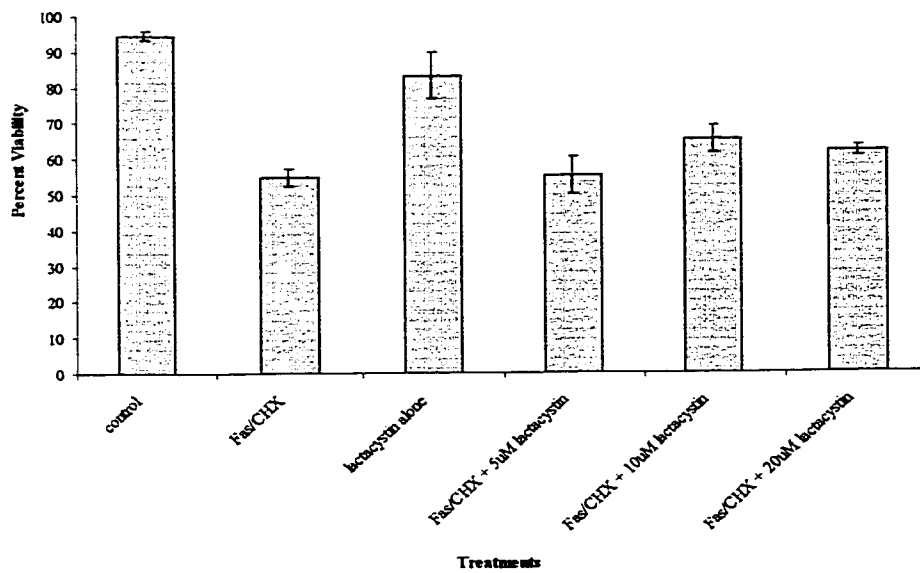
Figure 6: The Proteasome Inhibitor, Lactacystin has little or no effect on XIAP cleavage or cell viability in Fas/CHX treated Jurkat Cells Western blot using anti-XIAP polyclonal antibody of Jurkat whole cell lysates treated with Fas/CHX and lactacystin for 5 hours. Both the 30 kDa XIAP cleavage product and the full-length 55 kDa protein are present in all lanes where the Fas/CHX apoptotic trigger is used (lanes 2, 4-6). The contents of the lanes are as follows, lane 1 no treatment; lane 2 DMSO; lane 3 Fas/CHX; lane 4 lactacystin alone; lane 5 Fas/CHX + 5 uM lactacystin; lane 6 Fas/CHX + 10 uM lactacystin; Figure **6B** represents the viability of Jurkat cells during this experiment after 5 hours of treatment.

A

Anti-XIAP

**B**

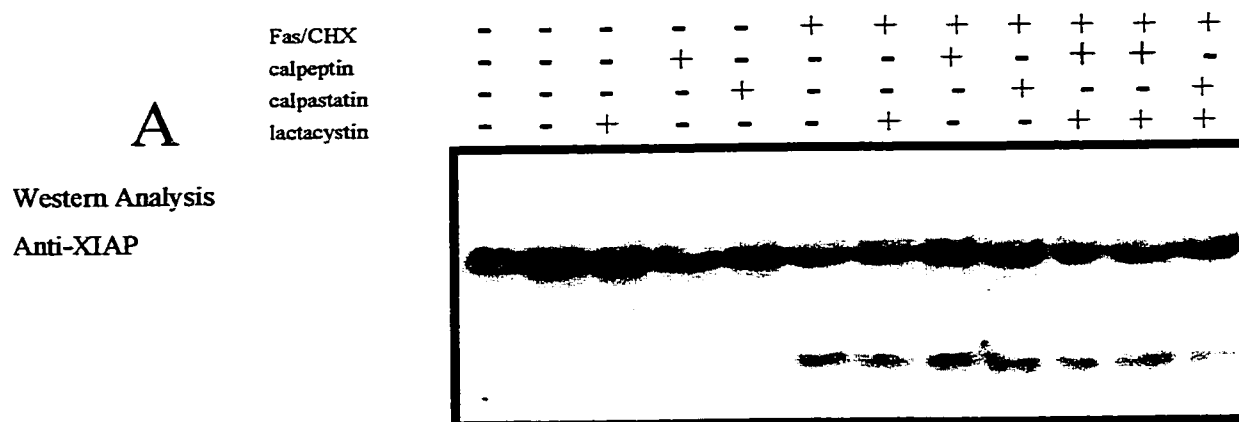
Viability Analysis



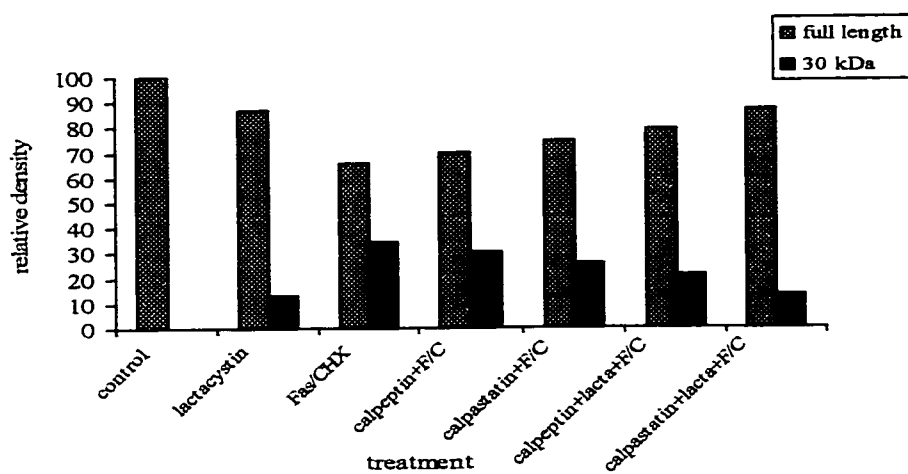
cleavage of XIAP. There appears to be a band at a higher size range than full length XIAP that may represent a ubiquitinated form of the protein. This band is not present in lanes where lactacystin was used.

The impetus for the next experiment (Figure 7) came from a study conducted by Keppler-Nicolai *et al* (1998) (Knepper-Nicolai, 1998), who investigated the effects of using a combination of proteasome and calpain inhibitors on constitutive neutrophil apoptosis. The two protease-family specific inhibitors used in combination had the ability to protect neutrophils from constitutive apoptosis in this previous study. In isolation, the two classes of inhibitors were found not to be cytoprotective. I sought to determine whether the combined protective effect of the two inhibitors was conserved between neutrophils and Jurkat T cells. Jurkat cells were treated with one or both of the inhibitor types (calpeptin or calpastatin and lactacystin) prior to Fas and CHX stimulation. After 4 hours of anti-Fas monoclonal antibody and CHX treatment, the cells were assessed for their apoptotic status (Figure 7C) and lysates were prepared for Western blots (Figure 7A). No evidence of the XIAP cleavage product was seen in the negative control lane (Figure 7A lane 1 and Figure 7B). However, there was small amounts of the ~30 kDa fragment present in the inhibitor alone lanes (lanes 3-5). This may be due to the effects of the individual inhibitors on cell viability. Conversely, there was a remarkable (~35%) quantity of the cleavage product in Jurkat cells treated with Fas/CHX (Figure 7A lanes 6 through 12). This is not surprising however as previous experiments have shown that calpeptin and calpastatin are ineffective in preventing death or XIAP cleavage in these cells (Figure 5). It is evident from both the densitometry data from Figure 7B and the

