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Investigations on apoptosis-associated caspase activation cascades

By

Brona Murphy

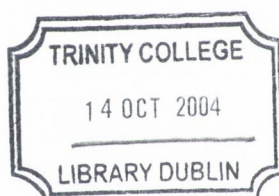
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the requirements for the degree of Doctor of Philosophy

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Research conducted in the Division of Molecular Cell Biology,
Department of Genetics, Trinity College Dublin, Dublin 2

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THESIS
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DECLARATION

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Brona Murphy

Brona Murphy

November, 2003

To my parents

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SUMMARY

Apoptosis is an important process in a wide variety of different biological systems such as the immune system, normal cell turnover and embryonic development (Stennicke *et al.*, 2002). Apoptosis is characterized by a series of distinct morphological and biochemical alterations such as DNA fragmentation, chromatin condensation, cell shrinkage and membrane blebbing (Adrain and Martin, 2001). In mammalian systems, the core component of the death machinery responsible for these stereotypic changes is a family of proteases known as caspases (Earnshaw *et al.*, 1999). The mammalian caspases may be divided into two broad subfamilies, the caspase-1 subfamily and the CED-3 subfamily. The CED-3 subfamily includes caspases -2, -3, -6, -7, -8, -9 and -10. These caspases are directly involved in apoptosis (Earnshaw *et al.*, 1999; Lamkanfi *et al.*, 2002). The research outlined in this thesis addresses these apoptotic caspases and in particular, the mechanisms governing the activation and regulation of these key proteases.

CHAPTER III

The work presented in this chapter examined the mitochondrial pathway of caspase activation. Initially we confirmed that the relative levels of each caspase and the activation kinetics of these enzymes are conserved in three cell types – two transformed cell lines – Jurkat and HEK293 and primary human neutrophils. We next revealed that the previously deduced order of caspase activation (Slee *et al.*, 1999b) was a general feature of apoptotic cells. Initial studies were performed by preparing cell-free extracts from healthy cells, triggering them to undergo apoptosis by the addition of cytochrome *c* and dATP and then examining the activation of the caspases with specific antibodies. In order to delineate the hierarchy of caspase activation events cell-free extracts were immunodepleted of specific caspases and then the effects of these depletions on the activation status of the remaining caspases were examined.

In the second section of chapter III we highlighted that cytochrome *c* is crucial for apoptosome mediated caspase activation in primary human neutrophils. We also showed however, that neutrophils have a substantially reduced requirement for cytochrome *c*, in comparison to other cell types, to induce apoptosome formation.

Techniques employed in this area of research included Western Blotting, RT-PCR, immunodepletions and immunofluorescence .

CHAPTER IV

The first section of chapter IV was dedicated to a second mechanism of caspase activation – granzyme B. The results highlighted that the granzyme B-mediated caspase cascade is very distinct from the previously discussed apoptosome-mediated cascade. This was the first analysis of the sensitivity of caspases to granzyme B proteolysis in a setting where the enzymes are present at physiological levels. This scheme demonstrated the caspases that are the direct targets of granzyme B and those that are not cleaved by this protease under physiological conditions.

The experiments in the second half of this chapter investigated whether interchain proteolysis was sufficient for the activation of procaspase-8. By using direct substrate cleavage assays we have revealed that caspase-8 proteolysis, in the absence of an adaptor platform, was sufficient for the formation of the active enzyme.

CHAPTER V

The research detailed in Chapter V was primarily concerned with caspase regulation via the IAPs – inhibitor of apoptosis proteins. The human IAPs, XIAP, c-IAP1 and c-IAP2 contain RING domains that permit these proteins to participate in *in vitro* ubiquitination reactions and therefore the IAPs are proposed to mediate their own degradation and the degradation of other substrates such as the caspases and Smac/Diablo *in vivo* (Yang *et al.*, 2000; Suzuki *et al.*, 2001; MacFarlane *et al.*, 2002). *In vitro* ubiquitination assays and overexpression studies in HEK293T cells have shown that XIAP is capable of autoubiquitination but IAP-mediated caspase ubiquitination was not detected. In addition, our results revealed that Smac attenuates the ubiquitin ligase activity of XIAP.

ABBREVIATIONS

ACINUS	apoptotic chromatin condenser in the nucleus
AIDS	Acquired Immune Deficiency Syndrome
Act D	actinomycin D
ALI	acute lung injury
ALPS	acute lymphoproliferative syndrome
Apaf-1	apoptotic protease activating factor
ARDS	acute respiratory distress syndrome
ATP	adenosine 5'-(tetrahydrogen triphosphate)
Bcl-2	B-cell lymphoma-2
BH	Bcl-2 homology region
BIR	Baculovirus IAP repeat
BSA	bovine serum albumin
CAD	caspase activated DNase
CAP	cytotoxicity-dependent APO-1-associated protein
CARD	caspase recruitmaent domain
CASPASE	cysteinyl aspartate-specific proteinase
CED	cell death defective
CFE	cell-free extract
cFLIP	cellular FLICE-like inhibitory protein
c-IAP1	cellular-inhibitor of apoptosis 1
c-IAP2	cellular-inhibitor of apoptosis 2
CL	cytotoxic lymphocyte
CRD	cysteine rich domain
CTL	cytotoxic lymphocyte
dATP	deoxyadenosine 5'-(tetrahydrogen triphosphate)
DD	death domain
DED	death effector domin
DIABLO	direct IAP binding protein with low pI
DISC	death-inducing signaling complex
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
DNA-PK _{CS}	DNA-dependent protein kinase (catalytic subunit)
DTT	dithiothreitol
EDTA	ethylinediaminetetraacetic acid
ER	endoplasmic reticulum
FADD	Fas associated protein with death domain
FAK	focal adhesion kinase
FasL	Fas ligand
FCS	foetal calf serum
FITC	flourescein isothiocyanate
FL	full length
FLICE	FADD-like ICE/CED-3 related protein
gld	generalized lymphoproliferative disease
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte macrophage-colony stimulating factor
GST	glutathione-S-transferase
HEK	human embryonic kidney
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]

Hid	Head involution defective
HBS	Hepes buffered saline
HRP	horse radish peroxidase
IAP	inhibitor of apoptosis protein
IBM	IAP binding motif
ICAD	inhibitor of caspase activated DNase
ICE	interleukin-1 β converting enzyme
Ig	immunoglobulin
IL	interleukin
IP	immunoprecipitation
IPTG	isopropylthio- β -D-galactoside
ITT	<i>in vitro</i> transcription and translation
kDa	kioldaltons
LB	Luria Bertani
lpr	lymphoproliferation
LPS	lipoplysaccharide
MPR	mannose 6-phosphate receptor
NBD	nucleotide binding domain
NF- κ B	Nuclear factor κ B
NK	Natural killer
NuMA	nuclear mitotic apparatus protein
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PARP	poly(ADP-ribose) polymerase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PIPES	Piperazine-N-N'-bis[2-ethanesulphonic acid]
PLAD	preligand binding assembly domain
PMSF	phenylmethysulphonylfluoride
RHG motif	Rpr-Hid-Grim motif
RING	really interesting new gene
RNA	ribonucleic acid
RNAi	RNA interference
RPMI	Roswell Park Memorial Institute
Rpr	Reaper
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecyl sulphate
siRNA	small interfering RNA
SIRS	systemic inflammatory response syndrome
SMAC	second mitochondrial activator of caspases
STS	staurosporine
TBST	Tris buffered saline with tween
TE	Tris EDTA
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TRADD	TNF receptor-associated death domain protein
TRAIL	TNF-related apoptosis-inducing ligand
Ub	Ubiquitin
UV	ultraviolet radiation
vFLIP	viral FLICE-like inhibitory protein

WDR	WD-40 repeat region
XIAP	X-linked inhibitor of apoptosis protein
Z-VAD.fmk	benzyloxycarbonyl-Val-Ala-Asp-(OMe) fluoromethylketone

PUBLICATIONS

Journals

Murphy BM, Creagh EM, Martin SJ (2004). Interchain Proteolysis, in the Absence of a Dimerization Stimulus, can initiate Apoptosis-Associated caspase-8 activation. *J Biol Chem* **279**, 36916-22.

Creagh EM, Murphy BM, Duckett DS, Martin SJ (2004). Smac/Diablo antagonizes IAP ubiquitin ligase activity of inhibitor of apoptosis proteins. *J Biol Chem* **279**, 26906-14.

Murphy BM, O'Neill AJ, Adrain C, Watson RWG, Martin SJ (2003). The Apoptosome Pathway to Caspase Activation in Primary Human Neutrophils Exhibits Dramatically Reduced Requirements for Cytochrome *c*. *J Exp Med* **197**, 625-632.

Book Chapter

Murphy BM, Martin SJ (2002). Caspases: Structure, Activation Pathways and substrates. Chapter 1 from *Essentials of Apoptosis: A guide for Basic and Clinical Research*. Edited by Dr. Xiao-Ming Yin and Dr. Zheng Dong. Published by Humana Press Inc, NJ, USA.

CHAPTER I

Introduction

1.1. INTRODUCTION

Apoptosis is characterized by a series of distinct morphological and biochemical alterations such as DNA fragmentation, chromatin condensation, cell shrinkage and membrane blebbing (Kerr *et al.*, 1972). Fig 1.1 highlights the morphological differences between a healthy and an apoptotic cell. Following cellular collapse *in vivo* (Fig 1.1B), such cellular fragments (apoptotic bodies) are engulfed by phagocytes, leaving no trace of the original cell behind (Adrain and Martin, 2001).

Apoptosis is an important process in a wide variety of different biological settings such as lymphocyte education within the immune system, normal cell turnover and embryonic development. Given that apoptosis is associated with key biological systems it is not surprising that deregulated apoptosis is associated with many diseases including AIDS, cancer, neurodegenerative disorders, autoimmune diseases, ischemic injury and restenosis (Stennicke *et al.*, 2002).

In mammalian systems, the death machinery responsible for these stereotypical changes involves an intricate web of molecules. The death machinery in *C. elegans* is not as complex however, and may be described in terms of four key players CED-3, CED-4, CED-9 and EGL-1 (Meier *et al.*, 2000).

As is shown in Fig 1.2 the ability of CED-3 to promote death is dependent on its activation by CED-4. In a healthy cell CED-4 is prevented from interacting with CED-3 due to its association with CED-9. However, upon receipt of a death-promoting stimulus, EGL-1 is thought to displace CED-9 from CED-4. CED-4 is thus free to recruit and activate CED-3, resulting in cell death (Conradt and Horvitz, 1998). The mammalian counterparts of these worm molecules have now been discovered. In humans, multiple homologs of such cell death proteins exist. Their role in apoptotic pathways, their interaction with one another, and their regulation, although highly complex, are currently being unraveled. The core components of the mammalian death machinery is a family of proteases known as caspases (Earnshaw *et al.*, 1999).

A



B

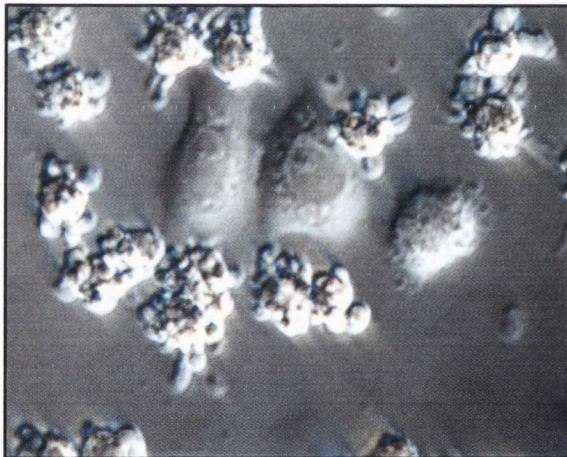


Fig 1.1. Phase contrast images of control and apoptotic HeLa cells at 40X magnification, kindly provided by Rebecca Taylor

A Typical HeLa Cell Morphology.

B Apoptotic morphological changes that occur after 4 h treatment with 10 μ M daunorubicin. Note the cell shrinkage and membrane blebbing.

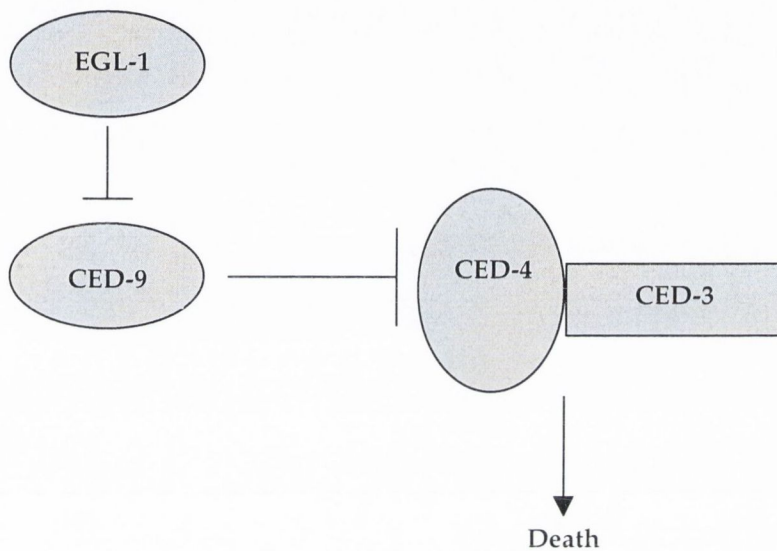


Fig 1.2. Schematic representation of the programmed cell death machinery in *C. elegans*

The activation of CED-3 is dependent upon interaction with CED-4. In a healthy cell, the CED-3/CED-4 interaction is inhibited by CED-9. Upon receipt of a death-promoting stimulus EGL-1 is thought to displace CED-9 from CED-4, thus freeing CED-4 to recruit and activate CED-3.

Caspases were first associated with apoptosis when it was discovered that the CED-3 protein of *C. elegans* was related to mammalian ICE (Interleukin-1 β -converting enzyme) or caspase-1 (Yuan *et al.*, 1993). It has since been discovered that caspase-1 does not directly contribute to an apoptotic pathway, this caspase is recognised as the founding member of this family whose members play a role in either apoptosis or inflammation (Thornberry and Lazebnik, 1998).

The research outlined in this thesis addresses the mammalian homologs of CED3 – members of the caspase family that participate in apoptosis.

1.2. CASPASES

1.2.1. Introduction

Currently, there are 11 human, 10 murine, 4 avian, 4 fish, 8 amphibian, 7 insect and 3 nematode caspases known (Lamkanfi *et al.*, 2002). Caspases, or cysteiny aspartate proteinases, use cysteine as the nucleophilic group for substrate cleavage and cleave the peptide bonds of their substrates after aspartic acid residues (Stennicke and Salvesen, 1999). The apoptotic phenotype illustrated in Fig 1.1, is orchestrated primarily by caspases cleaving key cellular substrates. Cleavage at internal aspartic acid residues also regulates caspase activation. Caspases may, therefore, be activated by other family members as part of an amplification cascade (Earnshaw *et al.*, 1999; Slee *et al.*, 1999a).

1.2.2. Caspase Classification

The mammalian caspases may be sub-divided into two broad subfamilies (Fig 1.3):

- Caspase-1 subfamily /Inflammatory caspases

This family includes caspases -1, -4, -5 and -11. These caspases do not play a direct role in apoptosis and are involved in the control of inflammation and the immune response. The prototype of this subfamily is caspase-1, which is implicated in the processing of inflammatory cytokines such as proIL-1 β and IL-18 (Earnshaw *et al.*, 1999; Lamkanfi *et al.*, 2002).

- CED-3 Subfamily

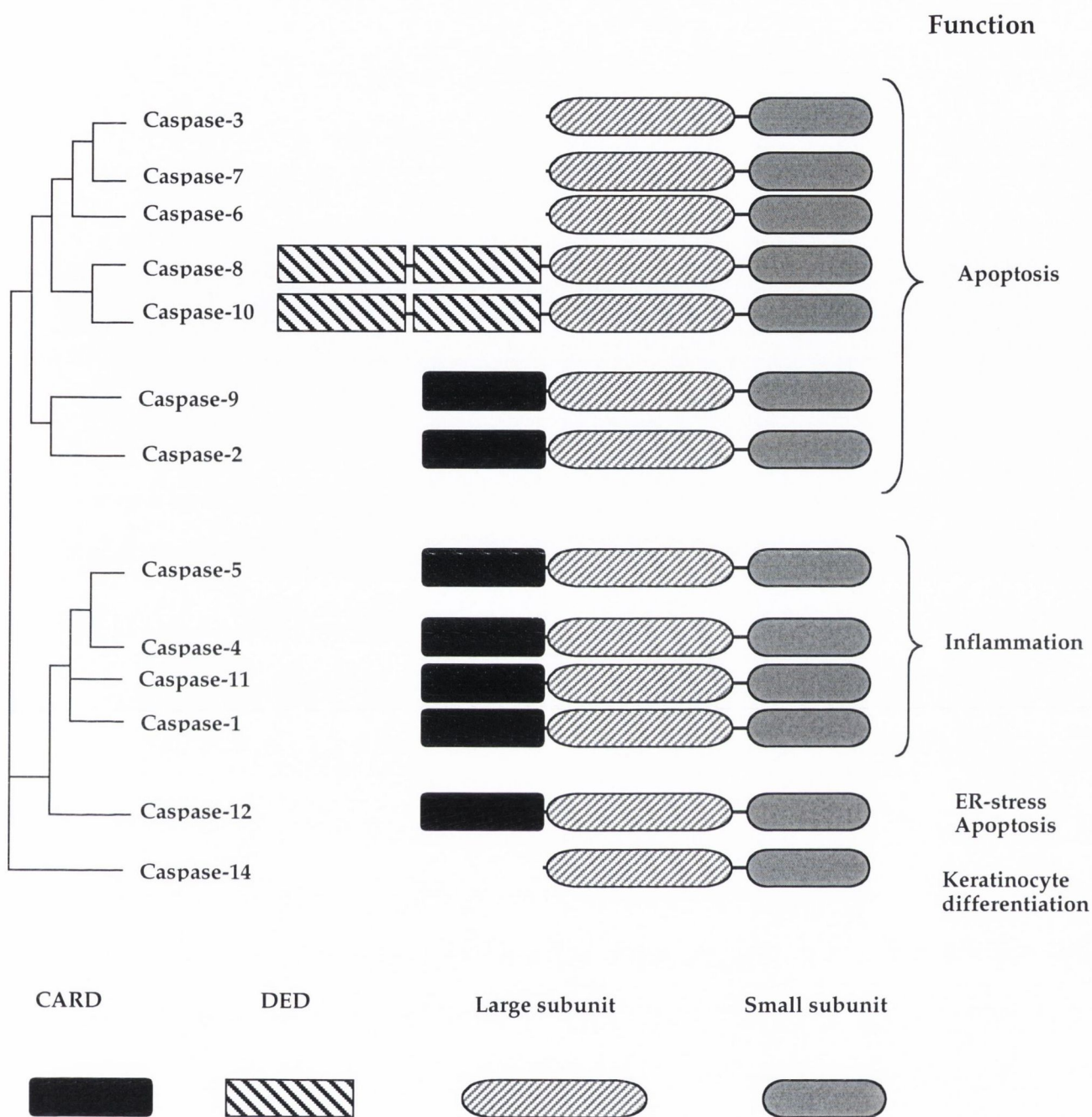


Fig 1.3. Schematic representation of the mammalian caspases adapted from Shi, 2002. Phylogenetic relationship of the mammalian caspases is illustrated on the left. The domain structure and proposed biological function of these proteases is highlighted on the right.

This sub-family includes caspases -2, -3, -6, -7, -8, -9 and -10. These caspases are directly involved in apoptosis. The prototype of this group is *C. elegans* CED-3 (Earnshaw *et al.*, 1999; Lamkanfi *et al.*, 2002).

Caspases-12 and -14 are also recognised members of the mammalian caspase family. Caspase-12, a murine caspase is believed to play a role in ER-stress induced apoptosis (Nakagawa *et al.*, 2000). However, the human homolog of caspase-12 does not appear to be expressed by the majority of individuals and therefore the role played by this protease in mammalian apoptosis is questionable (Fischer *et al.*, 2002). Caspase-14 is expressed in differentiating keratinocytes and is believed to participate in a unique form of programmed cell death in the skin (Chien *et al.*, 2002)

The CED-3 subfamily may be further subdivided on the basis of the length of their prodomains. Caspases that harbour long prodomains are believed to be involved in the initiation of apoptosis whereas those containing short prodomains are primarily involved in the execution or demolition phase (Earshaw *et al.*, 1999; Nicholson, 1999). Therefore the “initiators” include caspases -2, -8, -9 and -10 and the “executioners” include -3, -6 and -7. The initiator caspases contain either a DED (death effector domain) or a CARD (caspase recruitment domain) in their long prodomains (Earshaw *et al.*, 1999; Nicholson, 1999). Caspases -8 and -10 contain DEDs and caspases -2 and -9 contain CARDs (Fig 1.3). These domains are important for promoting interactions of these caspases within multimeric complexes, which ultimately leads to their activation (Li *et al.*, 1997; Medema *et al.*, 1997a; Srinivasula *et al.*, 1998). The executioner caspases are activated by the initiator caspases and are responsible for the majority of cleavage events which results in the eventual demise of the cell (Earshaw *et al.*, 1999; Nicholson, 1999). Interestingly, recent evidence suggests that the previous classification of caspases as “initiator” versus “executioner” somewhat oversimplifies a very diverse group. In certain instances the “initiator” caspases, such as caspase-8 and caspase-10 may be activated in a cascade-like manner by the “executioner” caspases (Slee *et al.*, 1999a). Indeed it has also been demonstrated that caspase-3 is the crucial caspase with respect to the cleavage of cellular substrates in the demolition phase of apoptosis (Slee *et al.*, 2001).

Evidence from *in vitro* combinatorial screening experiments suggests that all caspases recognise short tetrapeptide sequences within their target substrates (Thornberry *et al.*, 1997). These can be designated P₄-P₃-P₂-P₁. As previously stated, caspases cleave their substrates after aspartic acid residues and hence aspartic acid is required in the P₁ position. Caspases tend to prefer glutamic acid in the P₃ position. The residues required for substrate hydrolysis in P₂ and P₄ tend to vary from caspase to caspase and P₄ is believed to be the critical residue in determining the specificity of the cleavage reaction. As a result caspases may also be divided on the basis of the residue they tolerate in this P₄ position (Nicholson, 1999).

- Group I – caspases-1, -4 and -5.

These prefer bulky hydrophobic residues in P₄, such as tyrosine or tryptophan. Few, if any of the substrates cleaved during apoptosis contain such residues in the P₄ position and this is consistent with the role of these caspases in cytokine processing (Thornberry *et al.*, 1997; Nicholson, 1999).

- Group II – caspases-2, -3 and -7.

These require aspartic acid in P₄. Therefore, the preferred cleavage site of Group II caspases is DEXD (Thornberry *et al.*, 1997; Nicholson, 1999). This motif appears in many substrates cleaved during apoptosis and hence concurs with the observation that caspase-3 is responsible for significant substrate cleavage during cell death (Slee *et al.*, 2001).

- Group III – caspases-6, -8, -9 and -10.

This group prefers branched-chain aliphatic residues in P₄, such as valine, leucine and isoleucine. These residues are found at the processing sites in most group II and III caspases. Again this is in agreement with the classification of caspases-8, -9 and -10 as initiator caspases (Thornberry *et al.*, 1997; Nicholson, 1999).

1.2.3. Caspase structure

Caspases are synthesised as single chain inactive precursors – zymogens. They possess an N-terminal prodomain followed by a large and small subunit (Fig 1.4).

Generally, the caspase zymogen is activated by cleavage at internal aspartic acid residues. These cleavage events typically occur between (a) the prodomain and large subunit and (b) the large subunit and small subunit (Earnshaw *et al.*, 1999). The crystallographic X-ray structures of active caspase-1 (Walker *et al.*, 1994; Wilson *et al.*,

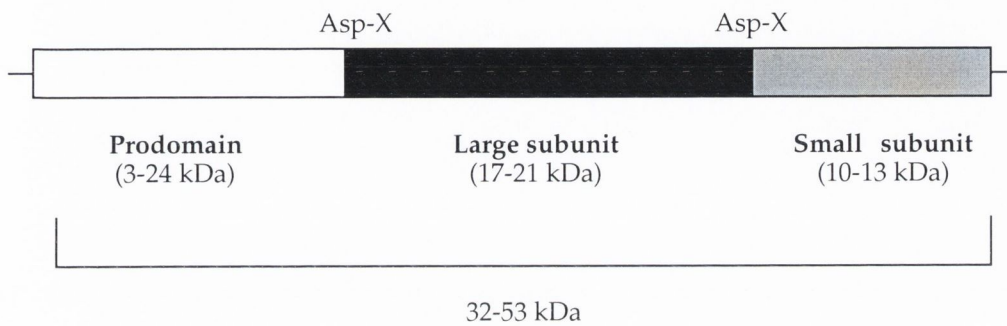


Fig 1.4. Procaspase domain structure

Caspases are synthesized as single chain precursors. The prodomain, large and small subunits are indicated. The range in sizes in kDa that these respective subunits comprise in different caspase members is shown. Cleavage at the indicated Asp-X bonds occurs during the proteolytic maturation of the pro-enzyme.

1994) caspase-3 (Rotonda *et al.*, 1996; Mittl *et al.*, 1997) and caspase-8 (Blanchard *et al.*, 1999) reveal that the active enzyme is a tetramer containing two large and two small subunits. The active sites are at opposite ends of the tetramer and are formed by four protruding loops (Fig 1.5).

The general characteristics of the active site are:

- The large subunit contains the active site cysteine residue.
- Both subunits contribute residues to form the substrate binding cleft – S_4 - S_3 - S_2 - S_1 . These sites bind the cleavage site of the target (P_4 - P_3 - P_2 - P_1).
- As P_4 is the major residue in determining substrate specificity, its binding site S_4 is also a critical factor. S_4 is found in the small subunit (Earnshaw *et al.*, 1999; Donepudi and Grutter, 2002).

S_4 is a large shallow depression on the protease surface of caspase-1, and accommodates the bulky hydrophobic residues preferred by caspase-1 in the P_4 position (Walker *et al.*, 1994; Wilson *et al.*, 1994). In caspase-3, S_4 is narrow, well defined and hence can accommodate the aspartic acid residue preferred by caspase-3 in this position (Rotonda *et al.*, 1996; Mittl *et al.*, 1997). Caspase-8 is slightly different however, as it appears that both S_3 and S_4 are critical in determining the substrate cleavage site required by this caspase (Blanchard *et al.*, 1999).

1.2.4. Caspase cleavage and activation

The crystal structure of procaspase-7 in comparison to active caspase-7 reveal important facts regarding the conformational changes required for procaspase activation (Chai *et al.*, 2001b; Riedl *et al.*, 2001a). Four loops, L_1 , L_2 , L_3 and L_4 form the substrate-binding groove on caspases. L_1 and L_4 form the sides of the groove. L_3 is located at its base. L_2 contains the catalytic cysteine residue and is located at one end of the groove with the side chain of cysteine pointing along the base of the groove (Chai *et al.*, 2001b; Riedl *et al.*, 2001a). At the active site, loops L_2 and L_4 from one subunit are stabilized by the N-terminus of the small subunit of the other heterodimer (loop L_2'), forming the so-called “loop bundle”. The formation of this structure is critical as the loop bundle stabilizes the position of L_2 in the active site and also contributes to the overall shape of the catalytic groove (Chai *et al.*, 2001b; Riedl *et al.*, 2001a). In the procaspase-7 zymogen the loop bundle is completely dissolved as L_2' is locked in a closed configuration (Chai *et al.*, 2001b; Riedl *et al.*,

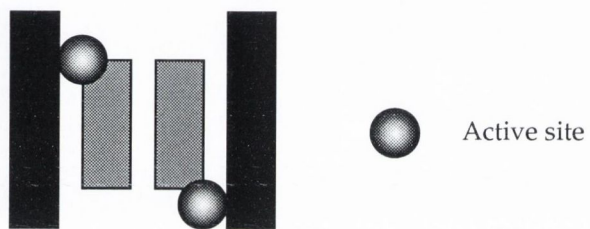


Fig 1.5. Active Caspase Dimer adapted from Donepudi and Grutter, 2001.

The active caspase enzyme is a heterodimer, comprising two large and two small subunits. Each separate subunit contains an active site that binds the target substrate. These active sites are diametrically opposed in the dimer. Each respective large and small subunit collectively contribute residues to form the substrate binding cleft.

2001a). Cleavage at aspartic acid-198 and subsequent substrate binding frees this loop, allowing it to rotate 180° and form the loop bundle. Cleavage at internal aspartic acid residues is critical to allow the active site of a caspase achieve the correct configuration for subsequent substrate hydrolysis. It is believed that this mechanism of activation holds true for all caspases (Chai *et al.*, 2001b; Riedl *et al.*, 2001a; Shi, 2002).

More recent studies however, have questioned whether cleavage is actually necessary to achieve catalytic competency, particularly in the instances of apical caspase activation. For example, apical caspase-8 activation may be explained by alternative models

- Induced proximity model and
- Allosteric Regulation Model.

In the induced proximity model the intrinsic catalytic activity of caspase-8 is believed to facilitate proteolytic processing and release of the active enzyme (Medema *et al.*, 1997a; Muzio *et al.*, 1998). However, in the allosteric regulation model dimerization is the critical event and interchain proteolysis is deemed unnecessary to achieve caspase-8 activation (Boatright *et al.*, 2003; Donepudi *et al.*, 2003). The debate regarding caspase cleavage as a means of achieving catalytic activity is discussed further in chapter V.

1.3. CASPASE ACTIVATION PATHWAYS

Many diverse stimuli promote apoptosis by engaging the core apoptotic machinery i.e. the caspases, but the exact nature of many of the upstream pathways leading to caspase activation has yet to be fully elucidated. However, it is known that many stimuli engage one of the pathways illustrated in Fig 1.6.

1.3.1. Mitochondrial pathway of Caspase Activation

Mitochondria act as important sensors of cellular damage. Various forms of stress such as DNA damage, heat shock and oxidative stress result in an increase in the permeability of the outer mitochondrial membrane. This allows cytochrome *c* to be released from the mitochondrial intermembrane space into the cytoplasm (Liu *et al.*, 1996; Adrain and Martin, 2001). In the cytoplasm cytochrome *c* associates with Apaf-1 in the presence of dATP or ATP. The resulting conformational change in Apaf-1

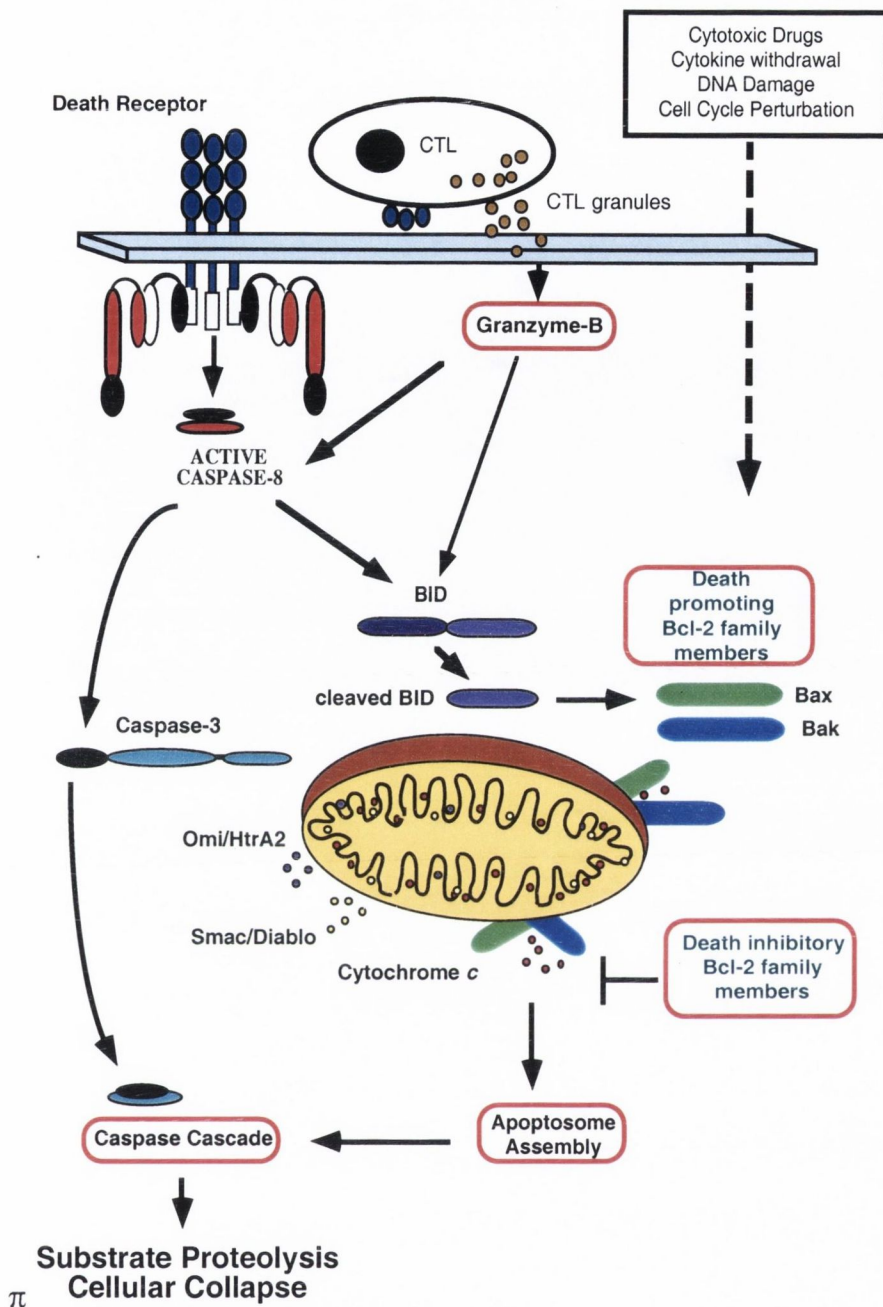


Fig 1.6. Caspase Activation Pathways, adapted from Adrain and Martin, 2001

(A) Engagement of plasma membrane-associated death receptors results in recruitment and activation of pro-caspase-8 via the adaptor molecule FADD. In type I cells, active caspase-8 can directly activate procaspase-3. Active caspase-3 can then initiate a caspase activation cascade. In type II cells, the reduced level of activated caspase-8 cleaves the BH3-only protein Bid. This 15kDa fragment, tBid, activates the mitochondrial pathway by stimulating cytochrome *c* release via Bax and/or Bak oligomerization and insertion into the outer mitochondrial membrane. Once in the cytoplasm cytochrome *c* promotes apoptosome assembly and thus caspase activation. (B)

Cytotoxic lymphocytes (CLs) can kill via the release of the serine protease, granzyme B into the cytoplasm of their target cells. Granzyme B can then either directly activate caspases or promote caspase activation via Bid cleavage. (C) Diverse forms of cellular stress, (DNA damage, cytotoxic drugs, cytokine withdrawal), may trigger the release of cytochrome *c* from mitochondria via the death-promoting Bcl-2 family members, such as Bax and/or Bak, oligomerization and insertion into the outer mitochondrial membrane. Cytochrome *c* release from mitochondria is inhibited by the death inhibitory Bcl-2 family members, such as Bcl-2 and Bcl-x. Cytochrome *c* is not the only pro-apoptotic molecule released from the mitochondria. Smac/Diablo and Omi/HtrA2 are also released.

promotes its oligomerization (Li *et al.*, 1997; Zou *et al.*, 1997; Hu *et al.*, 1999; Zou *et al.*, 1999). Procaspase-9 molecules can then bind to each of the Apaf-1 monomers via CARD-CARD interactions. This multi co-factor complex is called the apoptosome and its formation promotes caspase-9 activation (Li *et al.*, 1997; Zou *et al.*, 1997; Srinivasula *et al.*, 1998; Hu *et al.*, 1999; Zou *et al.*, 1999). The 3-dimensional structure of the apoptosome suggests there are approximately 7 Apaf-1 monomers in the apoptosome, each of which may have a caspase-9 dimer bound to it (Acehan, 2002). Upon activation, mature caspase-9 remains bound to the apoptosome and propagates the death signal through activation of a caspase cascade, illustrated in Fig 1.7, (Srinivasula *et al.*, 1998; Rodriguez and Lazebnik, 1999; Slee *et al.*, 1999). Caspase-3 and caspase-7 are the first caspases that are cleaved and activated by caspase-9 (Srinivasula *et al.*, 1998; Rodriguez and Lazebnik, 1999; Slee *et al.*, 1999b). Caspase-3 in turn cleaves and activates caspases-2 and -6 and the latter then promotes processing of caspases-8 and -10 (Slee *et al.*, 1999b). Caspase-3 also participates in a feed-back amplification loop to further process caspase-9 (Srinivasula *et al.*, 1998; Slee *et al.*, 1999b).

Once caspase-9 is activated there is rapid amplification of the death signal through a cascade of activated caspases. This important cascade has only ever been deduced in one particular cell line (Slee *et al.*, 1999b) and will be investigated further in chapter III. Unlike other caspases, the activation of caspase-9 does not appear to require inter-chain cleavage (Rodriguez and Lazebnik, 1999). Caspase-9 activation is believed to be achieved through recruitment to the apoptosome. Caspase-9 is recruited to the apoptosome via CARD-CARD interactions between the prodomain of caspase-9 and the NH₂-terminus of Apaf-1 (Qin *et al.*, 1999). There are believed to be seven Apaf-1 monomers in any one apoptosome and hence apoptosome formation results in a dramatic increase in the local concentration of caspase-9 monomers. This results in dimer formation between bound caspase-9 and free caspase-9 monomers found in the cytoplasm. It is this formation of dimers that is thought to be responsible for caspase-9 activation (Renatus *et al.*, 2001; Acehan *et al.*, 2002). The crystal structure of the active enzyme also revealed that only half of the active sites of the caspase-9 dimer adopt the correct configuration to achieve catalytic competency (Renatus *et al.*, 2001). It must be remembered that despite the apparent

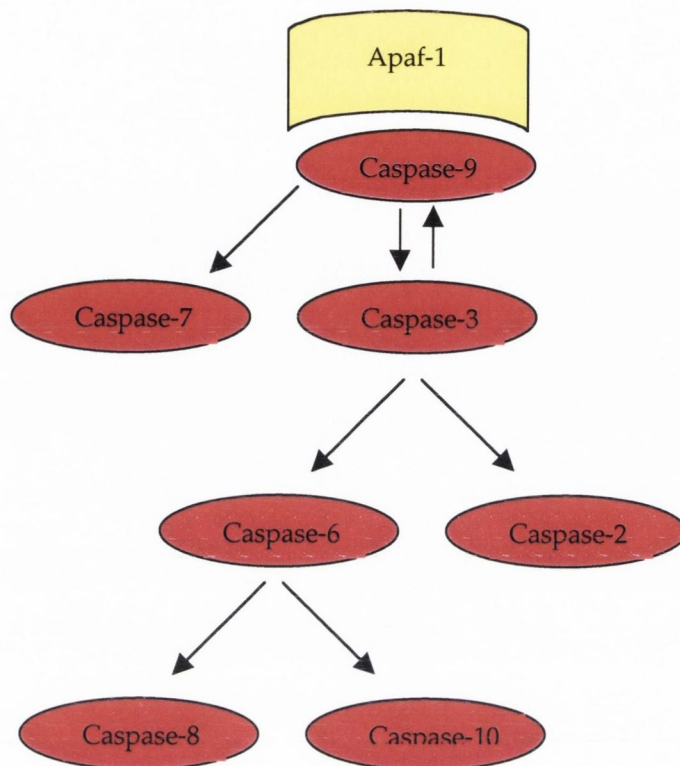


Fig 1.7. Apoptosome-mediated caspase cascade (Slee *et al.*, 1999b)

Caspase-3 and caspase-7 are the first caspases that are cleaved and activated by apoptosome-activated caspase-9. Caspase-3 in turn cleaves and activates caspases-2 and -6 and the latter then promotes activation of caspases-8 and -10. Caspase-3 also participates in a feed-back amplification loop to further process caspase-9.

ability of caspase-9 to forgo cleavage as a mechanism of activation, caspase-9 must be cleaved in order to be inhibited (Srinivasula *et al.*, 2001).

Apaf-1 Structure and Apoptosome assembly

Apaf-1 is a human homolog of *C. elegans* CED-4 (Zou *et al.*, 1997). As is illustrated in Fig 1.8, Apaf-1 consists of three functional domains an N-terminal CARD, responsible for binding caspase-9, a central CED-4 homology domain that facilitates nucleotide binding and oligomerization and a C-terminal region consisting of 12-13 WD-40 repeats (Zou *et al.*, 1997; Adrain *et al.*, 1999).

This C-terminal domain is an important negative regulator of Apaf-1 function. A truncated Apaf-1, lacking the WD-40 repeats constitutively promotes apoptosis and fails to bind cytochrome *c*. Simultaneously, addition of cytochrome *c* and dATP to cell-free extracts can overcome the inhibitory properties of the WD-40 region. These results suggest that cytochrome *c* triggers apoptosome assembly by interacting with the WD40 repeats of Apaf-1 (Hu *et al.*, 1998; Srinivasula *et al.*, 1998; Adrain *et al.*, 1999).

The 3-D structure of the apoptosome has revealed that the apoptosome is a wheel-like particle comprising of 7 Apaf-1 monomers (Fig 1.9 A). Each Apaf-1 monomer adopts a Y-conformation in the apoptosome. The WD40 repeats form the lobes of the Y. The CARD domain forms the base of the Y and is involved in the formation of a central hub (Fig 1.9 B) (Acehan *et al.*, 2002). Analysis of this 3-D structure, in conjunction with molecular modelling, provides us with a plausible explanation of the role of cytochrome *c* in apoptosome formation. The region between the two lobes of the Y is large enough to accommodate either a single cytochrome *c* or the CARD domain itself. It is hypothesised that in auto-inhibited Apaf-1, the CARD domain is buried between these lobes. Once cytochrome *c* binds to this region however, the CARD is released. Nucleotide binding within the NBD is proposed to then lock the Apaf-1 monomer in this extended conformation, allowing the CARD to recruit procaspase-9 to the central hub of the apoptosome. (Acehan *et al.*, 2002).

Cytochrome *c* plays an indispensable role in the mitochondrial pathway of caspase activation. The absolute requirement for cytochrome *c* in this pathway appears to be

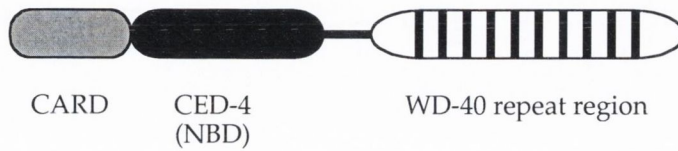


Fig 1.8. Domain structure of Apaf-1

Apaf-1 consists of three functional domains an N-terminal CARD, responsible for binding caspase-9, a central CED-4 homology domain that facilitates nucleotide binding and oligomerization and a C-terminal region consisting of 12-13 WD-40 repeats.

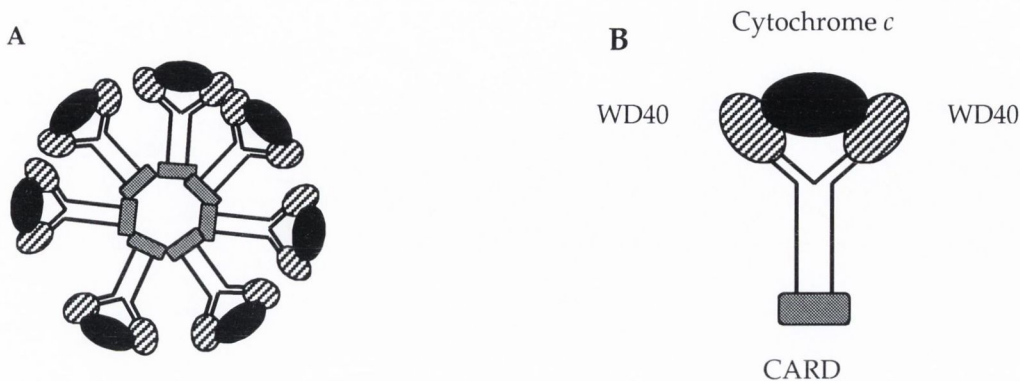


Fig 1. 9A. 7 Apaf-1 monomers arranged in the wheel-like apoptosome (Acehan *et al.*, 2002)

Analysis of the 3-D structure of the apoptosome has proposed that the apoptosome is a wheel-like particle comprising of 7-Apaf-1 monomers. Caspase-9 is believed to interact with the central hub formed by the CARD domains of Apaf-1.