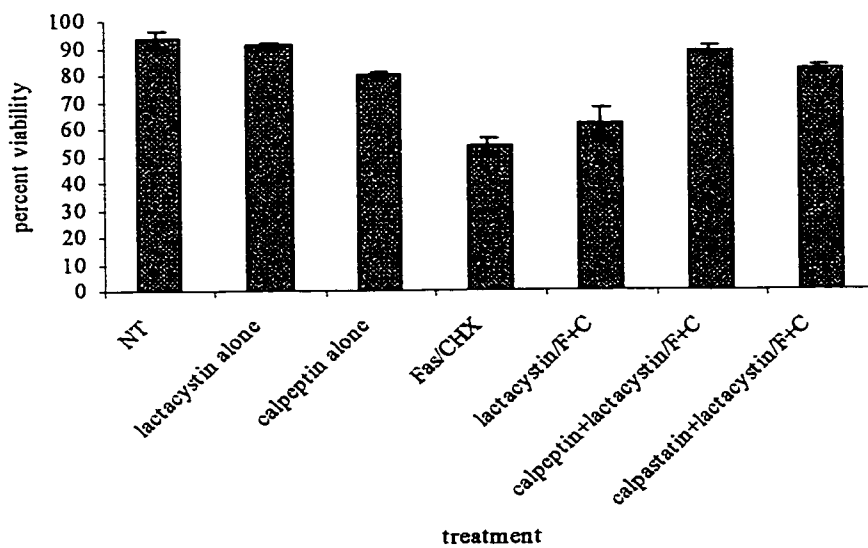
Figure 7: Calpain and Proteasome Inhibitors used together block death in Jurkat T cells treated with Fas/CHX, but not XIAP cleavage Calpain inhibitors, calpeptin and calpastatin peptides were used in conjunction and separately with the proteasome specific inhibitor, lactacystin in apoptotically triggered Jurkat cells. Western blots shown in **Figure 7A** were conducted using anti-XIAP polyclonal antibodies, on whole Jurkat cell lysates following these treatments. Lane 1 contains untreated cell lysates, after the 6 hour experiment. Lane 2, DMSO; lane 3: lactacystin alone; lane 4 calpeptin alone; lane 5 calpastatin alone; lanes 6-12 anti-Fas mAb + CHX; lane 6 alone; lane 7 lactacystin; lane 8 calpeptin; lane 9 calpastatin; lanes 10,11 calpeptin + lactacystin; lane 12 calpastatin + lactacystin. **Figure 7B** represents densitometry of the above Western blot, not including samples where inhibitors were used alone (without apoptotic stimuli). **Figure 7C** represents the viability of representative Jurkat cell samples in this experiment after 5 hours of treatment. Data is representative of two independent experiments.



B Densitometric Analysis



C Viability Analysis



viability study in Figure 7C, that the lactacystin used alone did not significantly protect cells from death or prevent XIAP cleavage.

Conversely, when used together lactacystin and either calpain inhibitor protect Jurkat cells from Fas/CHX stimulated cell death. However, in spite of this evidence of cytoprotection, the XIAP cleavage product was nevertheless present, though with slightly decreased amounts. According to the densitometric analysis, the decrease in cleavage product was much the same in the lactacystin treatment (without apoptotic stimuli) as in the double inhibitor lanes. It is difficult to assess whether this slight decrease in the amount of XIAP cleavage product is significant with a single densitometric analysis however. The results of this experiment argue that the apoptotic cascade may be conserved at the level of calpain and proteasome activation between neutrophils and Jurkat cells. I have shown that neither calpain inhibitors nor proteasome inhibitors are able to block the cleavage of XIAP in Fas/CHX treated Jurkat cells. In this experiment, I have been able to separate the incidence of death with the cleavage of XIAP.

Involvement of the Caspases in XIAP Cleavage

The final and arguably most important protease family activated during apoptosis are the caspases. Since their discovery (Cerretti, 1992), the caspases have been shown to be responsible for the proteolysis of a large number of diverse proteins during apoptosis, including those with such distinct classes as cytoarchitecture, transcription factors, and other proteases. The next stage of my investigation was focused upon assessing whether a member of the caspase family was responsible for the cleavage of XIAP. In the first experiment, I investigated the activation of caspase 3, an effector caspase in Fas/CHX

treated Jurkat cells. The experiment shown in Figure 8 was a five-hour time course assay of the cleavage of 32 kDa procaspase 3 into catalytically active 12 kDa and 17 kDa fragments during Fas/CHX mediated apoptosis. In this experiment, caspase cleavage was evident in cells that had undergone at least two hours of Fas/CHX treatment (Figure 8 lanes 5-8). This is the window of time when XIAP is also cleaved (time points following 1 hour-Figure 1A/II lanes 2-6). A previous study has also shown that in this system, the activation of caspase 8 and the cleavage of classic death substrates such as poly (ADP-ribose) polymerase (PARP) occurs in the same time frame (Scaffidi, 1998).

In the next stage of this investigation, I sought to assess the effects of the pan-caspase inhibitor, z-VAD-fmk on XIAP cleavage and cell viability in the Fas/CHX apoptotic system (Figure 9). Since calpain has been investigated as a potential candidate responsible for XIAP cleavage, two calpain inhibitors were used as negative controls for comparison. Again, calpain inhibitors calpeptin and calpastatin had no protective effect on the viability of these cells and did not prevent XIAP cleavage (Figure 9A, lanes 5 & 6 respectively and 9C). In the presence of anti-Fas mAbs and cycloheximide, the positive control, Jurkat cell viability decreased to ~20% after 6 hours and the 30 kDa cleavage product corresponds to 16% of the total XIAP protein according to densitometric analysis (Figure 9A lane 2, Figure 9B).

On the other hand, cells treated with Fas/CHX and the pan-caspase inhibitor z-VAD-fmk remained as healthy throughout the six-hour treatment as the negative control cells (Figure 9C). In the presence of z-VAD-fmk, Jurkat cells also contain 50% less of the 30 kDa XIAP cleavage product than cells which have undergone Fas/CHX treatment

Figure 8: Caspase 3 is Activated in Jurkat Cells during Fas/CHX mediated apoptosis in the same time period as XIAP cleavage Caspase 3 was activated, indicated by cleavage into 12 and 17 kDa fragments, in time points after one hour of treatment with anti-Fas mAbs and CHX. In this experiment, Jurkat T whole cell lysates were resolved on 12% SDS-PAGE and transferred to PVDF membranes. Western blotting was done using anti-Caspase 3 polyclonal antibodies, at a dilution of 1:2000 and fluorescence-conjugated secondary antibodies at a concentration of 1:2000. Contents of the lanes are as follows: lane 1, no treatment, lane 2 fifteen minutes, lane 3 thirty minutes, lane 4 one hour, lane 5 two hours, lane 6 three hours, lane 6 four hours, lane 7 five hours post-treatment. Data is representative of two independent experiments.

Western blot Analysis

Anti-caspase 3

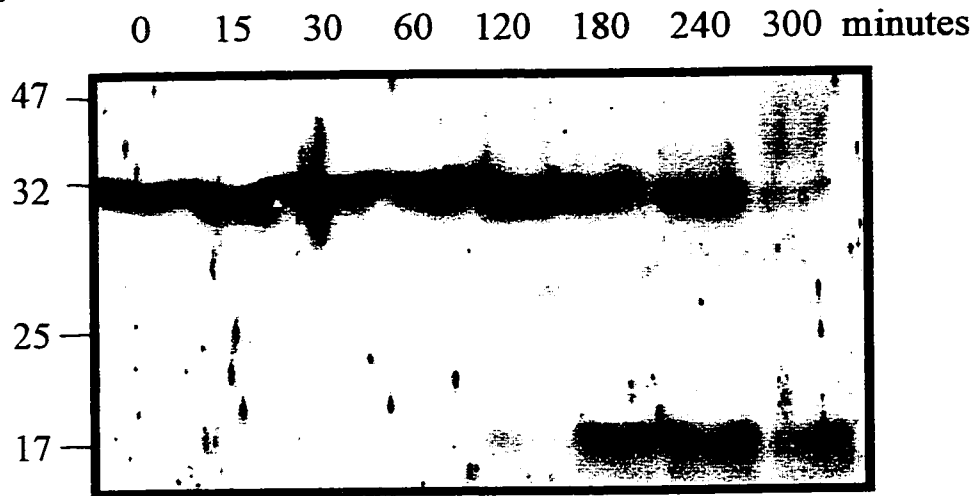
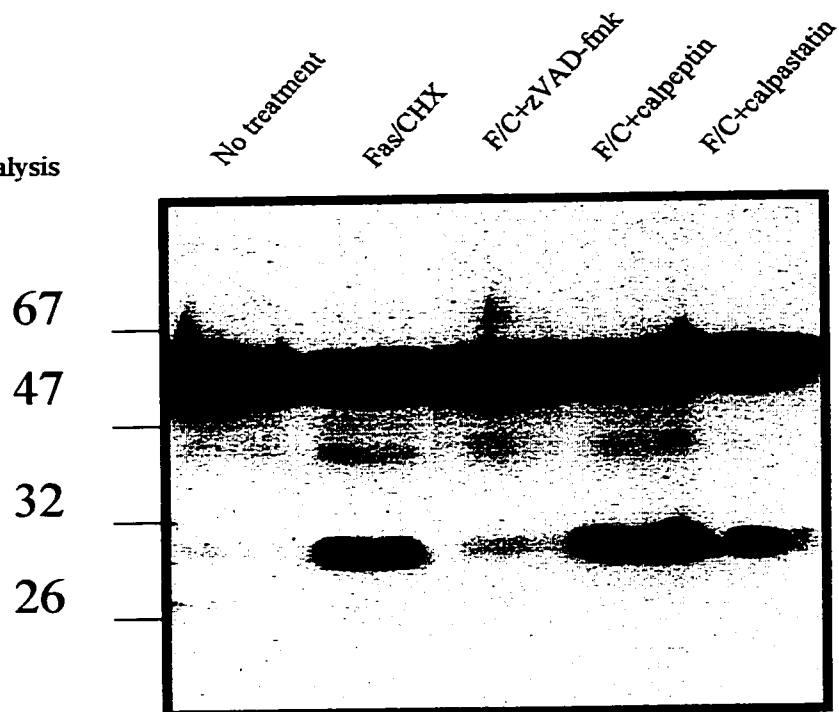
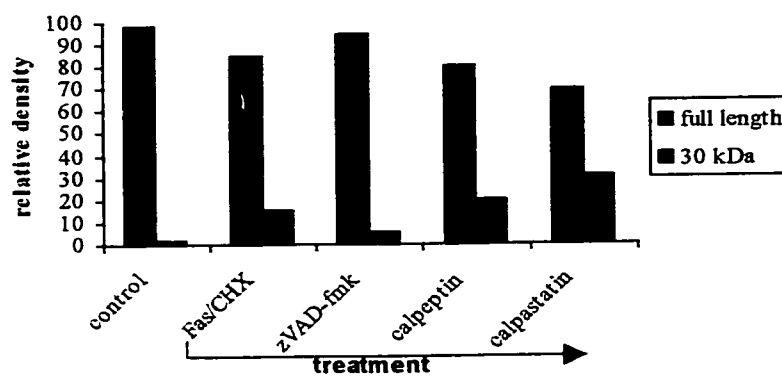


Figure 9: ZVAD-fmk, a pan-caspase Inhibitor blocks cell death and XIAP cleavage in Fas/CHX treated Jurkat T lymphocytes Figure 9A shows a Western blot using anti-XIAP monoclonal antibody using whole cell lysates of Jurkat cells treated with the following reagents. Little to no 30 kDa cleavage product is present in lanes where there zVAD-fmk has been used. Lane 1: no treatment control; lane 2 Fas and CHX; lane 3 zVAD-fmk + Fas and CHX; lane 4: calpeptin (ce) + Fas and CHX; lane 5: calpastatin (ca) + Fas and CHX; Figure 9B represents densitometry data from the above Western blot; Figure 9C represents viability of Jurkat cells during the experiment using trypan blue staining of the Jurkat cells. Data is representative of two independent experiments after 6 hours of treatment.

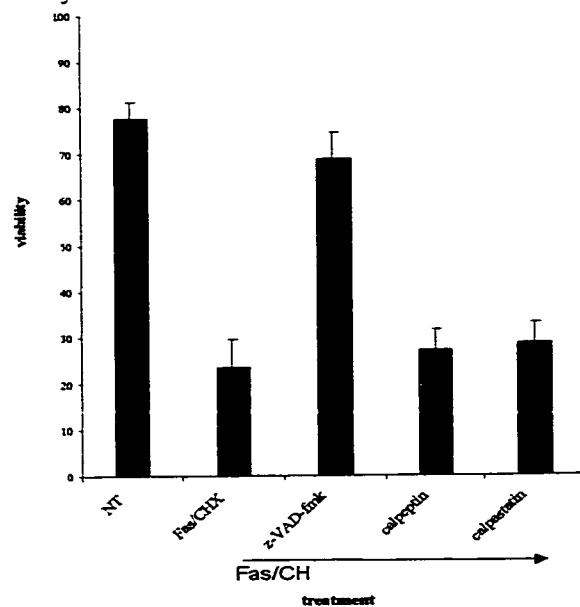
A
Western blot Analysis
Anti-XIAP



B Densitometric analysis



C Viability Analysis



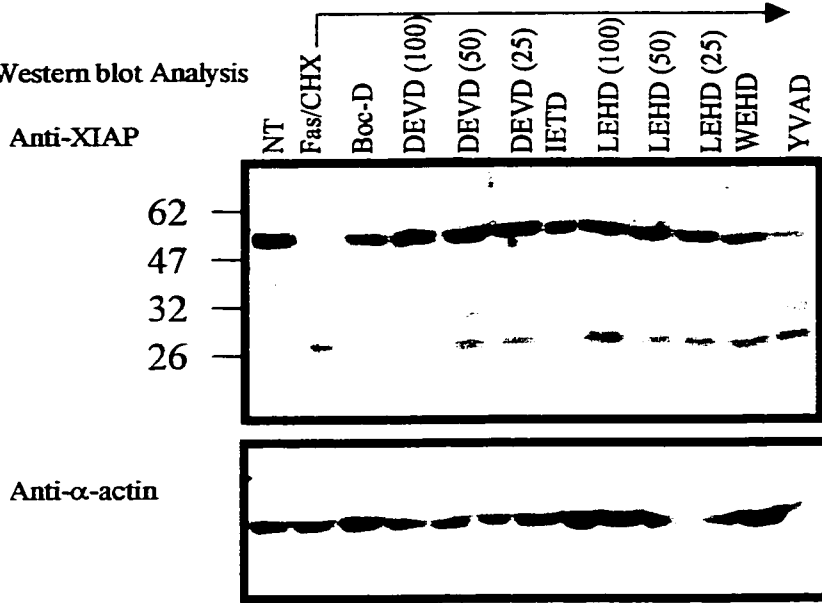
alone according to densitometric analysis, comprising less than 10% of the total XIAP proteins (Figure 9A compare lane 4 with lane 2, Figure 9B).