Fig 1.9B. Single Apaf-1 monomer of apoptosome in complex with cytochrome *c*

Each Apaf-1 monomer adopts a Y-conformation in the apoptosome. The WD40 repeats form the lobes of the Y. The CARD domain forms the base of the Y and is involved in the formation of the central caspase-9-binding hub. The region between the two lobes of the Y is large enough to accommodate either a single cytochrome *c* or the CARD domain itself. It is speculated therefore that in auto-inhibited Apaf-1, the CARD domain is buried between these lobes. Once cytochrome *c* binds to this region however, the CARD is released. Nucleotide binding is proposed to then lock the Apaf-1 monomer in this extended conformation, allowing the CARD to recruit procaspase-9 to the central hub of the apoptosome.

unique to mammalian systems. *C. elegans* does not require cytochrome *c* for apoptosome formation, nor does *Drosophila* (Dorstyn *et al.*, 2002; Martin, 2002; Zimmermann *et al.*, 2002). Recent studies in the fly have proposed that cytochrome *c* may be required for caspase activation during sperm differentiation (Arama *et al.*, 2003). The release of cytochrome *c* from mitochondria is generally recognised as the “point of no return” in the life of a cell (Fig 1.6). Studies using GFP- cytochrome *c* have highlighted, that once release is initiated cytochrome *c* is released from all mitochondria in a cell within approximately five minutes (Waterhouse *et al.*, 2002). With few exceptions, this rapid release seals the fate of the cell. The tightly regulated process of cytochrome *c* release is primarily achieved by members of the Bcl-2 family (Adams and Cory, 1998).

Regulation of cytochrome c release by Bcl-2 family proteins

Bcl-2 is the functional and structural homolog of the *C. elegans* protein, CED-9 (Hengartner and Horvitz, 1994). Cell death in *C. elegans* is inhibited by CED-9 (Fig 1.2; Hengartner and Horvitz, 1994). However, individual members of the mammalian Bcl-2 family, can be classified as either pro or anti-apoptotic (Fig 1.10). All members of the family contain at least one of four Bcl-2 homology (BH) domains, designated BH1, BH2, BH3 and BH4 (Fig 1.10). Some members also contain a transmembrane domain that tethers these proteins to the mitochondrial membrane (Adams and Cory, 1998; Kaufmann and Hengartner, 2001). Most anti-apoptotic members contain at least BH1 and BH2 and those most similar to Bcl-2, such as Bcl-x_L contain all 4 BH domains.

There are two distinct pro-apoptotic sub-families: Bax sub-family and the “BH3-only” proteins. Bax, Bak and Bok, which are very similar to Bcl-2 except they lack a BH4 domain. The “BH3-only” proteins such as Bik, Bim, Bad, Blk and Bid. These proteins just contain a central BH3 domain that is essential for their killing activity. The “BH3-only” proteins are a very diverse family. Even within this sub-family only Blk and Bik resemble each other (Adams and Cory, 1998; Kaufmann and Hengartner, 2001).

Bcl-2 and Bcl-x_L are potent inhibitors of apoptosis. These proteins act at the level of the mitochondria to prevent cytochrome *c* release (Heiden *et al.*, 1997; Kluck *et al.*,

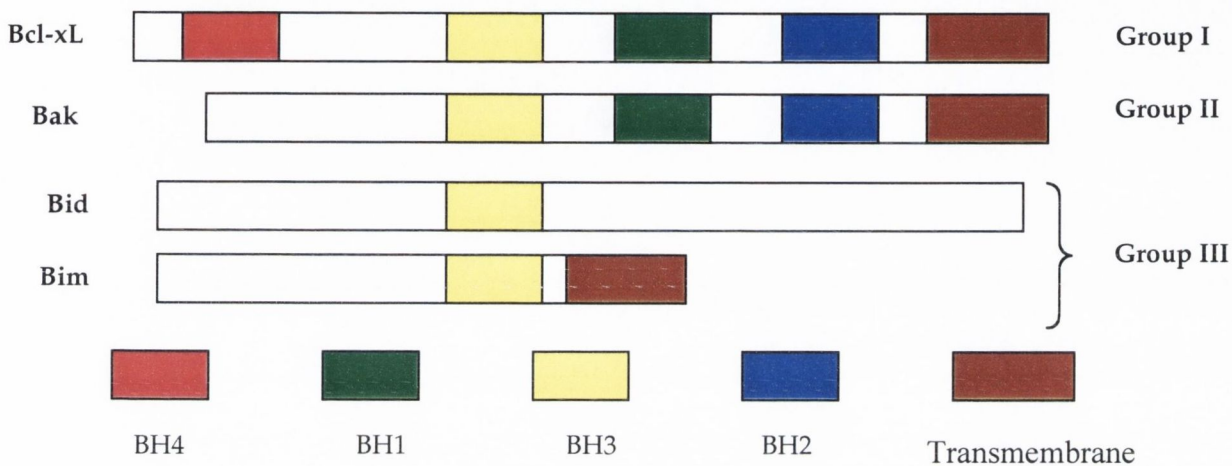


Fig 1.10. Schematic representation of some Bcl-2 family members

Bcl-2 family members are the key regulators of cytochrome *c* release from the mitochondria. As is illustrated, all members of the family contain at least one of four Bcl-2 homology (BH) domains, designated BH1, BH2, BH3 and BH4. Some members also contain a transmembrane domain that tethers these proteins to the mitochondrial membrane. Group I members are anti-apoptotic and contain at least BH1 and BH2 and those most similar to Bcl-2, such as Bcl-x_L, contain all 4 BH domains. There are two very distinct pro-apoptotic sub-families, group II and group III. Group II include Bax, Bak and Bok, which are very similar to Bcl-2 except they lack a BH4 domain. Group III are referred to as the “BH3-only” proteins such as Bik, Bim, Bad, Blk and Bid. These proteins just contain a central BH3 domain that is essential for their killing activity. The “BH3-only” proteins are a very diverse family.

1997; Yang et al., 1997). They are believed to achieve this by antagonizing the formation of pores in the outer mitochondrial membrane. These pores may be formed by the pro-apoptotic members of the Bcl-2 family Bak and Bax (Ferri and Kroemer, 2001; Korsmeyer *et al.*, 2000). In healthy cells, monomeric Bax is found in the cytosol or loosely attached to membranes. Upon receiving a death stimulus however, Bax translocates to the mitochondria and oligomerizes within the mitochondrial membrane, forming pores to facilitate cytochrome *c* release. Bak, which is constitutively found in the outer mitochondrial membrane, can also be triggered to oligomerize within the membrane and form pores (Fig 1.6; Ferri and Kroemer, 2001; Korsmeyer *et al.*, 2000). Bax and Bak oligomerization appears to be triggered by the post-translational modification of the BH3-only proteins (Korsmeyer *et al.*, 2000). The BH3-only-family is the mammalian homolog of EGL-1 in *C. elegans* (Conradt and Horvitz, 1998). Individual members of the mammalian BH3-only family act in cell type-specific manner (Huang and Strasser, 2000). These proteins promote apoptosis not only by directly inducing the oligomerization of the Bax-like proteins but also by facilitating oligomerization through inactivation of the Bcl-2-like proteins (Luo *et al.*, 1998; Yin *et al.*, 1999; Verma *et al.*, 2001; Wei *et al.*, 2001; Bouillet *et al.*, 2002; Kuwana *et al.*, 2002; Gillissen *et al.*, 2003).

1.3.2. Death receptor-mediated caspase activation

Death receptors play a critical role in apoptosis within the immune system. Death receptors belong to the tumor necrosis factor receptor (TNFR) superfamily. This family is characterised by the presence of cysteine rich domains (CRDs) in the extracellular domain of all members of this family (Ashkenazi and Dixit, 1998). Death receptors are expressed as pre-associated homotrimers on the cell surface. Trimerization is dependent upon the first CRD, within a region termed the PLAD or preligand binding assembly domain (Papoff *et al.*, 1999; Chan *et al.*, 2000; Siegel *et al.*, 2000). Trimerization of death receptors is critical for their function and mutations within the PLAD can inhibit the normal operation of the receptors (Chan *et al.*, 2000; Siegel *et al.*, 2000). Death receptors also contain a second domain within their cytoplasmic tails termed the death domain (DD). This protein interaction domain of approximately 80 amino acids is critical for allowing these receptors to transmit the death signal. Members of this death receptor family include CD95 (Apo-1/Fas), TNF

receptor I (TNFR-I), TRAIL receptor I (DR4) and TRAIL receptor 2 (DR5) (Ashkenazi and Dixit, 1998).

CD95/Fas

One of the best characterized members of the death receptor family is Fas/CD95 (Peter and Krammer, 2003). Fas/CD95 is a 317 amino acid transmembrane glycoprotein. Fas/CD95 and its ligand FasL/CD95L play an important role in three types of apoptosis, (a) killing of virus-infected cells or transformed cells by cytotoxic lymphocytes, (b) killing of inflammatory cells at “immune-privileged” sites such as the eye and (c) elimination of activated T-cells (Ashkenazi and Dixit, 1998).

The *lpr* (lymphoproliferation) and *gld* (generalized lymphoproliferative disease) defects in mice correspond to mutations in the genes encoding Fas/CD95 and FasL/CD95L respectively and unsurprisingly, are characterized by similar phenotypes, resulting in autoimmune-related disease (Watanabe-Fukunaga *et al.*, 1992; Takahashi *et al.*, 1994). In humans, mutations within the PLAD of Fas/CD95 are found in individuals with the autoimmune disorder ALPS – autoimmune lymphoproliferative syndrome – type 1a, and lymphocytes from these patients are resistant to Fas/CD95-induced apoptosis (Martin *et al.*, 1999; Siegel *et al.*, 2000). Mutations within FasL/CD95L are found in ALPS type 1b (Jackson and Puck, 1999). ALPS is a fatal autoimmune syndrome and therefore understanding how apoptosis is mediated by death receptors is critical for the treatment of such diseases. Upon ligand binding, Fas/CD95 transmits the death signal through its DD (Itoh *et al.*, 1993; Fig 1.6). In a yeast-two-hybrid screen the death domain of Fas was shown to bind to a second death domain containing protein, FADD, or Fas-associated protein with a death domain (Boldin *et al.*, 1995). Overexpression of full-length FADD induced apoptosis. It was discovered that the induction of death was not dependent on the death domain but on an N-terminal domain of about 80 amino acids termed the death effector domain (Boldin *et al.*, 1995). In 1995, Kischkel *et al.* reported the formation of a complex termed the DISC – death-inducing signaling complex - within seconds of CD95/Fas engagement. At the DISC the death domain of the activated receptor binds the death domain of FADD. FADD in turn recruits molecules to the DISC via death effector domain-death effector domain interactions. Caspase-8 was the first caspase to be identified as a member of the DISC (Boldin *et*

al., 1996; Muzio *et al.*, 1996). Caspase-8 contains two tandem death effector domains in its prodomain and these allow the enzyme to interact with FADD at the DISC (Medema *et al.*, 1997a). Two isoforms of the caspase-8 protein exist caspase-8/a and caspase-8/b. Both are recruited to the DISC (Scaffidi *et al.*, 1997). Caspase-8 recruitment to the DISC results in the activation of this protease (Fig 1.6; Medema *et al.*, 1997a). Caspase-8 processing and activation at the DISC had been proposed to be due to the increase in local concentration of the enzyme which facilitated autoproteolytic cleavage (Medema *et al.*, 1997a; Muzio *et al.*, 1998; Salvesen and Dixit 1999). There has recently been much debate over the exact mechanism of caspase-8 activation at the DISC (Boatright *et al.*, 2003; Donepudi *et al.*, 2003). Efforts to resolve this issue are included in the results section of this thesis and therefore caspase-8 activation pathways will be discussed in greater detail in Chapter V.

Once caspase-8 is activated the death of the cell is imminent. The exact mechanisms exerted by caspase-8 to achieve this result is cell-type dependent and hinges on the quantity of caspase-8 activation achieved at the DISC (Fig 1.6). In type I cells caspase-8 can activate sufficient procaspase-3 without any mitochondrial involvement (Scaffidi *et al.*, 1998). Active caspase-3 can then either cleave procaspase-9 or directly process procaspase-2 and procaspase-6 to initiate the caspase cascade. In type II cells, caspase-8 activation is insufficient to directly activate procaspase-3. Instead this reduced level of active caspase-8 cleaves Bid, to generate active tBid (Li *et al.*, 1998; Luo *et al.*, 1998). This 15kDa fragment activates the mitochondrial pathway by stimulating cytochrome *c* release via Bax and/or Bak oligomerization and insertion into the outer mitochondrial membrane (Wei *et al.*, 2001). Once in the cytosol cytochrome *c* promotes caspase activation as previously described (Fig 1.6). Studies have identified thymocytes and T-cells as type I cells and hepatocytes as type II (Peter and Krammer, 2003).

Undoubtedly, the roles played by caspase-8 and FADD in mammalian systems are critical as both knockout phenotypes are lethal *in utero*. Both phenotypes are similar displaying defective heart muscle development and lymphocyte abnormalities (Varfolomeev *et al.*, 1998; Yeh *et al.*, 1998; Zhang *et al.*, 1998). Caspase-8 and FADD are essential for mediating death signals induced by Fas/CD95 as embryonic fibroblasts derived from both knockout phenotypes and thymocytes isolated from

FADD^{-/-} chimaeras are completely resistant to Fas-induced cell death (Varfolomeev *et al.*, 1998; Yeh *et al.* 1998; Zhang *et al.*, 1998). In addition caspase-8-deficient and FADD-deficient Jurkat cell lines display similar resistance to Fas/CD95-induced apoptosis (Juo *et al.*, 1998; Juo *et al.*, 1999).

It has also been reported that caspase-10 can be recruited to the CD95/Fas DISC in a FADD dependent manner (Kischkel *et al.*, 2001; Sprick *et al.* 2002). Caspase-10 is the only other caspase known to contain death effector domains in its prodomain and therefore closely resembles caspase-8 (Fernandes-Alnemri *et al.*, 1996). Caspase-10 can cleave caspases-3 and -7 *in vitro* (Fernandes-Alnemri *et al.*, 1996) however, the functional importance of caspase-10 at the DISC is still open to some debate. In one report caspase-10 was unable to functionally replace caspase-8 in Fas-induced cell death in a caspase-8 deficient Jurkat cell line (Juo *et al.*, 1998). However, a subsequent study suggested that the antibody used was unreliable as it cross-reacted with Hsp-60 and gave evidence for death receptor recruitment and activation of caspase-10 at the DISC (Kischkel *et al.*, 2001). Another more recent report highlighted that cells devoid of caspase-8 cells were still susceptible to CD95/Fas-mediated death but required far higher concentrations of CD95L/FasL than caspase-10 deficient cells. Therefore, although caspase-10 is recruited and activated at the CD95/Fas DISC it cannot functionally replace caspase-8 (Sprick *et al.*, 2002). Obviously further research is needed to properly resolve the exact role played by caspase-10 at the DISC. This is complicated by the fact that a murine counterpart for caspase-10 has yet to be identified (Peter and Krammer, 2003).

Another member of the DISC is FLIP (FLICE-inhibitory protein). Viral FLIPs or v-FLIPS can inhibit FADD and caspase-8 and therefore inhibit apoptosis induced by death receptors. v-FLIPS have been identified in several herpesviruses and the tumorigenic human molluscipoxvirus (Bertin *et al.*, 1997; Thome *et al.*, 1997). The human homolog of the v-FLIPS is c-FLIP or cellular-FLIP. The c-FLIP protein is expressed as two different forms c-FLIP_L and c-FLIP_S – the long and short form respectively. Both have N-terminal death effector domains. c-FLIP_L also contains a C-terminal caspase-like domain which is missing critical residues, such as the active site cysteine, and therefore is not catalytically active. c-FLIP_L closely resembles caspase-8. c-FLIP_S is more structurally related to the v-FLIPS. Both forms are

recruited to the CD95/Fas DISC (Irmeler *et al.*, 1997). In the cell lines examined to date the most abundant isoform is c-FLIP_L (Scaffidi *et al.*, 1999). c-FLIP_S is an inhibitor of CD95/Fas-mediated apoptosis. It has been shown to inhibit procaspase-8 activation at the DISC and hence block CD95/Fas mediated apoptosis in BJAB cells (Krueger *et al.*, 2001). In addition, c-FLIP_S upregulation correlates with resistance to CD95/Fas-mediated apoptosis in reactivated T cells (Kirchoff *et al.*, 2000). The role played by c-FLIP_L at the DISC is open to debate. Studies have revealed that overexpression of c-FLIP_L can both protect cells against and sensitize cells to CD95/Fas-induced death (Irmeler *et al.*, 1997; Scaffidi *et al.*, 1999; Krueger *et al.*, 2001; Chang *et al.*, 2002). In an effort to resolve this debate Chang *et al.*, demonstrated that at low levels of expression, close to endogenous expression levels, c-FLIP_L is capable of facilitating caspase-8 activation at the DISC (Chang *et al.*, 2002). However, at higher levels of expression more c-FLIP_L is recruited to the DISC than pro-caspase-8 (Scaffidi *et al.*, 1999; Chang *et al.*, 2002). The presence of c-FLIP_L at the DISC prevents pro-caspase-8 recruitment and complete activation and hence inhibits death receptor-mediated cell death (Krueger *et al.*, 2001; Scaffidi *et al.*, 1999; Chang *et al.*, 2002). It would appear therefore, that the level of expression of c-FLIP_L in a cell is critical to the resulting sensitivity of the cell to CD95/Fas-induced death. Further investigation is warranted to explore the specific role played by c-FLIP in regulating death and survival signals in different cell and tissue types.

Alternative death receptor pathways: TNFR-I

Caspase-8 activation induced by TNF α engagement of TNFR-I was proposed to proceed in a similar manner to that previously described for Fas/CD95. The only difference was that the DD of the receptor initially recruited the adaptor protein – TRADD (TNF receptor-associated death domain protein) and TRADD in turn recruited FADD and caspase-8 (Ashkenzai and Dixit, 1998). However recent reports have highlights that neither caspase-8 or FADD is recruited to the native TNFR-1 signaling complex upon TNF α binding. However, there is rapid recruitment of other known members of the complex such as, TRADD, RIP and TRAF2 (Harper *et al.*, 2003; Micheau and Tschopp, 2003). TNF signaling via TRAF2 and RIP leads to the activation of NF-KB and JNK/AP1, which in turn induce the production of proinflammatory and immunomodulatory genes (Ashkenzai and Dixit, 1998).

However, it is known that fibroblasts derived from mice in which the *CASPASE-8* gene or the *FADD* gene has been disrupted are resistant to TNF α -induced apoptosis (Varfolomeev *et al.*, 1998; Yeh *et al.* 1998). Such resistance is also mirrored in FADD-deficient and caspase-8-deficient Jurkats (Harper *et al.*, 2003). Therefore, although caspase-8 and FADD are critical for the induction of apoptosis via TNF α , the hypothesis that these molecules are recruited to the same signaling complex as TRADD, RIP and TRAF2 needs to be re-evaluated.

1.3.3. Granzyme B-mediated caspase activation

Natural killer (NK) cells of the innate immune system and cytotoxic T lymphocytes (CTLs) of the adaptive immune system are collectively known as cytotoxic lymphocytes (CL). CLs can engage one of two distinct pathways to kill their target cells – virally infected and transformed cells (Lieberman, 2003).

- Engagement of the Fas/CD95 receptor pathway, as previously discussed.
- Release of cytotoxic granules onto the plasma membrane of target cells. These cytotoxic granules contain deadly arsenal including the membrane-perturbing protein – perforin and granule serine proteases or granzymes. Granzymes A and B are the most abundant granzymes in mice and humans. Of particular interest is granzyme B as it can directly and indirectly activate caspases (Lieberman, 2003). Granzymes A and B are critical in the fight against virally infected cells. Deficiency of either granzyme results in increased susceptibility to the mouse poxvirus, ectromelia and deficiency of both renders the mice incapable of controlling ectromelia infection (Mulbacher *et al.*, 1999). Cytotoxic granule-mediated cell death is also employed in eliminating tumour cells (Smyth *et al.*, 2000).

Perforin is a pore-forming protein and was initially identified from the cytolytic granules of T-lymphocytes (Masson and Tschopp, 1985). Perforin-deficient mice are viable, however the cytotoxicity exhibited by their T-cells and natural killer cells is greatly impaired as perforin is required for the delivery of granzymes into the cytoplasm of target cells (Kagi *et al.*, 1994; Lowin *et al.*, 1994). It was initially hypothesized that perforin facilitated the entry of granzyme B by forming pores on the target cell surface. However, it is now believed that perforin is required for the release of Granzyme B from endosomes once inside the target cell (Froelich *et al.*,

1996; Lieberman, 2003). This hypothesis was proposed as granzyme B, in the absence of perforin, may still gain entry to target cells. An endosomolytic agent such as adenovirus is required to compensate for the loss of perforin (Froelich *et al.*, 1996; Lieberman, 2003). The mannose 6-phosphate receptor (MPR) has been proposed as the cell-surface receptor for granzyme B (Motyka *et al.*, 2000).

Once inside the cell how does granzyme B wreak its havoc? Granzyme B, is a serine protease, which like caspases, cleaves its substrates after aspartic acid residues. Unsurprisingly therefore, granzyme B has been shown to cleave many caspases *in vitro* including caspase-3, -6, -7, -8, -9 and -10 (Chinnaiyan *et al.*, 1996; Duan *et al.*, 1996; Fernandes-Alnemri *et al.*, 1996; Gu *et al.*, 1996; Martin *et al.*, 1996; Muzio *et al.*, 1996; Orth *et al.*, 1996; Quan *et al.*, 1996). Exactly how granzyme B achieves caspase activation *in vivo* is currently an area of intense research and the relative importance of these caspase targets under physiological conditions is still unclear.

Granzyme B engages the mitochondrial pathway of caspase activation by cleaving the BH3-only protein Bid (Barry *et al.*, 2000; Heiben *et al.*, 2000; Sutton *et al.*, 2000;). Granzyme B specifically cuts Bid at Asp75 (Sutton *et al.*, 2000), which is distinct from its caspase cleavage site (Li *et al.*, 1998). This 14kDa truncated molecule, produced by granzyme B cleavage (gtBid), proceeds to translocate to the mitochondrial outer membrane and trigger release of cytochrome *c*. GtBid-mediated release of cytochrome *c* has been shown to be dependent on the presence of Bax and Bak in the outer mitochondrial membrane (Heiben *et al.*, 2000; Thomas *et al.*, 2001; Wei *et al.*, 2001). A separate study has indicated that during gtBid-induced apoptosome-mediated caspase activation, Bak is primarily responsible for triggering the release of cytochrome *c* from mitochondria (Wang *et al.*, 2001).

Cytochrome *c* is not the only pro-apoptotic molecule released from the mitochondria however. Smac and Omi/HtrA2 are also released (Fig 1.6) and these molecules have recently been proposed as critical regulators of granzyme B-mediated caspase activation (Goping *et al.*, 2003; Sutton *et al.*, 2003). Granzyme B has been shown to be capable of cleaving between the large and small subunit of caspase-3 but the caspase's own autocatalytic activity is required to remove its prodomain to produce the active enzyme (Martin *et al.*, 1996). The studies performed by Trapani and

Bleakley and their colleagues have proposed that *in vivo* the IAPs block this autoactivation of caspase-3 and hence Smac and Omi/HtrA2 release from mitochondria is required to displace the IAPs and allow complete caspase-3 activation (Goping *et al.*, 2003; Sutton *et al.*, 2003).

Embryonal fibroblasts of Bid-deficient mice remain susceptible to granzyme B-mediated cell death, despite the fact these cells do not release any cytochrome *c* (Thomas *et al.*, 2001). Granzyme B may be capable of initiating a cell death pathway, independently of Bid. In this regard, granzyme B has been shown to directly activate caspases-3 and -8 in intact cells (Chinnaiyan *et al.*, 1996; Froelich *et al.*, 1996; Medema *et al.*, 1997b; Atkinson *et al.*, 1998; Yang *et al.*, 1998). A more recent study from Metkar *et al.*, has proposed that in fact caspase-3 is the primary target of granzyme B. These authors claim that Bid cleavage is not even detected if attempts are taken to inhibit the protease during the solubilization of granzyme B-treated cells. This report reinstates caspase-3 as the essential mediator of granzyme B-initiated cell death (Metkar *et al.*, 2001). While the debate concerning the activation of caspases by granzyme B continues, it is certain that be it directly or indirectly, caspase activation via granzyme B results in the demise of the cell.

1.4. CASPASE REGULATION

Inadvertent caspase activation in a cell would wreak uncontrolled havoc as a result of spontaneous substrate cleavage. Therefore, cellular caspases are subjected to tight regulation. Both initiator and executioner caspases are synthesized as inactive precursors – zymogens. The initiator caspases essentially control the activation of the executioners and the activation of the initiators is determined by the formation of “activating scaffolds,” such as the DISC and the apoptosome. This strategy may be regarded as the first line of defence in a cell in order to prevent undesirable caspase activity (Earnshaw *et al.*, 1999; Martin, 2002).

1.4.1. IAPS

The second line of defense is in the guise of a family of proteins termed the IAPs or inhibitors of apoptosis proteins. IAPs were first discovered in baculoviruses as a result of a screen to identify genes that were functionally equivalent to the pan-caspase inhibitor p35 (Crook *et al.*, 1993; Birnbaum *et al.*, 1994). The defining

characteristic of the IAP family is the presence of a 70 amino acid zinc-binding fold termed the BIR (baculovirus IAP repeat) domain. BIR-containing proteins have since been discovered in *C. elegans*, yeast, mammals and insects. While a BIR domain is critical for an IAP to mediate its anti-apoptotic effects not all BIR-containing proteins (BIRPs) are IAPs (Miller, 1999). Fig 1.11 highlights the currently known members of the human IAP family. As is evident, all members contain 1-3 BIR domains, while others also share a C-terminal RING domain.

Survivin and Bruce only possess a single BIR. Survivin is expressed during mitosis and its primary role is in the regulation of cell division (Verhagen *et al.*, 2001). The importance of the role of survivin is highlighted by the fact that *SURVIVIN* knockout mice die *in utero* (Uren *et al.*, 2000). The ability of survivin to regulate cell division is a property shared with the BIRPs found in yeast and *C. elegans*. The BIR of survivin and Bruce is slightly larger than the BIR domain found in other mammalian IAPs and more closely resembles the BIR domains found in yeast and *C. elegans*. The function of Bruce is unknown but the presence of this larger BIR suggests that the role of Bruce is also in the regulation of cell division rather than the inhibition of cell death (Miller, 1999; Verhagen *et al.*, 2001). It is hypothesised that the original function of BIRPs was in the regulation of cell division and that their role as inhibitors of cell death arose later in evolution (Clem, 2001).

Of the remaining IAPs, XIAP, c-IAP1 and c-IAP2 are the most extensively studied. XIAP is the most potent inhibitor of cell death, which is attributed to its direct inhibition of caspases -3, -7 and -9 (Deveraux *et al.*, 1997; Deveraux *et al.*, 1998). c-IAP1 and c-IAP2 were originally identified as components of the TNF receptor 2 complex (Rothe *et al.*, 1995). These IAPs have also been reported to inhibit caspases -3, -7 and -9 (Roy *et al.*, 1997; Deveraux *et al.*, 1998). The inhibitory effects of XIAP are 100-1000-fold more potent than c-IAP1 or c-IAP2 however (Roy *et al.*, 1997; Deveraux *et al.*, 1998), (Fig 1.12). Structural data has provided important insights into the exact mechanisms of XIAP-mediated inhibition. The inhibition of the executioner caspases by XIAP differs fundamentally from the inhibition this IAP mediates on caspase-9. The BIR2 domain of XIAP was initially identified as the critical domain for caspase-3 and -7 inhibition (Takahashi *et al.*, 1998). It was later revealed that a linker region between BIR1 and BIR2 was also critical for this

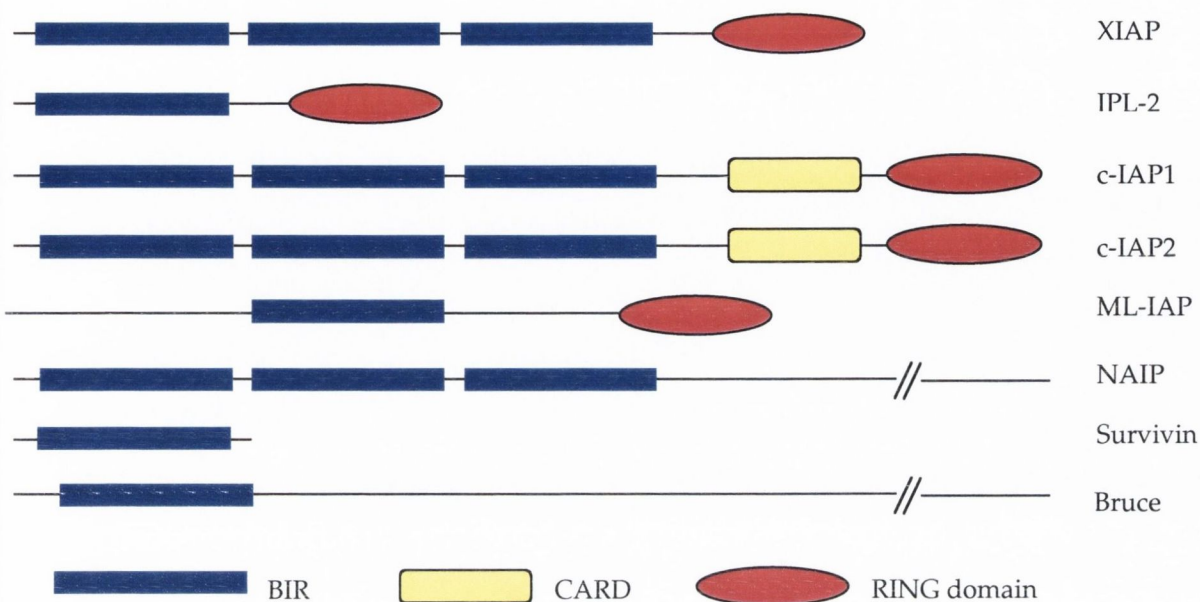


Fig 1.11. Schematic representation of the mammalian BIR-containing proteins (BIRPs), adapted from Salvesen and Duckett, 2002

All BIRPs contain between 1-3 BIR domains, while others also share a C-terminal RING domain. Survivin and Bruce are not believed to play a direct role in apoptosis but are believed to be involved in the regulation of cell division. Of the remaining proteins, XIAP c-IAP1 and c-IAP2 are the best characterised inhibitors of apoptosis proteins (IAPs).

inhibition (Sun *et al.*, 1999). More recent crystal structures of the BIR2-linker region of XIAP in complex with either caspase-3 or caspase-7 highlight that this linker segment provides the direct contact points - critical hydrogen bonds and hydrophobic interactions - with each caspase. The linker occupies the active sites of the caspases but in reverse orientation to that of peptidyl inhibitors such as DEVD-fmk, and other substrates (Chai *et al.*, 2001a; Huang *et al.*, 2001; Riedl *et al.*, 2001b). The linker segment is unable to inhibit the caspases on its own, suggesting that the BIR domain is important to help stabilize the interaction between the linker region and the caspase active site. To further support this theory, an experiment replacing the BIR2 region of XIAP with a GST moiety also results in inhibition of caspases-3 and -7 (Chai *et al.*, 2001a; Huang *et al.*, 2001; Riedl *et al.*, 2001b). Close examination of the contact residues in these complexes allows a greater understanding of the specificity of XIAP-mediated caspase inhibition. All of the contact residues in caspase-7 for the linker segment of XIAP are highly conserved in caspase-3, while they are far less conserved in caspase-1 and -8. This explains therefore, why caspases-3 and -7 are capable of being inhibited by XIAP, while caspases-1 and -8 are not (Huang *et al.*, 2001).

In the inhibition of caspase-9 by XIAP the BIR3 domain of XIAP binds to the NH₂-terminus of the small subunit of caspase-9 (Srinivasula *et al.*, 2001). Therefore, there is no direct contact between the BIR3 and the active site of the enzyme. This initial interaction facilitates the creation of a larger protein-protein interface between caspase-9 and the BIR3 of XIAP. Pro 336 and its adjacent residues in caspase-9 play a critical role in stabilizing the active site loops in the structure of caspase-9 homodimers (Renatus *et al.*, 2001). The inhibitory complex region is now involved in stabilizing the interactions between caspase-9 and XIAP. Hence the BIR3 of XIAP traps the active site loops of caspase-9 in a conformation that inhibits caspase-9 from achieving catalytic competency (Shiozaki *et al.*, 2003).

The loss of the fly IAP molecule, DIAP1 in *Drosophila* results in early embryonic death (Wang *et al.*, 1999). In contrast, XIAP-deficient mice are viable. Moreover, cells from the knockout animals respond as wild-type cells to various apoptotic stimuli. However, the levels of c-IAP1 and c-IAP2 are slightly increased in XIAP-deficient mice, which might suggest that the IAPs can compensate for one another *in vivo*

(Harlin *et al.*, 2001). Experiments to date however would not indicate that the IAPs can functionally substitute for one another (Deveraux *et al.*, 1998). Therefore, rather than playing a gross decision-making role in developmental cell death, the IAPs in mammals may be more critical in establishing sensitivity thresholds that regulate the concentration of active caspases present in a cell.

1.5. IAP REGULATION

The IAPs themselves are also subject to regulation. This is primarily achieved by (a) IAP regulatory proteins, (b) self-regulated stability and (c) caspase-mediated cleavage.

1.5.1. IAP Regulatory Proteins

DIAP1 is a *Drosophila* IAP and similar to mammalian IAPs, suppresses apoptosis by binding caspases. The inhibitory effects of DIAP1 are overcome by the *Drosophila* proteins, Reaper, Hid, Grim and Sickie (Wang *et al.*, 1999; Goyal *et al.*, 2000; Christich *et al.*, 2002; Srinivasula *et al.*, 2002; Wing *et al.*, 2002). Reaper, Hid, Grim and Sickie share a region of homology at their NH₂-termini termed the RHG motif - Rpr-Hid-Grim motif. This motif allows these proteins to induce apoptosis by binding to DIAP1, hence eliminating the inhibitory effect of this IAP on the caspases (Wang *et al.*, 1999; Goyal *et al.*, 2000; Christich *et al.*, 2002; Srinivasula *et al.*, 2002; Wing *et al.*, 2002). The mammalian functional homolog of Reaper, Hid, Grim and Sickie is Smac (second mitochondria-derived activator of caspase), or DIABLO (direct IAP binding protein with low pI) (Du *et al.*, 2000; Verhagen *et al.*, 2000).

Smac is a 239 amino acid nuclear-encoded protein. The first 55 amino acids of Smac contain a mitochondrial-targeting sequence that is cleaved during import. Upon apoptotic stimuli, mature Smac is released from mitochondria into the cytoplasm where it directly antagonises IAP inhibition of caspases (Fig 1.12), (Chai *et al.*, 2000; Du *et al.*, 2000; Verhagen *et al.*, 2000). Smac release from mitochondria has been shown to be a partially caspase-dependent phenomenon (Adrain *et al.*, 2001). The relief of IAP inhibition by Smac is also dependent on four residues at the NH₂-terminus of the mature protein – Ala-Val-Pro-Ile. This AVPI motif or IAP-binding motif (IBM) resembles the RHG motif found in *Drosophila* (Fig 1.13). As is evident in Fig 1.13, a similar motif is also found at the NH₂-terminus of the small subunit of

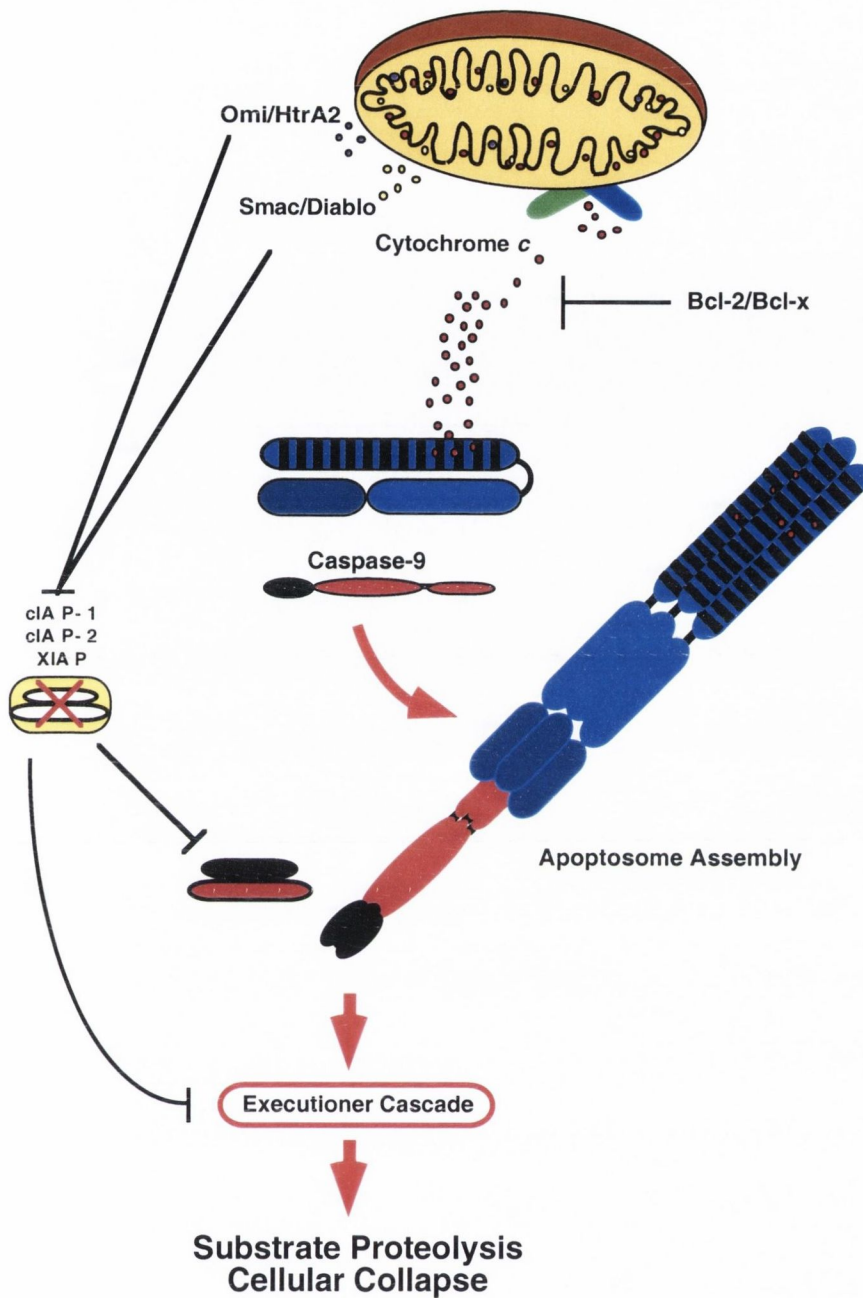


Fig 1.12. Schematic diagram of IAP-mediated caspase regulation and IAP regulation, adapted from Adrain and Martin, 2001.

IAPs may inhibit caspase activation downstream of apoptosome assembly. IAPs target the initiator caspase, caspase-9 and the executioners, caspase-3 and caspase-7. The proapoptotic molecules, Smac/Diablo and Omi/HtrA2 are also released from mitochondria during apoptosis and neutralize IAP function, thus promoting caspase activation.

caspase-9. Processing at Asp315 uncovers the motif of caspase-9 and XIAP binds caspase-9 at this site (Srinivasula *et al.*, 2001). Smac however competes for binding and thereby antagonises the inhibitory effects of XIAP (Srinivasula *et al.*, 2001). The initial processing of caspase-9 therefore, can result in both its inhibition and activation. It is not surprising that such a critical mediator of cell death is regulated by such a complex pathway. Caspase-9 is further processed by caspase-3 at Asp330. This processing enhances caspase-9 activity by (a) facilitating relief of inhibition by XIAP and (b) releasing the linker peptide into the cytoplasm where it can further antagonise IAP activity (Shi, 2002).

Surprisingly, Smac-deficient mice develop normally and their cells respond normally to a broad range of apoptotic stimuli (Okada *et al.*, 2002). This finding however should not allow the importance of Smac to be overlooked. The over-expression of Smac can sensitize cells to a broad range of apoptotic stimuli, such as TRAIL (Srinivasula *et al.*, 2000), vinblastine, cisplatin, etoposide and daunorubicin (Creagh *et al.*, submitted) and UV (Suzuki *et al.*, 2001a). In addition Smac peptides consisting of the first seven residues of the protein can mimic the effects of the full-length protein (Chai *et al.*, 2000; Srinivasula *et al.*, 2001). These findings make Smac an attractive therapeutic target as synthetic Smac peptides could be used in the treatment of cancers to increase a cell's susceptibility to death-inducing stimuli. Finally, Cohen and his colleagues have shown that a second Smac variant, also missing the IBM (Smac β), which does not interact with XIAP, can still potentiate apoptosis induced by a variety of apoptotic stimuli (Roberts *et al.*, 2001). These studies highlight that the ability of Smac to potentiate apoptosis may not solely rely on its role as an IAP-binding protein.

Omi/HtrA2

Omi/HtrA2 is a serine protease that together with Smac is released from the mitochondrial intermembrane space into the cytoplasm during apoptosis (Fig 1.12). This 36kDa protein has been shown to bind XIAP, c-IAP1 and c-IAP2. Its NH₂-terminus contains an IBM – AVPS – and this motif is critical for binding to the BIR2 and BIR3 domains of XIAP. Binding of Omi/HtrA2 to the BIRs diminishes the inhibitory effects of XIAP and mature Omi can promote procaspase-3 activation. Over-expression of Omi/HtrA2 sensitizes cells to UV-, TRAIL- and staurosporine-

Smac/DIABLO	<u>A</u> <u>V</u> <u>P</u> <u>I</u> A Q K S
Reaper	<u>A</u> <u>V</u> <u>A</u> <u>F</u> Y I P D
Grim	<u>A</u> <u>I</u> <u>A</u> <u>Y</u> F I P D
Hid	<u>A</u> <u>V</u> <u>P</u> <u>F</u> Y L P E
Sickle	<u>A</u> <u>I</u> <u>P</u> <u>F</u> F E E E
Casp-9-p12	<u>A</u> <u>T</u> <u>P</u> <u>F</u> Q E G L

Fig 1.13. An evolutionarily conserved family of IAP-binding motifs adapted from Srinivasula *et al.*, 2002

The *Drosophila* proteins, Reaper, Hid, Grim and Sickle share a region of homology at their NH₂-termini termed the RHG motif - Rpr-Hid-Grim motif. This motif allows these proteins to induce apoptosis by binding to DIAP1, hence eliminating the inhibitory effect of this IAP on the caspases. The relief of IAP inhibition by Smac is also dependent on four residues at the N-terminus of the mature protein – AVPI. This AVPI motif or IAP-binding motif (IBM) resembles the RHG motif found in the *Drosophila* proteins. A similar motif is also found at the NH₂-terminus of the small subunit of caspase-9. This site is revealed following processing at Asp315. XIAP binds caspase-9 at this site. Smac however competes with XIAP for binding to this site and thereby antagonises the inhibitory effects of XIAP

induced apoptosis. In addition to acting as an IAP antagonist, Omi/HtrA2 has also been shown to induce a caspase- and Apaf-1-independent cell death that relies on its serine protease activity (Suzuki *et al.*, 2001a; Hedge *et al.*, 2002; Martins *et al.*, 2002; van Loo *et al.*, 2002; Verhagen *et al.*, 2002).