The results from the experiments shown Figure 7 show that the prevention of apoptosis is not sufficient to stop XIAP cleavage. In the experiment shown in Figure 7, I was successfully able to slow apoptosis with calpain and proteasome inhibitors, but not completely block the cleavage event. The results from Figure 9 show that a wide spectrum caspase inhibitor was the most effective in preventing XIAP cleavage. Likewise, blocking the activity of the caspase family of proteases with an irreversible inhibitor such as zVAD-fmk has been shown in other studies to also block the cell's sensitivity to apoptotic stimuli (Juo, 1998; Longthorne, 1997; Zhivotzky, 1997).

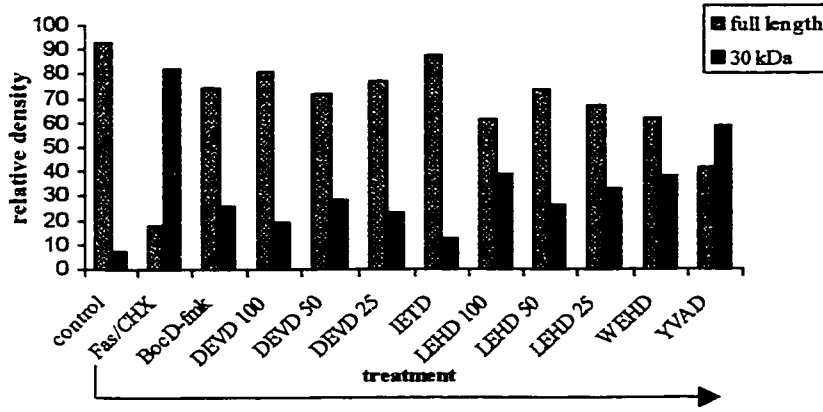
A more rigorous investigation of the specific caspase family member's involvement in the cleavage of XIAP is presented in Figure 10A. Many of the tetrapeptide caspase inhibitors with the exception of z-YVAD-fmk, (a caspase 1 inhibitor) can prevent Fas-mediated cell death in Jurkat T cells (Budihardjo, 1999; Cohen, 1997; Juo, 1998; Thornberry, 1997; Zhivotzky, 1997). Jurkat cells were treated with various caspase inhibitors one hour prior to apoptotic stimulation. Each of the caspase inhibitors partially blocks Jurkat cell apoptotic death and the proteolysis of XIAP (Figure 10A lanes 3-12) compared to the controls (lanes 1,2). In samples (lanes 3-12) that have been treated with the caspase specific inhibitors, full-length protein is present with small amounts of cleavage product. The results from this experiment indicated that a member of the caspase family is likely to be responsible for the XIAP cleavage event. From these data, the caspase 3 enzyme seems to be the most likely candidate.

Figure 10: Caspase Specific Inhibitors block death and XIAP cleavage to varying degrees in Jurkat cells undergoing Fas/CHX mediated apoptosis. Figure 10A show a Western blot of Jurkat whole cell lysates probed with 1:2000 anti-XIAP polyclonal antibody and probed with anti-actin monoclonal antibody. The experiment shows the relative effectiveness of each of the type specific caspase inhibitors in various concentrations, where lane 1 contains lysates from untreated cells. Lanes 2-12: α -Fas mAb (1 ug/mL) and CHX (20 ug/mL); lane 2: trigger alone; lane3 Boc-D-fmk [pancaspase inhibitor]; lane 4-6, DEVD-fmk [caspase 3 inhibitor] (25, 50 and 100 uM respectively); lane 7 IETD-fmk [caspase 8 inhibitor]; lanes 8-10, LEHD-fmk [caspase 9 inhibitor] (25, 50 100 uM respectively); lane 11 WEHD-fmk [caspase 5 inhibitor]; lane 12 z-YVAD-fmk [caspase 1inhibitor]; where inhibitor concentrations are not listed, 100 uM was used. Figures **10B** and **10C** represent densitometry data from the above anti-XIAP Western blot and viability of Jurkat cells respectively. Data is representative of two independent experiments.

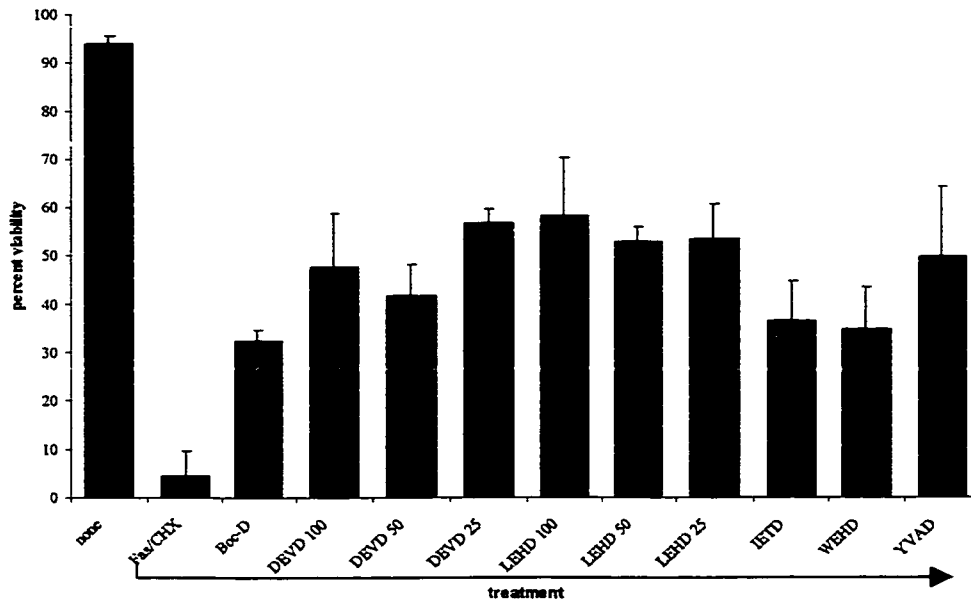
A Western blot Analysis



B Densitometric Analysis



C Viability Analysis



Discussion

This study has shown that XIAP is proteolytically processed from its native 55 kDa size to a fragment of 30 kDa during Fas/CHX mediated cell death in Jurkat T cells. The 30-kDa XIAP cleavage product was found after one hour of Fas/CHX treatment (Figure 1A) and six hours post-treatment with TNF α /CHX (Figure 1B). A single time point experiment was conducted using the TNF α /CHX apoptotic stimulus in order to demonstrate the universality of XIAP cleavage between receptor-mediated apoptotic cascades. Similarly, Deveraux *et al* (1999) and Johnson *et al* (2000) have also found that XIAP was cleaved into the 30-kDa fragment following apoptotic stimulus with anti-Fas monoclonal antibodies, etoposide or staurosporine. Etoposide (a chemotherapeutic inhibitor of the topoisomerase enzyme), and staurosporine (a general kinase inhibitor) are both potent inducers of apoptosis in Jurkat T cells (Deveraux, 1999; Johnson, 2000). Conservation throughout a number of different apoptotic pathways in Jurkat cells suggests the importance of the proteolysis of XIAP in the process of PCD.

In the first part of my study, I also sought to find which portion of the XIAP protein corresponded to the 30-kDa fragment by Western blot analysis (Figure 1A). The 30-kDa XIAP fragment was found to correspond to the carboxy-terminus of the protein, because this fragment was detected using a carboxyl-terminal specific antibody (BD Transduction Labs). Deveraux *et al* (1999) have shown that the 30 kDa fragment contains the carboxy terminal of the XIAP protein and they also mapped the cleavage site to a position between the second and third BIR domains (Deveraux, 1999). Interestingly, there was only a single fragment resultant from XIAP cleavage (see Figure IV for illustration). The fate of the amino terminus of the XIAP protein *in vivo* is as yet

uncertain. Deveraux and coworkers (1999) suggested that the amino terminus was degraded during apoptosis, leaving the carboxy terminal intact during Fas-mediated Jurkat cell death (Deveraux, 1999). As well, the amount of the full length XIAP protein did not decrease significantly during the time course assay, in parallel with the appearance of the cleavage product (Figure 1 A, lanes 1-6). Further investigation into possible variations in expression levels of XIAP during Fas/CHX mediated cell death will be necessary in order answer this question. Transient transfection studies could be conducted with an amino-terminal tagged XIAP expression vector in Jurkat T cells. Cells would be submitted to tag-specific antibody detection by Western blot analysis. Alternatively, *in vitro* studies using ³⁵S-methionine-labelled recombinant XIAP protein, truncated at amino acid position 241 (containing BIRs 1 and 2) could be undertaken. Exposure of the radiolabelled truncated XIAP protein to Fas/CHX-stimulated Jurkat cell lysates would indicate whether this portion of XIAP is degraded during Fas/CHX mediated apoptosis.

Apoptotic cell death seemed to be integrally associated with the incidence of the 30-kDa XIAP cleavage product. However, significant apoptotic death (>40%) has occurred prior to the appearance of the 30 kDa XIAP cleavage product. This observation raises questions as to the significance of XIAP proteolysis as a “gain-of-function” event. Whether or not the cleavage of XIAP changes its function is a question that remains unanswered. This is an issue that will be discussed further in later sections. Not surprisingly, Jurkat cells die rapidly following treatment with anti-Fas monoclonal antibodies and CHX (Figure 2). Within five hours, the cells underwent a decrease in viability of approximately 80% (Figure 2A). Nuclear morphology, as evidenced by

Hoescht staining of Fas/CHX treated cells, suggested that many of the cells were apoptotic after two hours (Figure 2B and 2C). Subsequent experiments in my study have shown the same results (Figures 5,9,10).

XIAP cleavage, having been established as a reproducible event within a defined system of apoptosis, was induced by an as yet, unidentified protease. In order to determine which were the candidate proteases responsible for XIAP proteolysis, XIAP localization studies were conducted (Figure 3). Here I show that the XIAP protein was present mainly in the cytoplasm. However, technically, one of the limitations of this apoptotic system was that in comparison to many other cultured cells, Jurkat cells contain mostly nuclear material. Therefore, the fine structure localization of proteins using Jurkat T cells was difficult to assess. Specifically, the proteins located within cytoplasmic membrane could not be distinguished from those located in the cytosol. In summary, the results with XIAP immunostaining of Jurkat cells (Figure 3) did not rule out the possibility that any of the three proteases, calpains, caspases and the 26S proteasome complex could be candidates for XIAP cleavage. It should be noted that the 26S proteasome is also present in the nucleus and therefore this experiment does not necessarily show the co-localization of XIAP and this protease complex (Driscoll, 1990).

Protein levels appear to drop following the initiation of apoptosis, evidenced by the decrease in the intensity of the XIAP-specific fluorescence staining in apoptotic cells (Figure 3). However, protein levels do not appear to decrease when visualized by Western blot analysis (Figure 1). In a cellular context, proteolysis of XIAP may decrease the affinity of the polyclonal antibodies for the protein, thereby causing a decrease in immunostaining. It would be interesting to be able to directly correlate apoptotic

morphology with the incidence of this decrease in XIAP fluorescence intensity by immunohistochemical staining.

There are a few examples where cytoplasmic proteases have been shown to translocate from the cytoplasm to the nucleus during apoptosis, including type I (μ) calpain (Mellgren, 1994) and caspase 3 (Zhvivotovsky, 1999). The significance of protease translocation has yet to be elucidated. The rationale for the experiment, shown in Figure 3 was to assess whether XIAP localization changed during the course of Fas/CHX-mediated death. Endogenous XIAP did not appear to translocate during Fas/CHX-mediated apoptosis.

A recent XIAP localization study conducted by Farahani *et al* (1997) showed that, when overexpressed in apoptotic Chinese Hamster Ovary (CHO) cells, the myc-tagged amino terminal portion of XIAP localizes to the nucleus (Farahani, 1997). Moreover, XIAP does not contain a consensus nuclear localization signal. Farahani's work (1997) suggests an interesting avenue of further investigation, in determining whether if in this scenario XIAP is cleaved, and whether the two fragments of XIAP were directed to different areas of the cell upon processing. In the present study, localization of the carboxy terminus was not established.

XIAP negatively regulates apoptosis and both localization and proteolysis may be involved in XIAP's control of apoptosis. Shuttling XIAP out of the cytoplasm into the nucleus may represent an alternative mechanism for controlling the cleavage of this protein. Should the presence of XIAP in the cytoplasm be essential for its inhibitory character, translocation to the nucleus would take the protein out of the context where it could properly function. As well, XIAP's ability to inhibit the caspases (possibly, with

the exception of caspase 3 (Zhivotovsky, 1999)) would be blocked if was translocated to the nucleus, cleaved or intact. The XIAP antagonist, XAF-1 (XIAP associating factor) also resides in the nucleus (Liston, 2000). Translocation of full-length or 30 kDa truncated XIAP to the nucleus could also block XIAP's inhibitory function by bringing it into contact with XAF. In summary, endogenous XIAP does not appear to translocate to the nucleus during Fas/CHX-mediated apoptosis (Figure 3) whereas in the mouse homolog (MIAP-3) when overexpressed in serum-deprived COS-1 cells translocates to the nucleus. More rigorous investigation will need to be conducted in order to elucidate the locale of XIAP fragments during apoptosis.

The first enzymes that I investigated in my study were the two ubiquitous isoforms of the calpains: type I (μ) and type II (m). I found that under *in vitro* conditions, the type I calpain enzyme was capable of cleaving XIAP (Figure 4A & 4B) into a product consistent in size to that observed in previous *in vivo* studies (Deveraux, 1999; Johnson, 2000). In the study conducted by Deveraux *et al* (1999), the non-calpain enzymes employed under *in vitro* conditions were used at a high molar excess (20-fold enzyme: substrate) and the assay was conducted over a period of several hours. In contrast, in my study, the type I (μ) calpain was able to process XIAP at a much lower ratio of enzyme to substrate and cleavage occurred in less than 10 minutes. The likelihood of concentrations such as 20 fold excess of enzyme to substrate within a cellular context is very low, suggesting the possibility that another protease, such as the type I calpain which requires a substantially smaller ratio, may be involved in the proteolysis of XIAP. The type I calpain able to process XIAP efficiently into discrete fragments (consistent in size with the 30-kDa fragment found in Fas/CHX-stimulated

Jurkat cells). Furthermore, when this reaction proceeded into longer time points, full-length GST-XIAP was degraded by this enzyme (protein levels dropped substantially) (Figure 4A, 4B). The GST-tag attached to the full-length XIAP protein (Figure 4A) may have stabilized the amino terminus of the XIAP protein explaining its presence. In summary, type I calpain can process and degrade GST-XIAP efficiently *in vitro*. In the cell, type I calpain may indeed be responsible for the degradation or specific cleavage of the amino terminal portion of XIAP.

There are two possible events which could account for the disappearance of the truncated XIAP protein following exposure to the type I (μ) calpain isoform. Firstly, the truncated form of XIAP, which contains BIR 2 and 3, could have been fully degraded. Alternatively, the results could be explained by the possibility that the epitope recognized by the anti-XIAP polyclonal antibodies was destroyed during the proteolysis of the truncated XIAP. The results from this experiment suggested that the region between BIR2 and BIR3 was unstable, a result that has been supported by others (Deveraux, 1999). Additional studies with the truncated XIAP (BIR23) protein and calpain will need to be conducted with sufficient amounts of protein to visualize on polyacrylamide gels (stained with Coomassie blue or silver stain) in order to assess whether this form was degraded or the epitope was destroyed.