Despite the similarities between the mechanisms of action of Omi and Smac, there are also striking differences. For instance active Smac is a homodimer (Chai *et al.*, 2000), while active Omi is a homotrimer. (Li *et al.*, 2002). More strikingly however, the serine protease activity of Omi has been shown to be capable of cleaving the IAPs, in particular XIAP and c-IAP1. This cleavage event is dependent on Omi binding the IAPs via its IBM. IAP cleavage by Omi/HtrA2 results in the inactivation of these proteins (Srinivasula *et al.*, 2003; Yang *et al.*, 2003).

1.5.2. Self-Regulated Stability

XIAP, C-IAP1 and C-IAP2 also possess C-terminal RING domains. This domain confers E3 ligase activity on the IAPs. Therefore, IAPs can participate in the ubiquitination of proteins within a cell and hence target them for degradation by the 26S proteasome (Weismann, 1997; Jesenberger and Jentsch, 2002). XIAP, c-IAP1 and c-IAP2 can mediate their own autoubiquitination and hence degradation *in vitro* (Huang *et al.*, 2000; Yang *et al.*, 2000). The *in vivo* relevance of this property is highlighted by the fact that over-expressed RING-less XIAP is not as potent an inhibitor as full length XIAP (Creagh *et al.*, submitted). The ability of IAPs to mediate the ubiquitination of other proteins, namely the caspases, and the role played by Smac in this pathway is further explored in Chapter V.

1.5.3. Caspase-mediated cleavage

Finally XIAP has been demonstrated to be a caspase substrate and is cleaved into two distinct fragments during apoptosis (Deveraux *et al.*, 1999). The first fragment corresponds to the BIR1-BIR2 domain of XIAP and the second corresponds to the BIR3-RING domain (Deveraux *et al.*, 1999). The significance of caspase-mediated cleavage for the function of this IAP remains unclear.

1.6. CASPASE SUBSTRATES

1.6.1. Introduction

The death of a cell by apoptosis is regulated by the activity of caspases. These proteases promote the coordinated demolition of a cell by cleaving key structural and regulatory proteins, that not only leads to the destruction and inactivation of these substrates, but in certain instances it actually induces the activation of these proteins. Many of these substrates (around 250) were serendipitously identified on a molecule per molecule basis (Fischer *et al.*, 2003). Analysis of the 2D-gel electrophoresis profiles of healthy cells in comparison with apoptotic cells suggests there are in fact over 500 caspases substrates (Adrain *et al.*, unpublished data). The exact role of each of these substrates is unknown. In fact it is believed that many are cleaved indiscriminately as they happen to contain a recognized caspase cleavage motif and hence are classified as “innocent bystanders” of the apoptotic machinery (Fischer *et al.*, 2003; Adrain *et al.*, unpublished data).

There is considerable conservation of the apoptotic phenotype from worm to mammals. It is likely that cleavage of a critical subset of substrates results in the stereotypical changes associated with apoptosis. Some of the caspase substrates currently established to be important are discussed below.

1.6.2. Key caspase-dependent cleavage events

- Cleavage of ICAD (Inhibitor of caspase-activated DNase) results in the activation of CAD (caspase-activated DNase). Once activated, CAD is partly responsible for DNA fragmentation, a hallmark of apoptosis (Enari *et al.*, 1998).
- Chromatin condensation and nuclear remodelling is dependent upon targeting of such proteins as acinus and helicard (Kovacsovics *et al.*, 2002; Sahara *et al.*, 1999).
- Proteolysis of core structural components of the cytoskeleton such as actin, fodrin, cytokeratin-18, vimentin, Gas2 and plectin contribute to the dramatic changes in cell shape witnessed during apoptosis (Fischer *et al.*, 2003).
- Cleavage of gelsolin and the kinases ROCK-1 and PAK2 may be factors involved in regulating membrane blebbing (Fischer *et al.*, 2003).

- Disruption of focal adhesion kinase (FAK) and paxillin is believed to permit cell shrinkage and detachment (Fischer *et al.*, 2003).

Morphological alterations aside, other key targets include:

- The caspases themselves, resulting in amplification of the death signal.
- The BH3 –only protein Bid, permitting the integration of the mitochondrial pathway of caspase activation with other death-inducing stimuli such as, death receptors and granzyme B (Earnshaw *et al.*, 1999).
- RasGAP, whose initial partially cleaved fragment is believed to protect cells from apoptosis but upon complete cleavage serves as a potent inducer of the death signal (Yang and Widmann, 2001).

It is evident from the above discussion that many diverse caspase substrates exist. This bewildering array of proteins however, also share some similarities.

As already stated, caspases prefer aspartic acid (D) in the P₁ position of the bond they are targeting. Indeed DXXD is one of the most common motifs found in cleaved substrates. A list of caspase substrates with DXXD cleavage sites is shown in Table 1.1. Substrates with non-DXXD motifs are presented in Table 1.2 (Nicholson, 1999; Fischer *et al.*, 2003).

1.6.3. Which caspases constitute the most important executioner enzymes

Studies using cell-free extracts depleted of specific caspases highlight caspase-3 as the primary executioner of substrate cleavage (Slee *et al.*, 2001). The sheer numbers of caspase-3 targets does not discount the possibility that caspase-6 and caspase-7 may also cleave critical substrates.

1.7. CONCLUDING REMARKS

The activity of the caspases is central for the execution of apoptosis. While it is evident from the preceding introduction that much is already understood concerning some critical proteases, debate still continues over many important issues, in particular the mechanisms leading to their activation and regulation.

Table 1.1. Caspase substrates possessing DXXD cleavage site

Substrate	Cleavage site
Acinus	DELD
Bcl-2	DAGD
Cytokeratin-18	DALD
FAK	DQTD
α II-Fodrin	DETD
β II-Fodrin	DEVD
Gelsolin	DQTD
Helicard	DNTD
ICAD	DETD, DAVD
RasGAP site I	DEGD
RasGAP site II	DTVD
ROCK-I	DETD
Vimentin	DSVD

(Nicholson, 1999; Fischer *et al.*, 2003)

Table 1.2. Caspase substrates possessing non-DXXD cleavage sites

Substrate	Cleavage site
Actin	ELPD
Bcl-x _L	HLAD, SSLD
Bid	LQTD
Cytokeratin-18	VEVD
Gas2	SRVD
Helicard	SCTD
PAK2	SHVD
Paxillin	NPQD, SQLD
Plectin	ILRD
Vimentin	IDVD, TNLD

(Nicholson, 1999; Fischer *et al.*, 2003)

1.8. AIMS OF THIS THESIS

The primary aim of this thesis was to compare caspase activation cascades in diverse cell types. In addition, we wished to further elucidate the regulation of caspases by the IAPs and Smac/Diablo.

Chapter III

Studies in the Jurkat T lymphocytic cell line have demonstrated that caspases may be activated in a co-ordinated manner within the apoptosome pathway of caspase activation (Slee *et al.*, 1999b). Our aim was to investigate if this hierarchy of caspase activation was conserved in two different cell types, a secondary transformed cell line, HEK293, and primary human neutrophils. During the course of this research we discovered that primary human neutrophils displayed an unusual requirement for cytochrome *c* in apoptosome formation. In the second section of this chapter this property was examined and the mitochondrial pathway of caspase activation in primary human neutrophils further explored.

Chapter IV

In this chapter a second mechanism of caspase activation, proteolysis via the serine protease, granzyme B, was examined in detail. Granzyme B has been shown to cleave many caspases *in vitro* including caspase-3, -6, -7, -8, -9 and -10 (Chinnaiyan *et al.*, 1996; Duan *et al.*, 1996; Fernandes-Alnemri *et al.*, 1996; Gu *et al.*, 1996; Martin *et al.*, 1996; Muzio *et al.*, 1996; Orth *et al.*, 1996; Quan *et al.*, 1996) but only caspase-3 and caspase-8 have been shown to be targets of this protease in intact cells (Chinnaiyan *et al.*, 1996; Froelich *et al.*, 1996; Atkinson *et al.*, 1998; Yang *et al.*, 1998; Medema *et al.*, 1997b; Sutton *et al.*, 2000). We wished to explore the direct and indirect caspase targets of granzyme B in Jurkat cell-free extract as these proteases are present at physiological levels in this system and therefore, we could examine the hierarchy of caspase activation in an alternative caspase cascade.

Granzyme B-mediated proteolysis of caspase-8 occurs in the absence of an adaptor platform. Recent literature has proposed that caspase-8 activation is in fact prompted by dimerization rather than proteolysis (Boatright *et al.*, 2003; Donepudi *et al.*, 2003) and argues that caspase-8 proteolysis via granzyme B or caspase-6 in the

apoptosome pathway yields inactive enzyme. Our aim was to examine the caspase-8 activity in both these instances and evaluate if the dimerization model is correct.

Chapter V

XIAP, c-IAP1 and c-IAP2 contain a C-terminal RING domain that has been reported to confer E3 ubiquitin ligase activity on these proteins, thereby enabling them to promote their own autoubiquitination and the ubiquitination of the caspases and Smac/Diablo (Huang *et al.*, 2000; Yang *et al.*, 2000; Suzuki *et al.*, 2001; MacFarlane *et al.*, 2002; Yang *et al.*, 2003). However, the majority of these studies have been performed *in vitro* and hence the *in vivo* relevance of IAP ubiquitin ligase activity has been speculative. In this chapter we explored the *in vitro* and *in vivo* ability of XIAP to function as an E3 ubiquitin ligase enzyme. We also examined whether Smac/Diablo mirrored its *Drosophila* functional homologs, Rpr and Hid, as a regulator of IAP ubiquitin ligase activity.

CHAPTER II

Materials and Methods

2.1.MATERIALS

2.1.1. Chemicals and general reagents

Unless otherwise stated, reagents were purchased from Sigma-Aldrich.

Biotin-VAD-FMK (Biotin-Val-Ala-Asp-[OMe]-fluoromethylketone) was purchased from ICN, (UK), biotin-YVAD-CMK (Biotinyl-Tyr-Val-Ala-Asp-chloromethylketone) was purchased from Bachem (UK). The caspase-8 synthetic substrate Ac-IETD-AFC (Ac-Ile-Glu-Thr-Asp-7-amino-4[trifluoromethyl]-coumaride) was purchased from ICN.

2.1.2. Antibodies

Table 2.1 lists the primary antibodies used during the course of the detailed experiments. Unless otherwise stated, the same primary antibody was used for both Western Blot analysis and Immunoprecipitation. Table 2.2 lists the secondary antibodies.

2.2. PURIFICATION AND MANIPULATION OF NUCLEIC ACIDS

2.2.1. Preparation of chemically competent cells

Typically *Escherichia coli* strain DH5 α was used to amplify plasmid DNA and these were made competent by the calcium chloride method. Untransformed DH5 α was streaked onto LB (Luria Bertani) agar (10g/L tryptone, 5g/L yeast extract, 10g/L NaCl, pH 7.0), supplemented with 15g/L agar and incubated overnight at 37°C. A well defined DH5 α colony was then picked from the LB agar plate and grown overnight in 3 ml LB (10g/L tryptone, 5g/L yeast extract, 10g/L NaCl). 1 ml of the overnight culture was then diluted to 50 ml with LB and grown until an OD₆₀₀ of 0.4 to 0.6 was achieved. Cells were harvested by centrifugation at 800 g for 10 min at 4°C, resuspended in 10 ml of ice-cold, sterile 0.1 M CaCl₂ and incubated on ice for 5 min. Following recentrifugation the cells were resuspended in 2 ml of 0.1 M CaCl₂ and 70 μ l of DMSO was then added. Cells were re-incubated on ice for 15 min. 200 μ l aliquots were stored at -70°C.

Table 2.1. Primary antibodies used during the detailed experiments.

Primary Antibody	Source
Anti-Actin	ICN Biomedicals, UK
Anti-Apaf-1 (Immunoprecipitation antibody)	Polyclonal antibody generated for the laboratory
Anti-Apaf-1 (Western Blot antibody)	R&D systems, USA
Anti-BRUCE	BD Biosciences, UK
Anti-Caspase-1	Santa Cruz Biotechnology Inc., USA
Anti-Caspase-2	BD Biosciences, UK
Anti-Caspase-3	BD Biosciences, UK
Anti-Caspase-3 (Active form only) (Western Blot antibody)	New England Biolabs, Inc., UK
Anti-Caspase-4	MBL, UK
Anti-Caspase-6 (immunoprecipitation antibody)	Neomarkers, UK
Anti-Caspase-6 (Western Blot antibodies)	Upstate Biotechnology Inc., USA and Neomarkers, UK
Anti-Caspase-7	BD Biosciences, UK
Anti-Caspase-8 (Immunoprecipitation antibodies)	Ab1, clone C15 (Apotech, UK); Ab2, clone 12F5 (Apotech, UK); Ab3, clone 5F7 (Upstate Biotechnology Inc., USA); Ab4, clone 1C12 (Cell Signaling Technology, UK).
Anti-Caspase-8 (Western Blot antibody)	clone 12F5 (Apotech, UK)
Anti-Caspase-9 (Immunoprecipitation antibody)	Polyclonal antibody generated for the laboratory
Anti-Caspase-9 (Western Blot antibody)	Upstate Biotechnology Inc, USA and Oncogene Research Products, UK
Anti-c-IAP1	R&D Systems, USA
Anti-Cytochrome c	BD Biosciences, UK

Primary Antibody	Source
Anti-Cytochrome <i>c</i> oxidase, subunit I	Molecular Probes, USA
Anti-Fas IgM (clone CH11)	Upstate Biotechnology Inc., USA
Fodrin	Chemicon, UK
Anti-Heat shock protein 60	StressGen Biotechnologies, Canada
Anti-Manganese superoxide dismutase	StressGen Biotechnologies, Canada
Anti-Smac	Polyclonal generated for the laboratory (Adrain <i>et al</i> , 2001)
Anti-Survivin	R&D Systems, USA
Anti-Tubulin	ICN Biomedicals, UK
Anti-Ub	Affiniti Research Products Limited, UK
Anti-X-linked inhibitor of apoptosis protein (XIAP)	BD Biosciences, UK

Table 2.2. Secondary antibodies used during the detailed experiments.

Secondary Antibody	Source
Peroxidase conjugated goat anti-mouse IgG	Jackson ImmunoResearch Laboratories Inc, USA
Peroxidase conjugated goat anti-rabbit IgG	Jackson ImmunoResearch Laboratories Inc, USA
Peroxidase conjugated rat anti-mouse kappa light chain (Used with caspase-8 immunoprecipiations to limit detection of the primary antibody heavy chain)	Zymed, USA
Peroxidase conjugated donkey anti-goat IgG	Santa Cruz, USA

2.2.2. Transformation of competent DH5 α

Frozen competent cells were thawed on ice. 50 μ l aliquots of bacteria were aseptically transferred to pre-chilled 13 ml bug culture tubes. 1 μ l of the desired plasmid was then added and the cell/plasmid mixture was incubated on ice for 30 min. Plasmid uptake was induced by heat-shocking the cells at 42°C for 40 seconds, followed by recovery on ice for 2 mins. 450 μ l of LB was then added and the cells were incubated for 1 h under low agitation (220 rpm) to allow for expression of the antibiotic resistance gene. Typically 30 μ l aliquots of cells were plated on LB agar plates containing either ampicillin (100 μ g/ml) or kanamycin (50 μ g/ml) to select for transformants harbouring the plasmids.

2.2.3. Plasmid DNA isolation procedures

Isolation of plasmid DNA from bacteria: a miniprep procedure

A single well-defined transformed bacterial colony was added to 3 ml of LB supplemented with the appropriate antibiotic, and grown at 37°C under constant agitation (260-280 rpm) until confluent, typically 8-12 h later. 1 ml of this culture was then harvested by centrifugation at 20,000 g for 1 min. The bacterial pellet was re-suspended in 100 μ l P1 buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA). The bacteria were lysed by the addition of 200 μ l P2 buffer (0.2 M NaOH, 1% SDS) and the tube was inverted 4-5 times to ensure thorough mixing. Following a 1 min incubation on ice, DNA was precipitated by the addition of 200 μ l P3 buffer (3M KAc, pH 5.5). Samples were again mixed inversion and incubated on ice for 5 min to ensure a thorough precipitation. Debris was pelleted by centrifugation at 20,000 g for 10 min. The supernatant contained the soluble plasmid DNA that was then precipitated by the addition of 0.7 volumes of isopropanol (DNA-grade). The tube was incubated for 20 min at room temperature. Samples were centrifuged for 10 min at 20,000 g. The resulting pellet was washed in 1 ml of ice-cold 70% ethanol and allowed air-dry before resuspension in 20 μ l sterile double-distilled H₂O, followed by 20 μ l sterile TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). 2 μ l sample was analysed by agarose gel electrophoresis.

Isolation of plasmid DNA from bacteria: a midiprep procedure

A single well-defined transformed bacterial colony was added to 3 ml of LB supplemented with the appropriate antibiotic and grown at 37°C under constant agitation (260-280 rpm) until confluent, typically 8-12 h later. 100 µl of this starter culture was then added to 50 ml LB supplemented with the appropriate antibiotic and grown overnight at 37°C under constant agitation (260-280 rpm). The 50 ml overnight culture was then harvested by centrifugation at 2,500 g in a Sorvall GSA rotor for 10 min at 4°C. The bacterial pellet was resuspended in 4 ml of P1 buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA) supplemented with 100 µg/ml RNase A (Qiagen, UK). Bacteria were lysed by the addition of 4 ml of P2 buffer (0.2 M NaOH, 1% SDS) and samples were gently inverted 4-5 times to ensure thorough mixing. 4 ml of P3 buffer (3M KAc, pH 5.5) was then added. Samples were again mixed by gentle inversion and incubated on ice for 5 min to ensure a thorough precipitation of SDS-complexes. Debris was pelleted for 20 min at 15,000 g in a Sorvall SS-34 rotor at 4°C. For applications such as transient transfection and *in vitro* transcription and translation (ITT) reactions that required ultra purified DNA, the Qiagen column procedure (Qiagen, UK) was performed at this point (Section 2.2.3.3.). The DNA was then precipitated by the addition of 0.7 volumes of isopropanol (DNA-grade) and allowed to incubate for 20 min at room temperature. Plasmid DNA was recovered by centrifugation at 15,000 g for 30 min in a Sorvall SS-34 rotor at 4°C. The resulting pellet was washed in 1 ml of ice-cold 70% ethanol and subsequently transferred into a 1.5 ml eppendorf. The pellet was re-centrifuged at 15,000 g for 10 min and the supernatant was discarded. The pellet was allowed air-dry before resuspension in 100 µl sterile double-distilled H₂O, followed by 100 µl sterile TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The pellet was allowed to dissolve completely in the TE by incubating it overnight at 4°C before quantification.

Qiagen Column Plasmid Purification Procedure

The supernatant obtained after centrifugation was loaded onto a Qiagen Tip-100 column that had previously been equilibrated with 4 ml of Buffer QBT (750 mM NaCl, 50 mM MOPS, pH 7.0, 15% isopropanol, 0.15% Triton X-100) and the sample was allowed to run through the column by gravity flow. The column was then washed with 2 x 10 ml of Buffer QC (1 M NaCl, 50 mM MOPS, pH 7.0, 15%

isopropanol). The plasmid DNA was then eluted with 5 ml of Buffer QF (1.25 M NaCl, 50 mM Tris-Cl, pH 8.5, 15% isopropanol).

2.2.4. Quantification of isolated plasmid DNA

The quantity of isolated DNA was estimated based on its absorbance at 260nm (UV range) on a spectrophotometer. The basic premise of this procedure is that an O.D. reading of 1 at 260nm represents a DNA concentration of 50 µg/ml. Therefore the reading obtained from the recently isolated plasmid DNA was multiplied by this factor. In addition each sample was diluted by a factor of 200 in sterile double-distilled H₂O prior to reading and this factor was also taken into account during the calculation. Sterile double-distilled H₂O was used to zero the spectrophotometer before use. Typically two readings were obtained for each plasmid and their mean derived.

2.2.5. Agarose Gel Electrophoresis

This procedure was also performed to verify the integrity of the isolated DNA. The gel was cast using typically an 0.6% agarose matrix - 0.6 g agarose in 100 ml TAE buffer (40 mM Tris-HCl, pH 8.0, 1 mM EDTA). This mixture was dissolved by heating in a microwave prior to pouring and was allowed to solidify and cool before loading any sample. Typically 1-2 µl of isolated DNA was brought to 10 µl with sterile double-distilled H₂O and 2 µl of 6X loading dye was then added. Samples were electrophoresed through the gel under a constant voltage of 75 V. The gel was then stained with ethidium bromide for 20 min before the isolated DNA was visualised under UV illumination.

2.2.6. Plasmid constructs

All constructs were obtained from laboratory plasmid stocks (Table 2.3). The source identifies the person/laboratory who originally created the plasmid.

2.2.7. Isolation of Total RNA from Neutrophils

Total RNA was isolated from freshly isolated primary human neutrophils using the RNeasy mini kit (Qiagen, UK), according to the manufacturer's guidelines. RNase-free pipette tips and eppendorfs were used throughout the protocol. Briefly 3×10^7 primary human neutrophils were disrupted by the addition of 600 µl Buffer RLT,

Table 2.3. Plasmid constructs used during the detailed experiments.

Construct	Source
pcDNA3	Invitrogen, USA
pcDNA3Apaf-1	From the laboratory of Dr. Xiadong Wang
pcDNA3Bax	Professor Seamus J Martin
pcDNA3YAMA/Caspase-3	From the laboratory of Dr. V M Dixit
pcDNA3Caspase-7	Professor Seamus J Martin
pcDNA3Caspase-9	Dr. Helen Egan
pcDNA3Smac	Dr. Helen Egan
pcDNA3Smac δ	Dr. Helen Egan
pCW7 (His ₆ -Ubiquitin plasmid)	From the laboratory of Dr. Ron Kopito
pEBG	From the laboratory of Dr. Colin Duckett
pEBG-XIAP constructs	From the laboratory of Dr. Colin Duckett

containing 6 μ l β -mercaptoethanol. The cells were then homogenised by vortexing. 600 μ l of 70% ethanol was then added to the homogenised lysate and the sample mixed well by pipetting. 700 μ l aliquot of the sample was added to an RNeasy mini column, previously placed in a 2 ml collection tube. The tube was then centrifuged at 8,000 g for 15 seconds. The flow-through was discarded. The remainder of the sample was then loaded onto the column and the tube was re-centrifuged. In order to wash the column, 700 μ l Buffer RW1 was added and the tube centrifuged at 8,000 g for 15 seconds. The flow-through was again discarded. The RNeasy mini column was transferred to a new collection tube. The column was rewashed by the addition of 500 μ l Buffer RPE, followed by spinning at 8,000 g for 15 seconds. The flow-through was discarded. Another 500 μ l aliquot of Buffer RPE was then added and the tube spun at maximum speed for 2 min. The RNeasy mini column was transferred to a new collection tube. To elute the RNA, 30 μ l RNase-free H₂O was added and the tube spun at 8,000 g for 1 min. This elution procedure was then repeated.

2.2.8. Quantification of isolated RNA

The quantity of isolated RNA was estimated based on its absorbance at 260nm (UV range) on a spectrophotometer. The basic premise of this procedure is that an O.D. reading of 1 at 260nm represents a RNA concentration of 40 μ g/ml. Therefore the reading obtained from the recently isolated RNA was multiplied by this factor. In addition the sample was diluted by a factor of 200 in sterile double-distilled H₂O prior to reading and this factor was also taken into account during the calculation. Sterile double-distilled H₂O was used to zero the spectrophotometer before use. Typically two readings were obtained for the sample of isolated RNA and their mean derived.

2.2.9. RT-PCR

First strand synthesis using reverse transcriptase

RNase-free pipette tips and eppendorfs were used throughout the protocol. This procedure was carried out using the Omniscript Reverse Transcriptase kit (Qiagen, UK). Except the RNase inhibitor, RNasin, all reagents were supplied by Qiagen. The RNasin inhibitor was supplied by Promega, USA. Briefly the following 20 µl reaction was prepared on ice:

10X RT Buffer	2 µl
5 mM dNTP mix	2 µl
10 µM oligo (dT) ₁₈ primer	2 µl
10 U/µl RNasin	1 µl
4 U/µl reverse transcriptase	1 µl
RNA Template	10 µl
RNase-free H ₂ O	2 µl

Incubated at 37°C for 1 h. The above reaction products were then immediately used in a subsequent Polymerase Chain Reaction (PCR).

PCR

Initially primers were designed for the amplification of full length Apaf-1. The forward primer was ATG-GAT-GCA-AAA-GCT-CGA-AAT-TGT-TTG. The reverse primer was TTA-TTC-TAA-AGT-CTG-TAA-AAT-ATA-TAA-AAT-ACC. The forward and reverse primers were created by Sigma Genosys Ltd., UK. The primers were brought to 100 µM concentration with TE Buffer, pH 8.0.

Each PCR reaction was assembled on ice as follows:

cDNA template	3 μ l
2.5 mM dNTPs (Roche, Germany)	4 μ l
Taq Buffer (Qiagen, UK)	5 μ l
Taq enzyme (Qiagen, UK)	0.5 μ l
Forward primer	to final concentration of 100/200/300/400 nM
Reverse primer	to final concentration of 100/200/300/400 nM
Distilled H ₂ O	to 50 μ l final volume

50 ng pcDNA3 empty vector or 50 ng pcDNA3Apaf-1 was used as the template in two separate reactions respectively. The primer concentration in these instances was 300 nM.

The PCR reaction conditions were:

- 3 min at 94°C
- 35 cycles of 1 min at 94°C, 1 min at 52°C (annealing temperature for the primers) and 5 min at 72°C (elongation period)
- 10 min at 72°C.

10 μ l of each reaction was then subjected agarose gel electrophoresis to allow for visualisation of the reaction products.

2.3. MAMMALIAN CELL CULTURE BASED METHODS

2.3.1. Cell culture

The Jurkat (human T lymphocytic leukaemic) cell line was cultured in RPMI supplemented with 5% FCS. The Human Embryonic Kidney 293 and Human Embryonic Kidney 293T cells were cultured in DMEM containing 10% FCS.

2.3.2. Calcium phosphate precipitation transfection

Cells were transfected with plasmids according to the standard calcium phosphate precipitation method. HEK293T cells were seeded at a density of 2×10^6 cells/10cm plate 24h prior to transfection. Precipitates were prepared by mixing the appropriate amount of DNA made up to 200 μ l with sterile 0.5X TE (5 mM Tris-Cl, pH 8.0, 0.5 mM EDTA) with 50 μ l sterile CaCl_2 . The DNA/ CaCl_2 mixture was added drop-wise 250 μ l 2X HBS (280 mM NaCl, 10 mM KCl, 1.5 mM Na_2HPO_4 , 12 mM dextrose, 50 mM HEPES, pH 7.1). Following a 30 min incubation period the complexes were added to the cells. DNA complexes were typically allowed to remain on cells for 6h before replacing with fresh medium

Following transfection, cells were lysed on ice for 15 min in 800 μ l triple-detergent lysis buffer (50mM Tris-Cl, pH 8.0, 150mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 0.02% sodium azide) containing 100 μ M PMSF, 10 μ g/ml leupeptin and 2 μ g/ml aprotinin.

2.3.3. GST-pulldown assays

HEK293T cells were seeded at a density of 2×10^6 cells/10cm plate 24h prior to transfection. Cells were transfected as described by the calcium phosphate precipitation method. 24 h later cells were lysed on ice for 15 min in 800 μ l triple-detergent lysis buffer (50mM Tris-Cl, pH 8.0, 150mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 0.02% sodium azide) containing 100 μ M PMSF, 10 μ g/ml leupeptin and 2 μ g/ml aprotinin. Lysates were cleared by centrifugation at top speed at 4°C for 15 min. Lysates were added to new eppendorfs and incubated with 40 μ l glutathione sepharose beads (Amersham Pharmacia, Sweden). Samples were rotated at 4°C for 4h. Complexes were pelleted at 1,000 g and were washed three times in IP lysis buffer (50mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.1% NP-40). Immunoprecipitates were then analysed by immunoblotting using appropriate primary and secondary antibodies.

2.3.4. Preparation of Subcellular Fractions

Cytosolic and mitochondrial fractions of neutrophils and Jurkat cells were prepared as follows: 10^7 cells were harvested by centrifugation at 800 g, washed in PBS, pH 7.2, and recentrifuged. Cells were digitonin permeabilized for 5 minutes on ice at a density of 3×10^7 /ml in CLAMI Buffer (250 mM sucrose, 70 mM KCl, 137 mM NaCl, 4.3 mM Na_2HPO_4 , 1.4 mM KH_2PO_4 , pH 7.2, 100 μM PMSF, 10 $\mu\text{g}/\text{ml}$ leupeptin, 2 $\mu\text{g}/\text{ml}$ aprotinin, containing 250 $\mu\text{g}/\text{ml}$ digitonin). Cells were then centrifuged at 1,000 g for 5 minutes at 4°C. The supernatants (cytosolic fractions) were saved and the pellets solubilized in the same volume of mitochondrial lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 2mM EGTA, 0.2% Triton X-100, 0.3% NP-40. 100 μM PMSF, 10 $\mu\text{g}/\text{ml}$ leupeptin, 2 $\mu\text{g}/\text{ml}$ aprotinin) followed by centrifugation at 10,000 g for 10 minutes at 4°C. The recovered supernatants were the mitochondrial fractions. For the detection of MnSOD or cytochrome *c*, equal volumes (32 μl) of cytosolic and pellet fractions were supplemented with 5X SDS-PAGE loading buffer and subjected to standard 12% SDS-PAGE

2.3.5. Immunostaining of Neutrophils

Freshly isolated primary human neutrophils were incubated for 45 mins at 37°C with Mitotracker CMXRos (Molecular Probes) followed by washing in 1X PBS, pH 7.2. Neutrophils were cytopun onto glass slides at a density of 5×10^5 cells/ml and immediately fixed on the slides by incubation for 10 min in 3% paraformaldehyde/PBS. The fixed cells were washed briefly x3 in 1X PBS, pH 7.2 and permeabilized for 15 mins in 0.15% Triton X-100/PBS. Cells were blocked for 30 mins in 2% BSA/PBS and probed with a 1:40 dilution of anti-cytochrome *c* (BD Biosciences) for 2 h at room temperature. The cells were once again washed x3 (10 min per wash) in 1X PBS, pH 7.2, followed by probing for 1 h with a 1:500 dilution of Alexafluor 488-conjugated anti-mouse antibody (Molecular Probes). The immunostained cells were then washed x3 in IX PBS, pH 7.2 and briefly stained with 20 μM Hoechst 33358 in PBS. The cells were mounted in Slowfade Light Antifade mounting medium (Molecular Probes) before visualisation.

2.3.6. Immunoprecipitation (IP) of Cytochrome *c*

Cell lysates were prepared by resuspending the indicated number of cells in 800 µl IP lysis buffer (50mM Tris-Cl, pH 8.0, 150 mM NaCl, 1% NP-40) containing 100 µM PMSF, 10 µg/ml leupeptin, 2 µg/ml aprotinin. Lysates were cleared by centrifugation at top speed at 4°C for 15 min. Lysates were added to new eppendorfs and incubated with 1 µg of anti-cytochrome *c* antibody and 30 µl of a 50% slurry of protein A/G agarose beads (Santa Cruz Biotechnology, Inc). The eppendorfs were rotated continuously at 4°C for 4-6 hours. The resulting IP complexes were washed three times in IP lysis buffer containing 0.1% NP-40 (50mM Tris-Cl, pH 8.0, 150 mM NaCl, 1% NP-40). Immunoprecipitates were then analysed by immunoblotting.

2.3.7. FACS Analysis

600 µl sample of control and Fas-treated cells were spun at 600 g for 5 min. The medium was aspirated and the cells were resuspended in Annexin V binding buffer (10 mM Hepes-NaOH, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂) containing FITC (avidin-fluorescein isothiocyanate)-labelled Annexin V at a final concentration of 1 µg/ml. Cell fluorescence was measured using CellQuest software on a fluorescence-activated flow cytometer (FACSCalibur; Becton Dickinson, CA) using the FL-1 channel, with excitation and emission settings of 488 and 600 nm, respectively.

2.4. CELL-FREE EXTRACT PRODUCTION AND MANIPULATION

Mammalian cell-free systems have proven to be extremely advantageous in the study of programmed cell death (Martin *et al.*, 1995; Liu *et al.*, 1996; Martin *et al.*, 1996). These systems can faithfully reproduce the apoptotic changes witnessed in intact cells during apoptosis (Martin *et al.*, 1995; Liu *et al.*, 1996; Martin *et al.*, 1996). In addition, these extracts are not constrained by a plasma membrane and therefore, it is relatively simple to add exogenous constituents or even deplete endogenous components. Therefore, cell-free extracts are indispensable in the elucidation of apoptotic cascades and substrate cleavage events (Slee *et al.*, 1999; Slee *et al.*, 2001)

2.4.1. Cell-free extract preparation

Approximately 5×10^8 cells were pelleted at 400 g and washed three times with PBS, pH 7.2. The first wash was with 50 mls PBS, the second wash was with 30 mls PBS and the final wash was with 1.5 mls PBS which allowed the cells to be packed into a 2-ml Dounce-type homogeniser. After this final wash the cells were pelleted at 800 g for 10 minutes to ensure complete removal of the PBS. Two volumes of cell extract buffer were then added (CEB: 20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5mM $MgCl_2$, 1mM EDTA, 1mM EGTA, 1mM dithiothreitol, 100 μ M PMSF, 10 μ g/ml leupeptin, 2 μ g/ml aprotinin). Cells were allowed to swell on ice for 20 minutes and then were lysed by 20-30 strokes of a B-type pestle. The crude extracts were centrifuged at 15,000 g for 15 minutes to remove debris such as nuclei and unbroken cells. Extracts were then either used immediately or frozen in 50 μ l aliquots at -70°C . The concentration of the extract was determined using the Biorad Protein Assay.

2.4.2. Preparation of immunodepleted cell-free extracts

The following describes the immunodepletion of 100 μ l of cell-free extract. All quantities were halved when only 50 μ l of cell-free extract was immunodepleted. The only time this protocol was not adhered to was when the anti-caspase-6 antibody (Neomarkers) was used. In that instance 10 μ g of antibody was used to immunodeplete 100 μ l of extract.

40 μ l of a 50 % slurry of protein A/G agarose beads (Santa Cruz Biotechnology, Inc.) was brought to a final volume of 200 μ l with PBS, pH 7.2. In order to coat the beads 5 μ g of a particular depletion antibody was added and rotated continually for 3 h at 4°C . As a control, purified rabbit or mouse IgG – 5 μ g (Sigma-Aldrich) was used in a

mock depletion. Antibody-coated beads were washed twice with cell extract buffer. 100 μ l cell-free extract was then added to the beads and rotated overnight at 4°C to ensure efficient immunodepletion. The beads were removed by centrifugation at 1,000 g for 2 minutes and careful removal of the extract.

2.4.3. Assembly of cell-free reactions

Cell-free reactions were set up in 10-100 μ l reaction volumes. All were assembled on ice. Extract concentration was typically ~30 mg/ml.

- For 100 μ l control reactions, 50 μ l cell-free extract was added to 50 μ l cell-extract buffer.
- For 100 μ l cytochrome c/dATP-treated reactions 50 μ l cell-free extract was added to 30 μ l cell-extract buffer. 10 μ l of a 10X stock of bovine heart cytochrome c (final concentration 50 μ g/ml) and 10 μ l of a 10X stock of dATP (final concentration 1 mM) were then added.
- For 100 μ l Granzyme B-treated reaction 50 μ l cell-free extract was added to 40 μ l cell-extract buffer. 10 μ l of a 10X stock of purified human Granzyme B (Calbiochem UK) was then added to final concentration of 100 nM.

Reactions were incubated at 37°C as indicated in text, typically 60-120 mins. All volumes were scaled down according to final reaction volume.

2.4.4. Immunoprecipitation of Caspase-8

After a two-hour incubation at 37°C, 50-100 μ l cell-free reactions were diluted to 250 μ l with cell-extract buffer. 1-2 μ g of the appropriate caspase-8 monoclonal antibody and 40 μ l of a 50% slurry of protein A/G agarose beads (Santa Cruz Biotechnology, Inc) were then added, followed by constant rotation at 4°C for four hours. The immunocomplexes were pelleted at 1000 g and washed three times in 1 ml cell-extract buffer.

2.5. METHODS RELATED TO ASSESSMENT OF CASPASE-8 ACTIVITY

2.5.1. Initiation of Caspase-8 Proteolytic Processing

Caspase-8 processing was triggered in cell-free extract by the addition of either cytochrome *c* (final concentration 50 µg/ml)/dATP (final concentration 1 mM) or purified human Granzyme B (final concentration of 100 nM). Fas/CD95-driven caspase-8 processing was initiated in Jurkat cells by stimulation with 100 ng/ml anti-Fas IgM for 4 h at 37°C after which cell-free extract was prepared.

2.5.2. Assessment of Catalytic Activity

Caspase-8 was assessed for catalytic activity using both affinity labelling and substrate hydrolysis assays.

2.5.2.1. Affinity-labeling of caspases

In this technique Jurkat cell-free extract was activated by the addition of cytochrome *c*/dATP or granzyme B (Ref Section 2.4.3). Control and activated extracts (30 µl) were then incubated at 37°C with either 10 µM biotin-VAD-FMK or 10 µM biotin YVAD-CMK, and samples were removed after 10, 20 and 30 min. Biotinylated proteins were captured by dilution of reactions to 250 µl with CEB, followed by addition of 30 µl agarose-streptavidin beads. These were then incubated at 4°C under continuous rotation for 3 h. Bound proteins were washed three times with CEB (1 ml per wash) and were eluted from beads by addition of SDS-PAGE sample buffer followed by boiling for 7 min.

2.5.2.2. Synthetic peptide hydrolysis assays

In the synthetic peptide hydrolysis assays protein A/G-agarose-immobilized caspase-8 complexes were resuspended in 50 µl CEB and transferred to black 96-well fluotrac plates. Ac-IETD-AFC (50 µl) was then added to each well (to a final concentration of 50 µM) and liberation of free AFC was monitored for 1 h at 37°C at excitation and emission wavelengths of 430 nm and 535 nm, respectively.

2.5.2.3. Radiolabelled substrate cleavage assays with immunoprecipitated caspase-8 (Ref Section 2.4.4).

In the radiolabelled substrate cleavage assays A/G-agarose-immobilized caspase-8 complexes were resuspended in 15 µl CEB containing 0.5µl *in vitro* transcribed and translated caspase-3. Reactions were allowed to proceed for 3 h at 37°C and were stopped by the addition of SDS-PAGE sample buffer followed by analysis by SDS-PAGE/autoradiography.

2.6. PROTEIN ANALYSIS – PURIFICATION AND DETECTION

2.6.1. Protein Purification

Purification of GST fusion proteins

GST fusion proteins were induced and purified in *E. coli* DH5α using the pGEX system. *E. coli* DH5α was transformed with the plasmid, pGEX4T2, as previously described. A single colony was used to inoculate a 3 ml culture of LB containing ampicillin. The overnight culture was then diluted to OD₆₀₀ of 0.1 and grown to OD₆₀₀ of 0.4-0.6. The culture was induced with 100 µM IPTG at 37°C for 3-4 h. Cells were pelleted by centrifugation at 10,000 g for 10 min. The cell pellet was then lysed in NETN buffer (20 mM Tris-Cl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40) containing 100 µM PMSF, 10 µg/ml leupeptin, 2µg/ml aprotinin. The volume of NETN buffer used was 2% of the original culture volume. Cells were disrupted by sonication on ice. The sonication procedure was 5x30 second pulses at 50% output on the sonicator, allowing the cells to recover on ice for 30 seconds between continuous pulses. The resulting lysates were centrifuged at 15,000 g for 30 min at 4°C to pellet inclusion bodies and debris. The supernatant was incubated with 50% slurry of sepharose glutathione (Amersham Pharmacia, Sweden). The volume of sepharose glutathione used was typically 100 µl/ml of bacterial lysate. The lysate was incubated at 4°C under constant rotation to allow for capture of the GST fusion protein. The glutathione beads were collected by centrifugation at 800 g and washed three times in cold PBS, pH7.2 and resuspended to the original bead volume in PBS, pH 7.2 containing 100 µM PMSF, 10 µg/ml leupeptin, 2 µg/ml aprotinin.

Detection of Purified Protein by Coomassie Staining

Purified protein, typically 10 μ l, 5 μ l and 2.5 μ l aliquots, were resolved on SDS-PAGE. The gel was then stained by gently rocking overnight in Coomassie Blue Solution (45% methanol, 10% glacial acetic acid, 0.25% brilliant blue) and destained by gently rocking in destain solution (45% methanol, 10% glacial).

Elution of GST fusion protein

GST fusion proteins were eluted in elution buffer (10 mM reduced glutathione in 50 mM Tris-Cl, pH 8.0) at 1 ml/100 μ l beads. Each elution was carried out at 37°C/RT/4°C for 30 min. Again eluted proteins were resolved on SDS-PAGE and detected by Coomassie Staining. The simultaneous electrophoresis of BSA allowed the protein concentration to be estimated. Eluted proteins were concentrated using spin filters of appropriate molecular weight cut-off (Sartorius, UK). Proteins were spun through the filter in a microfuge at 4°C, at 5,000 g, until the protein had been concentrated to the desired amount. The concentrated protein was then washed to remove the elution buffer, with cell-extract buffer,

Estimation of Protein Concentration – Biorad Protein Assay

The concentration of protein was finally measured using the Biorad Protein assay (Biorad, UK). A series of dilutions of BSA were prepared in double-distilled H₂O, within the concentration range of 0.2-0.9 mg/ml (linear range of BSA). The protein sample was also diluted, usually by 1/50 – 1/100. The dye reagent was prepared by making a 1/5 dilution of the Dye Reagent Concentrate, again with double-distilled H₂O. Typically 3 x 20 ml aliquots of each protein sample and BSA standard was made up to 1 ml with the diluted dye reagent. The samples were well mixed and allowed incubate at RT for 15 min. The absorbance of each sample was read at 595 nm. A mean reading was derived for the protein sample.

2.6.2. *In vitro* transcription and translation (ITT)

Radio-labelled proteins were produced using an *in vitro* transcription and translation (ITT) system termed T_NT T7 coupled Reticulocyte Lysate System (Promega, UK). This rabbit reticulocyte lysate was missing the amino acid, methionine that was replaced in the reaction mixture with ³⁵S-labelled translation grade methionine (10 mCi/ml) (ICN, UK). A typical ITT reaction was as follows:

25 µl rabbit reticulocyte lysate
2 µl TNT Buffer
1 µl RNA polymerase (typically T7)
1 µg plasmid template (Qiagen cleaned)
1 µl RNasin
1 µl amino acid mixture (minus methionine)
1-2 µl ³⁵S-labelled methionine

This was brought to a final volume of 50 µl with nuclease-free H₂O.

Reactions were typically carried out at 30°C for 1-2 h. ³⁵S-labelled proteins were visualised by subjecting 0.5-2.0 µl of the final product to SDS-PAGE, followed by fluorography.

2.6.3. SDS-PAGE

Standard SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as detailed by Sambrook and Maniatis (Sambrook and Maniatis, 1989). 8-15% polyacrylamide gels were prepared as described. The most common gel cast was 12%, with 15% gels used for the electrophoresis of smaller sized proteins (<20 kDa) and 8% gels used for the electrophoresis of larger proteins (>50 kDa). Protein samples were prepared for electrophoresis by denaturing at 90°C for 7 min, typically in the presence of 2X SDS loading buffer (100 mM Tris-Cl, pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol). The initial voltage applied to the gel was 55 V to drive the protein samples through the stacking gel and then this was increased to 75 V to drive the samples through the resolving gel. The running buffer composition was 25 mM Tris-Cl, pH 8.3, 250 mM glycine and 0.1% SDS.