The other method that I employed in order to investigate the candidate proteases involved in XIAP cleavage was to use cell permeable, protease specific inhibitors on intact Fas/CHX treated Jurkat cells. In this study, I tested the effectiveness of a number of different inhibitors in blocking the XIAP cleavage event, including calpain (Figures 5, 7 and 9), 26S proteasome (Figures 6 and 7) and caspase (Figures 9 and 10) specific

inhibitors. However, these results must be interpreted carefully as the effectiveness of these inhibitors was not assessed in these assays. Therefore the only indication of protease inhibitor effectiveness was in those samples where a biological effect was noted. This point will be addressed in more detail in the following sections.

In order to be certain what the conclusions of the inhibitor studies shown in this thesis, the most important control will be to assess the activity of calpains and caspases using fluorescent or colorimetric tagged peptides. In such experiments, active enzymes would be able to cleave the tag from the substrate, thereby inducing a fluorescent or colorimetric signal. In order to assess the effectiveness of the 26S proteasome inhibitor, lactacystin, a Western blot against ubiquitin could be conducted on each of the samples. A smear of staining would indicate that ubiquitination mediated protein degradation was inhibited. Future studies on the subject XIAP cleavage could include controlling for the activation of the three proteases following treatment with the various inhibitors used in my study. There are a number of protein substrates for each of the protease classes that can be used as markers for protease activation during apoptosis. For instance, the classic calpain substrate is a structural protein, termed fodrin (Vanags, 1996). The substrate most often associated with the 26S proteasome is NF κ B (Dallaporta, 2000), and the canonical caspase 3 substrate is PARP (Thornberry, 1992). Western blotting techniques using lysates from inhibitor treated apoptotic cells detected with antibodies against fodrin, NF κ B or PARP would demonstrate whether the substrates were indeed cleaved, indicating specific protease activity.

The first protease group that was investigated using specific inhibitors was the calpains. In this study, calpeptin (a synthetic peptide inhibitor) and calpastatin (a

commercially available, cell permeable form of the endogenous calpain inhibitor) were used (Figures 5, 7 and 9) because both had the highest specificity as inhibitors of calpain. Several of the synthetic calpain inhibitors used in earlier studies have had dual specificity for calpains and the 26S proteasome, including the original aldehyde leucinyll derivative peptide inhibitors, LLnL (calpain inhibitor I) and LLM (calpain inhibitor II) (Jenson, 1995). Calpeptin is a more recently developed synthetic inhibitor and shares similar aldehyde peptide structure (with LLnL and LLM). As an inhibitor, calpeptin has been shown to be very specific for calpains (Tsujiyama, 1988). Finally, I used the endogenous calpain inhibitor, calpastatin which has absolute specificity for the type I and II calpain isoforms (Murachi, 1989). In other studies, calpastatin effectively blocked the activation of calpains in Jurkat cells during apoptosis (Knepper-Nicolai, 1998; Squire, 1999).

The results from the calpain inhibitor assays suggested that calpain was probably not centrally involved in the direct cleavage of XIAP. In the experiment shown in Figure 5, treatment of Fas-triggered Jurkat cells with calpeptin caused apoptosis and XIAP cleavage while cells treated with the trigger alone did not (Figure 5 lane 3). Although this appears to be an anomalous result, previous studies have shown that Jurkat cells are prone to apoptotic death following treatment with calpeptin at concentrations between 50-100 μ M (Vanags, 1996). As I have mentioned above, the effectiveness was not assessed in this assay, allowing the possibility that the inhibitor did not enter the cell or come in contact with the enzyme. However, a biological effect was observed in the case of the calpeptin inhibitor, suggesting that this inhibitor penetrated the cell, causing this effect. In summary, these results demonstrate that Fas-mediated apoptosis is likely

calpain-independent. This interpretation was supported by the previous study conducted by Vanags *et al* (1996), where, in Fas/CHX-treated Jurkat cells, calpain inhibitors did not effectively block cell death.

The activity of calpain in CHX-treated was not clearly defined in the results of my study. In Jurkat T cells, CHX was not a potent inducer of apoptosis. However, in neutrophils (other immune cells), CHX induces a rapid potent apoptotic response, similar to *in vivo* constitutive apoptosis (55). Neutrophils undergo apoptosis constitutively when cultured *ex vivo* for periods of greater than 48 hours. Knepler-Nicolai *et al* (1998) showed that CHX-mediated apoptosis was calpain-dependent in neutrophils. Further experiments using Jurkat cells and calpain inhibitors may require higher concentrations of the protein synthesis inhibitor, CHX in order to induce a potent apoptotic response (Squire, 1999). Given the results from the *in vitro* studies (Figure 4) and the uncertainty pertaining to the importance of calpain during CHX-mediated death, the involvement of calpain (possibly indirect) in XIAP cleavage cannot be fully dismissed.

In order to further assess the importance of calpain in XIAP cleavage, calpain levels could be lowered using anti-sense RNAs or by transfection with cDNAs encoding calpastatin. By this anti-sense approach, the effectiveness of enzyme inhibition could be more easily relied upon. One must keep in mind that there may be technical difficulties with assessing the effectiveness of calpain inhibitors. Specific problems may include poor cell permeability, differences in substrate colocalization and variability in temporal expression.

The second protease group that was investigated with respect to XIAP cleavage was the 26S proteasome complex. At concentrations of 5 μ M, the 26S proteasome

inhibitor lactacystin had little effect on the cell viability or XIAP cleavage. Increasing concentrations of lactacystin improved cell viability in Fas/CHX treated cells, although no controls were conducted to assess the effectiveness of the 26S proteasome inhibitor, lactacystin.

The interaction between the 26S proteasome and XIAP has already been established in glucocorticoid induced apoptosis, by Yang *et al* (2000). In this study, overall XIAP levels were dramatically reduced by 26S proteasome activity following dexamethasone (DEX) treatment of primary thymocyte cultures (Yang, 2000). This effect was abrogated with treatment with lactacystin and other specific proteasome inhibitors. As mentioned, in primary thymocytes, dexamethasone mediated killing follows a distinctly different pathway than Fas-mediated apoptosis. The pattern of XIAP proteolysis was markedly different in these two pathways. Yang *et al* (2000) investigated primary thymocytes treated with DEX, where XIAP was fully degraded (Yang, 2000). In Jurkat T cells treated with Fas/CHX, XIAP was specifically processed into a 30-kDa product. Likely, therefore the pathways affecting cells treated with DEX are different from those in cells treated with Fas/CHX.