2.6.4. Fluorography

The polyacrylamide gel was fixed for 1-2 h or else overnight in autorad fixative solution (45% methanol, 45% double-distilled H₂O, 10% glacial acetic acid). The fixed gel was then re-hydrated for 30 min in double-distilled H₂O and incubated for 20 min in Amplify (Amersham, UK). The gel was then washed briefly in double-distilled H₂O and vacuum dried onto blotting paper (Whatman, UK) for 1.5 h at 80°C

on a gel dryer (Biorad, UK). To visualise ^{35}S -labelled proteins the autorad was exposed to Hyperfilm (Ameraham, UK) for 12-36 h at -70°C .

2.6.5. Western Blotting

Transfer

Following electrophoresis, proteins were transferred onto a 0.2 mM nitrocellulose membrane (Schleicher and Schuell, Germany) as follows. Four sheets of 3MM blotting paper (Whatman, UK) were cut to the same size as the scotch-brite pads used in the transfer cassette. The cassette was assembled with these four pieces of Whatman paper placed inside two scotch-brite pads and this cassette was then immersed in Transblot Buffer (39 mM glycine, 48 mM Tris-Cl, pH 8.3, 0.037% SDS, 20% methanol). The membrane was then soaked for 5 min in distilled H_2O and then for 5 min in Transblot buffer. The cassette was then opened and the membrane was placed onto one side of the cassette, stacked with two pieces of Whatman paper on top of one scotch-brite pad. The gel was then carefully placed on top of the membrane, ensuring no air bubbles were trapped in the process. The remaining two pieces of Whatman paper and the final scotch-brite pad were placed on top of the gel and the cassette was reassembled and placed in a transfer cassette and covered with Transblot buffer. The proteins were transferred overnight at a constant current of 35 mA.

Immunoblotting

The nitrocellulose membrane was initially blocked for 30 min in blocking solution (1 mM Tris-Cl, pH 8.0, 15 mM NaCl, 0.005% Tween, 5% non-fat dried milk, 0.05% NaN_3). The primary antibody and blocked membrane were then incubated together for at least 2 h under constant rotation in a heat-sealed polythene bag at room temperature. The primary antibody was typically diluted 1:1000 i.e. 5 μl /5 ml blocking solution. The membranes were then washed at least three times in TBST (1 mM Tris-Cl, pH 8.0, 15 mM NaCl, 0.005% Tween). The typical wash volume was 30-50 ml and each wash was approximately 10 min. The secondary antibody was incubated for 1 h in a similar fashion to the primary antibody. The secondary antibody was also diluted 1:1000 but the blocking solution did not contain NaN_3 as this would have inhibited the horse-radish-peroxidase enzyme, coupled to the secondary antibody. The membranes were washed once more in TBST. The wash

volume and the duration of each wash typically remained constant but the number of washes varied from 2-5.

Protein Detection

Proteins were detected by briefly pipetting both sides of the membrane with equal volumes of Supersignal West Pico Stable Peroxide and Luminol/Enhancer solutions (Pierce, UK). The membrane was then transferred to an exposure cassette, covered in saran wrap, and images obtained by exposing the membrane to autoradiography film (Hyperfilm, Amersham Biosciences, UK). On occasions where it was difficult to obtain a signal using these conditions, the Supersignal West Dura reagent (Pierce, UK) was used to improve sensitivity.

2.7. DETECTION OF UBIQUITINATED SPECIES

2.7.1. *In Vitro* Ubiquitination Assay

In vitro ubiquitination assays were typically set up in 10 μ l reaction volumes. 1 μ l *in vitro* transcribed and translated caspase was added to 2 μ l 5X Ubiquitination Buffer (250 mM Tris-HCl, pH 7.5 and 12.5 mM $MgCl_2$) in the presence of 500 μ M DTT, 2 mM ATP and 250 μ g/ml His₆-ubiquitin (Affinity, UK). 10 μ g/ml or 50 μ g/ml E1, 20 μ g/ml or 100 μ g/ml E2 (Affinity, UK) and 10 μ M GST-XIAP/GST-cIAP1 (Lab stocks) were added as indicated. Reactions were incubated at 25°C or 37°C for 3 h. Equal volumes of 2X SDS-PAGE sample buffer were then added. SDS-PAGE separation followed by fluorography allowed for assessment of caspase ubiquitination.

2.7.2. *In Vitro* Ubiquitination Assay using Cell-Free Extracts

The reaction volume in this instance was typically 40 μ l. Control or cytochrome *c*/dATP activated extracts were supplemented with 10 μ g/ml E1 and 20 μ g/ml E2 (Affinity, UK) as indicated. The reactions were incubated at 37°C for 90 min and equal volumes of 2X SDS-PAGE sample buffer were added. The extracts were then subjected to subsequent Western blot analysis.

The reaction volume in this instance was typically 40 μ l. Control or cytochrome *c*/dATP activated extracts were supplemented with 250 μ g/ml His₆-ubiquitin (Affinity, UK) and 1 μ l of *in vitro* transcribed and translated caspase-3. 10 μ g/ml E1, 20 μ g/ml E2 (Affinity, UK) and 10 μ M GST-XIAP (Lab stocks) were added as indicated. The reactions were incubated at 37°C for 120 min. Samples were taken at 60 and 120 min and equal volumes of 2X SDS-PAGE sample buffer were added. SDS-PAGE separation followed by fluorography allowed for assessment of caspase ubiquitination.

2.7.3. *In Vitro* Ubiquitination Assay using Cell-Free Extracts with added Reticulocyte Lysate

In vitro ubiquitination assays were typically set up in 100 μ l reaction volumes. For 100 μ l scale reactions, 25 μ l of Jurkat cell-free extract (~30 mg/ml protein) and 25 μ l rabbit reticulocyte lysate (Promega, UK) were supplemented with 10 μ g/ml E1, 15 μ g/ml E2, 250 μ g/ml His₆-ubiquitin (Affinity, UK), 10 μ M lactacystin, 1 mM ATP and were brought to a final volume of 100 μ l in CEB. Caspase activation was initiated by addition of 50 μ g/ml bovine heart cytochrome *c* (Sigma, UK) and 1 mM dATP. Samples (10-20 μ l) of cell-free reactions were removed at times indicated in the figure and frozen at -70°C for subsequent Western blot analysis.

CHAPTER III

Cytochrome *c*-initiated caspase activation cascades in transformed and primary cells

3.1. INTRODUCTION

3.1.1. Neutrophils

Neutrophils (polymorphonuclear leukocytes) are crucial to the innate or natural immune response. They are the most abundant leukocyte in blood and are the first line of defence against invading micro-organisms (Mollinedo *et al.*, 1999). Neutrophils are terminally differentiated cells and hence are incapable of cell division. They are generated in the bone marrow at a rate of 10^{11} /day in a normal adult (Mollinedo *et al.*, 1999; Akgul *et al.*, 2001). During maturation neutrophils acquire granule proteins. Different granules perform different functions and hence there is variation in the protein composition of the granules. Granules can be divided into primary or azurophilic granules, secondary granules and gelatinase or tertiary granules. Generally, primary and secondary granules contain the antimicrobial arsenal of the neutrophil. Neutrophils also possess a storage organelle termed the secretory vesicle and this organelle in conjunction with the gelatinase granules are involved in neutrophil mobilization and migration (Borregard and Cowland 1997; Levy, 2000).

3.1.2. Role of Neutrophils *in vivo*

Phagocytosis, or internalization of micro-organisms into a membrane-bound vacuole (phagosome) is the critical event in a neutrophil's fight against microbes. Shortly after formation the phagosome fuses with granular endomembrane compartments within the neutrophil. This enables the arsenal required to kill the invading microbes, be delivered to the phagosome (Levy, 2000). Following phagocytosis, neutrophils undergo a process termed a respiratory burst. During the respiratory burst consumption of oxygen by the enzyme, NADPH oxidase, donates electrons to oxygen. Reduction of oxygen results in the formation of superoxide anion (O_2^-). This initial product is not toxic to bacteria but is converted to other reactive oxygen intermediates such as hydrogen peroxide (H_2O_2), its derivative hypochlorous acid, and hydroxyl radical (OH) which are involved in killing bacteria (Chapman *et al.*, 2002).

3.1.3. Disposal of Neutrophils

Neutrophils are equipped with deadly cytotoxic enzymes and reactive oxygen species and their careful removal is, therefore, essential. Neutrophils have a very short half-life in circulation (8-20 h; Akgul *et al.*, 2001). In the absence of external stimuli, such as those found at the site of infection, aged neutrophils undergo spontaneous apoptosis and are removed by macrophages (Savill *et al.*, 1989). Pro-inflammatory stimuli such as bacterial lipopolysaccharide (LPS; Lee *et al.*, 1993), or cytokines, including granulocyte macrophage-colony stimulating factor (GM-CSF), granulocyte-colony stimulating factor (G-CSF), IL-1 β , interferon- γ and interleukin-8 (Colotta *et al.*, 1992; Cox *et al.*, 1992; Lee *et al.*, 1993; Leuenroth *et al.*, 1998) increase half-life of neutrophils in inflamed or infected tissues.

One of the major mechanisms the latter agents use to increase neutrophil survival rate is to interfere with the constitutive apoptotic pathways of neutrophils. For example GM-CSF and LPS have both been shown to increase the expression of the anti-apoptotic protein Mcl-1 (Moulding *et al.*, 1998). These agents also upregulate expression of IL-1 β , that is thought to inhibit the neutrophil cell death programme (Watson *et al.*, 1998). G-CSF has also been shown to inhibit caspase-3 activation and Bax translocation to mitochondria (Maiani *et al.*, 2002). Neutrophil apoptosis and subsequent macrophage-mediated disposal of these cells is critical for the resolution of inflammation. If neutrophil clearance is inhibited, these cells undergo secondary necrosis and release their toxic cargo - reactive oxygen species and granule enzymes - which serves to aggravate the inflammation even further.

3.1.4. Neutrophil apoptosis

Deregulated neutrophil apoptosis is associated with diseases such as systemic inflammatory response syndrome (SIRS; Jimenez *et al.*, 1997), acute respiratory distress syndrome (ARDS; Matute-Bello *et al.*, 1997) and acute lung injury (ALI; Lee *et al.*, 2001). Delayed neutrophil apoptosis also causes complications for burn victims (Chitnis *et al.*, 1996) and is implicated in the pathogenesis of sepsis (Oberholzer *et al.*, 2001). Understanding neutrophil apoptosis, both spontaneous and delayed, would be of great benefit to patients who suffer as a result of deregulated neutrophil death (Akgul *et al.*, 2001).

Certain aspects of neutrophil apoptosis are beginning to be unravelled. The death receptor pathway, via engagement of TNFRI or Fas, is known to be functional in neutrophils. Neutrophils express both Fas and Fas ligand (FasL) and undergo apoptosis when treated with anti-Fas IgM (mAb CH-11). Indeed it was initially believed that it was this pathway that elicited spontaneous apoptosis in neutrophils (Liles *et al.*, 1996). More recent studies have reported that neutrophils do not express surface FasL and indeed that neutrophils from FasL-deficient-mice maintain their constitutive apoptotic programme (Brown and Savill, 1999). The role played by Fas in spontaneous neutrophil apoptosis is therefore a matter of some debate. Inflammatory neutrophils are resistant to Fas-induced cell death (Watson *et al.*, 1997). This resistance is associated with an inhibition of the activity of caspase-3 and -8 and an increase in mitochondrial stability (Watson *et al.*, 1999). The role played by the cytokine TNF α in neutrophil apoptosis is complicated. Initial reports gave conflicting results – TNF α was reported to both prolong survival and also induce death of neutrophils (Murray *et al.*, 1997). It has since been reported that, at low concentrations, TNF α evokes a survival response in neutrophils and apoptosis occurs at higher concentrations of the cytokine (van den Berg *et al.*, 2001). TNF α -induced apoptosis in neutrophils is accompanied by caspase-3 and -8 activation (Yamashita *et al.*, 1998).

While the death receptor-mediated apoptosis in neutrophils has been well studied, very little is known about the role played by the mitochondrial pathway in neutrophil apoptosis. Neutrophil cytosols have been shown to be capable of triggering caspase-3 and -8 activation upon addition of exogenous cytochrome *c* (Yamashita *et al.*, 1998). The presence of other caspases involved in the cell death programme and their responsiveness to cytochrome *c* remains elusive however. Indeed reports regarding the mere presence of intact functional mitochondria in neutrophils are inconclusive with some authors arguing the presence of mitochondria (Pryde *et al.*, 2000; Maiani *et al.*, 2002), while others argue neutrophils only possess few mitochondria that lack the ability to respire (Peachman *et al.*, 2001). In addition it has been shown that mature neutrophils do not possess the full repertoire of caspase substrates. For example, PARP, NuMA, U1-70kDa and DNA-PK α are absent (Sanghavi *et al.*, 1998). Likewise, the anti-apoptotic protein Bcl-2 is not expressed (Moulding *et al.*, 1998).

The observations prompted us to investigate if neutrophils were also deficient in critical mediators of the mitochondrial pathway and furthermore to question if this pathway was functional in neutrophils.

3.2. CHAPTER AIMS

One of the major pathways of caspase activation is the mitochondrial pathway. The exact order of caspase activation events in this pathway has been deduced (Slee *et al.*, 1999b). However this cascade has been deduced largely in one cell type – the Jurkat T lymphocytic cell line. The conservation of this cascade in other transformed cell lines and indeed, in primary cells has not been examined in detail. One of the aims of this chapter, was to perform a detailed analysis of the caspases present in three cell types, the transformed cell lines Jurkat and HEK293, and primary human neutrophils, to determine whether the “Jurkat” cascade represents a general mechanism of caspase activation. The relative levels of each caspase, the kinetics of their activation and their order of activation was determined for each cell type. Initial studies were performed by preparing cell-free extracts from healthy cells, triggering them to undergo apoptosis by the addition of cytochrome *c* and dATP and then examining the activation of the caspases with specific antibodies. In order to delineate the hierarchy of caspase activation events, cell-free extracts were immunodepleted of specific caspases and the effects of these depletions on the activation status of the remaining caspases was examined. Finally, the threshold for caspase activation, in terms of a requirement for cytochrome *c* and dATP, in the cell-free extracts from the three cell types was determined.

Work then focused on deducing whether neutrophils possessed an intact mitochondrial pathway to caspase activation. We explored whether critical components of the mitochondrial pathway such as Apaf-1 and cytochrome *c* were present and whether the pathway was dependent on such factors in this cell line.

3.3. RESULTS

3.3.1. Relative levels of caspases present in Jurkat, HEK293 and neutrophil cell-free extracts

To investigate the repertoire and relative abundance of caspases present in Jurkat, HEK293 and primary human neutrophils, cell free extracts were prepared. These were subjected to SDS-PAGE separation and immunoblotted with specific caspase antibodies. As is illustrated in Fig 3.1, caspases -3, -6, -7, and -9 are present in all cell types. However neutrophils have elevated levels of caspases-1 and -4, consistent with their role in inflammation (Fig 3.1). Caspase-2 is absent from neutrophils, which is in agreement with previous studies (Santos-Beneit and Mollinedo, 2000). Fodrin and Tubulin levels were analysed as loading controls (Fig 3.1).

3.3.2. The kinetics of cytochrome *c*/dATP induced caspase activation in Jurkat, HEK293 and Neutrophil cell-free extracts

To initiate caspase activation, cytochrome *c* (50 µg/ml final concentration) and dATP (1 mM final concentration) were added to the extracts. These were then incubated at 37°C for various periods of time. At 0, 15, 30, 60, 90 and 120 minutes samples were taken for subsequent SDS-PAGE separation and Western Blot analysis. Using caspase-specific antibodies the kinetics of cytochrome *c*/dATP induced caspase activation was determined. As can be seen in Fig 3.2 the caspases present in Jurkat, HEK293 and neutrophil cell-free extracts are all capable of being activated in an apoptosome-dependent manner, with similar kinetics. In addition, the same cleavage pattern can be observed for all caspases, indicating that proteolytic processing of the caspases occurs in these cell types. Finally, this figure confirms that the neutrophil caspases are indeed capable of being activated in a cytochrome *c*-inducible manner.

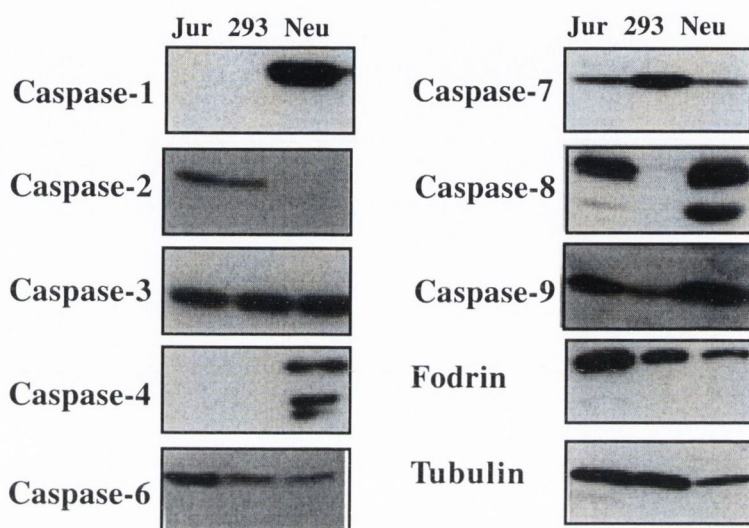


Fig 3.1. Relative levels of caspases present in Jurkat, HEK293 and neutrophil cell-free extracts
 Cell-free extracts were prepared as described in Materials and Methods. Equal amounts (approximately 125 µg) of protein lysate were then separated by SDS-PAGE and replicate blots were probed with the indicated caspase antibodies. Data are representative of at least four independent experiments.

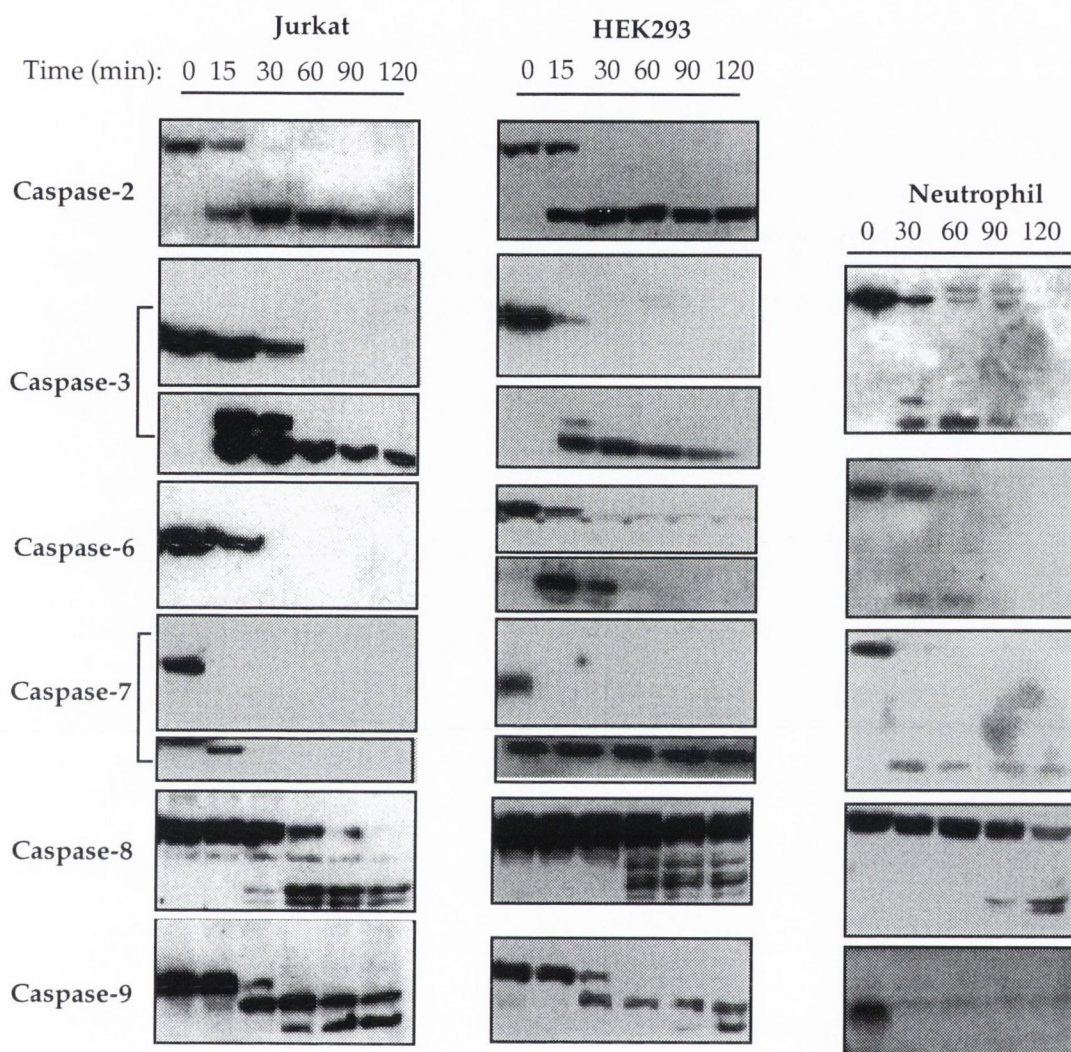


Fig 3.2. The kinetics of cytochrome *c*/dATP induced caspase activation in Jurkat, HEK293 and Neutrophil cell-free extracts

Cell-free reactions were assembled as described in Materials and Methods and incubated at 37°C for the times indicated. The kinetics of cytochrome *c*/dATP induced caspase activation was determined by probing the extracts with caspase-specific antibodies. Data are representative of at least four independent experiments.

3.3.3. Determination of the order of apoptosome-mediated caspase activation in HEK293 and Neutrophil cell-free extract

The apoptosome-mediated caspase cascade has previously been deduced in the Jurkat cell line (Slee *et al.*, 1999b) and is illustrated in Fig 3.3.

Caspase-3 and caspase-7 are the first caspases to be cleaved and activated by the apoptosome-activated caspase-9 (Srinivasula *et al.*, 1998; Rodriguez and Lazebnik, 1999; Slee *et al.*, 1999b). Caspase-3 in turn cleaves and activates caspases-2 and -6 and the latter then promotes activation of caspases-8 and -10 (Slee *et al.*, 1999b). Caspase-3 also participates in a feed-back amplification loop to further process caspase-9 (Srinivasula *et al.*, 1998; Slee *et al.*, 1999b). Therefore, once caspase-9 is activated within the apoptosome there is rapid amplification of the death signal through a cascade of activated caspases (Fig 3.3).

The previous experiments confirmed that the caspases present in HEK293 cells and neutrophils were all capable of undergoing cytochrome *c*/dATP-inducible activation, suggesting that the apoptosome pathway was active in all three cell types. In order to investigate if the hierarchy of caspase activation seen in Fig 3.3, was the same in all three cell types, cell-free extracts were immunodepleted of caspases -3, -6 and -7 using caspase specific antibodies. Mock depletions were also performed to ensure the depletion procedure had no inhibitory effects on the extracts. The extracts were then triggered to undergo apoptosis by addition of cytochrome *c* and dATP. As is illustrated in Fig 3.4, the order of caspase activation events in HEK293 and neutrophil cell-free extract is identical and mimics that of Jurkat cell-free extract. As anticipated, mock-depletion had no effect on caspase-2, -3, -6, -7 and -8 processing. Caspase-3 depletion blocked caspase-2 processing in HEK293 extract and caspase-6 and caspase-8 processing in both neutrophil and HEK293 extracts, consistent with the original model based upon Jurkat cell-free extracts. Caspase-6 depletion prevented caspase-8 processing in both cell types. Caspase-7 depletion had no effect on caspase processing. Therefore the previously deduced caspase cascade is conserved between cell types. The observation that the caspase activation cascade is maintained not only in a secondary transformed cell line but also a primary cell type reveals that this cascade is conserved between cell types and can be considered a generalized feature of apoptosis.

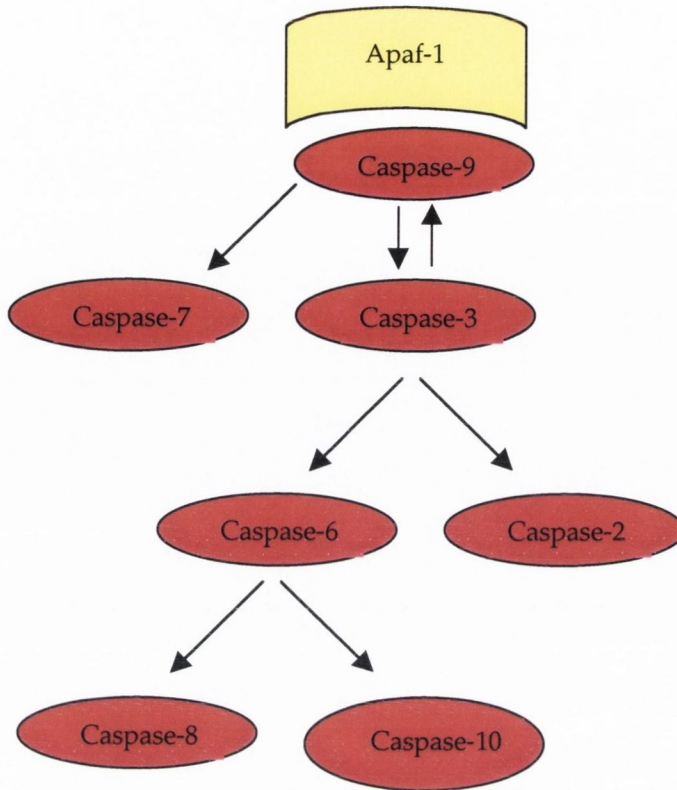


Fig 3.3. Apoptosome-mediated caspase cascade (Slee *et al.*, 1999b)

Caspase-3 and caspase-7 are the first caspases that are cleaved and activated by apoptosome-activated caspase-9. Caspase-3 in turn cleaves and activates caspases-2 and -6 and the latter then promotes activation of caspases-8 and -10. Caspase-3 also participates in a feed-back amplification loop to further process caspase-9.

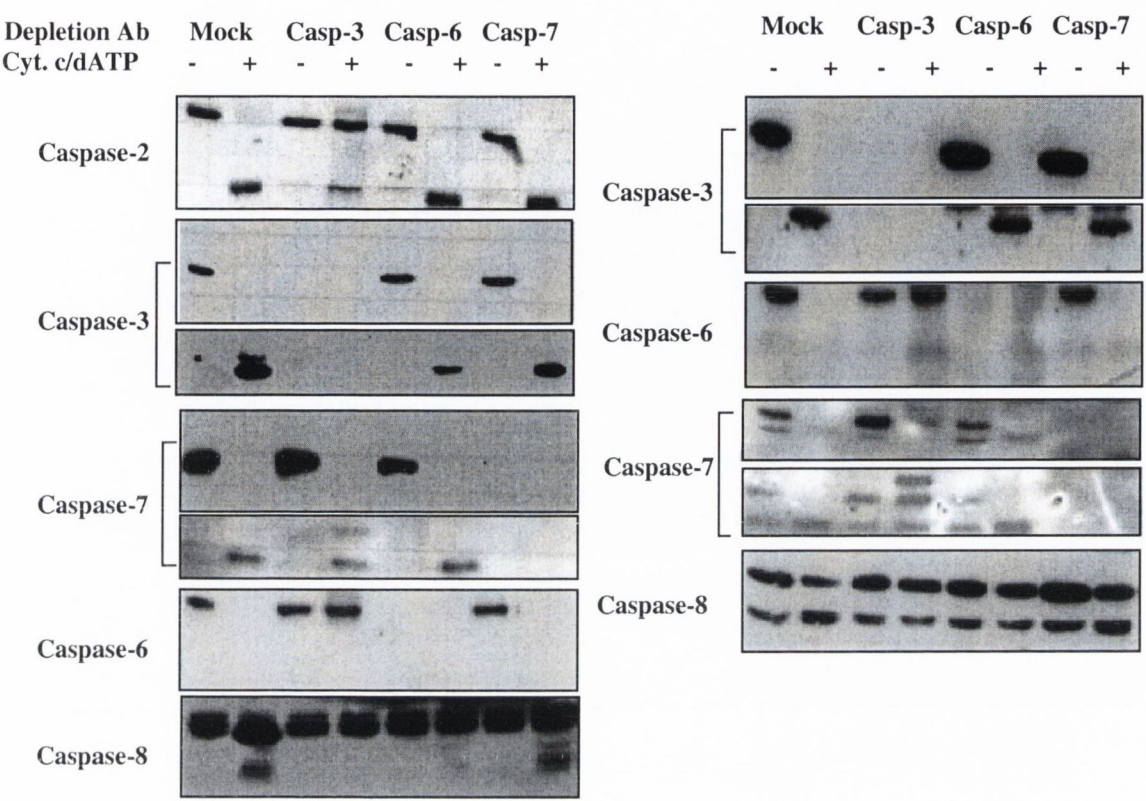


Fig 3.4. Determination of the order of apoptosome-mediated caspase activation events in HEK293 and Neutrophil cell-free extracts

HEK293 (on left) and neutrophil (on right) extracts were depleted of caspases-3, -6 and -7 as indicated in Materials and Methods. The extracts were incubated at 37°C for two hours. Samples taken at 0 and 120 minutes were subjected to Western Blot analysis with the indicated antibodies. Data are representative of at least three independent experiments.

As the order of caspase activation events in the apoptosome pathway was conserved between cell types, we next examined whether these extracts also behaved similarly in terms of their requirement for cytochrome *c* and dATP to initiate apoptosome formation.

3.3.4. Analysis of the levels of cytochrome *c* and dATP required to trigger caspase activation in Jurkat, HEK293 and Neutrophil cell-free extracts

To assess whether extracts prepared from Jurkat, HEK293 and neutrophils were equally sensitive to the triggering of a cytochrome *c*-driven cascade, apoptosome formation was initiated in Jurkat, HEK293 and Neutrophil cell-free extracts by the addition of 1 mM dATP and decreasing amounts of cytochrome *c*. The extracts were incubated at 37°C for one hour, followed by probing for caspase-3 to examine the processing status of the enzyme. Cytochrome *c* is generally added to cell free extracts at a final concentration of 50 µg/ml to ensure robust caspase activation. As is shown in Fig 3.5, partial caspase activation occurred in Jurkat cell-free extracts at 5 µg/ml concentration of cytochrome *c*. However in HEK293 cell-free extracts partial activation of caspase-3 occurred across a greater range of cytochrome *c* concentrations. In neutrophil cell-free extracts where cytochrome *c* concentration was dropped to 78 ng/ml complete caspase-3 activation occurred. Thus, neutrophil cell-free extract seemed to require far lower levels of cytochrome *c* than either Jurkat or HEK293 extract and therefore appeared to be significantly more sensitive to apoptosome-mediated caspase activation. We then investigated whether this low tolerance was also seen with respect to dATP requirements.

To examine the requirement for dATP for caspase activation, cell-free extracts were incubated with decreasing amounts of dATP in the absence of cytochrome *c*. As Fig 3.6 shows, when no cytochrome *c*/dATP was added caspase-3 activation was not seen in any extract. Upon addition of dATP (1 mM), in the absence of cytochrome *c*, complete caspase-3 processing occurred only in neutrophil cell-free extract. The latter result was surprising as it suggested that caspase activation in neutrophil cell-free extracts could proceed independently of cytochrome *c*. Addition of both cytochrome *c* (50 µg/ml) and dATP (1 mM) ensures robust caspase-3 activation in all three extracts as expected. Of note again is the fact that addition of dATP alone did not result in caspase-3 activation in Jurkat or HEK293 cell-free extracts. However,

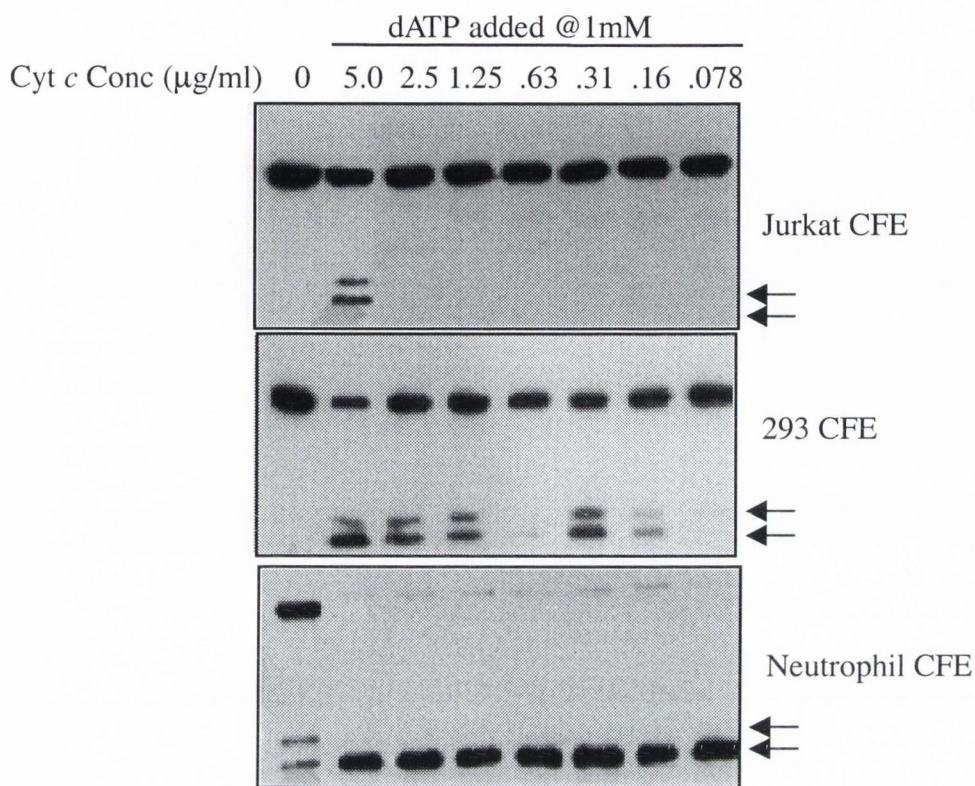


Fig 3.5. Threshold levels of cytochrome *c* required to trigger caspase activation in Jurkat, HEK293 and Neutrophil cell-free extracts

Jurkat, HEK293 and neutrophil extracts were incubated at 37°C for 1 h in the presence or absence of 1 mM dATP and decreasing amounts of cytochrome *c* as indicated. The extracts were then probed with anti-caspase-3 antibodies. Data are representative of at least three independent experiments.  Indicates processed caspase-3.

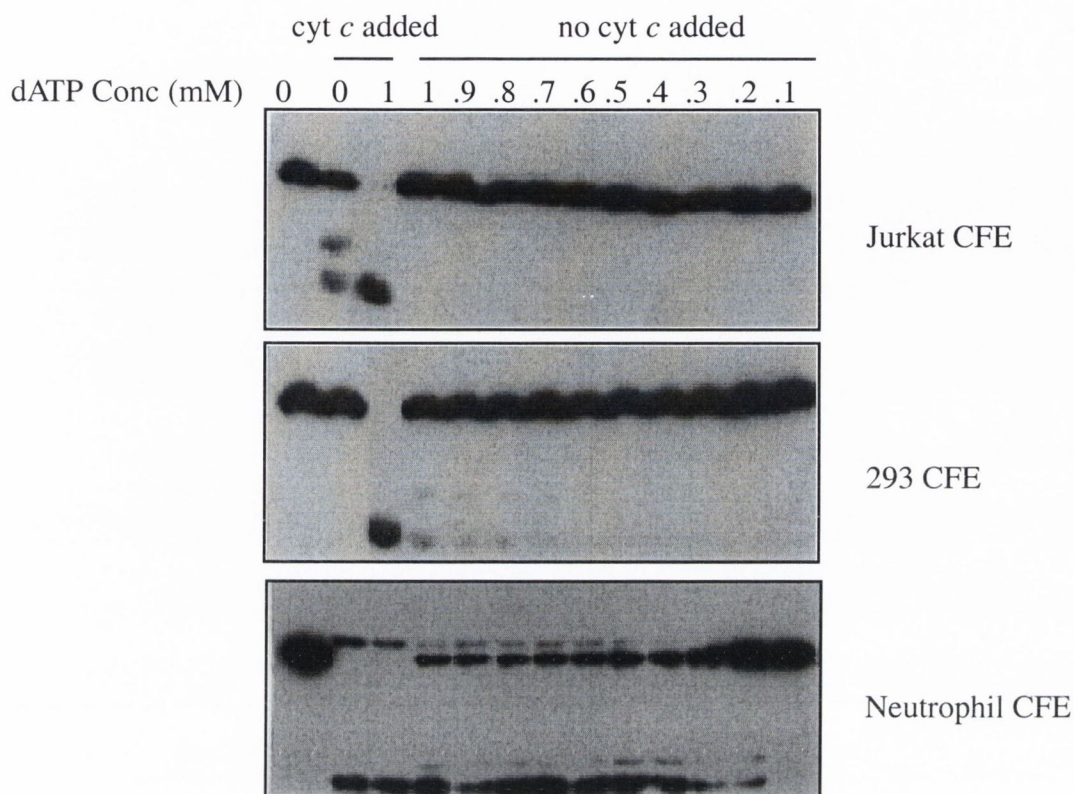


Fig 3.6. Levels of dATP required to trigger caspase activation in Jurkat, HEK293 and Neutrophil cell-free extracts

Apoptosome formation was triggered in Jurkat, HEK293 and neutrophil extracts by the addition of decreasing amounts of dATP. Following incubation at 37°C for 1 h, the extracts were probed with anti-caspase-3. The first three lanes on the left in every panel are control lanes that have been incubated in the presence or absence of 50 $\mu\text{g/ml}$ cytochrome *c* along with 1 mM dATP to ensure that all extracts permit apoptosome formation. Data are representative of at least three independent experiments.

dATP concentration could be titrated right down to 0.1mM before caspase-3 activation was completely blocked in neutrophil extract.

Three potential explanations for the apparent cytochrome *c*-independency displayed by neutrophil cell-free extracts were considered. (A) The levels of cytochrome *c* already present in neutrophil extract were sufficient for apoptosome formation upon addition of dATP alone. During cell-free extract preparation it is possible that a fraction of mitochondria was ruptured and released cytochrome *c*. Therefore it was possible that the level of cytochrome *c* contamination in the neutrophil extracts was already sufficient for apoptosome formation upon dATP addition. (B) Neutrophils may express a novel Apaf-1 isoform that assembles in a cytochrome *c*-independent manner. The activation of Apaf-1 is regulated by 12-13 WD-40 repeats in the C-terminal region of the protein (Fig 3.7). Binding of cytochrome *c* to these motifs induces Apaf-1 oligomerization and hence promotes recruitment of caspase-9 to the apoptosome (Adrain and Martin 2001). An Apaf-1 construct, missing this portion of the molecule is constitutively active (Adrain *et al.*, 1999). We speculated if the Apaf-1 isoform present in primary human neutrophils was mutated in this region and hence did not rely on cytochrome *c* for its oligomerization. (C) Neutrophils may possess a novel cytochrome *c*-and Apaf-1-independent pathway for apoptosome formation. Thus it was possible dATP was triggering a completely novel Apaf-1-independent pathway to caspase activation in neutrophil extracts.

3.3.5. Exploring cytochrome *c*-dependency for caspase activation in neutrophil cell-free extracts

To explore whether neutrophil extracts were contaminated with cytochrome *c*, Jurkat, HEK293 and neutrophil cell-free extracts were subjected to SDS-PAGE and Western Blot analysis using a cytochrome *c* specific antibody. As is shown in Fig 3.8, cytochrome *c* was not detectable in neutrophil cell-free extracts by Western Blot. Thus, the neutrophil extracts were profoundly sensitive to dATP addition, in spite of having little or no endogenous cytochrome *c*.

To explore whether neutrophils may express an aberrant form of Apaf-1, Jurkat, HEK293 and Neutrophil cell-free extracts were probed using an Apaf-1 antibody (Fig 3.9). This revealed that neutrophil extracts had higher levels of Apaf-1 in

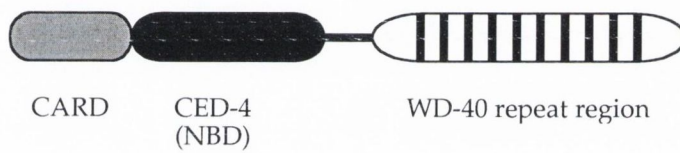


Fig 3.7. Domain structure of Apaf-1

Apaf-1 consists of three functional domains an N-terminal CARD, responsible for binding caspase-9, a central CED-4 homology domain that facilitates nucleotide binding and oligomerization and a C-terminal region consisting of 12-13 WD-40 repeats.

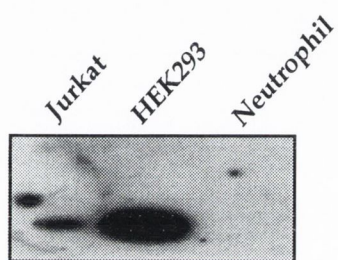


Fig 3.8. Neutrophil extracts contain undetectable amounts of cytochrome *c*

Equal amounts (120 µg) of protein from Jurkat, HEK293 and neutrophil cell-free extracts were separated by SDS-PAGE followed by immunoblotting for cytochrome *c*.

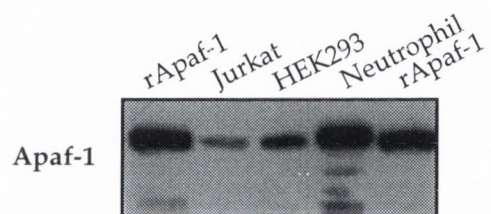


Fig 3.9. Neutrophils contain elevated levels of Apaf-1

Equal amounts (100 μ g) of protein were separated by SDS-PAGE followed by probing with anti-Apaf-1.

Recombinant Apaf-1 was obtained by transiently overexpressing an Apaf-1 expression plasmid in HEK293 cells. Data are representative of at least five independent experiments.

comparison to Jurkat or HEK293 extracts. Interestingly, however there was also the appearance of lower molecular weight forms of Apaf-1 on these blots. We wondered whether these lower molecular weight bands represented novel isoforms of Apaf-1.

Accordingly, RNA was isolated from 3×10^7 neutrophils and used as a template to create cDNA. Apaf-1 primers, flanking the first and last exons of the Apaf-1 gene, were then designed to allow amplification of the Apaf-1 message in a subsequent PCR reaction. The result in lane 3 in Fig 3.10, shows that neutrophils do not display any abnormal Apaf-1 isoforms at the mRNA level and only possess the full-length Apaf-1 isoform. It is not unreasonable to conclude that the Apaf-1 present in neutrophils was likely to be of the same domain structure as is illustrated in Fig 3.7, and the lower molecular weight bands in Fig 3.9 were most likely non-specific proteins that cross-reacted with the antibody used or degradation products of Apaf-1.

At this stage it was apparent that caspase processing did occur in neutrophils in response to dATP and that the Apaf-1 isoform found in neutrophils was most likely the same as that found in other cell types. We next explored whether the Apaf-1/caspase-9 apoptosome was in fact responsible for the caspase activation observed by exogenous dATP addition.

To explore whether Apaf-1 and caspase-9 were required for dATP-induced caspase activation, we immunodepleted Jurkat and neutrophil extracts of both of these constituents. Depletion was confirmed by immunoblotting (data not shown). Mock-depleted extracts were also generated. Both extracts were then treated with (a) no stimulus, (b) dATP (1 mM) alone or (c) cytochrome *c* (50 $\mu\text{g}/\text{ml}$)/dATP (1 mM) and incubated at 37°C for two hours. The treated extracts were then subjected to SDS-PAGE and Western Blot analysis with anti-caspase-3. As is illustrated in Fig 3.11 mock-depletion has no effect on the extracts, as expected. Cytochrome *c* and dATP were both required for efficient caspase-3 activation in mock-depleted Jurkat cell-free extract whereas dATP was sufficient for caspase-3 activation in mock-depleted neutrophil extract, as before. However, immunodepletion of Apaf-1 and caspase-9 resulted in complete abrogation of caspase-3 activation in both Jurkat and neutrophil extracts. Thus, dATP-induced caspase activation in neutrophils required an intact



Neutrophil Apaf-1 cDNA was generated as detailed in Materials and Methods. This was subsequently used as the template for a PCR reaction using Apaf-1 specific primers. Lane 2 shows the optimal primer concentration to be 300 nM. Lane 5, contains a positive control which highlights the PCR result when 50 ng pcDNA3Apaf-1 was used as the template. Lane 6 is the negative control and here 50 ng pcDNA3 empty vector was used as the template. Data are representative of two independent experiments.

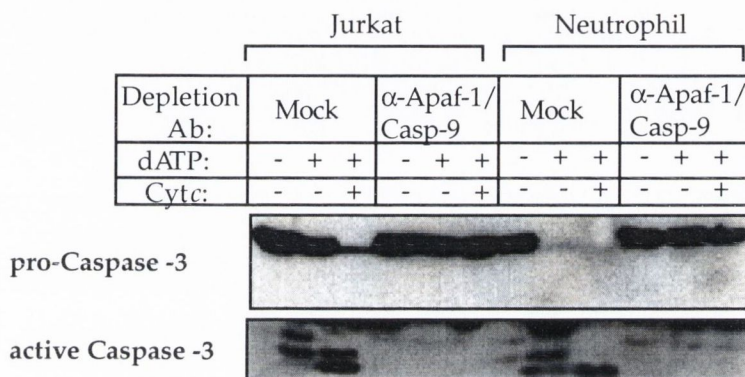


Fig 3.11. dATP-inducible caspase activation in neutrophil cell-free extract is Apaf-1 and Caspase-9 dependent

Jurkat and neutrophil cell-free extracts were depleted of Apaf-1/caspase-9 using a polyclonal antibody generated for the laboratory. Briefly 40 μ l of a 50 % slurry of protein A/G agarose beads (Santa Cruz Biotechnology, Inc.) was brought to a final volume of 200 μ l with PBS, pH 7.2. In order to coat the beads 5 μ g of the depletion antibody was added and rotated continually for 3 h at 4°C. As a control, purified rabbit - 5 μ g (Sigma-Aldrich) was used in a mock depletion. Antibody-coated beads were washed twice with cell extract buffer. 100 μ l Jurkat/neutrophil cell-free extract was then added to the beads and rotated overnight at 4°C to ensure efficient immunodepletion. Depleted extracts were then incubated for 2 h at 37°C in the presence or absence of 50 μ g/ml cytochrome *c* and 1 mM dATP. The extracts were then probed with anti-caspase-3.

Apaf-1/caspase-9 apoptosome pathway. This suggests that although neutrophils possess the conventional apoptosome machinery, they differed radically from other cell types in terms of their requirement for cytochrome *c*.

Western blot analysis of neutrophil cell-free extracts indicated that these extracts did not contain cytochrome *c*, however, it is possible that traces of cytochrome *c* were present in these extracts that were sufficient to assemble apoptosomes upon addition of dATP. In order to establish whether this was a genuine cytochrome *c*-independent pathway, immunodepleted Jurkat and Neutrophil extracts were once again generated. Now the extracts were depleted of cytochrome *c* and subsequently treated with either (a) no stimulus, (b) dATP (1 mM) alone or (c) cytochrome *c* (50 µg/ml) and dATP (1 mM). After incubation at 37°C for one hour the samples were subjected to SDS-PAGE. The blots were again probed with anti-caspase-3 to examine the activation status of this caspase. The results are shown in Fig 3.12.

As expected, in both mock and cytochrome *c*-depleted Jurkat extract exogenous cytochrome *c* addition was required for caspase activation. However, neutrophil cell-free extract displayed an unexpected requirement for cytochrome *c*. In the mock-depleted extract, caspase-3 activation and fodrin cleavage occurred upon dATP addition alone. In cytochrome *c*-depleted neutrophil extract addition of dATP was insufficient to allow caspase-3 activation and fodrin cleavage. Re-addition of cytochrome *c* and dATP to cytochrome *c*-depleted extract allowed caspase activation and substrate cleavage to occur as normal. Thus, neutrophils do require cytochrome *c* to allow functional apoptosome formation. However, the levels of cytochrome *c* required to form functional apoptosomes in these cells seemed to be significantly lower than in the other cell types examined.

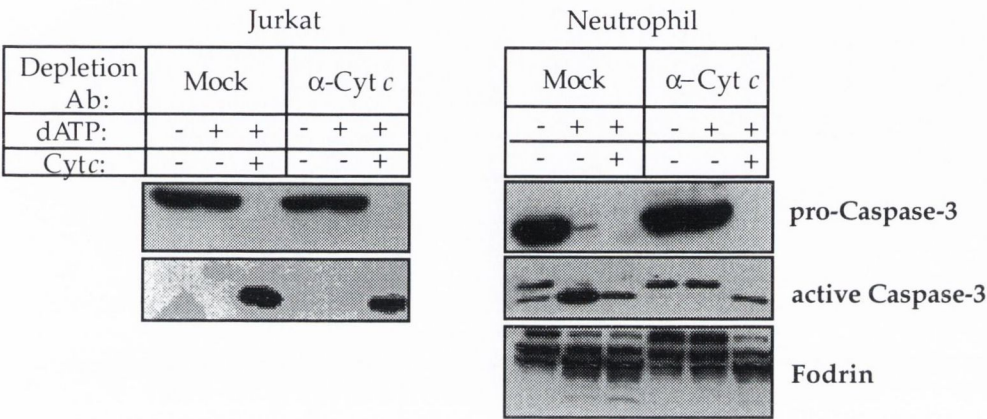


Fig 3.12. dATP-inducible caspase activation in neutrophil cell-free extracts is cytochrome *c* dependent

Jurkat and neutrophil cell-free extracts were depleted of cytochrome *c* using anti-cytochrome *c* (BD Biosciences, UK). Briefly 40 μ l of a 50 % slurry of protein A/G agarose beads (Santa Cruz Biotechnology, Inc.) was brought to a final volume of 200 μ l with PBS, pH 7.2. In order to coat the beads 5 μ g of the depletion antibody was added and rotated continually for 3 h at 4°C. As a control, purified mouse - 5 μ g (Sigma-Aldrich) was used in a mock depletion. Antibody-coated beads were washed twice with cell extract buffer. 100 μ l Jurkat/neutrophil cell-free extract was then added to the beads and rotated overnight at 4°C to ensure efficient immunodepletion. Mock and depleted extracts were then incubated for 1 h at 37°C in the presence or absence of 50 μ g/ml cytochrome *c* and 1 mM dATP. The extracts were then probed with anti-caspase-3. Data are representative of three independent experiments.

3.3.6. Assessment of the relative levels of cytochrome *c* in neutrophils

In an effort to determine the actual amounts of cytochrome *c* in primary neutrophils, concentrated cell lysates were prepared. These were separated by SDS-PAGE in parallel with Jurkat or HEK293 cell lysates as controls. As is evident in Fig 3.13 cytochrome *c* was readily detected in Jurkat and HEK293 lysates with as few as 1×10^5 cells loaded per lane. Cytochrome *c* failed to be detected by Western Blot analysis, in neutrophil lysates of up to 4×10^5 cells per lane. Reprobing the blots with anti-manganese superoxide dismutase (MnSOD) highlighted that the neutrophil lysates were rich in other mitochondrial marker proteins.

In another attempt to detect cytochrome *c* in neutrophils we treated both Jurkat cells and neutrophils with two different cytotoxic agents known to trigger cytochrome *c* release from mitochondria – staurosporine and actinomycin D. Samples were taken at varying time-points over a 24 h period. Concentrated subcellular fractions (mitochondrial versus cytosolic) were prepared at each time point using a digitonin-based technique as described in Materials and Methods. Equal volumes of these fractions were subjected to SDS-PAGE and probed with anti-cytochrome *c* antibody. Replicate blots were also probed with anti-MnSOD. As is highlighted in Fig 3.14 cytochrome *c* release from mitochondrial fractions into cytosolic fractions was readily detectable in Jurkat cells as early as 7 h after treatment with both drugs. However, cytochrome *c* was not detected in neutrophil samples – even in the concentrated mitochondrial fractions. Again MnSOD was readily detected in both Jurkat and neutrophil mitochondrial fractions. The release of MnSOD into the cytosol was also evident at later time-points, confirming that apoptosis-associated mitochondrial damage occurred in these cells.

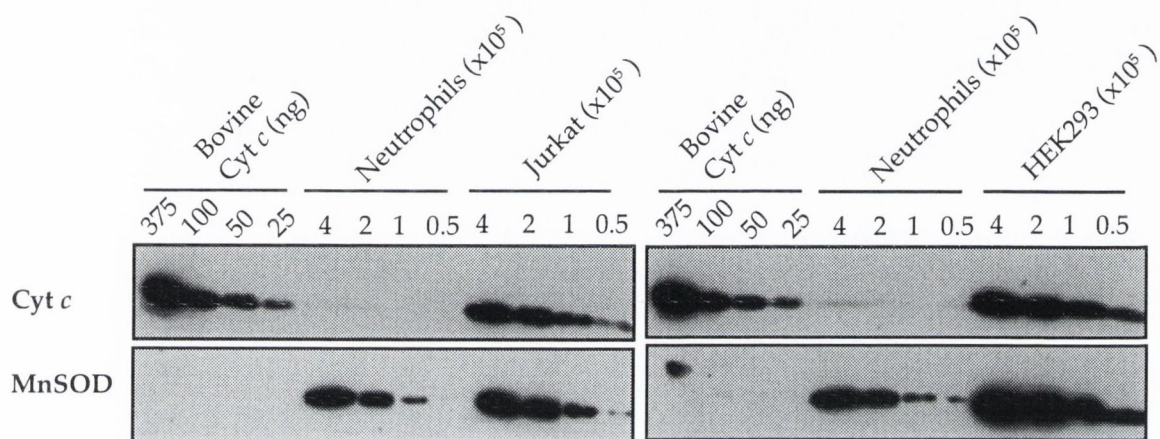


Fig 3.13. Relative levels of cytochrome *c* in primary neutrophils, Jurkat and HEK293 cells
 Total protein lysates were prepared from equal numbers of Jurkat, HEK293 and neutrophils (10^7 cells / ml). The indicated cell equivalents were loaded on 15% SDS-PAGE gels followed by Western Blot analysis of cytochrome *c* and MnSOD levels in the lysates. Bovine cytochrome *c* was electrophoresed to allow quantification of cytochrome *c*.

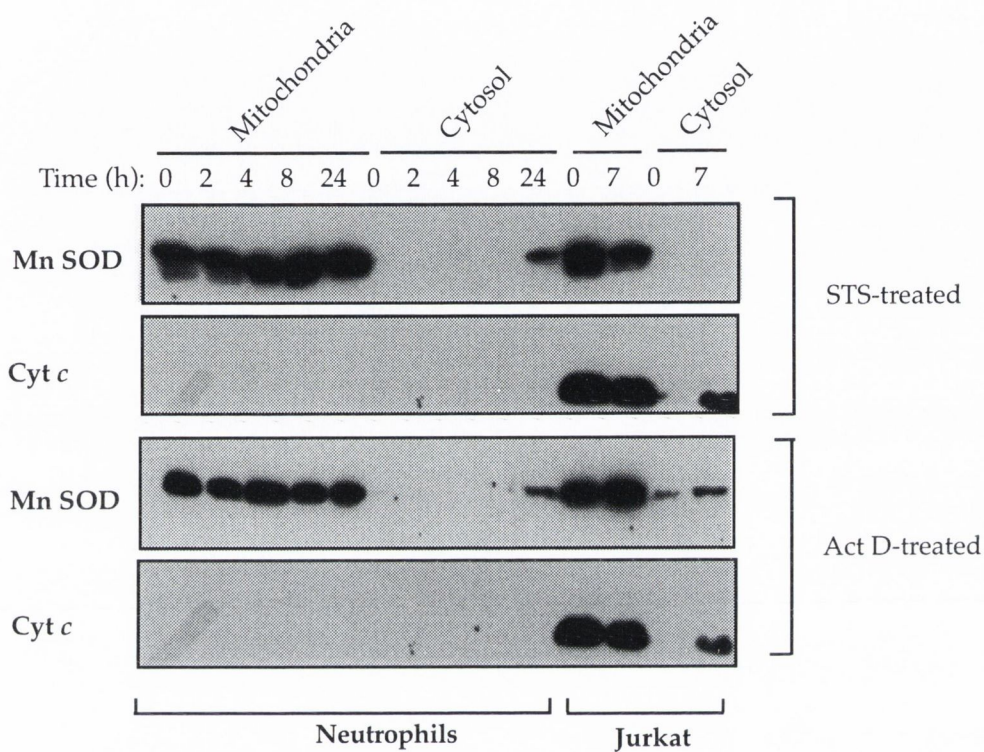


Fig 3.14. Apoptosis-associated cytochrome *c* release in Jurkat cells but not primary human neutrophils

Primary human neutrophils and Jurkat cells were treated with 500 nM staurosporine (STS) or 20 μ M actinomycin D (Act D). Mitochondrial and cytosolic fractions were prepared at the indicated time-points, as detailed in Materials and Methods, using a digitonin-based permeabilization technique. Cell fractions were then probed for cytochrome *c* and MnSOD.

In a further attempt to detect cytochrome *c* in primary neutrophils, immunoprecipitation experiments were performed on both Jurkat and neutrophil lysates. As is illustrated in Fig 3.15, this technique was successful in detecting cytochrome *c* in neutrophils. However approximately ten times more neutrophils than Jurkats were required to saturate the antibody used for immunoprecipitation. Thus relative to Jurkat cells, primary human neutrophils contain very modest amounts of cytochrome *c* (approximately 10% the amount of Jurkat cells).

3.3.7. Detection of both mitochondrial structures and cytochrome *c* in primary human neutrophils

Mitochondrial structures had previously been reported to be present in neutrophils (Pryde et al, 2000; Maiani *et al.*, 2002). However, no extensive studies had been conducted on the presence of cytochrome *c* in these structures. Reports have suggested that neutrophils are incapable of oxidative phosphorylation (Peachman *et al.*, 2001), suggesting that cytochrome *c* levels in neutrophils are too low to support a functional respiratory chain. Western Blot analysis of neutrophil lysates confirmed that whereas neutrophils contained relatively comparable levels of caspases-1, -3, -8, Actin and MnSOD, they were deficient in other mitochondrial proteins such as Smac, COX I and HSP-60 (Fig 3.16).

We decided to examine neutrophils for the presence of both mitochondria and cytochrome *c* to clearly define the location of cytochrome *c* within primary human neutrophils. When mitotracker Red, a dye that is preferentially incorporated into mitochondria, was incubated with primary human neutrophils, punctate mitochondrial-like structures were labelled (Fig 3.17). Hoechst dye allowed visualisation of the distinctive multi-lobed nuclei present in neutrophils. The mitochondrial staining pattern co-localised well with the pattern obtained from cytochrome *c* immunostaining experiments (Fig 3.17). Neutrophils do possess cytochrome *c* that is contained within mitochondrial-like structures.

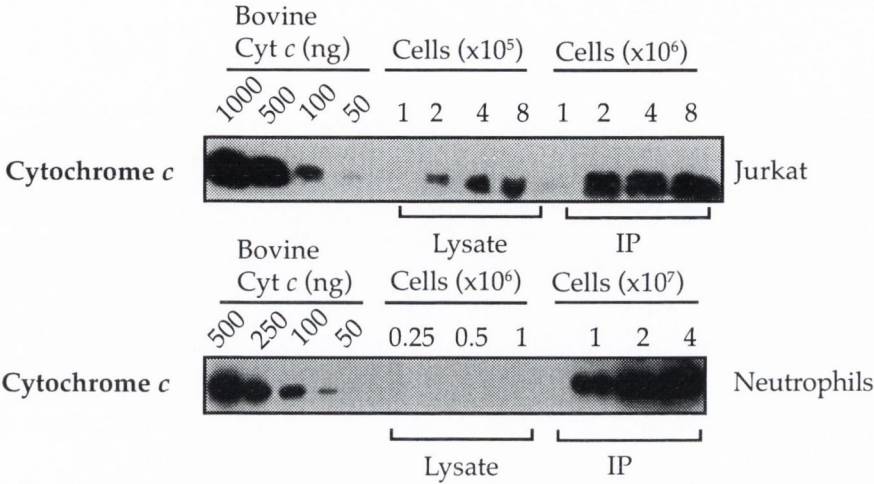


Fig 3.15. Relative levels of cytochrome *c* in primary human neutrophils and Jurkat cells

Cytochrome *c* was immunoprecipitated from lysates prepared from the indicated numbers of cells. Immunoprecipitations were performed as detailed in Materials and Methods. Briefly, 800 µl clarified lysates were incubated with 1 µg anti-cytochrome *c* antibody and 30 µl of a 50% slurry of protein A/G agarose beads (Santa Cruz Biotechnology, Inc). The lysates were rotated continuously at 4°C for 4-6 h. The resulting immunoprecipitated complexes were washed three times in IP lysis buffer containing 0.1% NP-40 and then analysed by Western Blotting. Bovine cytochrome *c* was electrophoresed to allow for quantification of cytochrome *c*.

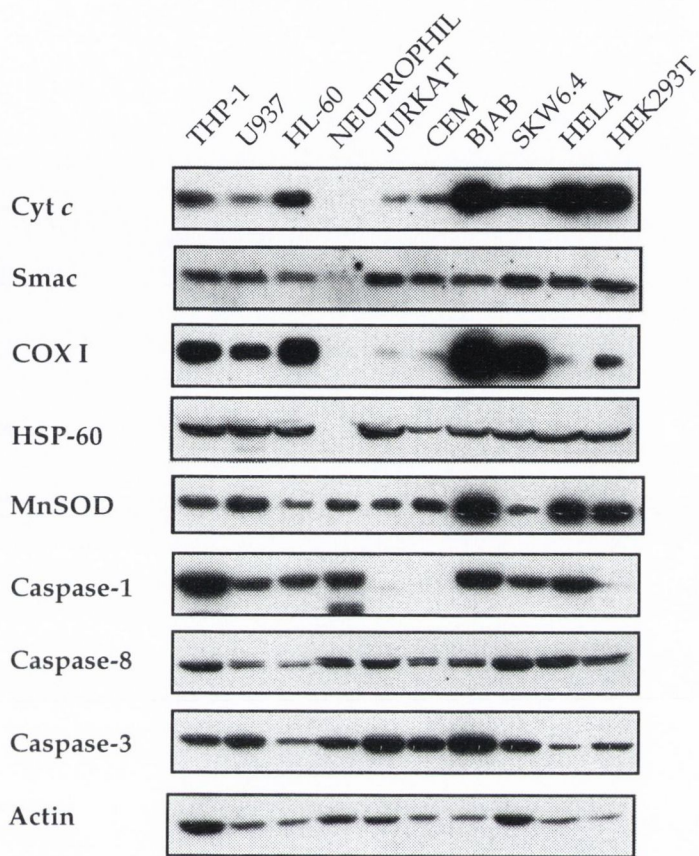


Fig 3.16. Neutrophils are deficient in cytochrome *c* and other mitochondrial proteins

Lysates (10^7 cells/ml) were prepared from primary human neutrophils and the indicated cell lines. Equal amounts of protein (100 μ g) were separated by SDS-PAGE followed by probing replicate blots with antibodies against the indicated proteins.

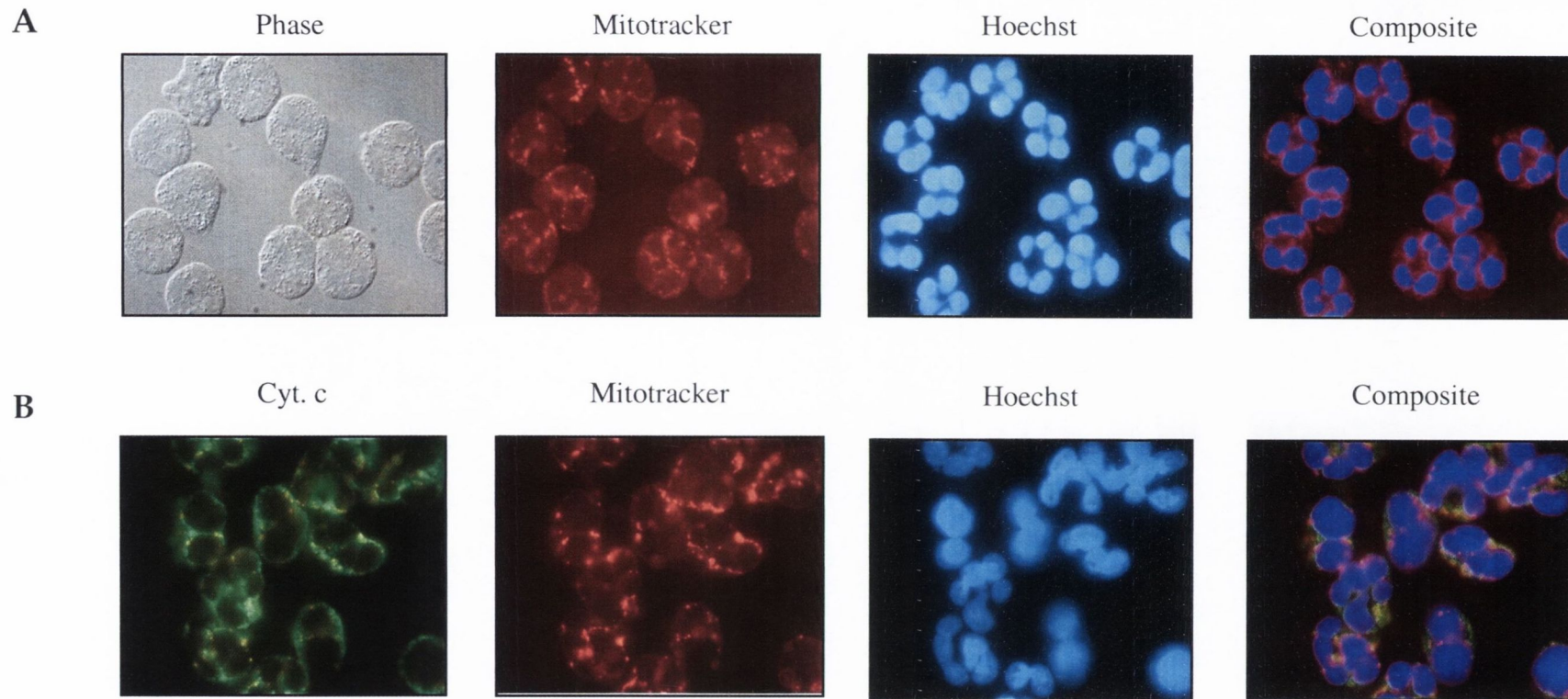


Fig 3.17. Immunolocalization of cytochrome *c* in primary human neutrophils.

(A) Purified human neutrophils were incubated for 45 min at 37°C in Mitotracker Red followed by fixation and staining with Hoechst dye to visualize nuclei.

(B) Neutrophils were labeled with Mitotracker Red followed by immunostaining for cytochrome *c* as described in Materials and Methods.

3.3.8. Lowered threshold for cytochrome *c*-apoptosome-mediated activation of caspases in primary human neutrophils

On the basis of cytochrome *c* immunodepletion experiments, it was clear that neutrophils displayed an absolute requirement for cytochrome *c* to initiate apoptosome-mediated caspase activation. In an effort to quantify exactly how little cytochrome *c* was required for apoptosome assembly in neutrophils, Jurkat, HEK293 and neutrophil cell-free extracts, depleted of cytochrome *c*, were generated. Exogenous cytochrome *c* was titrated back into these extracts in the presence of 1 mM dATP. Following SDS-PAGE, the resulting Western Blots were probed with anti-caspase-3 (Fig 3.18) . This experiment suggested that in primary human neutrophils the concentration of cytochrome *c*, required to trigger caspase activation can be lowered to approximately 5% of that required by Jurkat and HEK293 cells. This may be due to the fact that neutrophils contain very high levels of Apaf-1 in comparison to Jurkat or HEK293 extracts (Fig 3.9). It remains feasible that these high levels of Apaf-1 compensate for the lack of cytochrome *c* and hence, minimal cytochrome *c* is sufficient to trigger apoptosome formation because of the high abundance of Apaf-1 present. It is worth noting that the levels of IAPs found in neutrophils were also significantly lower than in other cell types (Fig 3.19). The latter observation could also explain the lowered threshold for apoptosome assembly in primary human neutrophils.

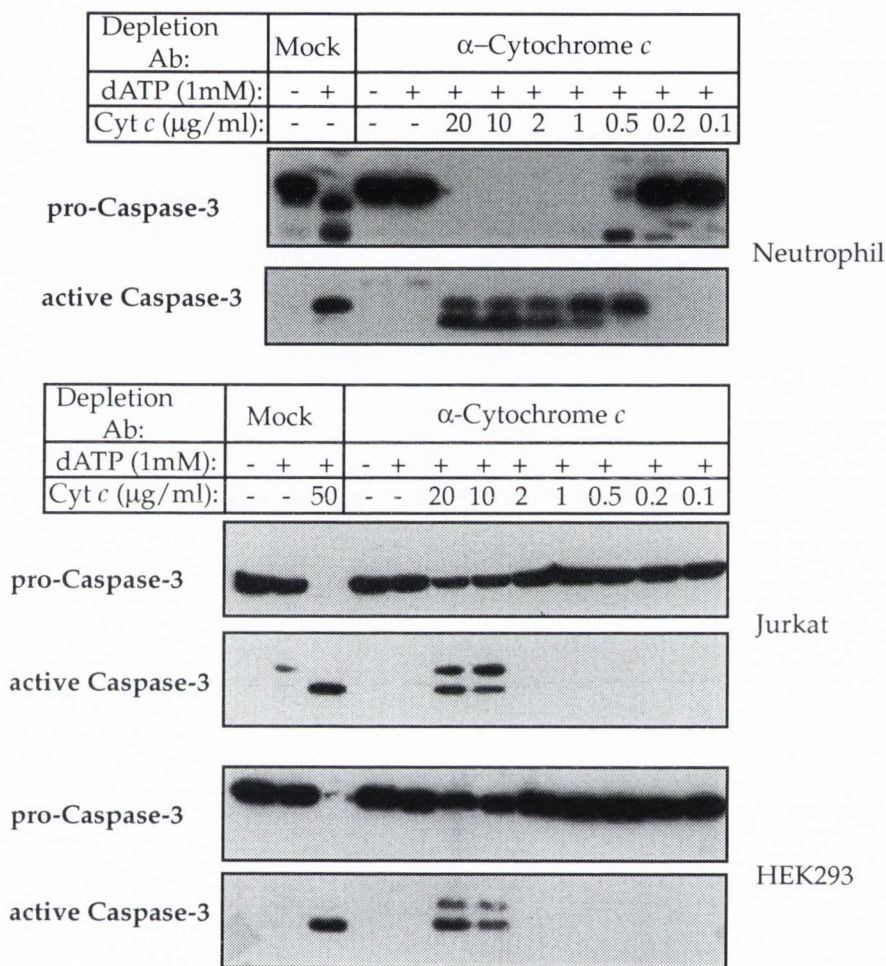


Fig 3.18. Neutrophil cell-free extracts exhibit a dramatically lowered threshold for cytochrome *c*-triggered caspase activation

Cell-free extracts were immunodepleted of cytochrome *c* as previously described. The concentration of exogenous cytochrome *c* required to trigger Apaf-1-dependent caspase activation was then assessed by incubation of the extracts for 1 h at 37° C in the presence of 1 mM dATP as indicated.

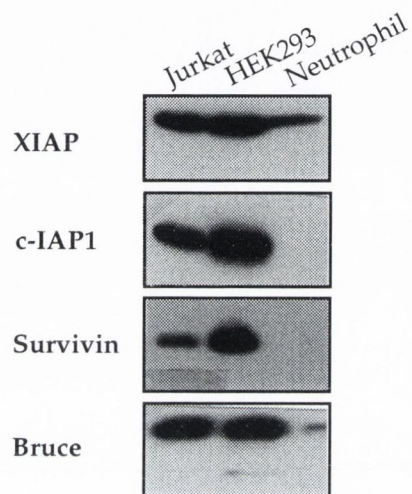


Fig 3.19. Neutrophils contain reduced levels of Inhibitor of Apoptosis Proteins (IAPs)

Equal amounts of Jurkat, HEK293 and primary human neutrophil cell-free extracts were separated by standard 12% SDS-PAGE followed by probing with the indicated IAP antibodies.

3.4 DISCUSSION

3.4.1. Caspase activation cascades

The order of caspase activation events in apoptosome-mediated apoptosis has previously been deduced from Jurkat cell-free extracts (Slee *et al.*, 1999b) and is illustrated in Fig 3.3. The results of this study revealed that this cascade is conserved not only in the transformed cell line – HEK293 but also in primary human neutrophils. These findings highlight that the key apoptotic caspases are present at similar levels in all cell types examined and undergo activation in a similar stepwise manner upon apoptosome assembly. Thus, the caspase cascade is likely to be a generalized feature in all cells undergoing apoptosis.

In the apoptosome caspase cascade, caspase-2 is a downstream target of caspase-3 (Slee *et al.*, 1999b; Fig 3.3). Recent reports have suggested that caspase-2 acts upstream of apoptosome formation to promote cytochrome *c* release from mitochondria (Guo *et al.*, 2002; Lassus *et al.*, 2002; Robertson *et al.*, 2002). Using RNA interference (RNAi) to downregulate caspase-2 expression, Lazebnik and colleagues showed a reduction in the level of apoptosis induced by the DNA-damaging agents, etoposide, cisplatin and UV (Lassus *et al.*, 2002). In addition these authors claimed that silencing caspase-2 expression reduced Bax translocation to mitochondria and subsequent release of cytochrome *c* and Smac/Diablo (Lassus *et al.*, 2002). Other groups have reported that recombinant caspase-2 can trigger cytochrome *c* and Smac/Diablo release from isolated mitochondria (Guo *et al.*, 2002). The hypothesis that caspase-2 acts at the mitochondria to regulate cytochrome *c* release contradicts the demonstrated downstream position of caspase-2 in the apoptosome-mediated caspase cascade. A number of issues need to be addressed before this “initiator” role of caspase-2 is accepted.

The exact mechanism used by caspase-2 to induce downstream caspase activation needs to be defined. One possible explanation, suggested by Kumar and colleagues, was that in response to certain death-inducing stimuli caspase-2 was incorporated into a high molecular weight complex, similar to the apoptosome (Read *et al.*, 2002). However, this hypothesis remains to be conclusively proven. Furthermore, the *CASP-2* knockout phenotype is essentially normal and lymphocytes and neurons

derived from such knockout mice are sensitive to pro-apoptotic stimuli *in vitro* (Bergeron *et al.*, 1998; O'Reilly *et al.*, 2002). This phenotype would not suggest that caspase-2 is a critical regulator of the mitochondrial pathway of caspase activation. Indeed, cells derived from *APAF-1*- or *CASP-9*-null animals fail to process caspase-2 (Bergeron *et al.*, 1998; O'Reilly *et al.*, 2002). Finally, neutrophils die in response to agents that provoke the mitochondrial pathway of caspase activation, such as actinomycin D and staurosporine, even though these cells appear to be deficient in caspase-2 (Fig 3.1; Santos-Beneit and Mollinedo, 2000). Collectively these findings argue in favour of a downstream role for caspase-2 in the apoptosome caspase cascade. A further issue that needs to be addressed is the complication associated with the use of siRNA to ablate gene expression. siRNA was used by Lazebnik and colleagues to show a requirement for caspase-2 in stress-induced apoptosis prior to mitochondrial permeabilization (Lassus *et al.*, 2002). Recent reports have shown however, that the small interfering RNAs (siRNAs), used to manipulate gene expression, can activate an interferon response in mammalian systems (Bridge *et al.*, 2003; Sledz *et al.*, 2003). A secondary concern with the use of RNAi is that the siRNAs may in fact cross-react with targets of limited sequence similarity (Jackson *et al.*, 2003). These non-specific effects of RNAi were not considered by Lazebnik and colleagues and cast doubt upon the conclusions drawn from these experiments.

In *C. elegans*, all programmed cell death events require CED-3 (Yuan *et al.*, 1993). In contrast, in mammalian systems, multiple homologs of the CED-3 protein exist (Earnshaw, 1999; Nicholson *et al.*, 1999) and the exact contribution made by each individual caspase to the apoptotic process remains to be determined. In the apoptosome-mediated caspase cascade, caspase-9 is the most apical caspase and the absence of such an initiator protease in this system inhibits any further caspase processing events (Li *et al.*, 1997; Hakem *et al.*, 1998; Kuida *et al.*, 1998; Slee *et al.*, 1999b). While these studies clearly highlight the importance of caspase-9 in the apoptosome pathway of caspase activation, the status of the downstream caspases in this cascade remains controversial. Caspase-3 and caspase-7 are the direct targets of caspase-9 in the cascade and these caspases, in association with caspase-6, are often collectively referred to as the executioner caspases (Earnshaw, 1999; Nicholson *et al.*, 1999). Such a generalisation is misleading however as these caspases cannot functionally substitute for each other (Slee *et al.*, 1999b). For instance, in the absence

of caspase-3, caspase-7 fails to substitute for caspase-3, and caspase-2 and caspase-6 are no longer processed (Slee *et al.*, 1999b). Indeed, caspase-8 and caspase-10 may only be processed by caspase-6 in this cascade (Slee *et al.*, 1999b; Cowling and Downward, 2002). In addition, there are clear distinctions between the knockout phenotypes of the executioner caspases. Deletion of the *CASPASE-3* gene in mice results in perinatal lethality due to a decrease in neuronal cell death during development (Kuida *et al.*, 1996). Deletion of the *CASPASE-7* gene in mice is reported to result in embryonic lethality (Zheng *et al.*, 1999). Earnshaw and colleagues have recently examined the induction of apoptosis in *CASPASE-7*^{-/-} DT40 chicken β -lymphoma cells and propose that these cells are more resistant to apoptosis-inducing agents than wild-type DT40 cells (Korfali *et al.*, 2003). These authors argue that these results place caspase-7 upstream of caspase-3 and caspase-6, but this hypothesis demands a more critical assessment (Korfali *et al.*, 2003). In stark contrast deletion of the *CASPASE-6* gene in mice is reported to have no adverse effects (Zheng *et al.*, 1999). *CASPASE-6*^{-/-} DT40 chicken β -lymphoma cells undergo apoptosis in a similar manner to wild-type cells (Ruchaud *et al.*, 2002). The same study claims that caspase-6 is required for completion of chromatin condensation and apoptotic body formation in nuclei containing lamin A, the only substrate currently known to be specifically cleaved by caspase-6 (Slee *et al.*, 2001; Ruchaud *et al.*, 2002). Therefore, the *CASPASE-3* knockout phenotype would suggest a critical role for this protease in the brain. The *CASPASE-7* knockout phenotype would suggest this enzyme is critical during embryonic development, while the *CASPASE-6* knockout phenotype would not propose any particular significant role for this caspase. Such stark contrasts severely undermine the possibility of functional redundancy between the executioner caspases. In fact, the remarkable similarity between the caspase-3 knockout phenotype (Kuida *et al.*, 1996) and the caspase-9 knockout phenotype (Hakem *et al.*, 1998; Kuida *et al.*, 1998), would argue that caspase-3 may be functionally more equivalent to caspase-9 rather than caspase-6 or caspase-7. The role of caspase-3 as an initiator-type caspase is highlighted by studies that have identified caspase-3 as the primary executioner of substrate cleavage events during apoptosis (Slee *et al.*, 2001).

3.4.2. Apoptosome formation in primary human neutrophils

In our examination of apoptosome formation in primary human neutrophils we established that neutrophils required both Apaf-1 and caspase-9 for functional apoptosome formation. However, reducing the levels of exogenous cytochrome *c* added to Jurkat, HEK293 and primary human neutrophil extracts revealed that neutrophils could tolerate substantially lower levels of cytochrome *c* for apoptosome formation, in comparison to Jurkat or HEK293 cells. Further analysis revealed that the levels of cytochrome *c* in neutrophil lysates were approximately 10-fold lower than in Jurkat or HEK293 lysates. Furthermore, the levels of cytochrome *c* required to trigger apoptosome formation in primary human neutrophils can be lowered to approximately 5% of that required by Jurkat and HEK293 extracts.

The initial discovery of the involvement of cytochrome *c* in apoptosome formation was extremely surprising and is unique to mammalian systems (Liu *et al.*, 1996). In the worm, CED-3 activation is regulated by CED-9, which sequesters CED-4 until a signal to die is received (Conradt and Horvitz, 1998). Two separate studies have also proposed that the fly apoptosome forms without cytochrome *c* involvement (Dorstyn *et al.*, 2002; Zimmermann *et al.*, 2002). Cytochrome *c* release was not observed in *Drosophila* cells during apoptosis triggered by various stimuli such as, growth factor deprivation, exposure to cytotoxic drugs or UV (Zimmermann *et al.*, 2002). These agents readily initiate cytochrome *c* release in mammalian cells. The second study used RNAi to ablate expression of cytochrome *c* and demonstrated that despite a massive reduction in cytochrome *c* expression, there was no interference with the assembly of the fly apoptosome (Dorstyn *et al.*, 2002). A recent study has reported a requirement for a specific form of cytochrome *c*, encoded by the *cyt-c-d* gene, to facilitate effector caspase activation at the onset of sperm differentiation in *Drosophila* (Arama *et al.*, 2003). This suggests that cytochrome *c* may play a role in certain caspase activating contexts in *Drosophila*. DARK, the Apaf-1/CED-4 related molecule in *Drosophila*, contains multiple WD-40 repeats but this domain does not act as a negative regulator of DARK function as it does in mammalian Apaf-1 (Zou *et al.*, 1997). Therefore the regulatory function of Apaf-1 is dependent on the ability of the WD-40 repeat region to act as a cytochrome *c* sensor within the cytoplasm. The results presented in this chapter highlight that even a trickle of cytochrome *c* in the cytoplasm of primary human neutrophils was detected by Apaf-1 and hence caspase

activation in neutrophils was subjected to the same level of regulation as in other mammalian systems.

Immunofluorescence analysis also successfully detected cytochrome *c* in neutrophils (Fig 3.17). Cytochrome *c* co-localised with punctate mitochondrial-like structures, thereby arguing that neutrophils do in fact possess mitochondria. This finding is in agreement with Pryde *et al.*, 2000 and Maianski *et al.*, 2002. Neutrophil mitochondria have been reported to be incapable of oxidative phosphorylation (Peachman *et al.*, 2001). The results presented here support this theory as cytochrome *c* is a central component of the mitochondrial electron transport chain and it is doubtful that the low levels of cytochrome *c* present in neutrophils could support mitochondrial respiration. Eosinophils, like neutrophils, have also been reported to be incapable of oxidative phosphorylation, possessing few mitochondria and little cytochrome *c* (Dewson *et al.*, 2001; Peachman *et al.*, 2001). It has been suggested that eosinophils use their cytochrome *c* exclusively for initiating apoptosis (Peachman *et al.*, 2001). The results presented here would argue that neutrophils also use the little cytochrome *c* retained in mitochondrial-like structures, solely for initiating apoptosis. A recent report by Kuijpers and colleagues supports this proposal (Maianski *et al.*, 2003).

Neutrophils have a very short half-life in circulation (8-20 h; Akgul *et al.*, 2001). In the absence of external stimuli, such as those found at the site of infection, aged neutrophils undergo spontaneous apoptosis (Savill *et al.*, 1989). While death receptor mediated-apoptosis could be a primary trigger of such spontaneous programmed cell death, it must be remembered that neutrophils from FasL-deficient-mice maintain their constitutive apoptotic programme (Brown and Savill, 1999). The *in vivo* relevance of apoptosome-mediated caspase activation in neutrophil apoptosis remains to be determined. However, in addition to having a substantially reduced requirement for cytochrome *c*, in comparison to other cell types, to induce apoptosome formation, neutrophils also have elevated levels of Apaf-1 and lowered levels of IAPs. It is interesting to speculate that cells with little cytochrome *c* evolved to have such aberrant levels of other apoptosis-related proteins as a compensatory mechanism thus ensuring that the crucial balance between cell death and cell proliferation is maintained.

CHAPTER IV

Caspase-8 Activation Mechanisms

4.1. INTRODUCTION

4.1.1. The identification of caspase-8

Caspase-8 was initially identified as the protease involved in the death signal induced by death receptor ligation (Boldin *et al.*, 1996; Muzio *et al.*, 1996). In 1995, Kischkel *et al.*, reported the formation of the death-inducing signalling complex (DISC) at the activated CD95/Fas receptor (Kischkel *et al.*, 1995). The DISC was comprised of the activated receptor and four proteins, which the authors termed CAP1, CAP2, CAP3 and CAP4. (CAP: cytotoxicity-dependent APO-1-associated protein). CAP1 and CAP2 were identified as two forms of FADD, differing only in terms of their phosphorylation state. CAP3 and CAP4 remained unidentified (Kischkel *et al.*, 1995). CrmA, a serpin isolated from the cowpox virus that inhibits ICE/caspase-1, inhibits cell death induced by CD95/Fas and TNF (Enari *et al.*, 1995; Los *et al.*, 1995). It was hypothesised that an ICE-like protease /caspase was involved in receptor-mediated cell death. This hypothesis was confirmed when CAP4 was identified as FLICE or caspase-8. CAP4 was the full-length enzyme and CAP3 was an N-terminal fragment (Fig 4.1a; Muzio *et al.*, 1996).

4.1.2. Caspase-8 Structure

The domain structure of caspase-8 is shown in Fig 4.1a. The crystal structure highlights that the active caspase-8 enzyme, like caspase-1 and -3, is comprised of two (p18-p12) subunits. Each p18-p12 sub-unit is composed of twisted β -sheet formed by six β -strands (Fig 4.1b). Five of the β -strands run parallel (a, b, c, d and e) to each other and the remaining (f) runs anti-parallel. Two α -helices (H2 and H3) are on side of the β -sheet and three α -helices (H1, H4 and H5) are on the other. The active site of each subunit is located at the C-terminal end of the five parallel β -strands. The active sites are diametrically opposite one another in the dimer – Fig 4.1b (Blanchard *et al.*, 1999; Shi, 2002).

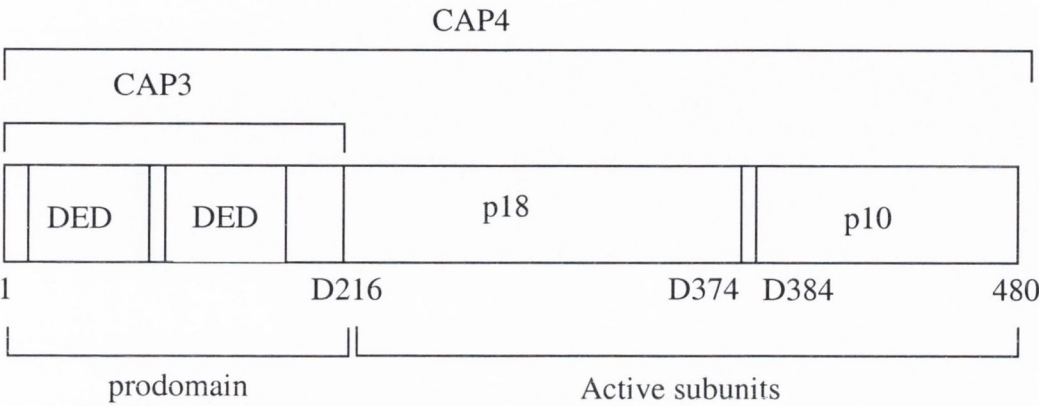


Fig 4.1(a). Domain structure of caspase-8 adapted from Muzio *et al.*, 1996; Chang *et al.*, 2002.