Although 26S proteasome function is required for a number of forms of cell death, the importance of the 26S proteasome during apoptosis in Jurkat T cells has not been elucidated. However, a recent study demonstrated that 26S proteasome function was dispensable during Fas-mediated cell death in primary cultured thymocytes (Dallaporta, 2000). In this study, Dallaporta *et al* (2000) distinguished between the endogenous (including apoptotic stimuli such as dexamethasone and etoposide) and the exogenous (including Fas) pathways of death in primary thymocytes. Dexamethasone is a

glucocorticoid that causes death by activating the expression of a number of proapoptotic genes, whereas etoposide is a topoisomerase inhibitor that prevents DNA replication (Cidlowsky, 1996). Dallaporta *et al* (2000) showed that endogenous forms of death required the activation of the 26S proteasome which was activated upstream of the caspases, whereas the exogenous pathway did not. According to Scaffidi *et al* (1998), apoptosis in Jurkat T cells follows the type II death pathway (where the activation of the apoptosome [caspase 9, Apaf-1 and cyt c] at the mitochondria is necessary) whereas primary thymocytes follow the type I pathway (where caspases 3 and 7 are immediately activated). The difference between the two pathways of Fas-mediated death, as discussed by Scaffidi *et al* (1998) were focussed on the activation of the mitochondrial events (activation of the apoptosome), much like the differences between the endogenous and exogenous pathways discussed in the study conducted by Dallaporta *et al* (2000). The activation of the apoptosome is important in both the type II Fas-mediated pathway (Scaffidi, 1998) and the endogenous pathway (Dallaporta, 2000), suggesting the possibility that there are more similarities between type II (Jurkat) Fas death and the endogenous pathway than with the Fas-mediated death of primary thymocytes. This sort of crosstalk between the different apoptotic mechanisms provides an additional impetus for studying the XIAP cleavage event in the Jurkat system and provided the rationale for my next experiment.

Kneppler-Nicolai *et al* (1998) established that there was a synergism between calpains and the 26S proteasome complex in constitutively apoptotic neutrophils. The purpose of the next set of experiment was to investigate whether this synergism was also activated in Fas/CHX-treated Jurkat cells. Kneppler-Nicolai *et al* (1999) showed that in

neutrophils, inhibitors against these two protease groups when used together blocked constitutive apoptosis. In my study, I demonstrate that, as found in neutrophils, there is a synergy between the activity of the 26S proteasome and calpain during Fas-mediated apoptosis in Jurkat T cells. The results suggest that the two proteases work in parallel pathways, being functionally redundant but necessary for death. Interestingly, XIAP cleavage is not blocked even while cell viability is preserved. The synergism between the 26S proteasome and calpain is activated downstream of caspase activation, suggesting that this is a late stage event in the apoptotic cascade. Figure V illustrates a putative model summarizing the activities of the various proteases and their involvement with the XIAP protein during apoptosis.

The third and final protease group that was investigated in my study was the caspases (reviewed in (Cohen, 1997)). Previous studies had shown that caspases could not process XIAP in *in vitro* assays (Deveraux, 1998; Duckett, 1998), probably due to the fact that XIAP is a potent inhibitor of several caspase family members. Caspase-independent apoptotic cascades are rare, but are reliant on alterations in mitochondrial membrane structure, and the release of various control proteins (Green, 1998). The central executioner, caspase 3, is activated in most apoptotic cascades, including Fas/CHX mediated PCD in Jurkat cells. Caspase 3 activation occurred after 2 hours of Fas/CHX-stimulation in my study demonstrating that this was a late stage event in the apoptotic cascade (Figure 8). Activation of caspase 3 temporally parallels the cleavage of XIAP in Jurkat cells, suggesting that this enzyme may be responsible for XIAP processing.

The possibility that caspases were responsible for XIAP cleavage was investigated using irreversible caspase inhibitors on Fas/CHX-treated Jurkat cells. Not

surprisingly, the pancaspase inhibitor zVAD-fmk was able to block PCD in Fas/CHX treated Jurkat cells. Notably, XIAP cleavage was also blocked by zVAD-fmk.

In Knepper-Nicolai's study (1998) z-VAD-fmk has also been found to block death in apoptotic neutrophils. In comparison, inhibitors against calpain and 26S proteasome did not block the cleavage of XIAP, but did block death in Jurkat cells. This result indicated that the caspases were activated upstream of the point of synergism between the 26S proteasome and calpain in both apoptotic neutrophils and Jurkat T cells (Knepper-Nicolai, 1998). Results from my study (Figures 8 and 9) have established that in Fas/CHX induced apoptosis, the cleavage of XIAP was an event that occurred upstream of the events which were blocked using 26S proteasome and the calpains inhibitors (see Figure V for schematic of protease activation). This interpretation was based on the fact that in z-VAD-fmk and Fas/CHX treated Jurkats both PCD and the incidence of the XIAP cleavage product were blocked simultaneously.

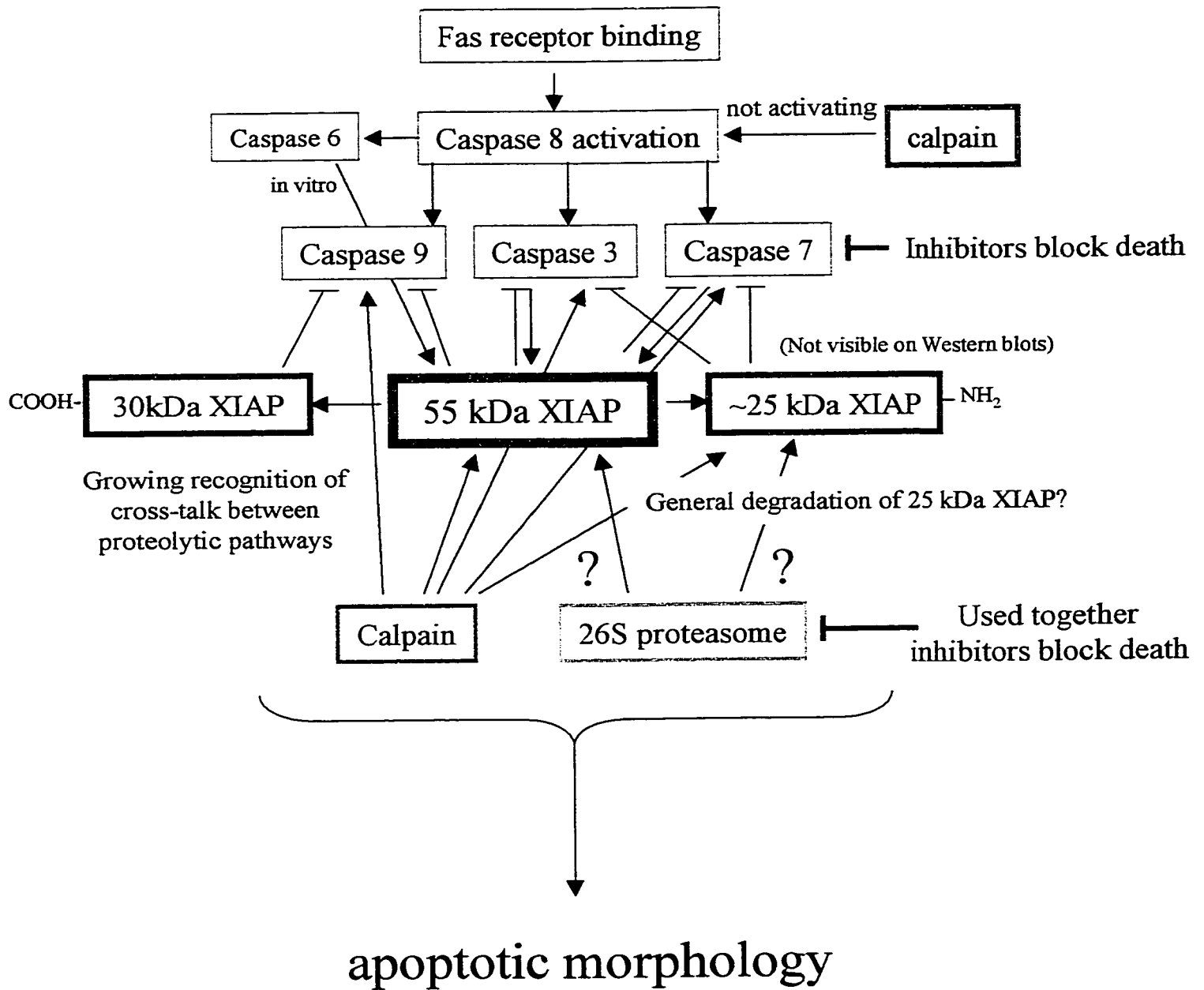
The investigation of proteases in apoptosis is an expanding field. Interactions between the three major cytoplasmic protease families involved in apoptosis have been established. XIAP cleavage occurs amidst the activation of each of the protease groups, and may be related to the interactions between the protease families. Figure V outlines the activation of various proteases in the Fas-mediated PCD pathway in relation to the cleavage of XIAP. Cleavage events occur between the protease families. Namely, calpain is capable of cleaving several of the caspase family proteases. Cleavage of the caspases by calpain isoform did not result in caspase activation or destroy the active site (Chua, 2000). In the other direction, caspases are capable of proteolytically processing calpastatin, the endogenous inhibitor of the calpains, thereby inactivating it (Wang, 1998). However, to

date no direct interactions between the 26S proteasome and the caspases have been reported.