The prodomain consists of two tandem DEDs. The large subunit is p18 and the small subunit is p10. The sequence of cleavage events proposed by the Induced Proximity Model to liberate the active enzyme is, cleavage at D374 between the large and small subunits, to generate p43 and p12 fragments and subsequently, cleavage at D216 and D384 to generate the active p18/p10 subunit and the prodomain.

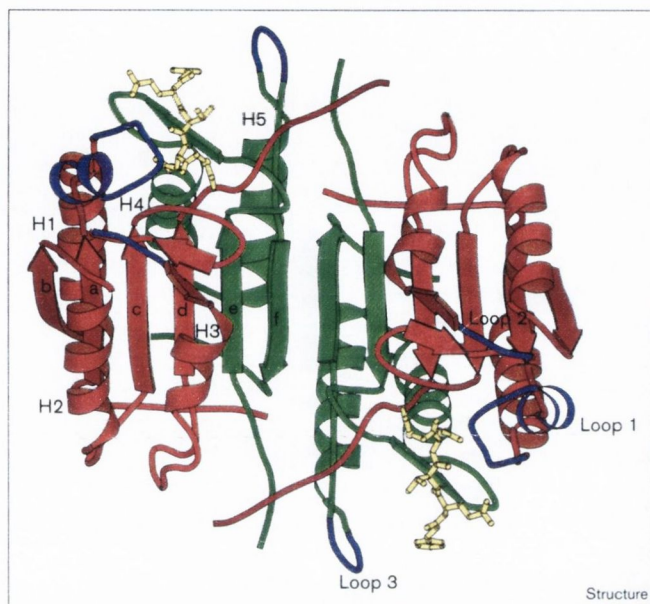


Fig 4.1(b). Ribbon diagram of the (p18-p12)₂ caspase-8 dimer structure taken from Blanchard *et al.*, 1999.

The active caspase-8 enzyme is comprised of two (p18-p12) subunits. Each p18-p12 is composed of twisted β -sheet formed by six β -strands. Five of the β -strands run parallel (a, b, c, d and e) to each other and the remaining (f) runs anti-parallel. Two α -helices (H2 and H3) are on side of the β -sheet and three α -helices (H1, H4 and H5) are on the other. The active site of each subunit is located at the C-terminal end of the five parallel β -strands. The active sites are diametrically opposite one another in the dimer

4.1.3. Caspase-8 Activation

The sequence of events required to achieve apical caspase-8 activation may be explained by alternative models

- Induced proximity model
- Allosteric Regulation Model.

Induced proximity model

Caspase-8 zymogens are unable to be purified from *E. coli* because the enzyme rapidly undergoes autoprocessing due to its intrinsic catalytic activity (Salvesen and Dixit, 1999). It is this intrinsic catalytic activity that is responsible for caspase-8 activation at the DISC. The increase in the local concentration of caspase-8 at the DISC allows for clustering of the enzyme and the intrinsic activity of the caspase facilitates proteolytic processing and release of the active (p18-p10) dimer (Medema *et al.*, 1997 a; Muzio *et al.*, 1998). Cleavage at Asp374 between the large and small subunits generated p43 and p12 fragments. Subsequent cleavage at Asp216 and Asp384 generated the active p18/p10 subunit and the prodomain (Medema *et al.*, 1997a; Fig 4.1a. It had been proposed that the caspase molecules processed each other *in trans* i.e. one zymogen processing the other (Salvesen and Dixit, 1999). It has since been shown however, that a dimerized caspase-8 molecule can process itself by intramolecular processing and subsequently process other caspase-8 molecules *in trans* (Chen *et al.*, 2002). Initial intramolecular processing is supported by crystal structure information. The active sites in the caspase-8 dimer are on opposite sides of the molecule, and therefore intramolecular processing is sterically more favourable (Chen *et al.*, 2002).

Allosteric Regulation Model

The intrinsic catalytic activity of caspase-8 is also the focus of this model. However, dimerization is thought to be the critical event required to harness this intrinsic power and interchain proteolysis is unnecessary to achieve caspase-8 activation.

This theory is supported by the following evidence:

- Full-length, inactive caspase-8 exists predominantly as a monomer (Boatright *et al.*, 2003).

- Dimerization, even in the absence of proteolysis, is sufficient to induce enzymatic activity (Boatright *et al.*, 2003; Chang *et al.*, 2003; Donepudi *et al.*, 2003).

Grutter and colleagues recently showed that increasing the concentration of procaspase-8 molecules lacking the DED domains (Δ DED), or substrate binding, triggered dimerization and enhanced the activity of this construct. This study also highlighted that an uncleavable Δ DED procaspase-8 construct cannot form dimers and is 100-fold less active than its dimeric counterpart (Donepudi *et al.*, 2003). Salvesen and colleagues employed kosmotropes, salts with the ability to stabilize protein structures, to emphasize that dimerization of Δ DED procaspase-8 enhanced the catalytic activity of this construct (Boatright *et al.*, 2003). In addition, mutation of key residues that contribute to dimer formation in the procaspase zymogen, abolished catalytic activity (Boatright *et al.*, 2003; Chang *et al.*, 2003). However, it must be stated that these mutations would also interfere with formation and activity of the processed dimer.

Caspase-8 Activation in the absence of a dimerization stimulus

The allosteric regulation model maintains that in order for caspase-8 to undergo activation, dimerization of inactive monomers must be achieved (Boatright *et al.*, 2003; Donepudi *et al.*, 2003). This model predicts that proteolytic processing of caspase-8 alone is insufficient to achieve activation of this caspase (Boatright *et al.*, 2003; Donepudi *et al.*, 2003). There are at least two well-defined contexts in which caspase-8 processing is seen in the absence of a dimerization stimulus, granzyme B-mediated activation (Muzio *et al.*, 1996; Medema *et al.*, 1997b; Sutton *et al.*, 2000) and caspase-6-mediated activation (Slee *et al.*, 1999; Cowling and Downward 2002). The allosteric model predicts that proteolysis of caspase-8 by granzyme B or caspase-6 will fail to yield an active enzyme, a hypothesis we wished to explore in this chapter.

4.1.4. Granzyme B-initiated caspase activation

The cytotoxic T cell protease, granzyme B, can directly process at least two caspases; caspase-8 and caspase-3 (Chinnaiyan *et al.*, 1996; Froelich *et al.*, 1996; Medema *et al.*, 1997b; Atkinson *et al.*, 1998; Yang *et al.*, 1998; Sutton *et al.*, 2000). Processing of several other caspases occurs as a response to the delivery of granzyme B into intact cells, in cell-free systems or *in vitro* (Chinnaiyan *et al.*, 1996; Duan *et al.*, 1996;

Fernandes-Alnemri *et al.*, 1996; Gu *et al.*, 1996; Martin *et al.*, 1996; Muzio *et al.*, 1996; Orth *et al.*, 1996; Quan *et al.*, 1996; Yang *et al.*, 1998). However, it remains unclear which of the caspases are preferentially processed and activated by this CTL/NK granule protease and the hierarchy of caspase activation events that follow downstream. Previous studies have suggested that caspase-3 and caspase-8 are the physiological targets of granzyme B (Chinnaiyan *et al.*, 1996; Froelich *et al.*, 1996; Medema *et al.*, 1997b; Atkinson *et al.*, 1998; Yang *et al.*, 1998; Sutton *et al.*, 2000) but, as discussed in the previous section, recent data from the Salvesen and Grutter laboratories have challenged the view that proteolytic processing of caspase-8 leads to activation of this caspase (Boatright *et al.*, 2003; Donepudi *et al.*, 2003).

4.2. CHAPTER AIMS

The aims of this chapter were twofold.

(A) Determination of the hierarchy of caspase activation in granzyme B-mediated caspase cascade

Granzyme B can activate caspases both directly and indirectly. The caspases directly targeted by granzyme B are proposed to be caspases-3 and -8 (Chinnaiyan *et al.*, 1996; Froelich *et al.*, 1996; Medema *et al.*, 1997b; Atkinson *et al.*, 1998; Yang *et al.*, 1998; Sutton *et al.*, 2000). As was shown in previous chapter the hierarchy of apoptosome-mediated caspase activation events is conserved among different cell types. As caspase-9 is the most apical caspase in the apoptosome-mediated caspase cascade we hypothesised that a granzyme B initiated caspase cascade would differ significantly from the apoptosome-mediated pathway. Our primary aim was to determine the order of caspase activation events in granzyme B-stimulated Jurkat cell-free extracts. This was achieved by immunodepleting Jurkat cell-free extracts of either caspase-3, -6, -7, -8 or -9 and then monitoring the activation of the remaining caspases upon the addition of purified human granzyme B.

(B) Determining whether the allosteric regulation model holds true for all instances of caspase-8 activation

If the proposed Allosteric Regulation Model is correct, then granzyme B-processed caspase-8 will fail to produce active enzyme. Caspase-8 can also be directly processed by caspase-6 in the apoptosome-mediated caspase cascade (Slee *et al.*, 1999; Cowling and Downward 2002). The allosteric model brings the activity of caspase-8 into question in this context as well. A secondary objective of this chapter was to investigate the activity of caspase-8 in both these instances and to determine whether dimerization was in fact a prerequisite for caspase-8 activation.

4.3. RESULTS

4.3.1. Granzyme-B initiated Caspase cascade

To explore the order of caspase activation events initiated by granzyme B we used Jurkat cell-free extracts immunodepleted of caspases-3, -6, -7, -8 and -9. Depleted extracts were then left untreated or supplemented with 100 nM granzyme B and incubated at 37°C for two hours. Following SDS-PAGE blots were probed with antibodies specific to various caspases. Samples of each depleted extract were also subjected to SDS-PAGE and probed with the relevant antibody to ensure efficient depletion of specific caspases. As shown in Fig 4.2, all caspases were successfully depleted, whereas mock-depletions, using a mouse IgM antibody, had no effect on caspase levels.

We next examined the ability of these caspase-depleted extracts to support granzyme B-mediated caspase activation. As is evident in Fig 4.3 depletion of caspases-6, -7, -8 and -9 had no effect on caspase-3 processing. Hence, caspase-3 processing via granzyme B is direct and not reliant on any other caspase. The status of caspase-8 processing in the various depleted extracts was then examined (Fig 4.4). As anticipated from previous studies, granzyme B was able to directly process caspase-8 (Medema *et al.*, 1997b). This effect was not abolished by depletion of caspases -6, -7 or -9. Intriguingly however, caspase-3 depletion prevented complete caspase-8 processing. The release of p18 was evidently impaired in the absence of caspase-3 (Fig 4.4). Hence, even though caspase-8 is a direct target of granzyme B, there is also a dependence on caspase-3 in order to complete the processing. This result reveals that granzyme B is capable of processing caspase-8 at its first cleavage site (D374) but caspase-3 is required to process the protease at the second cleavage site (D216; Fig 4.1a). Caspase-7 has also been proposed to be another direct physiological target of granzyme B (Metkar *et al.*, 2003). A separate study has reported that activation of this caspase is dependent on caspase-3 in a granzyme B-mediated cascade (Yang *et al.*, 2003). In the cell-free system, immunodepletion of caspases-3, -6, -8 or -9 had no effect on caspase-7 processing (Fig 4.5). Thus, caspase-7 represents another direct physiological target of granzyme B in agreement with a recent study (Metkar *et al.*, 2003).

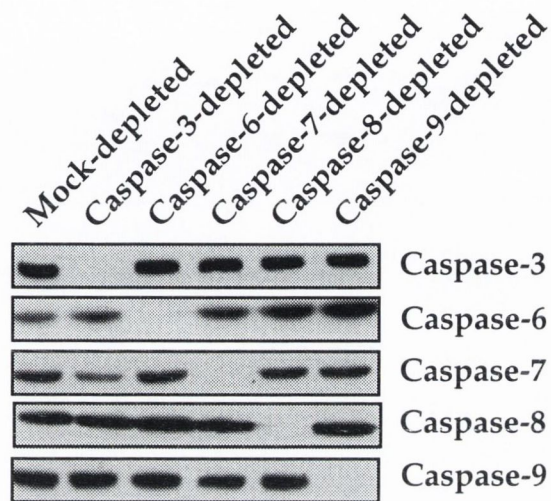


Fig 4.2. Immunodepletion of Caspases from Jurkat cell-free extracts

Immunodepletions were performed as detailed in Materials and Methods. Briefly, 40 μ l of a 50% slurry of protein A/G agarose beads (Santa Cruz Biotechnology, Inc.) were brought to a final volume of 200 μ l with PBS, pH 7.2. 5 μ g of the specific caspase depletion antibody was added and rotated continually for 3 h at 4°C. As a control, purified mouse - 5 μ g (Sigma-Aldrich) was used in a mock depletion. Antibody coated beads were washed twice with cell extract buffer. 100 μ l Jurkat cell-free extracts were then added to the beads and rotated overnight at 4°C to ensure efficient immunodepletion. Equal amounts of depleted Jurkat cell-free extracts were separated by SDS-PAGE followed by probing with antibodies against the indicated caspases.

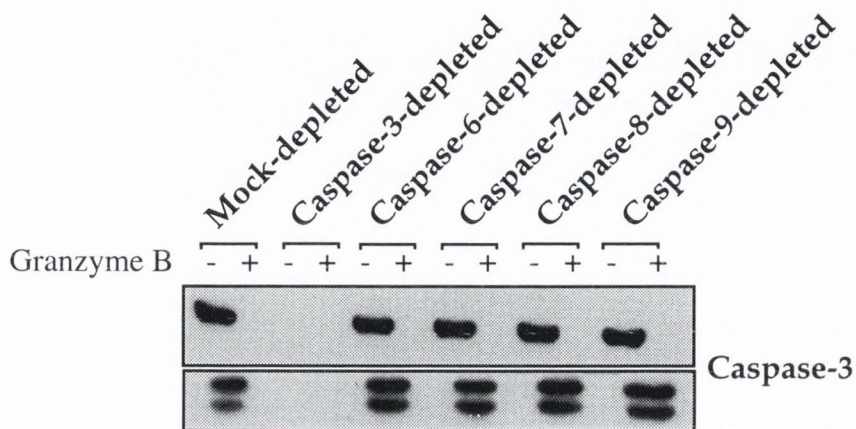


Fig 4.3. Caspase-3 is a direct target of granzyme B

Depleted Jurkat cell-free extracts were incubated at 37°C for 2 h in the presence or absence of 100 nM granzyme B. The extracts were then separated by SDS-PAGE and probed with anti-caspase-3 antibody. Data are representative of three independent experiments.

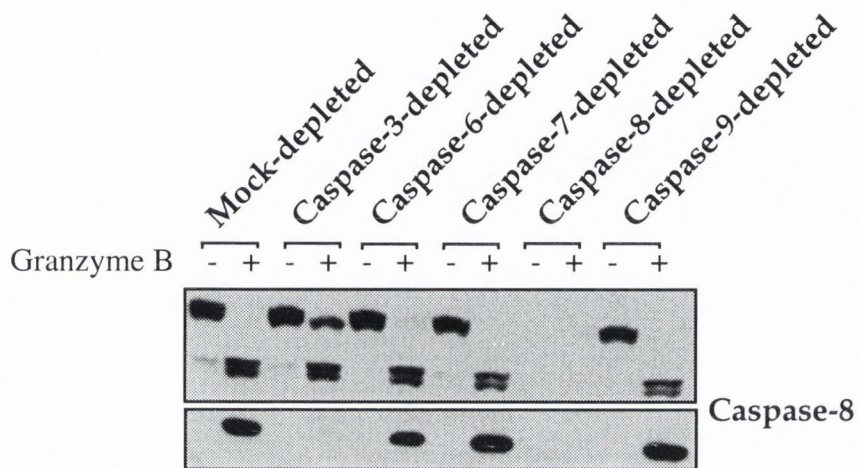


Fig 4.4. Caspase-8 is a direct target of granzyme B but requires active caspase-3 for complete processing

Depleted Jurkat cell-free extracts were incubated at 37°C for 2 h in the presence or absence of 100 nM granzyme B. The extracts were then separated by SDS-PAGE and probed with anti-caspase-8 antibody. Note the absence of caspase-8 p18 formation in caspase-3 depleted extract. Data are representative of three independent experiments.

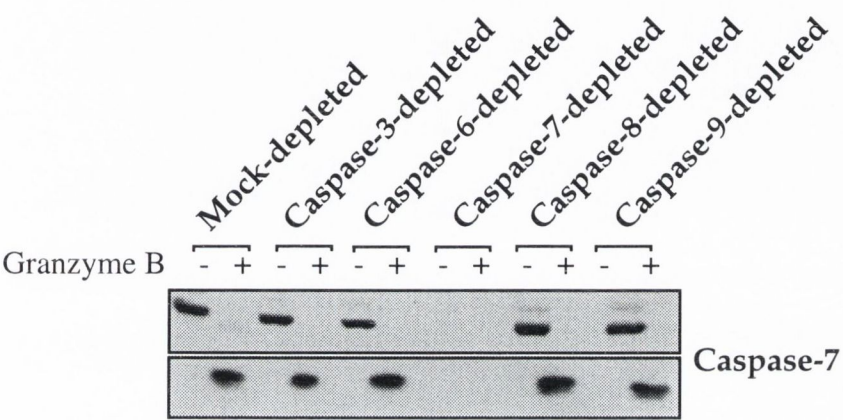


Fig 4.5. Caspase-7 is a direct target of granzyme B

Depleted Jurkat cell-free extracts were incubated at 37°C for 2 h in the presence or absence of 100 nM granzyme B. The extracts were then separated by SDS-PAGE and probed with anti-caspase-7 antibody. Data are representative of three independent experiments.

The next caspase examined was caspase-2 (Fig 4.6). Mock-depletion or depletion of caspases -6, -7, -8 or -9 had no effect on caspase-2 processing. However, depletion of caspase-3 completely abrogated caspase-2 processing. Thus caspase-2 is not a direct target of granzyme B but is activated in a cascade via caspase-3, in a manner similar to that originally proposed for caspase-7 (Yang *et al.*, 1998). Caspase-6 and caspase-9 have both been reported to be *in vitro* targets of granzyme B (Duan *et al.*, 1996; Orth *et al.*, 1996). However, in both these instances there were no other caspase targets in the reaction milieu and therefore we sought to determine whether these proteases remained direct granzyme B targets at physiological levels. These results mirror the caspase-2 result and are shown in Fig 4.7 and 4.8 respectively. Mock-depletion or depletion of caspases-6, -7, -8 or -9 did not hinder caspase-6 or -9 processing, caspase-3 depletion did, however, prevent their activation. These caspases are not therefore the direct physiological targets of granzyme B and require caspase-3 as the mediator of their proteolytic processing.

On the basis of these observations, the order of caspase activation events that are triggered by entry of granzyme B into a target cell can be represented as illustrated in Fig 4.9. In this cascade caspases-3 and -7 are direct physiological targets of granzyme B. Caspase-8 may be processed at its first processing site (D374) by granzyme B but cleavage at the second site (D216) is dependent on the proteolytic activity of caspase-3. Under our experimental conditions, caspases-2, -6 and -9 require processing by caspase-3 in a granzyme B-mediated cascade.

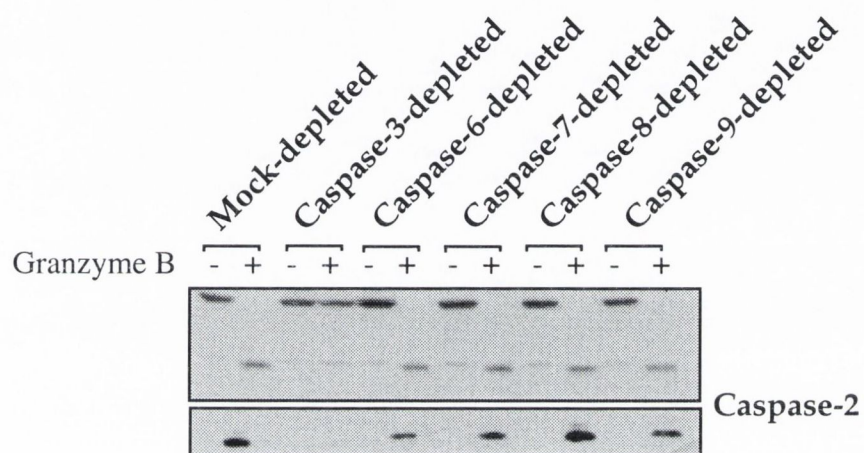


Fig 4.6. Caspase-2 is activated via caspase-3 in a granzyme B-mediated caspase cascade

Depleted Jurkat cell-free extracts were incubated at 37°C for 2 h in the presence or absence of 100 nM granzyme B. The extracts were then separated by SDS-PAGE and probed with anti-caspase-2

antibody. Note the absence of caspase-2 activation in caspase-3 depleted extract. Data are representative of three independent experiments.

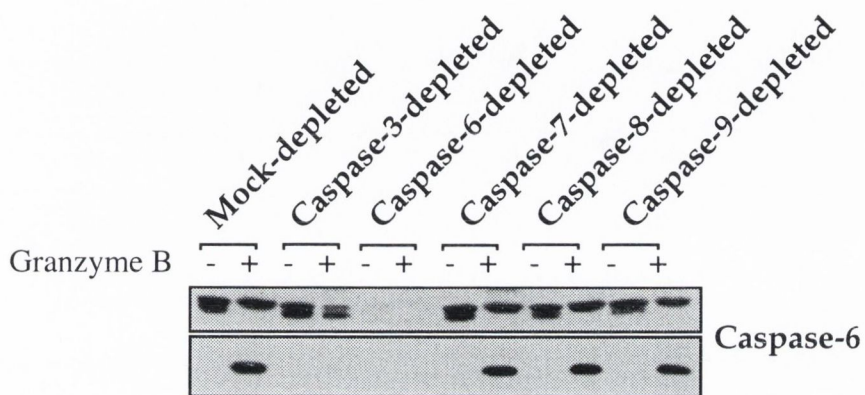


Fig 4.7. Caspase-6 is activated via caspase-3 in a granzyme B-mediated caspase cascade

Depleted Jurkat cell-free extracts were incubated at 37°C for 2 h in the presence or absence of 100 nM granzyme B. The extracts were then separated by SDS-PAGE and probed with anti-caspase-6 antibody. Note the absence of caspase-6 activation in caspase-3 depleted extract. Data are representative of three independent experiments.

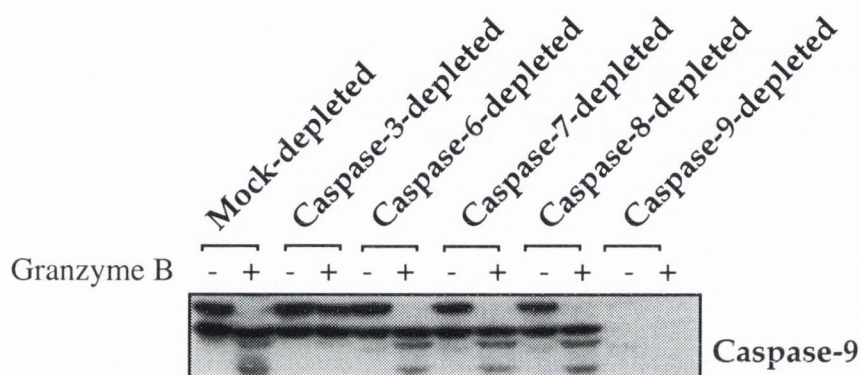


Fig 4.8. Caspase-9 is activated via caspase-3 in a granzyme B-mediated caspase cascade

Depleted Jurkat cell-free extracts were incubated at 37°C for 2 h in the presence or absence of 100

nM granzyme B. The extracts were then separated by SDS-PAGE and probed with anti-caspase-9

antibody. Note the absence of caspase-9 activation in caspase-3 depleted extract. Data are representative

of three independent experiments.

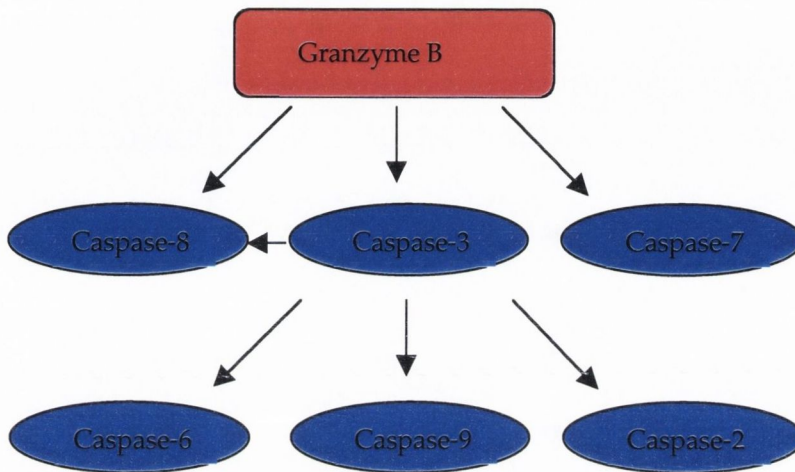


Fig 4.9. Schematic representation of the granzyme B-mediated caspase cascade

Caspases-3 and -7 are direct physiological targets of granzyme B. Caspase-8 may be processed at its first processing site (D374) by granzyme B but cleavage at the second site (D216) is dependent on the proteolytic activity of caspase-3. Caspases-2, -6 and -9 also require processing by caspase-3.

4.3.2. Can direct interchain proteolysis result in the activation of caspase-8?

To date, it has generally been assumed that the appearance of processed forms of caspases correlated with their activation status, i.e. their conversion from a single chain inactive enzyme to their active two chain form (illustrated in Fig 4.10). As previously stated, recent studies have cast doubt upon this hypothesis, suggesting that dimerization is a pre-requisite for the activation of “initiator” caspases, in particular caspase-8 (Boatright *et al.*, 2003; Donepudi *et al.*, 2003). As stated in the introduction to this chapter there are two instances when caspase-8 is clearly processed in the absence of a dimerization platform - granzyme B-mediated or apoptosome-mediated (Fig 4.10). The illustrated cleavage patterns are the same as those observed in the presence of a dimerization platform (Fig 4.13b) but in light of the recently proposed dimerization model we sought to explore whether caspase-8 processing was a true indication of its activation status. Our efforts to resolve this issue are detailed below.

4.3.3. Affinity-labelling of active caspase-8

To explore whether granzyme B or caspase-6 could activate caspase-8 we utilised two different biotin-tagged affinity labels –biotin-VAD-FMK and biotin-YVAD-CMK. These were incubated with caspase-8 after processing of the enzyme in response to the addition of granzyme B or cytochrome *c*/dATP to cell-free extracts. The caspase recognition sequence – VAD or YVAD – should allow binding between the label and the cysteine in the active site of the enzyme to occur. The functional group – FMK (fluoromethylketone) or CMK (chloromethylketone)– ensured the binding was irreversible. The biotin tag then allowed capture of the active enzyme/label complex (Kohler *et al.*, 2002). We treated Jurkat cell-free extract with either (a) no stimulus (b) 100 nM granzyme B or (c) 50 µg/ml cytochrome *c* and 1 mM dATP. The extracts were incubated at 37°C for two hours. 10 µM biotin-VAD-FMK or 10 µM biotin-YVAD-CMK was then added and the extracts were again incubated at 37°C. Samples were taken at 10, 20 and 30 minutes. Biotin-tagged active enzymes were captured with agarose-streptavidin beads. Following SDS-PAGE replicate blots of the biotin-VAD/biotin-YVAD-labelled pull-downs were probed with anti-caspase-8 and anti-caspase-3.

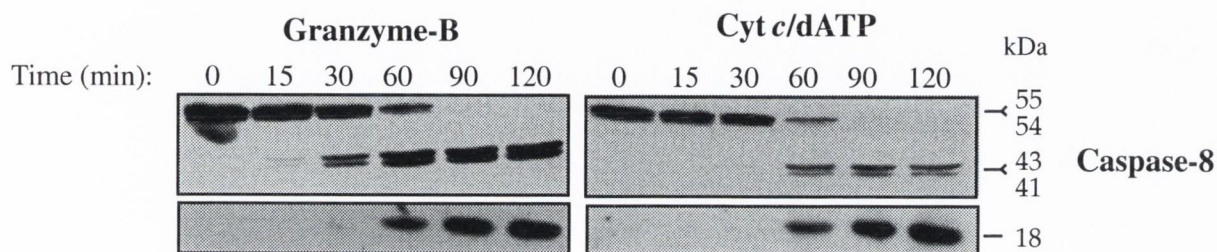


Fig 4.10. Addition of Granzyme B or Cytochrome *c*/dATP triggers caspase-8 proteolysis in Jurkat cell-free extract

Jurkat cell-free extracts were incubated at 37°C in the presence or absence of 100nm Granzyme B, or 50 µg/ml cytochrome *c* and 1 mM dATP. Samples were removed at the indicated timepoints and analysed for caspase-8 processing by Western Blotting.

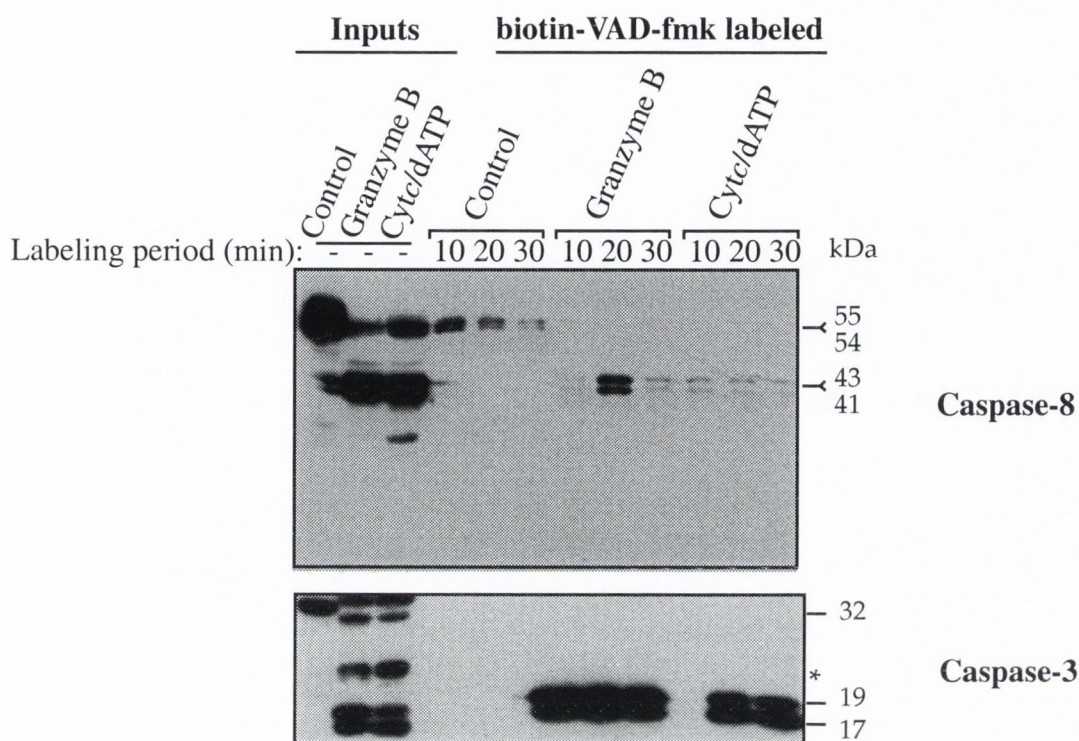


Fig 4.11(a). Peptide Affinity labelling of Caspase-8 and Caspase-3 after proteolytic processing by Granzyme B or Caspase-6

Jurkat cell-free extracts were incubated at 37°C for 2 h in the presence or absence of Granzyme B or cytochrome *c*/dATP, as shown. 10 μ M Biotin-VAD-FMK was then added and samples were taken at 10, 20 and 30 mins. Affinity-labeled caspases were captured as described in Materials and Methods. Bead complexes were probed for either caspase-8 (upper) or caspase-3 (lower). Inputs represent 20% of the cell-free lysate volume used for labelling reactions. Asterixes denote a non-specific band.

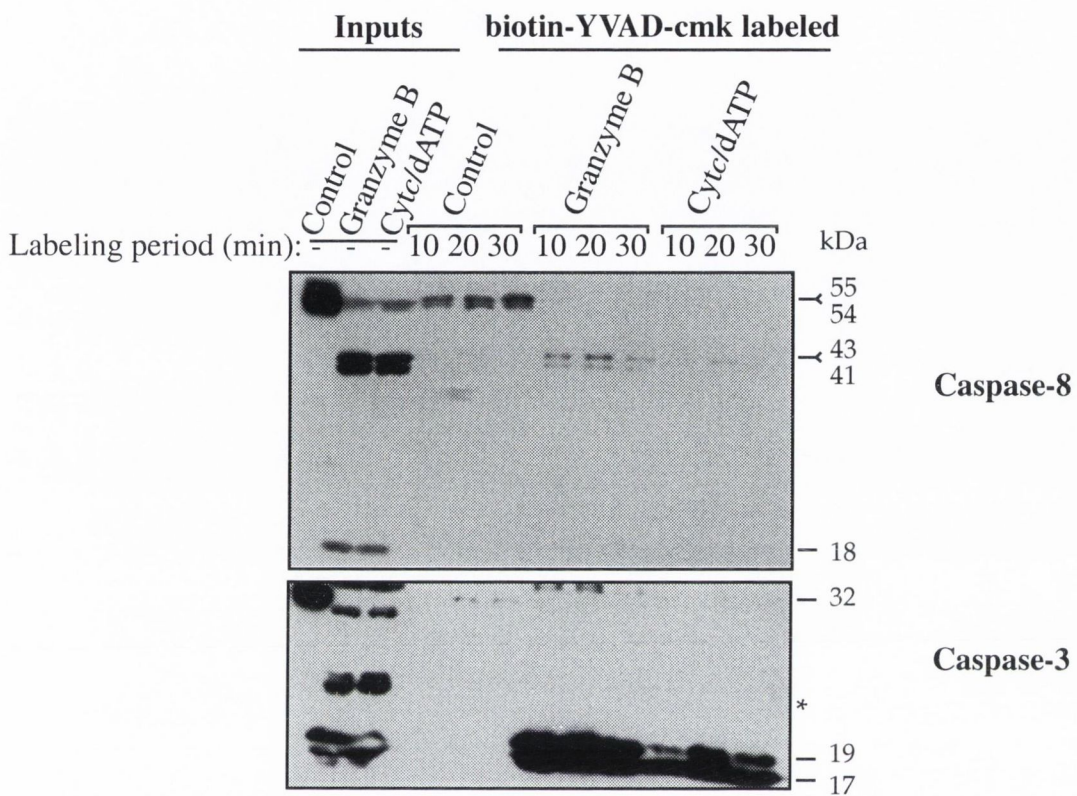


Fig 4.11(b). Peptide Affinity labelling of Caspase-8 and Caspase-3 after proteolytic processing by Granzyme B or Caspase-6

Jurkat cell-free extracts, were treated as described in (a) except 10 μ M Biotin-YVAD-CMK used as the affinity-label. Bead complexes were probed for either caspase-8 (upper) or caspase-3 (lower). Inputs represent 20% of the cell-free lysate volume used for labelling reactions. Asterixes denote a non-specific band.

Figs 4.11(a) and 4.11(b) demonstrate that proteolytically-processed caspase-3 was labelled very effectively by both affinity labels in granzyme B-mediated and apoptosome-mediated caspase activation pathways, as confirmed by extensive capture of the p20 and p17 forms of the enzyme. In sharp contrast, there was no significant labelling of caspase-8, despite the fact the enzyme is clearly processed in the input lanes. This result is consistent with Boatright *et al.*, 2003, and cast doubt on the activity of the processed caspase-8 in the absence of adaptor-driven aggregation of caspase-8 molecules. However, to ensure that this labelling method could label caspase-8 in a dimerization-driven context we decided to explore the effectiveness of this labelling technique in the context of Fas-driven caspase activation.

As previously outlined, caspase-8 can be activated via aggregation of death receptors i.e. ligation of the Fas receptor at the cell surface leads to recruitment of the adaptor protein, FADD, which in turn recruits and activates caspase-8 (Boldin *et al.*, 1996; Muzio *et al.*, 1996). In this context caspase-8 is dimerized and activated and therefore one would expect efficient labelling of the enzyme under these conditions. To explore whether this was the case, Jurkat cells were left untreated or treated with 100 ng/ml of anti-Fas mAb for four hours. As is illustrated in Fig 4.12, ~70% of the cells were annexin V-positive at this time-point and thus the culture was undergoing extensive apoptosis. Cell-free extracts were prepared and labelled under the same conditions as the previous experiments. The resulting Western Blots (Fig 4.13) revealed the same results as Figs 4.11(a) and (b) i.e. effective labelling of processed caspase-3 and poor labelling of processed caspase-8. In light of the fact that the efficiency of active caspase-8 labelling was similar under all caspase-8 activating regimes tested, we concluded that this assay was unable to accurately determine caspase-8 activity. Thus we decided to use the more reliable approach of assaying substrate cleavage events.

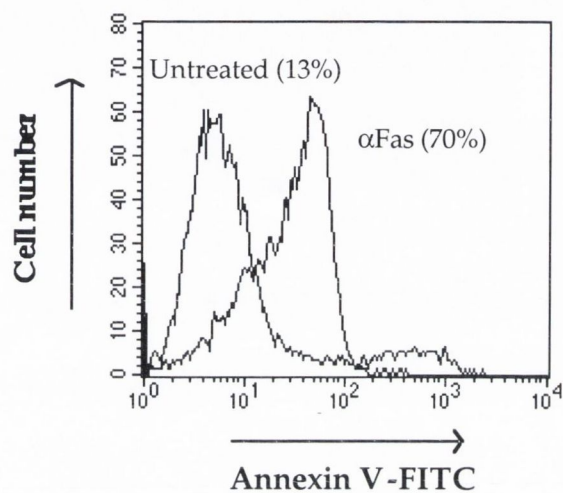


Fig 4.12. α Fas-treated Jurkat cells die by apoptosis

Jurkat cells were cultured in the presence or absence of anti-Fas IgM (100 ng/ml) for 4 h, followed by assessment of apoptosis by staining with FITC-conjugated annexin V and analysis by flow cytometry. Percentages of apoptotic cells are indicated in brackets.

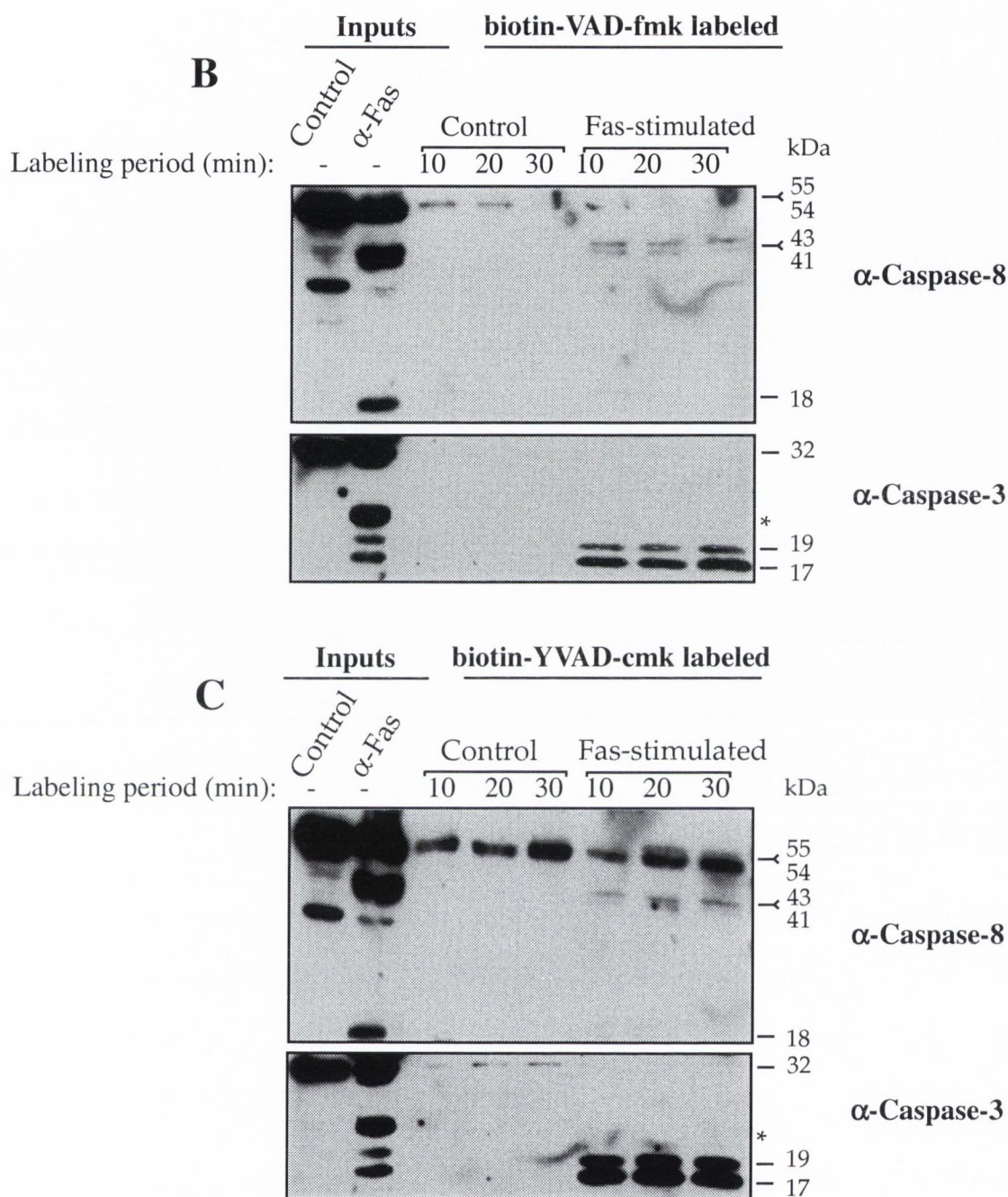


Fig 4.13. Peptide Affinity labelling of Caspase-8 after Fas-stimulation

Cell-free extracts, generated from control or Fas-stimulated Jurkat cells, were affinity-labeled as previously described in Fig 4.11. Biotinylated complexes were probed for caspase-8 or caspase-3. Inputs represent 33% of the cell-free lysate volume used for labelling reactions. Asterixes denote a non-specific band. Results are representative of two separate experiments.

4.3.4. Caspase-8 immunoprecipitation and measurement of catalytic activity

In an effort to more directly assess the activity of the processed form of the enzyme, caspase-8 was immunoprecipitated from control or activated cell-free extracts. As is illustrated in Fig 4.14(a), four different monoclonal antibodies were used. Each antibody successfully immunocaptured both the full-length and the proteolytically processed forms of the enzyme. Blots were reprobed with anti-FADD and subsequently anti-caspase-3 antibodies to ensure the immunoprecipitations were specific. Thus, the caspase activity captured on the beads could only be attributed to caspase-8.

The immunoprecipitated enzyme was then used in a fluorometric substrate peptide hydrolysis assay. As is highlighted in Fig 4.14(b) only the processed form of the enzyme, activated via granzyme B or caspase-6, was able to cleave the synthetic substrate – Ac-IETD-AFC, which is the preferred substrate of caspase-8. Contrary to recently published results the analysis of the data in this study indicates that interchain proteolysis of caspase-8, in the absence of a dimerization platform, is sufficient to activate the enzyme.