Previous *in vitro* studies showed that XIAP binds and potently inhibits the activity of active caspases 3 and 7, and blocks the activation of procaspase 9 (Deveraux, 1997; Roy, 1997; Takahashi, 1998). The inhibitory character of XIAP against caspases 3 and 7 *in vitro* was found to be dependent on the first two BIR domains (Takahashi, 1998). Events in the Fas-induced Jurkat cell apoptotic cascade are mediated by caspase 3 and 7, which bind to XIAP *in vivo* (Deveraux, 1997; Deveraux, 1999; Takahashi, 1998). Deveraux *et al* (1999) also showed that the 5' truncated XIAP cDNA (including the first two BIR units), when transiently transfected into 293T (embryonic kidney) cells was able to block Fas-mediated apoptosis. In my study, inhibitors of caspase 3 and 9 (DEVD-fmk and LEHD-fmk respectively) were able to block death and the cleavage of XIAP in Fas/CHX treated Jurkat cells (Figure 10 lanes 4-6, 8-10). As well, the caspase 8 inhibitor, IETD-fmk was also able to block death and XIAP cleavage in a weaker fashion. This was not a surprising result because caspase 8 is the apical protease in the Fas-pathway of death. Blocking its activation prevents the activation of all other protease activation in this pathway. The results of my study and others (Deveraux, 1999; Johnson, 2000) suggest that a member of the caspase family was the most likely candidate for the proteolysis of XIAP. My study using Fas/CHX-treated Jurkat T cells supports the results of Deveraux *et al* (1999), where they showed that showed caspases 3, 6 and 7 were capable of cleaving XIAP *in vitro*.

Figure V: Schematic model of molecular ordering of protease activation during Fas-mediated apoptosis in relation to the inhibitor of apoptosis protein XIAP; pathway begins with Fas receptor binding, followed by activation of caspases, 26S proteasome and calpains. Arrowheads represent proteolytic events/activation of enzymes, whereas blunt ends represent inhibition activity. The end of the pathway (apoptotic morphology) implies the initiation of cellular morphological changes (Budihardjo, 1999; Chua, 2000; Ciechanover, 1998; Cohen, 1997; Cryns, 1998; Knepper-Nicolai, 1998; Squire, 1994; Wang, 1998; Widmann, 1998).

**Model of Molecular Ordering of Protease Activation
During Fas-Mediated Apoptosis**



Deveraux *et al* (1999) were also able to show that the carboxy terminal protected the same cells against Bax-overexpression induced apoptosis (Deveraux, 1999). The Fas pathway of PCD is dependent on the activation of caspases 3 and 7, whereas the Bax pathway is reliant on the activity of mitochondria and caspase 9 (Deveraux, 1999). The portion of XIAP that is protective against Fas-mediated apoptosis, (amino terminal + BIR2) was degraded during Fas/CHX induced death, leaving only the BIR3 and RING Zn finger motifs intact (Roy, 1997). The amino terminus, shown to have cytoprotective properties against Fas-mediated apoptosis was not found. Therefore degradation of the amino terminus of the XIAP protein in Fas/CHX-mediated apoptotic cells may represent a mechanism of tipping the balance of signals towards death.

The relevance of proteolytic processing in relation to XIAP's function during apoptosis has yet to be assessed. During apoptosis, proteolysis may result in a loss or a gain of protein function. Examples of proteins that lose their function following cleavage are PARP and FAK (focal adhesion kinase) (Widmann, 1998). PARP, when cleaved loses its ability to repair DNA breaks, thereby committing the cell to death. The function of FAK is ablated by cleavage and therefore cell matrix contact is lost (van de Water, 1999). On the other hand, there are also numerous proteins whose functions are enhanced or changed by proteolysis. Two examples of these proteins are baculoviral inhibitors of apoptosis, P35 and CrmA (Widmann, 1998). These two inhibitors gain their function through cleavage, and remain bound to their respective proteases, a process known as suicide substrate inhibition. Upon processing, the larger product of p35 (termed p25) remains bound in a stable dimer with caspase 3. This cleavage event induces a conformational change that is required for caspase inhibition (Fischer, 1999).

Suicide substrate inhibitors are an interesting paradigm that may be relevant in the case of the interaction between XIAP and the caspases. Johnson *et al* (2000) showed that in Jurkat cells the cleaved form of XIAP was capable of remaining attached to the caspase that cleaves it following treatment with various apoptotic stimuli. As well, in one case, XIAP was found to be associated with the p24 partially processed form of caspase 3 (Deveraux, 1998), suggesting the possibility that the protein may act in the same manner as the baculoviral p35 inhibitory protein. Deveraux *et al* (1999) have shown that mutation of the XIAP cleavage site slightly enhanced XIAP's inhibitory character (Deveraux, 1999). Although there is no sequence homology between the P35 and XIAP proteins, these two proteins are able to functionally complement one another. However, there is no sequence or structural homology between XIAP and p35 (Fischer, 1999). Both proteins potently inhibit the caspases and are cleaved by the enzymes they inhibit. Future studies will be focussed on determining whether XIAP functions as a suicide substrate inhibitor or whether cleavage inactivates the protein's inhibitory character in the context of the Fas/CHX pathway of death in Jurkat cells.

Conclusions

During Fas/CHX-induced apoptosis in Jurkat T lymphocytes, the endogenous anti-apoptotic protein XIAP was cleaved from its native 55 kDa form into a single observable fragment of 30 kDa. The 30 XIAP fragment (Figure 1) was derived from the carboxyl terminus of the protein. Apoptotic cell death corresponds temporally with the appearance of this proteolytic product (Figure 2). XIAP was cleaved by a cytoplasmic protease (Figure 3). The calcium-dependent endoprotease, calpain (type I) is capable of cleaving XIAP under in vitro conditions (Figure 4), but inhibitors against this protease do not block death or XIAP cleavage (Figure 5). Inhibitors against the 26 proteasome complex also do not block cell death or XIAP cleavage (Figure 6). However, when used in conjunction calpain and 26S proteasome inhibitors were able to block cell death in Fas/CHX treated Jurkat cells, but not XIAP cleavage (Figure 7). The primary effector caspase, caspase 3 was activated in the same temporal period as XIAP cleavage in Fas/CHX-treated Jurkat cells (Figure 8). Additionally, caspase inhibitors block Jurkat cell death and XIAP cleavage (Figure 9 and 10). According to the data in my study, the most likely candidate proteases for XIAP cleavage are caspases 3 or 9. Recently, it was shown that the 30 kDa XIAP fragment remains bound to the caspases which cleave it (Johnson, 2000). Johnson *et al*'s observations (2000) and those from my study suggested that XIAP could act as a suicide substrate inhibitor.

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