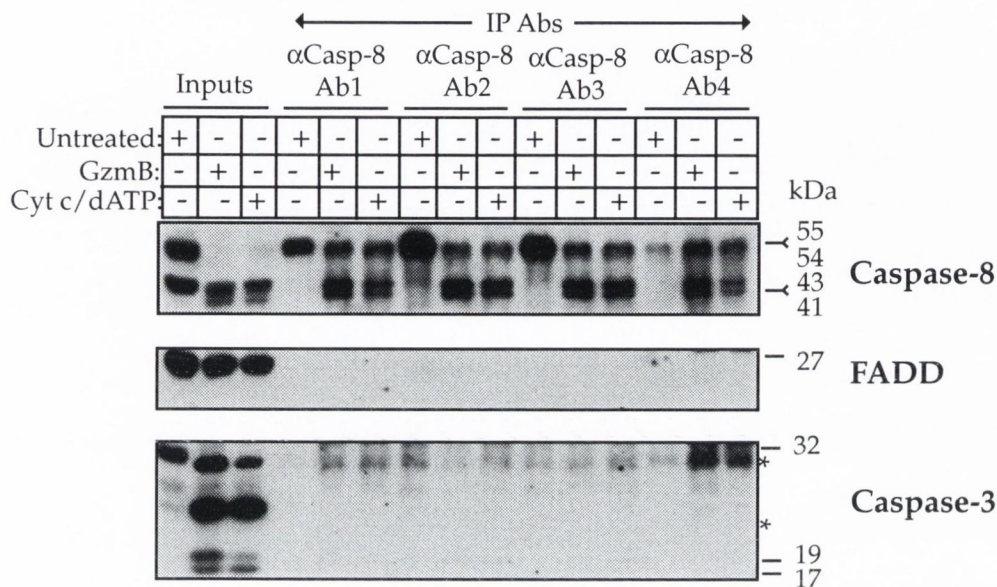


Fig 4.14(a). Immunoprecipitation of proteolytically processed caspase-8 after treatment with granzyme B or cytochrome c/dATP.

Jurkat cell-free extracts (200 µl) were incubated at 37°C in the presence or absence of granzyme B (100 nM), or cytochrome c/dATP. After the 2 h incubation period, each reaction was divided into four aliquots (50 µl each) and caspase-8 was immunoprecipitated using four different anti-caspase-8 monoclonal antibodies as indicated. The caspase-8 monoclonal antibodies were as follows: Ab1, clone C15 (Apotech, UK); Ab2, clone 12F5 (Apotech, UK); Ab3, clone F57 (Upstate Biotechnology Inc., USA); Ab4, clone 1C12 (Cell Signaling Technology, UK). Caspase-8 immunocomplexes were washed (x3) and aliquots were assessed for capture efficiency as indicated. Asterixes indicate non-specific bands.

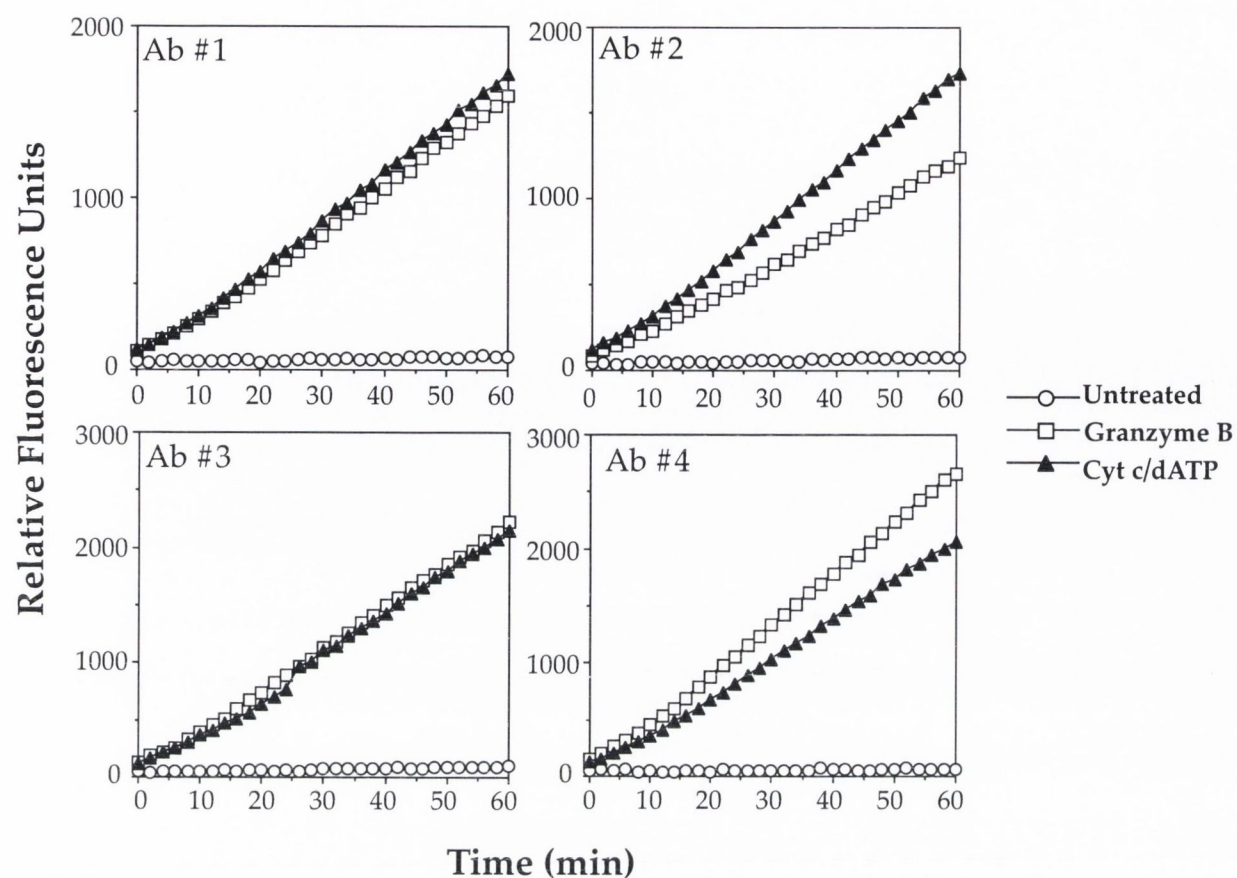


Fig 4.14(b). Interchain proteolysis is sufficient to activate caspase-8

Jurkat cell free extracts were incubated for 2 h in the presence or absence of granzyme B or cytochrome *c*/dATP followed by immunoprecipitation of caspase-8 using four different caspase-8 mAbs as detailed previously. Caspase-8 immunocomplexes were then assessed for their ability to hydrolyse Ac-IETD-AFC as described in materials and methods. Results are representative of four separate experiments.

In a parallel experiment, the immunocaptured enzyme was also examined for its ability to cleave a natural caspase-8 substrate – caspase-3. As is shown in Fig 4.14(c), only the processed form of the enzyme was able to cleave caspase-3. This result further confirms that the catalytic competence of the processed form of caspase-8 is activated in the absence of an adaptor molecule. Thus, proteolytically cleaved caspase-8 is active.

Undoubtedly, caspase-8 activated in the absence of adaptor-mediated aggregation displays catalytic activity. However, it was unclear from these assays whether the activity observed was equivalent to that observed during dimerization-mediated caspase-8 activation. We subsequently used immunoprecipitation to compare the catalytic activity of caspase-8 processed in the context of Fas stimulation with the activity of this caspase in the context of engagement of the apoptosome pathway by cytochrome *c*/dATP.

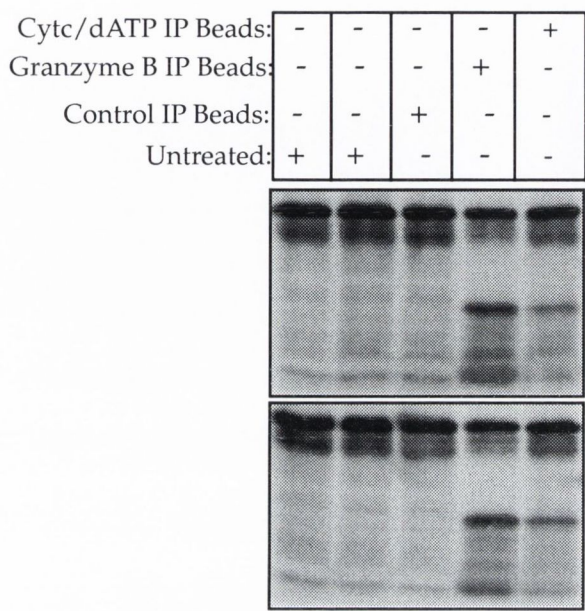


Fig 4.14(c). Interchain proteolysis is sufficient to activate caspase-8

Caspase-8 was immunoprecipitated from Jurkat cell-free extracts (Upper panel Ab, clone 1C12, (Cell Signaling Technology, UK); Lower panel Ab, clone 5F7 (Upstate Biotechnology Inc., USA): after treatment with granzyme B or cytochrome *c*/dATP, as indicated. Immunocomplexes were then washed, and resuspended in CEB containing [³⁵S]-labelled procaspase-3. Reactions were incubated at 37°C for 3 h followed by SDS-PAGE and fluorography for assessment of caspase-3 proteolysis. Results are representative of three separate experiments.

Jurkat cells were treated with anti-Fas mAb for four hours at which time 81% of the population were Annexin V-positive (Fig 4.15a).

Caspase-8 was then immunocaptured from control and Fas-treated cell-free extracts and used in the Ac-IETD-AFC hydrolysis assay as previously described. As a control, caspase-8 was immunoprecipitated from control and cytochrome *c*/dATP-activated cell-free extract, generated from an equivalent number of cells. The result shown in Fig 4.15(b) illustrates that active caspase-8 immunoprecipitated from Fas-treated extract displays catalytic activity, which is equivalent to that seen in the absence of adaptor-mediated aggregation. Thus caspase-8 activated by direct proteolysis displays equivalent activity to caspase-8 activated by dimerization.

One remaining possibility was that the adaptor protein, FADD, promoted caspase-8 dimerization during the previous “adaptor-less” contexts. Therefore, we also examined caspase-8 processing and activation in cell-free extracts generated from a FADD-deficient Jurkat cell line.

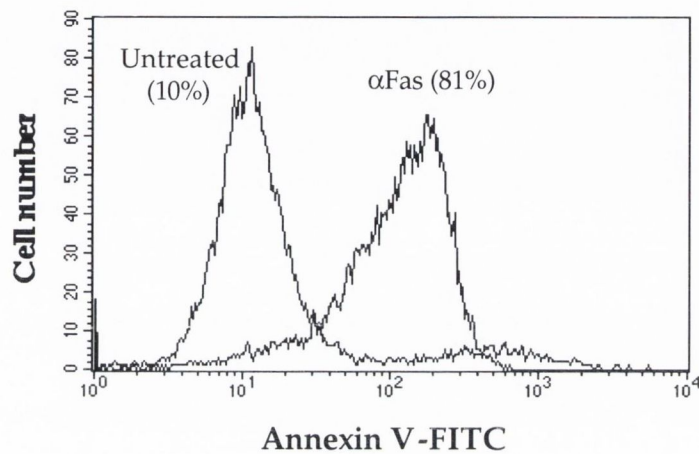


Fig 4.15(a). α Fas-treated Jurkat cells die by apoptosis

Jurkat cells were cultured in the presence or absence of anti-Fas IgM (100 ng/ml) for 4 h, followed by assessment of apoptosis by staining with FITC-conjugated annexin V and analysis by flow cytometry. Percentages of apoptotic cells are indicated in brackets. Cell-free extracts were then generated from the control and anti-Fas-stimulated cell populations.

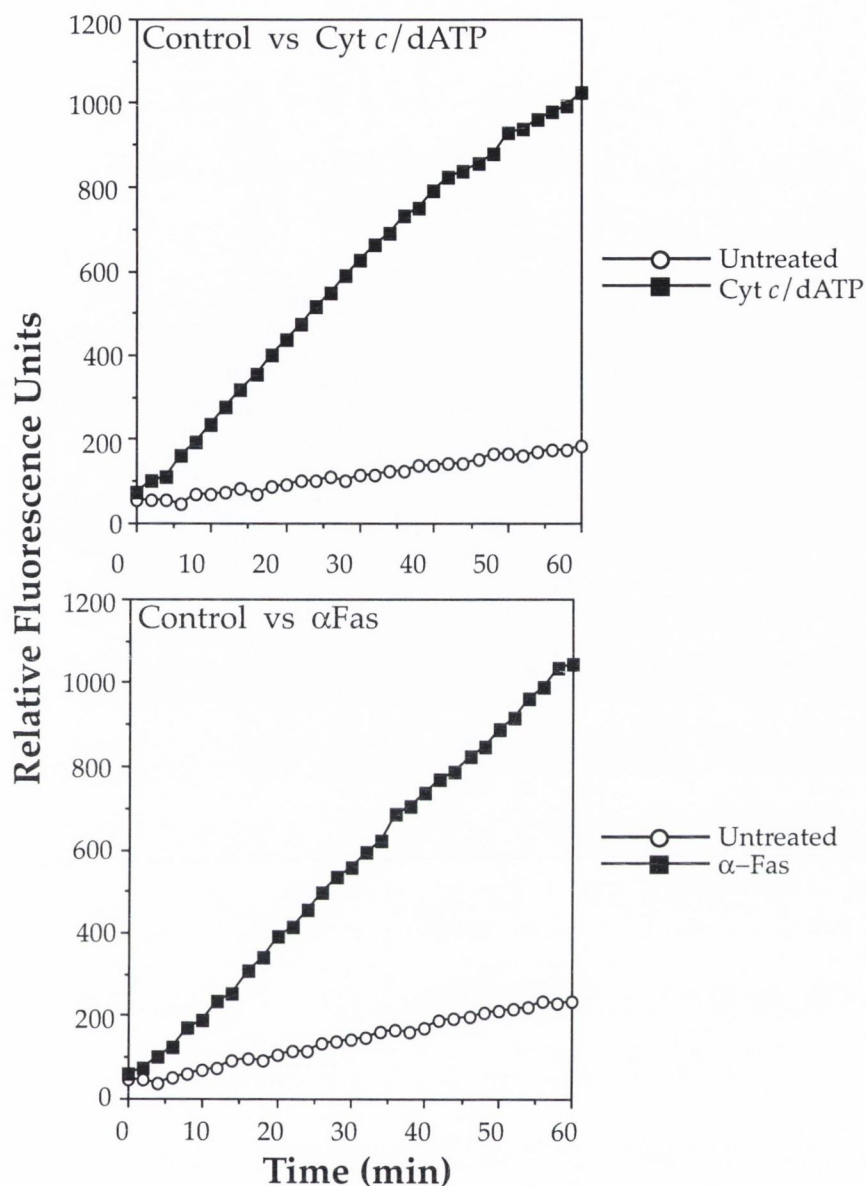


Fig 4.15(b). Direct proteolysis yields similar levels of active caspase-8 to dimerization-driven activation

Upper panel, Jurkat cell-free extracts were incubated for 2 h in the presence or absence of cytochrome *c*/dATP followed by immunoprecipitation of caspase-8 and assessment of Ac-IETD-AFC hydrolysis activity as described in Materials and Methods. Lower panel, caspase-8 was immunoprecipitated from Jurkat cell-free extracts prepared from untreated versus anti-Fas IgM-treated Jurkat cell populations as shown in 4.16(a). Caspase-8 immunocomplexes were then assessed for Ac-IETD-AFC hydrolysis activity. Note that caspase-8 immunocomplexes were prepared from the same volumes of cell-free lysates in all cases.

4.3.5. Caspase-8 immunoprecipitation and measurement of catalytic activity in a FADD-deficient background

Fig 4.16(a) illustrates that FADD-deficient Jurkat cells, in comparison to the wild-type Jurkat cell line, expressed minimal amounts of FADD while still maintaining relatively equal levels of caspase-8 –3 and actin.

Caspase-3 and –8 processing occurred as normal in FADD-deficient cells, in response to granzyme B and cytochrome *c*/dATP (Fig 4.16b). Caspase-6-mediated processing of caspase-8 was somewhat delayed in these extracts in comparison to wild-type extracts (Fig 4.10). Fig 4.16(c) illustrates that processed caspase-8 immunocaptured from FADD-deficient Jurkat cell-free extracts still maintained catalytic activity in an Ac-IETD-AFC hydrolysis assay. Thus, interchain proteolysis of caspase-8 in the absence of a FADD dimerization platform was still sufficient to mediate activation of the protease.

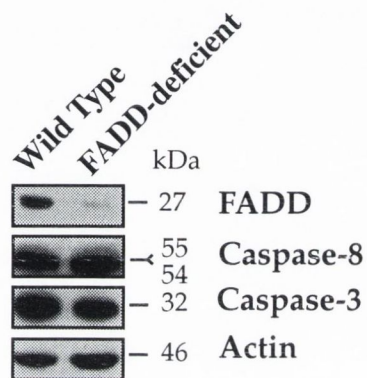


Fig 4.16(a). Analysis of FADD-deficient Jurkat cells

Equal concentrations of wild-type and FADD-deficient Jurkat cell free extracts were separated by SDS-PAGE and subsequently probed with the indicated antibodies. Note the virtual absence of FADD in FADD-deficient cells, while the levels of caspases and actin remain constant in both cell-lines.

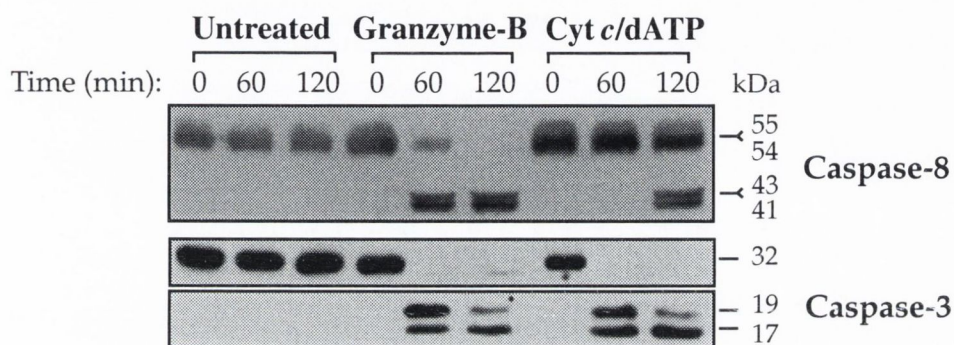


Fig 4.16(b). Addition of granzyme B or cytochrome *c*/dATP triggers caspase-8 and caspase-3 activation in FADD-deficient Jurkat cell-free extracts

FADD-deficient Jurkat cell-free extracts were incubated at 37°C in the presence or absence of 100 nM granzyme B, or 50 µg/ml cytochrome *c* and 1 mM dATP. Samples were removed at the indicated time-points and analysed for caspase-8 and caspase-3 processing by Western Blotting.

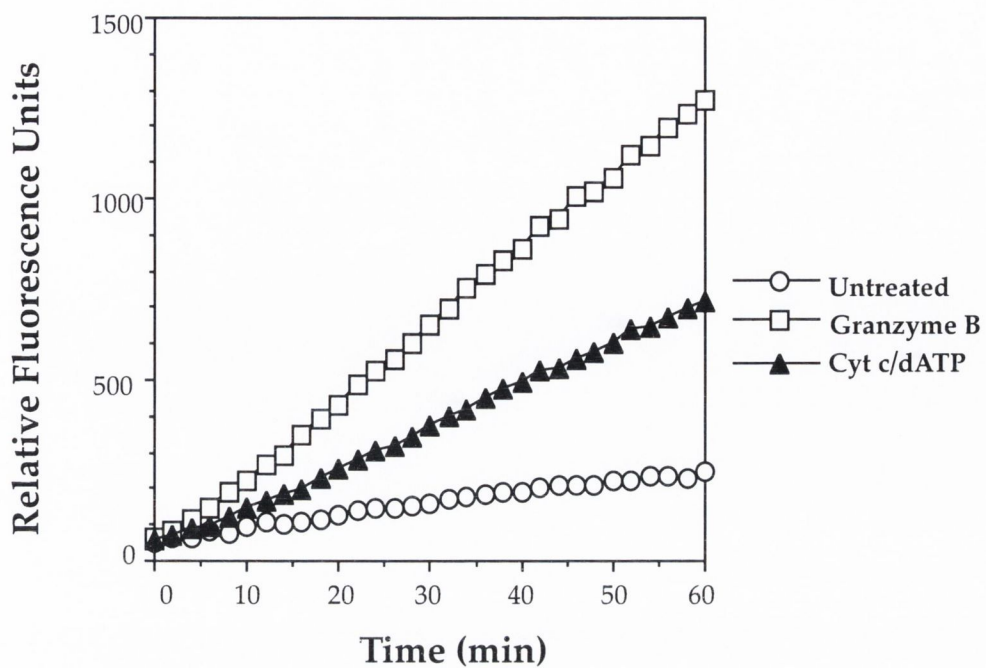


Fig 4.16(c). Interchain proteolysis is sufficient to activate caspase-8 in a FADD-deficient background

FADD-deficient Jurkat cell-free extracts were incubated for 2 h in the presence or absence of cytochrome *c*/dATP followed by immunoprecipitation of caspase-8 and assessment of Ac-IETD-AFC hydrolysis activity as described in Materials and Methods.

4.4. DISCUSSION.

4.4.1. Granzyme B-mediated cell death

Cytotoxic lymphocytes (CLs) deficient in both granzyme A and B have a profound defect in cytotoxicity as compared to wild-type cells (Shresta *et al.*, 1999). CLs derived from mice deficient in either granzyme A or granzyme B are as cytotoxic as wild-type CLs, suggesting the granzymes may substitute for one another (Heusel *et al.*, 1994; Ebnet *et al.*, 1995;). However, granzyme B^{-/-} CLs are deficient in the early induction of apoptosis and DNA fragmentation in target cells (Heusel *et al.*, 1994), while granzyme A-deficient mice are more susceptible to the mouse poxvirus, ectromelia (Mullbacher *et al.*, 1996). These results highlight that the granzymes induce target cell death via distinct mechanisms. Granzyme A induces a caspase-independent cell death, while granzyme B may induce a caspase-dependent cell death (Lieberman, 2003).

The results presented in the first section of this chapter reveal that granzyme B induces caspase activation in a hierarchical manner. The granzyme-B initiated caspase cascade is quite different to the apoptosome-mediated cascade (Slee *et al.*, 1999b). The most apical caspases are a trio of caspases - caspases-3, -7 and -8 and the downstream targets are caspases-2, -6 and -9. This cascade is somewhat in reverse orientation to the cytochrome *c*/dATP-initiated cascade, and contradicts the theory of “initiator” and “executioner” caspase (Nicholson, 1999). The “executioners” (caspases-3 and 7) are apical in the granzyme B cascade and the “initiators” (caspases-2 and -6) are activated by caspase-3. However, similar to the apoptosome cascade, caspase-3 plays a pivotal role in the granzyme B cascade and is a major effector caspase. In addition, just as caspase-3 can feed-back and activate caspase-9 in the apoptosome-mediated cascade, caspase-3 is also critical for the complete proteolytic processing of caspase-8 in the granzyme B-initiated cascade.

From our analysis we have concluded that caspases-3 -7 and -8 are the preferred substrates of granzyme B amongst the array of caspases tested. The direct cleavage

of caspase-3 by granzyme B is in agreement with previous studies (Chinnaiyan *et al.*, 1996; Fernandes-Alnemri *et al.*, 1996; Froelich *et al.*, 1996; Martin *et al.*, 1996; Quan *et al.*, 1996; Atkinson *et al.*, 1998; Yang *et al.*, 1998). Granzyme B-mediated caspase-8 processing has also been reported previously (Muzio *et al.*, 1996; Medema *et al.*, 1997(b); Sutton *et al.*, 2000). However, our studies suggest that caspase-8 requires active caspase-3 to achieve complete proteolytic activation. Incomplete caspase processing by granzyme B has been reported previously (Martin *et al.*, 1996). Indeed, Trapani and colleagues have demonstrated that Bcl-2 overexpression also limits granzyme B-mediated caspase-8 proteolysis to the creation of the p43/41 fragment (Sutton *et al.*, 2000). Presumably, in this instance, there was an insufficient level of caspase-3 activation to allow caspase-8 prodomain removal. Caspase-7 has been reported to be activated by caspase-3 in the granzyme B-activated caspase cascade (Yang *et al.*, 1998). However, direct caspase-7 activation by granzyme B is confirmed in this chapter and has recently been highlighted by Metkar *et al.*, who have shown that in the absence of caspase-3, caspase-7 is still activated by granzyme B and is capable of cleaving PARP (Metkar *et al.*, 2003). The absence of caspase-3 blocked the activation of caspases-9, -2 and -6 by granzyme B. Hence, these caspases are direct targets of caspase-3 in the granzyme B-mediated caspase cascade. The lack of affinity of granzyme B towards caspase-9 *in vivo* has recently been confirmed (Sutton *et al.*, 2003) but there is scant information regarding the ability of the protease to cleave caspases-2 and -6 *in vivo* and our studies suggest that they are not physiological targets of granzyme B.

The existence of such a radically different cascade suggests that if the concentration of the protease is not limiting, granzyme B is capable of initiating a caspase cascade independently of mitochondrial involvement. Titrating the concentration of granzyme B in cell-free extract could identify the threshold levels of the protease required to initiate a caspase cascade independently of mitochondrial involvement. The ability of granzyme B to initiate a caspase cascade, with or without mitochondrial involvement, is similar to the ability of activated caspase-8 at the DISC. In type I cells, caspase-8 can activate sufficient procaspase-3 without any mitochondrial involvement (Scaffidi *et al.*, 1998). In type II cells, caspase-8 activation is insufficient to directly activate procaspase-3. Instead this reduced level of active caspase-8 cleaves Bid (Li *et al.*, 1998; Luo *et al.*, 1998). This 15kDa fragment in turn

activates the apoptosome pathway of caspase activation by stimulating cytochrome *c* release from mitochondria (Wei *et al.*, 2001). In contrast to these distinct cell type-specific pathways, the *in vivo* model we propose for granzyme B-mediated caspase activation is the simultaneous activation of these two distinct cascades via (a) direct caspase proteolytic processing and (b) the activation of the apoptosome caspase cascade via cleavage of Bid.

Bid plays a pivotal role in granzyme B-mediated cell death (Barry *et al.*, 2000; Heiben *et al.*, 2000; Sutton *et al.*, 2000;). However, embryonal fibroblasts from Bid-deficient mice remain susceptible to granzyme B-mediated cell death, despite the fact they do not release any cytochrome *c* (Thomas *et al.*, 2001). Indeed granzyme B can induce cell death in the presence of the pan-caspase inhibitor, z-VAD-fmk (Sarin *et al.*, 1997; Talanian *et al.*, 1997). Therefore, granzyme B can initiate cell death without any direct caspase involvement. It is known that the CAD inhibitor, ICAD and PARP, DNA-PKcs and NuMA are all cleaved by granzyme B (Lieberman, 2003). However, these are all previously identified caspase substrates (Lieberman, 2003). In an effort to identify granzyme B-specific cellular substrates a proteome-based screen was undertaken in our laboratory (Adrain *et al.*, unpublished results). Using a cell-free system in which caspase activity was blocked by the addition of the pan-caspase inhibitor z-VAD-fmk, 10 novel granzyme B substrates were identified. The identification of these targets will provide novel insights into the mechanisms of granzyme B-mediated apoptosis. It will be interesting to decipher whether these critical substrates are activated or inactivated as a result of granzyme B-mediated processing and what exactly are the downstream implications of such activation. It is interesting to speculate that some of these targets may be capable of processing caspases downstream of their activation, that in turn could lead to the discovery of novel caspase activating pathways (Adrain *et al.*, unpublished results).

Granzyme A and B are the most extensively studied granzymes of CLs (Lieberman, 2003). A recent report from Trapani and colleagues suggest that neither granzyme A or B is involved in tumour rejection by CTL and NK cells (Smyth *et al.*, 2003). This study suggests that either perforin alone or perforin in conjunction with another granule component is responsible for tumour cell killing *in vivo* (Smyth *et al.*, 2003). This study serves as a reminder of the other serine proteases found in the granules of

CLs, granzymes C, D, E, F, G, H, K and M. The cell death pathways of these granzymes are only beginning to be deciphered (Lieberman, 2003). It will be interesting to examine if any of these remaining granzymes are involved in tumour cell killing and whether there is any caspase involvement in their cytotoxic mechanisms of action.

4.4.2. A dimerization-inducing platform is not a prerequisite for Caspase activation

Processed caspase-8 fragments are of the same molecular size, irrespective of whether they are derived from death receptor engagement or direct caspase-6/granzyme B-mediated proteolysis. This indicates that the same cleavage events occur in both instances. However, if the Allosteric Regulation Model is correct this predicts that proteolysis of caspase-8 alone is insufficient to achieve catalytic activity. However, the results presented in the second part of this chapter demonstrate that caspase-8 processed in the absence of a dimerization platform retained catalytic activity. Initially we investigated the activity of caspase-8 by employing an affinity labelling technique also used by Salvesen and colleagues (Boatright *et al.*, 2003). Active caspase-8 is labelled quite poorly with both biotin-VAD-FMK and biotin-YVAD-FMK, while active caspase-3 is labelled very effectively. Such poor labelling was reflected in both instances of caspase-8 activation, i.e. in the absence and presence of an aggregation platform. As it was obvious that active caspase-8 was a poor target in this labelling assay, we deemed this technique unsuitable for the determination of the activation status of this enzyme. These data are important as they highlight that caution should be applied to affinity labels as these may not be definitive indicators of the activation status of caspases. Salvesen and colleagues used affinity-labelling to demonstrate that cleavage does not result in caspase-8 activation (Boatright *et al.*, 2003). Our results cast serious doubt upon the conclusions drawn from these experiments and suggest that the affinity labelling method produced artificial results in this instance.

As affinity labelling failed to provide us with a conclusive answer to the caspase-8 activation question we decided to employ the more reliable approach of examining substrate cleavage events. Caspase-8 processed by either granzyme B or caspase-6 cleaved both synthetic (IETD-AFC) and natural (caspase-3) substrates. Indeed, even

in a FADD-deficient background, caspase-8 was still processed by both granzyme B and cytochrome *c*/dATP and is also capable of cleaving Ac-IETD-AFC. Therefore, caspase-8 activated in the absence of an adaptor platform was catalytically active.

These results do not exclude the possibility that caspase-8 dimerization is a method of caspase activation nor do they exclude the possibility that dimer formation occurs subsequent to cleavage. The formation of caspase-8 dimers/oligomers is the fundamental principle underlying the induced proximity model (Medema *et al.*, 1997; Muzio *et al.*, 1998). In addition, the enhancement of caspase-8 activity via the dimerization between (a) wild-type caspase-8 and the protease-deficient c-FLIP_L (Chang *et al.*, 2002; Chang *et al.*, 2003) or (b) wild-type caspase-8 and a protease-deficient caspase-8 (Chan *et al.*, 2001; Chang *et al.*, 2003) has been reported. These results clearly demonstrate that dimerization does enhance the intrinsic activity possessed by procaspase-8.

The most recent reports from Boatrigh *et al.*, 2003 and Donepudi *et al.*, 2003 however argue that dimerization is sufficient to achieve full catalytic activity, and processing is only a means of enhancing the stability of the pre-formed dimer. However, one of the key experiments is the use of affinity labelling and our results demonstrate that this technique is unacceptable as a means of providing concrete evidence regarding caspase-8 activity. To further bolster the dimerization model, monomeric caspase-8 was shown to be inactive, possessing 1% of the activity of the dimer (Boatrigh *et al.*, 2003; Donepudi *et al.*, 2003). However, this construct is also incapable of autocatalytic cleavage and therefore one cannot exclude the possibility that it is this property that results in the formation of an inactive species.

It must also be recalled that at the DISC, active caspase-8 does not activate procaspase-8 but rather cleaves other substrates (Medema *et al.*, 1997; Chang *et al.*, 2003). Therefore, dimer formation via aggregation is a prerequisite to facilitate procaspase-8 activation at the DISC. Pro-caspase-8 activation can also occur independently of DISC formation. The arguments presented in the Allosteric Regulation Model do not consider these alternative caspase-8 activation stimuli. The results provided in this chapter argue that granzyme B and caspase-6 generate proteolytically active caspase-8 and hence caspase-8 activation is not always

dependent on its dimerization. Crystal structure analysis of the caspase-7 zymogen, in comparison to the active enzyme, has highlighted that processing is required for the active site to achieve a catalytically favourable configuration (Chai *et al.*, 2001b; Riedl *et al.*, 2001a). Similar crystal structure analysis of caspase-8 would yield greater insights into the sequence of events required by this protease to achieve catalytic competency.

Finally these results must also be considered when commenting on the activities of caspases-2 and -10 in the apoptosome-mediated caspase cascade and caspase-9 in the granzyme B-mediated cascade. The Allosteric Regulation Model predicts that these protease are inactive as an adaptor molecule is not present. However, the results presented in this chapter suggest that proteolytic processing does in fact result in the formation of an active enzyme and therefore caspases-2 and -10 in the apoptosome-mediated caspase cascade and caspase-9 in the granzyme B-mediated cascade are also catalytically active.

Chapter V

Role of the IAPs as Ubiquitin Ligases towards caspases

5.1 INTRODUCTION

5.1.1. Introduction

IAPs –inhibitor of apoptosis proteins - were first recognised in baculoviruses due to their ability to inhibit cell death (Crook *et al.*, 1993; Birnbaum *et al.*, 1994). The proteins responsible for mediating this inhibition were termed Cp-IAP and Op-IAP. These proteins share a conserved domain termed the BIR or baculoviral IAP repeat domain. This domain is approximately 70 amino acids and is a zinc-binding fold. Since this initial discovery, BIR-containing proteins have been identified in viruses, yeast, insects and mammals. While at least one BIR is required for the IAPs to mediate their anti-apoptotic effects, not all BIR-containing proteins can be termed inhibitors of apoptosis (Miller, 1999; Verhagen *et al.*, 2001). Fig 5.1 illustrates the known members of the human IAP family.

5.1.2. Inhibitory effects of the IAPs

The human IAPs, XIAP, c-IAP1 and c-IAP2 are the most extensively studied members of this family to date. These IAPs can directly inhibit caspases –3, -7 and –9, highlighting that their inhibitory properties encompass both initiator and executioner caspases (Deveraux *et al.*, 1997; Roy *et al.*, 1997; Deveraux *et al.*, 1998). XIAP is the most potent of these inhibitors (Roy *et al.*, 1997; Deveraux *et al.*, 1998). It was initially proposed that the BIR2 domain of XIAP inhibited caspases-3 and –7 and the BIR3 domain inhibited caspase-9 (Takahashi *et al.*, 1998; Deveraux *et al.*, 1999). More recent crystal structures of the BIR2-linker region of XIAP complexed with either caspase-3 or caspase-7 demonstrate that this linker segment provides the direct points of contact between XIAP and the active site of caspase-3 or –7, while the BIR2 is important for maintaining the correct conformation of this linker segment (Chai *et al.*, 2001a; Huang *et al.*, 2001; Riedl *et al.*, 2001b). In contrast to XIAP inhibition of caspase-3 and caspase-7, caspase-9 inhibition does not involve active site blockade. Rather, initial binding between the BIR3 of XIAP and the NH₂-terminus of the small subunit of caspase-9 results in the formation of a larger protein-protein interaction domain between the two molecules. In this conformation, caspase-9 is trapped as a monomer and its active sites are blocked from achieving the correct conformation to allow catalytic activation (Shiozaki *et al.*, 2003). No structural data is currently available on c-IAP1 or c-IAP2.

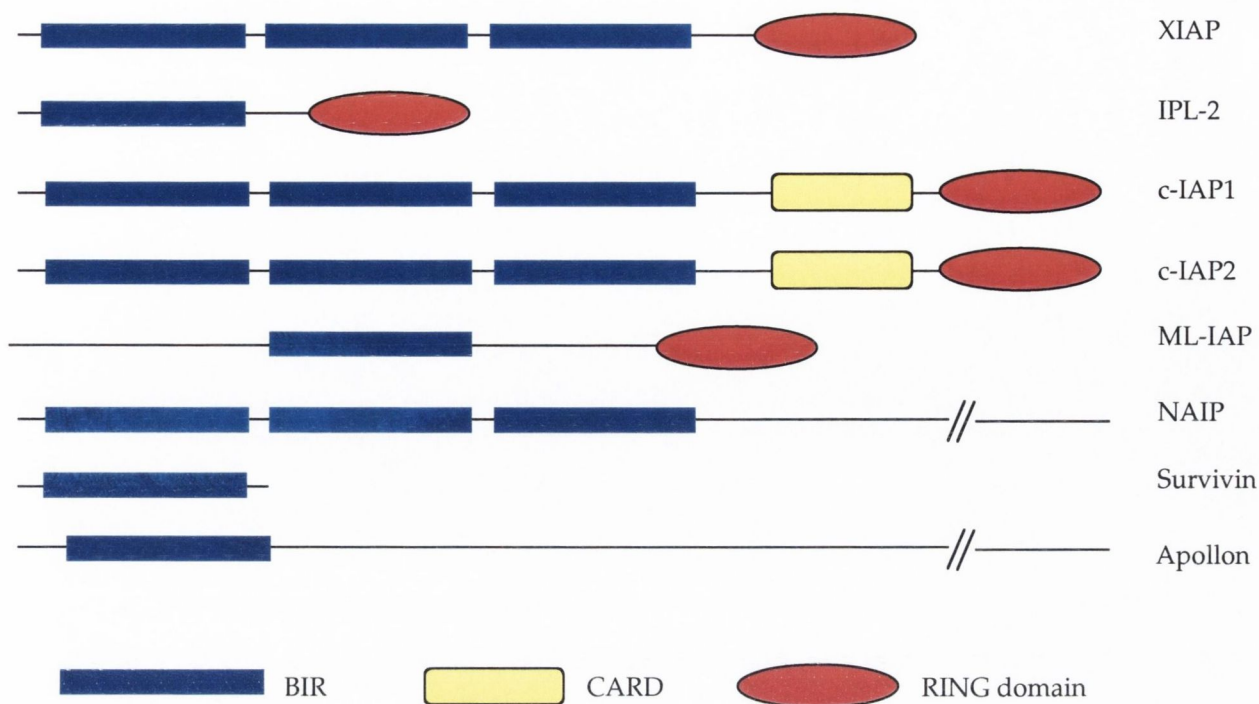


Fig 5.1. Mammalian BIR containing proteins (BIRPs), adapted from Salvesen and Duckett, 2002

All BIRPs contain between 1-3 BIR domains, while others also share a C-terminal RING domain. Survivin and Bruce are not believed to play a direct role in apoptosis but are believed to be involved in the regulation of cell division. Of the remaining proteins, XIAP c-IAP1 and c-IAP2 are the best characterised inhibitors of apoptosis proteins (IAPs).

In addition to the BIR domain, human IAPs also contain a motif termed the RING domain (Fig 5.1). Experiments using overexpressed RING-less XIAP, suggest that this protein is not as potent an inhibitor of apoptosis as full length XIAP (Creagh *et al.*, submitted). This result suggests that the IAP RING domain could also play a role in mediating their inhibitory effects and it is this aspect of IAP function that is the focus of the studies detailed in this chapter.

5.1.3. RING-domain proteins and Ubiquitination

RING domain-containing proteins are involved in the process of ubiquitination within a cell (Salvesen and Duckett, 2002). Ubiquitination involves the transfer of ubiquitin, a 76 amino acid globular protein, to lysine residues on a target protein. Polyubiquitination of a protein in such a manner primarily targets the protein for degradation by the 26S proteasome, a multicatalytic protease. Ubiquitination involves three distinct enzymes

- E1 – ubiquitin-activating enzyme,
- E2 – ubiquitin-conjugating enzyme and
- E3 – ubiquitin ligase.

In the first step free ubiquitin (Ub) is complexed with E1 in an ATP-dependent manner, i.e. complex formation requires E1 to hydrolyse ATP. This results in the formation of a thioester bond between a reactive cysteine in E1 and the C-terminal end of Ub. In the second step, Ub is transferred to a conserved 14-16 kDa Ub-conjugating (UBC) domain of an E2 enzyme. E2 subsequently transfers Ub to the target protein with the aid of an E3 ligase. E3s are primarily responsible for providing substrate specificity in the ubiquitination reaction (Weismann, 1997; Jesenberger and Jentsch, 2002). E3s may belong to one of two families, either those containing a HECT (homology to E6-AP carboxyl terminus) domain or those containing a RING domain (Joazeiro and Weissmann, 2000). These families differ in their exact mode of Ub transfer i.e. HECT-domain E3s may bind Ub to their HECT domain before transferring Ub to the target protein, while RING-domain E3s simply mediate direct transfer of Ub between E2 and the target protein (Salvesen and Duckett, 2002).

5.1.4. IAP Ubiquitin Ligase Activity

Can the RING domain-containing IAPs also be classified as E3 enzymes? Evidence to date suggests that this may be the case. XIAP, c-IAP1 and c-IAP2 can undergo autoubiquitination *in vitro* and this process is dependent upon an intact RING domain (Huang *et al.*, 2000; Yang *et al.*, 2000). In addition, c-IAP2 has been shown to monoubiquitinate caspases-3 and -7 *in vitro* (Huang *et al.*, 2000) and c-IAP1 can mediate the polyubiquitination of caspases-9 and -3 *in vitro* (Yang *et al.*, 2003). Takahashi and colleagues also provide some evidence to suggest that XIAP can promote proteasomal degradation of caspase-3 in cells. However, these results were based on overexpression studies with a synthetic caspase-3 construct where the position of the large and small subunits had been reversed, so the actual relevance of these results is open to debate (Suzuki *et al.*, 2001b). XIAP has also been proposed to ubiquitinate Smac *in vitro*, which may contribute to proteasome-mediated degradation of Smac *in vivo* (MacFarlane *et al.*, 2002).

The putative ubiquitin ligase activity of mammalian IAPs is also reflected in *Drosophila* IAPs. Similar to XIAP, c-IAP1 and c-IAP2, the *Drosophila* IAP, DIAP1, is capable of autoubiquitination, a property that is dependent on an intact RING domain (Ryoo *et al.*, 2002; Wilson *et al.*, 2002; Yoo *et al.*, 2002). Meier and colleagues have also shown that DIAP1 is capable of promoting ubiquitination of the *Drosophila* caspase DRONC (Wilson *et al.*, 2002).

5.1.5. New insights into the regulation of ubiquitin ligase activity

Three proteins, Rpr, Hid and Grim, induce cell death in *Drosophila* (Wang *et al.*, 1999; Goyal *et al.*, 2000; Lisi *et al.*, 2000). These proteins share a region of homology at their NH₂-termini termed the RHG motif and it has previously been shown that this RHG motif enables Rpr, Hid and Grim bind to DIAP1, thereby allowing caspase activation (Wang *et al.*, 1999; Goyal *et al.*, 2000). Besides this direct inhibition however, Rpr and Hid have also recently been proposed to contribute to DIAP1 degradation by stimulating autoubiquitination of the latter (Ryoo *et al.*, 2002; Yoo *et al.*, 2002). Indeed it was demonstrated that Rpr could even mediate mammalian XIAP and c-IAP1 autoubiquitination (Holley *et al.*, 2002).

As stated in the introduction to this thesis, the mammalian functional homolog of Rpr, Hid and Grim is Smac (Chai *et al.*, 2000; Wu *et al.*, 2000). Similar to the *Drosophila* RHG proteins, the NH₂-terminus of Smac also contains an IAP-binding motif, that plays a critical role in the relief of IAP inhibition mediated by Smac. (Chai *et al.*, 2000; Wu *et al.*, 2000). It is currently unknown whether Smac also mimics the *Drosophila* RHG proteins in terms of being able to modulate IAP ubiquitin ligase activity in mammalian systems.

5.2. CHAPTER AIMS

As outlined in the introduction to this chapter, the presence of a RING domain in several mammalian IAPs suggests that these proteins may act as E3 ligases towards their binding partners. However, to date evidence in support of this hypothesis is weak. We decided to explore whether XIAP, the most potent inhibitor of apoptosis (Roy *et al.*, 1997; Deveraux *et al.*, 1998), could act as an ubiquitin ligase towards the caspases, that this IAP is well established to interact with, i.e. caspase-3, caspase-7 and caspase-9 (Deveraux *et al.*, 1997; Deveraux *et al.*, 1998; Takahashi *et al.*, 1998; Deveraux *et al.*, 1999; Chai *et al.*, 2001a; Huang *et al.*, 2001; Riedl *et al.*, 2001b; Shiozaki *et al.*, 2003). We also wished to explore whether XIAP could act as an E3 ligase towards another major binding partner, Smac (Chai *et al.*, 2000; Du *et al.*, 2000; Verhagen *et al.*, 2000).

Mammalian Smac and *Drosophila* Rpr, Hid and Grim share an evolutionary conserved IBM or RHG motif to mediate their pro-apoptotic effects (Chai *et al.*, 2000; Wu *et al.*, 2000). The *Drosophila* functional homologs of Smac (Rpr, Hid and Grim) have also been shown to promote autoubiquitination of D-IAP1, and therefore we explored whether the ubiquitin ligase activity of XIAP could also be modulated by Smac in a similar manner.

5.3. RESULTS

5.3.1. *In vitro* ubiquitination of caspase-3, caspase-7 and caspase-9

To explore whether caspases could undergo IAP-mediated ubiquitination we used a standard ubiquitination buffer supplemented with recombinant E1 ubiquitin activating enzyme, E2 ubiquitin conjugating enzyme and either GST-XIAP or GST-c-IAP1 as the E3 ligase component. Reactions were performed as detailed in Materials and Methods. The result shown in Fig 5.2 was performed at 25°C and the concentration of E1 was 10 µg/ml and E2 was 20 µg/ml. In Fig 5.3 the concentration of E1 and E2 remained constant but the temperature was raised to 37°C. In Fig 5.4 the temperature was kept at 37°C but the concentration of E1 and E2 was increased 5-fold to 50 µg/ml and 100 µg/ml respectively. Protein bands having a ladder or smeared appearance typically portrays a polyubiquitinated protein. Generally these ladder species will occur approximately every 7 kDa, reflecting the step-wise transfer of ubiquitin to the target. Unfortunately despite trying various combinations of E1, E2 and E3 constituents we failed to detect caspase ubiquitination in any of these instances (Figs 5.2-5.4). This suggested either some components of the ubiquitin machinery are missing from the experimental system chosen or that caspases are poor targets for ubiquitination.

Jurkat cell-free extracts can readily support apoptosome formation, caspase activation and subsequent substrate cleavage upon the addition of cytochrome *c* and dATP. We then explored whether this system would also allow the detection of ubiquitinated species upon addition of recombinant E1 and E2. This system had the added advantage that it also permitted caspase activation and caspase-mediated IAP cleavage. This allowed us to investigate whether activated caspases or IAP breakdown products were more easily susceptible to ubiquitination than their pro and unprocessed forms respectively.

5.3.2. Ubiquitination in a cell-free system

As is shown in Fig 5.5, while the addition of cytochrome *c* and dATP activated caspase-3 that subsequently cleaved the IAPs, there was no apparent ubiquitination of either pro or processed caspase-3, neither was there any observable autoubiquitination of XIAP or c-IAP1.

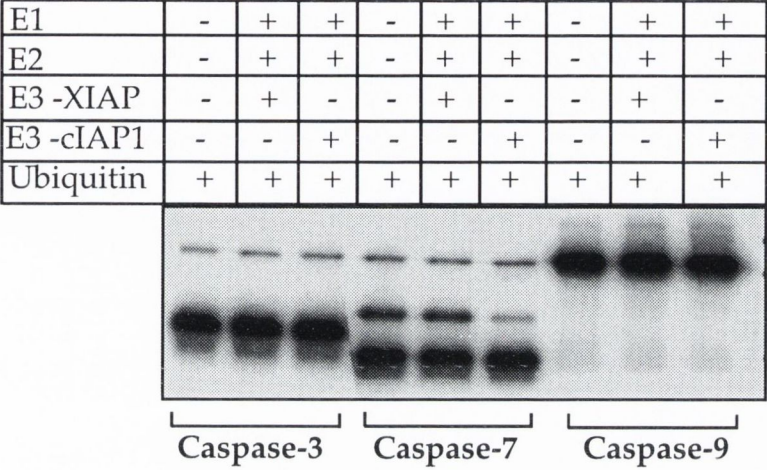


Fig 5.2. *In vitro* ubiquitination of caspases-3, -7 or -9

The reactions were assembled as described in Materials and Methods. [³⁵S]-labelled caspases were the target species and either 10 μM GST-XIAP or GST-cIAP1 was the E3 ligase enzyme. Reactions were incubated at 25°C for 3 h followed by SDS-PAGE and fluorography to allow for assessment of possible caspase ubiquitination.

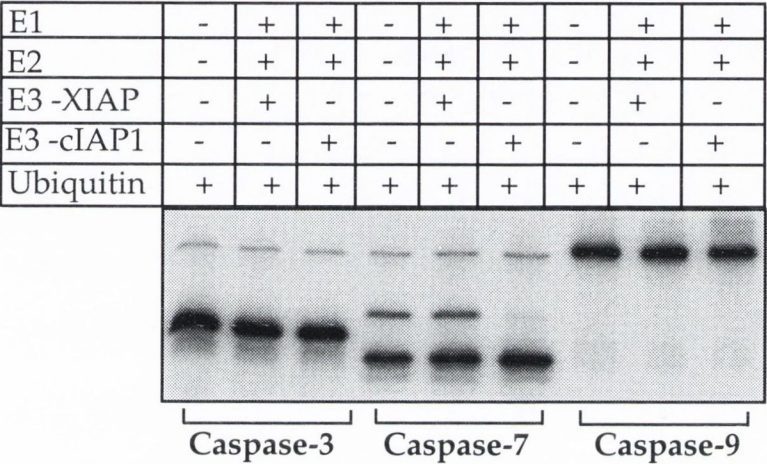


Fig 5.3. *In vitro* ubiquitination of caspases-3, -7 or -9

The reactions were assembled as described in Materials and Methods. [³⁵S]-labelled caspases were the target species and either 10 μM GST-XIAP or GST-cIAP1 was the E3 ligase enzyme. Reactions were incubated at 37°C for 3 h followed by SDS-PAGE and fluorography to allow for assessment of possible caspase ubiquitination.

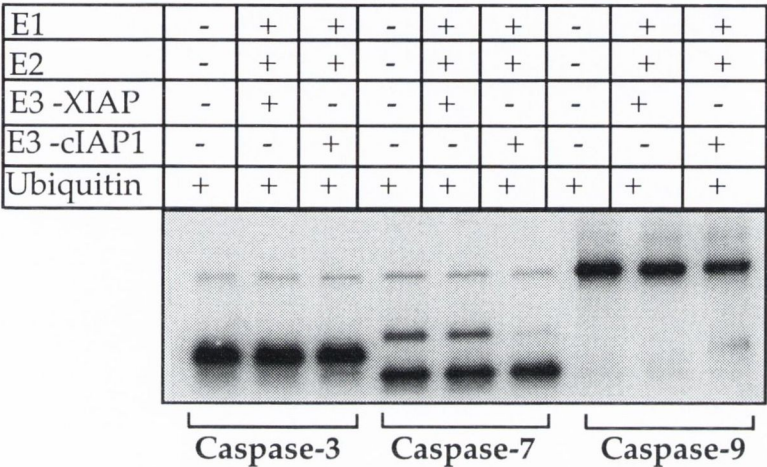


Fig 5.4. *In vitro* ubiquitination of caspases-3, -7 or -9

The reactions were assembled as described in Materials and Methods. [³⁵S]-labelled caspases were the target species and either 10 µM GST-XIAP or GST-cIAP1 was the E3 ligase enzyme. E1 and E2 concentrations were increased 5-fold relative to previous experiments (50 µg / ml and 100 µg / ml respectively). Reactions were incubated at 37°C for 3 h followedby SDS-PAGE and fluorography to allow for assessment of possible caspase ubiquitination.

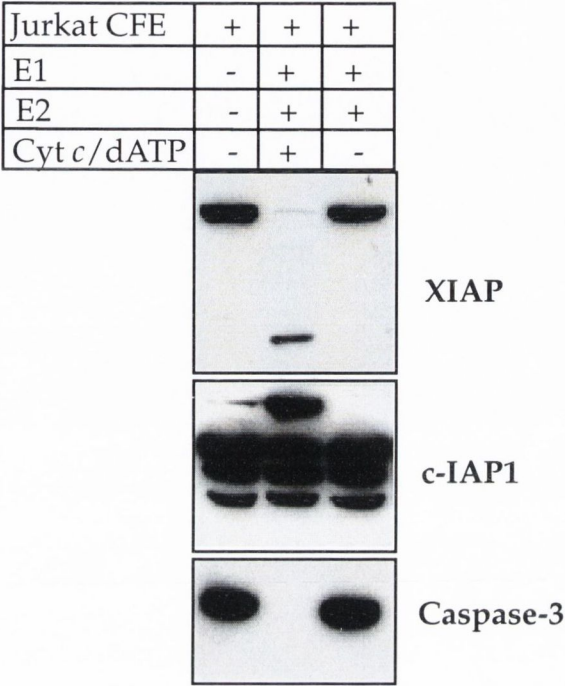


Fig 5.5. *In vitro* ubiquitination assay in Jurkat cell-free extracts
Control and cytochrome *c*/dATP-treated cell-free reactions were supplemented with recombinant E1 and E2 and incubated at 37°C for 90 min. Following SDS-PAGE the replicate blots were probed with the indicated antibodies.

We extended our experimental design by supplementing the extracts not only with recombinant E1 and E2 but also with recombinant GST-XIAP as an E3 ligase and ³⁵S labeled caspase-3 as the target species. Caspase activation was triggered with cytochrome *c*/dATP as indicated. As shown in Fig 5.6 caspase-3 activation was not as robust as normally seen, indicating XIAP-mediated inhibition of the protease. At 120 min, the majority of the inhibitory effects of XIAP had been overcome and caspase-3 was fully active. However, no evidence of ubiquitination was seen.

Coinciding with our efforts to detect ubiquitin ligase activity associated with mammalian IAPs, several reports were published reporting such activities with respect to *Drosophila* IAPs. It was noted that proteins translated in the presence of rabbit reticulocyte lysate were readily ubiquitinated (Ryoo *et al.*, 2002). We reasoned that the reticulocyte lysates might serve as a source of components of the ubiquitin machinery that had been lacking in previous experiments. Therefore, Jurkat cell-free extracts were supplemented with rabbit reticulocyte lysate in addition to recombinant E1, E2 and ubiquitin for further experiments.

5.3.3. Ubiquitination in cell-free extract with added reticulocyte lysate

As is evident in the left hand lanes of the upper and lower panels in Fig 5.7, this approach allowed the *in vitro* detection of XIAP autoubiquitination in a time-dependent manner. The autoubiquitination of XIAP was detected in both control and cytochrome *c*/dATP-activated extracts. This result indicates that autoubiquitination may not merely be a means of down-regulating XIAP levels in cells undergoing apoptosis but may also be involved in regulating the natural turnover of XIAP in healthy cells. However, under the same conditions, we failed to detect any evidence consistent with XIAP-mediated polyubiquitination of caspases.

5.3.4. Smac/DIABLO inhibits XIAP autoubiquitination *in vitro*

As stated in the introduction to this chapter studies on *Drosophila* IAPs have suggested that the RHG proteins can enhance the autoubiquitination of DIAP1. We investigated whether this were also true for the mammalian functional homolog of the RHG proteins – Smac/Diablo. Rather surprisingly, Smac/Diablo inhibited XIAP autoubiquitination both in control and activated extracts (Fig 5.7). This result was rather puzzling as Smac is perceived as an antagonist of the IAPs. We therefore

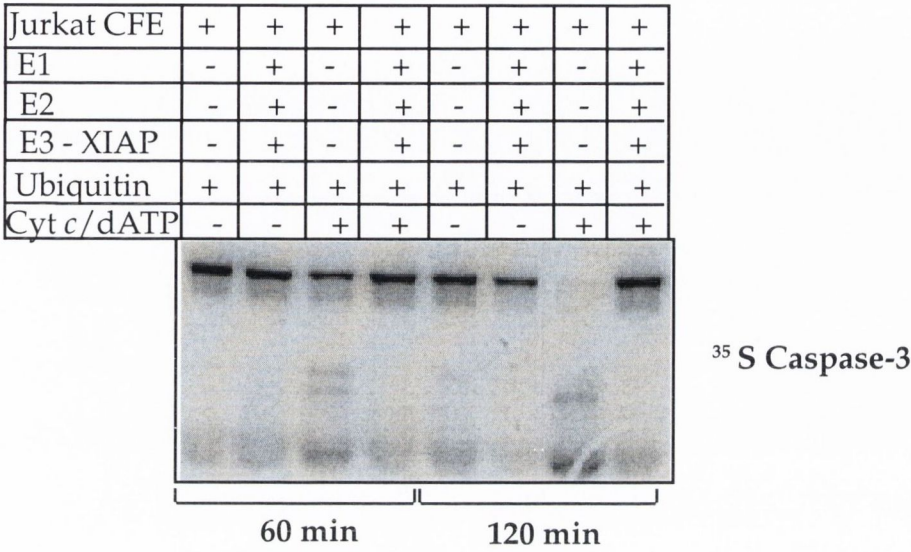


Fig 5.6. *In vitro* ubiquitination assay in Jurkat cell-free extracts

Control and cytochrome *c*/dATP-treated cell-free extracts were supplemented with recombinant ubiquitin, E1, E2, GST-XIAP as E3 and [³⁵S]-labelled caspase-3 as the target species. Reactions were incubated at 37°C and samples taken at the indicated time-points were subjected to SDS-PAGE separation followed by fluorography to allow for assessment of possible caspase ubiquitination.

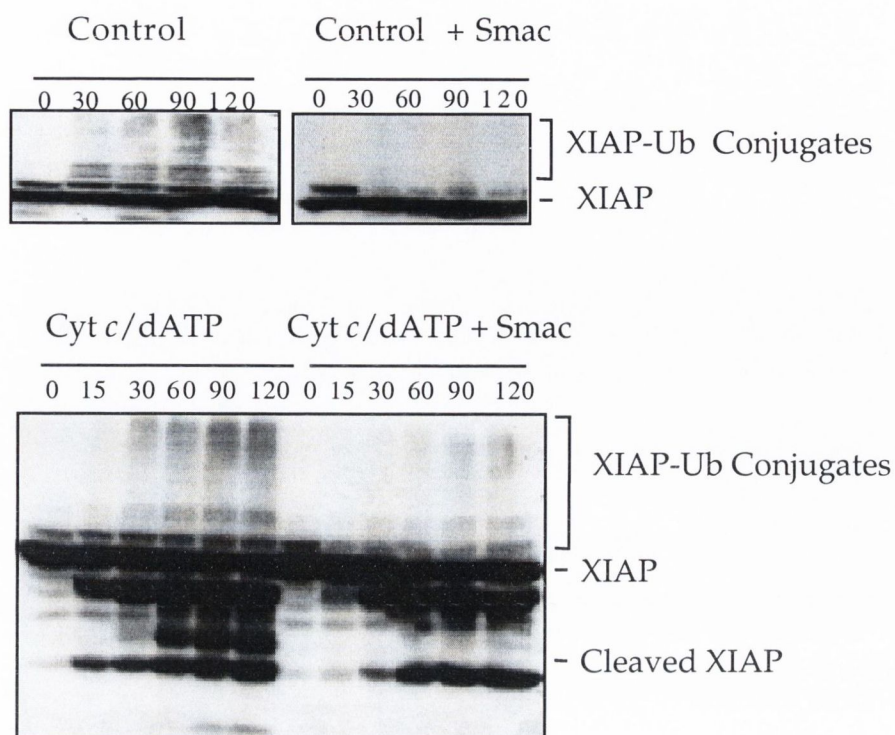


Fig 5.7. *In vitro* ubiquitination assay in Jurkat cell-free extracts with added rabbit reticulocyte
 Control and cytochrome *c*/dATP-treated cell-free extracts were supplemented with rabbit reticulocyte in the presence or absence of 5 μ M recombinant His₆-Smac Δ 1-55. Reactions were incubated at 37°C and samples taken at the indicated time-points were subjected to SDS-PAGE separation followed immunoblotting for XIAP.

decided to explore whether Smac could inhibit XIAP autoubiquitination in a cellular context also.

5.3.5. XIAP ubiquitin ligase activity *in vivo*

Initially, we examined XIAP autoubiquitination in isolation by overexpressing polyhistidine-tagged ubiquitin (His-Ub) and varying amounts of XIAP in human embryonic kidney (HEK) 293T cells. As is evident in Fig 5.8, a ladder of polyubiquitinated XIAP species was readily detected *in vivo*. Titration of XIAP levels reduced the level of ubiquitinated species, consistent with XIAP acting as an E3 ligase towards itself. Thus XIAP is capable of undergoing autoubiquitination *in vivo*.

To verify that the RING domain of XIAP was required for its ubiquitin ligase activity we co-transfected HEK293T cells with His-Ub and GST vector or one of a panel of GST-XIAP constructs. The pEBG-XIAP3xBIR construct contained only the three BIR domains of XIAP. The pEBG-XIAP Δ RING construct lacks the RING domain and the pEBG-XIAP_{H467A} construct is mutated at an important histidine residue within the RING domain. Fig 5.9 (upper panel) illustrates that only the construct harboring an intact RING domain, pEBG-XIAP, effectively autoubiquitinates. This result confirms that XIAP autoubiquitination requires an intact RING domain. Fig 5.9 (lower panel) shows that all XIAP constructs were expressed at similar levels in HEK293T cells. However, the construct missing the RING domain (GST-XIAP Δ RING) was highly unstable and rapidly degraded *in vivo*. Thus the RING domain appears to be important for maintaining the protein in a stable configuration.

Having confirmed the ability of XIAP to autoubiquitinate *in vivo*, we next investigated the role of Smac in this system.

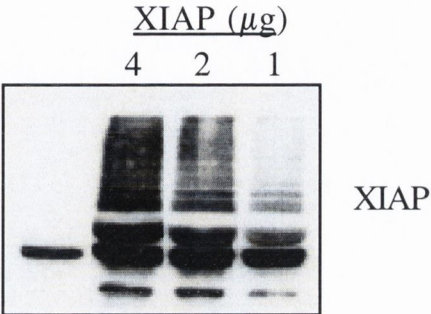


Fig 5.8. XIAP autoubiquitination in HEK293T cells
HEK293T cells were co-transfected with His₆-tagged ubiquitin (2 μg) and the indicated amounts of a XIAP expression plasmid or empty vector. Cell lysates were prepared 24 h later and a Western Blot was probed for XIAP.

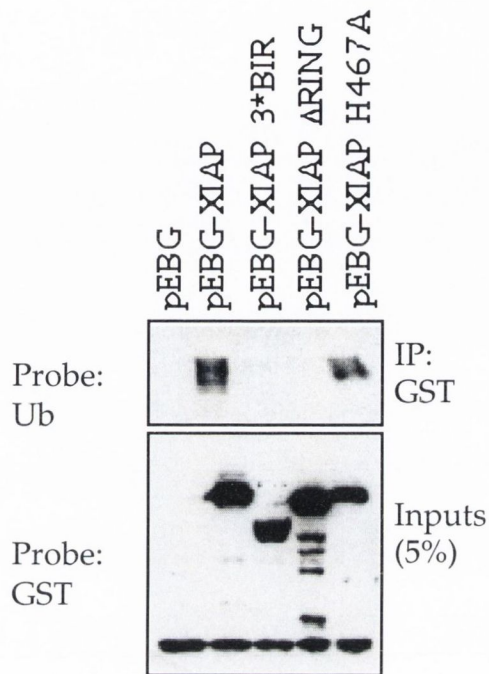


Fig 5.9. XIAP autoubiquitination is RING domain-dependent

HEK293T cells were transfected either with GST vector, or the indicated GST-XIAP constructs, along with His₆-tagged ubiquitin (2 μ g). Cell lysates, prepared 24 h post-transfection, were incubated with glutathione-conjugated sepharose beads followed by probing for ubiquitin. Cell lysates (5% of input) were probed for GST, as shown.

5.3.6. Smac/Diablo inhibits XIAP autoubiquitination

In order to confirm our *in vitro* observations, we co-expressed Smac along with XIAP and His-Ub in HEK293T cells. As is illustrated in Fig 5.10b, in accordance with the *in vitro* results, Smac strongly inhibited XIAP autoubiquitination *in vivo*. A splice variant of Smac - Smac δ , which lacks amino acids 62-105 but retains the IAP-binding domain (Figure 5.10b), was even more potent than full-length Smac in repressing XIAP autoubiquitination. As a further confirmation of this result we co-transfected HEK293T cells with GST or GST-XIAP in combination with either full-length Smac or Smac δ . The lysates were then subjected to immunoprecipitation with glutathione-sepharose beads and the resulting immunoprecipitates were probed with anti-Ub antibody. As is shown in the upper panel of Fig 5.11, the presence of Smac effectively repressed the ability of XIAP to autoubiquitinate. Again Smac δ was even more effective than wild-type Smac.

Collectively, the *in vitro* and *in vivo* observations convincingly argue that Smac/Diablo does indeed repress XIAP autoubiquitination. However, we were still unable to reconcile this property of Smac with its ability to act as IAP antagonist. We questioned how could Smac promote death by stabilizing IAPs?

One solution to this problem was that by inhibiting XIAP autoubiquitination, Smac could promote the E3 ligase activity of XIAP towards itself. However, as Fig 5.10 (lower panel) and Fig 5.12 illustrate, no evidence of Smac ubiquitination could be detected *in vivo*.

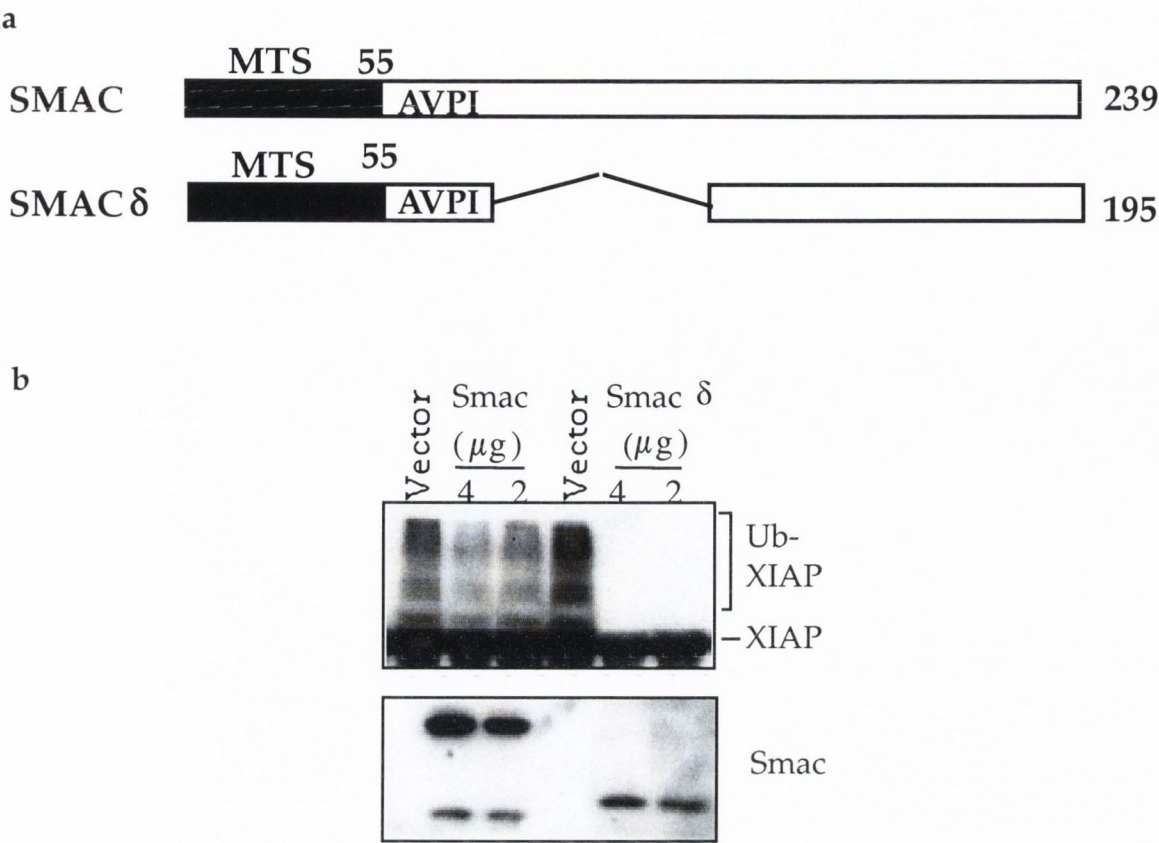


Fig 5.10(a). Schematic representation of wild-type Smac and Smac δ

Both the illustrated Smac isoforms, full-length Smac and Smac δ , contain a mitochondrial targeting sequence (MTS) and the IAP binding motif, AVPI. Smac δ is lacking amino acids 62-105 immediately adjacent to the IAP binding motif (IBM). Smac δ potentially induces apoptosis upon transient overexpression in a number of cell types, which contrasts sharply with the activity of full-length Smac.

Fig 5.10(b). Smac inhibits XIAP autoubiquitination *in vivo*

HEK293T cells were transfected with His₆-ubiquitin (2 μ g) and XIAP (1 μ g) plasmids, in combination with the indicated amounts of plasmids encoding Smac or Smac δ . Lysates were immunoblotted for XIAP (upper panel) and Smac (lower panel).

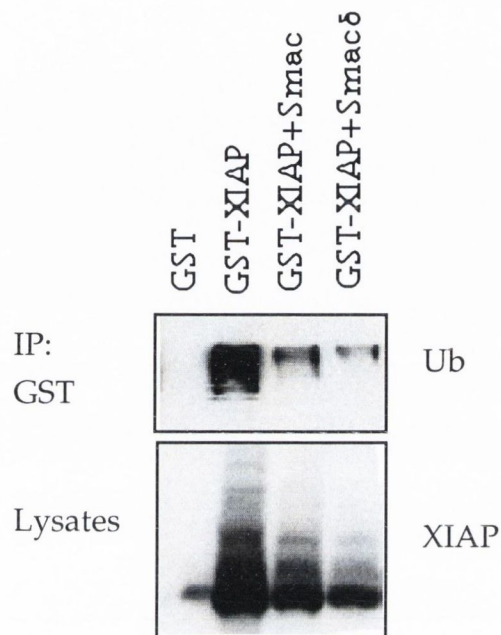


Fig 5.11. Smac inhibits XIAP autoubiquitination *in vivo*

HEK293T cells were transfected with GST or GST-XIAP expression plasmids, in combination with plasmids encoding Smac or Smac δ (3 μ g). Lysates were subjected to glutathione-sepharose beads and subsequently probed for ubiquitin (upper panel). Cell lysates (5% of input) were also probed for XIAP (lower panel).

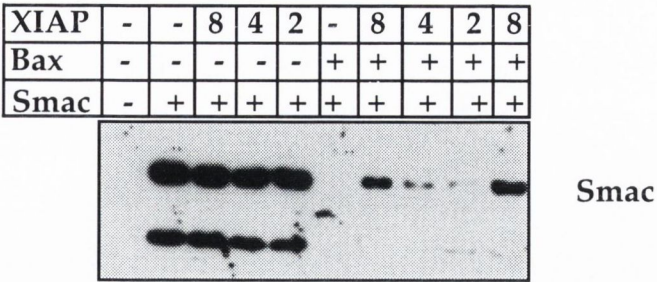


Fig 5.12. Smac ubiquitination *in vivo*
HEK293T cells were transfected with plasmids encoding Smac (3 μ g), in combination with decreasing amounts of GST-XIAP and 0.75 μ g of Bax expression plasmid as indicated. In the final lane z-VAD was added to a final concentration of 10 μ M. Lysates were prepared 24 h later. Following SDS-PAGE separation the lysates were probed with anti-Smac antibody.

5.1. DISCUSSION

5.1.1. IAP ubiquitin ligase activity

The presence of a RING domain in several mammalian IAPs suggests that these proteins may act as E3 ligases towards their binding partners. However, to date evidence in support of this hypothesis is weak. We wanted to determine the importance of the RING domain of the mammalian IAPs in the context of apoptosis. Initially we investigated IAP autoubiquitination or IAP-mediated caspase ubiquitination *in vitro*, as we reasoned that this approach would provide a more controlled environment and hence would permit easier detection of ubiquitinated species, as proteasome-mediated degradation would be avoided. Our initial attempts in establishing an *in vitro* ubiquitination assay failed however, despite testing various parameters such as temperature, concentration of reaction constituents and the presence or absence of control and activated cell-free extract. Upon addition of rabbit reticulocyte lysate, we successfully detected IAP autoubiquitination. Obviously the presence of the reticulocyte lysate creates a more favourable environment for the detection of ubiquitinated species, perhaps by contributing key reaction constituents. This environment was sensitive to the concentration of added recombinant E1 and E2 however, as decreasing their concentration abolished the detection of ubiquitinated species (data not shown).

IAP-mediated caspase ubiquitination was not detected in any of these experimental settings. Caspases-3, -7 and -9 were examined as it is well established that the IAPs, namely XIAP, c-IAP1 and c-IAP2, interact with and inhibit the activities of these caspases (Deveraux *et al.*, 1997; Deveraux *et al.*, 1998; Takahashi *et al.*, 1998; Deveraux *et al.*, 1999; Chai *et al.*, 2001a; Huang *et al.*, 2001; Riedl *et al.*, 2001b; Shiozaki *et al.*, 2003). Our failure to detect IAP-mediated caspase ubiquitination was not particularly surprising as definitive evidence of IAP-mediated caspase ubiquitination in mammalian systems is lacking. Hunter and colleagues have proposed that c-IAP2 is capable of promoting the ubiquitination of caspase-7 and caspase-3 (Huang *et al.*, 2000). These results are based on an *in vitro* assay however, using recombinant histidine-tagged c-IAP2 and either recombinant procaspase-7 or a mixture of recombinant inactive and active caspase-3. Furthermore this analysis only revealed the monoubiquitination of these targets (Huang *et al.*, 2000). Takahashi and

colleagues have also claimed that caspase-3 ubiquitination may be mediated by XIAP (Suzuki *et al.*, 2001). These results are based on overexpression studies with an artificial caspase-3 construct, so once again the actual relevance of these findings is questionable (Suzuki *et al.*, 2001). Therefore, despite the proposed potential of the IAPs to harbour a dual means of blocking caspase function through direct inhibition and through ubiquitination leading to degradation, convincing evidence of the latter hypothesis has yet to be provided. However, as our analysis was confined to an *in vitro* setting, and as *in vitro* experiments do not always reflect the *in vivo* environment, our results do not exclude the possibility that IAP-mediated caspase ubiquitination occurs *in vivo*.

XIAP autoubiquitination was successfully detected using this *in vitro* system and overexpression studies in HEK293 cells confirmed the *in vitro* capability of XIAP to autoubiquitinate. XIAP autoubiquitination *in vivo* has only ever been demonstrated once before (Holley *et al.*, 2002). Our studies also displayed that the E3 ubiquitin ligase activity of XIAP was dependent on the presence of an intact RING domain.

The *in vivo* significance of the proteasomal-mediated degradation of XIAP in mammalian systems is unclear. XIAP-deficient mice are viable (Harlin *et al.*, 2001). Moreover, cells from the knockout animals respond as wild-type cells to various apoptotic stimuli (Harlin *et al.*, 2001). Disruption of *c-IAP1* and *c-IAP2* has been reported to have no deleterious consequences in the mouse either (unpublished data). Such knockout phenotypes suggest that IAPs may be able to compensate for one another during development. The fact that XIAP is a far more potent inhibitor of apoptosis than either *c-IAP1* or *c-IAP2* would argue against this proposal however (Deveraux *et al.*, 1998). Therefore, rather than playing a gross decision-making role in developmental cell death, the IAPs in mammals may be more critical in establishing sensitivity thresholds that regulate the concentration of active caspases present in a cell. The generation of a mouse deficient in all IAPs would allow a more critical assessment of the role of the IAPs *in vivo*. The presence of several mammalian homologs may make this very difficult to create however. In sharp contrast to the viable XIAP-deficient mice (Harlin *et al.*, 2001), the loss of DIAP1 in *Drosophila* is sufficient to trigger widespread apoptosis, resulting in embryonic lethality (Wang *et al.*, 1999). However, similar to mammalian IAPs, DIAP1 is capable of autoubiquitination, a property that is dependent on an intact RING domain (Ryoo

et al., 2002; Wilson *et al.*, 2002; Yoo *et al.*, 2002). Meier and colleagues have also shown that DIAP1 is capable of promoting ubiquitination of the *Drosophila* caspase DRONC (Wilson *et al.*, 2002). This study did not conclusively show that this post-translational modification actually targets DRONC for proteasomal-mediated degradation however (Wilson *et al.*, 2002).

It is not altogether surprising that the regulation of apoptosis in mammalian systems is not mirrored exactly in *Drosophila*. Striking differences between these two systems have previously been discussed. For example, two separate studies have proposed that the fly apoptosome forms without cytochrome *c* involvement (Dorstyn *et al.*, 2002; Zimmermann *et al.*, 2002), while this co-factor is critical for mammalian apoptosome formation (Liu *et al.*, 1996). Recent reports have indicated that the actual trigger for apoptosome assembly in the fly is in fact the loss of DIAP1 (Rodriguez *et al.*, 2002; Zimmermann *et al.*, 2002). Therefore, it would appear that IAP autoubiquitination is in fact more critical for *Drosophila* systems than for mammalian systems as the loss of DIAP1 promotes apoptosome assembly and subsequent apoptosis.

It is interesting to note that mammalian IAPs still retain an ability to autoubiquitinate even though the loss of these proteins in this particular setting is not as deleterious as it is in the fly. IAPs were first discovered in baculoviruses as a result of a screen to identify genes that were functionally equivalent to the pan-caspase inhibitor p35 (Crook *et al.*, 1993; Birnbaum *et al.*, 1994). The defining characteristic of the IAP family is the presence of a 70 amino acid zinc-binding fold termed the BIR (baculovirus IAP repeat) domain. BIR-containing proteins have since been discovered in *C. elegans*, yeast, mammals and insects. While a BIR domain is critical for an IAP to mediate its anti-apoptotic effects not all BIR-containing proteins (BIRPs) are IAPs (Miller, 1999).

Survivin and Bruce only possess a single BIR. The primary role of survivin is in the regulation of cell division (Verhagen *et al.*, 2001). The ability of survivin to regulate cell division is a property shared with the BIRPs found in yeast and *C. elegans*. The BIR of survivin and Bruce is slightly larger than the BIR domain found in other

mammalian IAPs and more closely resembles the BIR domains found in yeast and *C. elegans*. The function of Bruce is unknown but the presence of this larger BIR suggests that the role of Bruce is also in the regulation of cell division rather than the inhibition of cell death (Miller, 1999; Verhagen *et al.*, 2001). It is hypothesised that the original function of BIRPs was in the regulation of cell division and that their role as inhibitors of cell death arose later in evolution (Clem, 2001). It is interesting to speculate that once mammalian IAPs acquired the ability to inhibit cell death via the BIR domain, the function of the RING domain became somewhat redundant in the more complex mammalian systems while remaining important in the simpler *Drosophila* systems, where the actual loss of the IAP is sufficient to trigger apoptosis. It must still be noted however, that the full length XIAP is a more potent inhibitor of apoptosis than a deletion mutant of the protein, lacking the RING domain (Creagh *et al.*, 2004), suggesting that the RING domain, although not essential in mammalian systems does indeed play a role in the inhibition of apoptosis.

The *Drosophila* proteins, Reaper, Hid and Grim share a region of homology at their NH₂-termini termed the RHG motif - Rpr-Hid-Grim motif. This motif allows these proteins to induce apoptosis by binding to the *Drosophila* IAP, DIAP1, hence eliminating the inhibitory effect of this IAP on the caspases (Wang *et al.*, 1999; Goyal *et al.*, 2000). Rpr, Hid and Grim, may be described as the functional homologs of mammalian Smac (Wang *et al.*, 1999; Goyal *et al.*, 2000). The relief of IAP inhibition by Smac is also dependent on four residues at the NH₂-terminus of the mature protein – Ala-Val-Pro-Ile. This AVPI motif or IAP-binding motif (IBM) resembles the RHG motif found in *Drosophila* (Srinivasula *et al.*, 2001). A similar motif is also found at the NH₂-terminus of the small subunit of caspase-9. XIAP binds caspase-9 at this site. Smac also binds to this site and thereby antagonises the inhibitory effects of XIAP (Srinivasula *et al.*, 2001). Rpr and Hid have been shown to promote DIAP1 autoubiquitination (Ryoo *et al.*, 2002; Yoo *et al.*, 2002). We also examined whether Smac/Diablo modulated mammalian IAP ubiquitin ligase activity in a manner similar to Rpr and Hid.

5.1.2. Smac inhibits XIAP autoubiquitination

Surprisingly, addition of Smac to our *in vitro* assay severely attenuated XIAP autoubiquitination. Our initial *in vitro* observation was repeated *in vivo*, whereupon

Smac overexpression inhibited XIAP autoubiquitination in HEK293T cells. Indeed a splice variant of Smac - Smac δ , which lacks amino acids 62-105 but retains the IAP-binding domain, was even more potent than full-length Smac in repressing XIAP autoubiquitination. Interestingly Smac δ is also more efficient than Smac wild-type in the sensitization of cells to apoptosis-inducing stimuli (data not shown). This observation would argue favorably that inhibition of IAP ubiquitin ligase activity serves an important role in the global mechanisms utilized by Smac to act as an IAP antagonist.

We were puzzled by the ability of Smac to attenuate XIAP autoubiquitination as we were unable to reconcile this property with the role of Smac as an IAP antagonist. How could the prevention of IAP degradation be perceived as an inhibitory mechanism. Cohen and colleagues had proposed that XIAP promotes Smac ubiquitination (MacFarlane *et al.*, 2002), and therefore we reasoned that the inhibition of XIAP autoubiquitination simultaneously promotes Smac ubiquitination. As the results published by Cohen and colleagues were based on an *in vitro* assay using recombinant GST-XIAP and recombinant histidine-tagged Smac (MacFarlane *et al.*, 2002), we tested our hypothesis *in vivo*. Our analysis did not detect any evidence of XIAP-mediated Smac ubiquitination *in vivo* and thus, this scheme was deemed inappropriate. However, subsequent experiments in our laboratory highlighted that promotion of Smac release from mitochondria resulted in the inhibition of XIAP-mediated caspase-7 polyubiquitination in HEK293T cells (Creagh *et al.*, 2004). These latest results suggest that Smac attenuates the global ubiquitin ligase activity of XIAP (Fig 5.13).

As previously discussed Smac associates with the BIR3 of XIAP, via the IAP-binding motif at the amino-terminus of Smac (Chai *et al.*, 2000; Wu *et al.*, 2000). This association disrupts the caspase-9/XIAP BIR3 interaction (Srinivasula *et al.*, 2001). Therefore, the function of Smac is thought to be mediated essentially by the physical displacement of caspases from XIAP. It is interesting to note however, that the linker-BIR2 region of XIAP, caspase-7 and Smac are capable of forming a ternary complex (Huang *et al.*, 2003). One could speculate that the formation of such a complex may distort the RING domain of XIAP, eliminating the E3 ligase activity of

this domain and thereby explaining how Smac antagonises XIAP-mediated caspase ubiquitination.

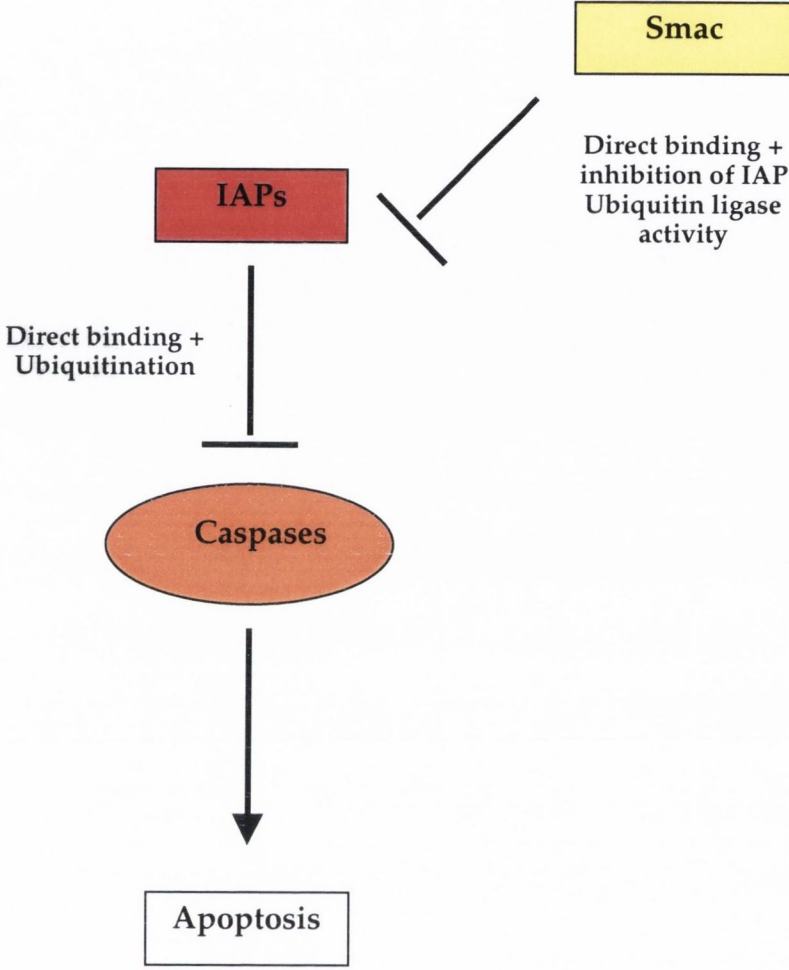


Fig 5.13. Smac mediated regulation of IAP caspase-inhibitory activity

Chapter VI

General Discussion

The *C. elegans* CED-3 protein was the first gene product identified as being essential for programmed cell death (Yuan and Horvitz, 1990). The research presented in this thesis is centred on the mammalian homologs of the *C. elegans* CED-3 protein – the cell death associated caspases and in particular, the mechanisms governing the activation and regulation of these key proteases.

It is evident from the results presented in this thesis that caspases may be activated by other family members as part of an amplification cascade. During the course of our research, we have examined two such cascades, the apoptosome-mediated cascade and the granzyme B-mediated cascade. We have revealed that the order of activation events in the apoptosome-mediated cascade is conserved not only in the transformed cell line, HEK293, but also primary human neutrophils. The maintenance of this hierarchy across varying cell types highlights that these crucial proteases, that can cleave up to 500 cellular substrates (Adrain *et al.*, 2003, unpublished data), are themselves subject to tight regulation and their activation is a highly ordered and consistent event. This is not surprising when one considers that deregulated caspase activation would wreak havoc in a cell. We have also proposed the order of caspase activation in the granzyme B-mediated cascade. This is the first analysis of the sensitivity of caspases to granzyme B proteolysis in a setting where all enzymes are present at physiological levels. This cascade is distinct from the apoptosome-mediated cascade. However, despite the differences between the exact sequence of events in these two cascades, the actual caspases involved are identical. Are these the only caspases that are activated in a cascade-like manner during the execution of cell death? Recent studies suggest that additional caspases are involved in apoptotic cascades activated by various stimuli, as will be discussed below.

Caspase-12 has been proposed to act as an initiator caspase in the endoplasmic reticulum (ER) stress-associated caspase cascade (Nakagawa *et al.*, 2000; Morishima *et al.*, 2002; Rao *et al.*, 2002). Accumulation of misfolded proteins and alterations in calcium homeostasis in the ER provoke ER stress and may result in cell death. Exactly how caspase-12 is activated by this pathway remains a unknown but caspase-9 has been reported to be the downstream target of caspase-12 (Morishima *et al.*, 2002 Rao *et al.*, 2002). The *in vivo* functional relevance of the ER stress-associated

caspase cascade is questionable as *CASPASE-12* null mice develop normally (Nakagawa *et al.*, 2002). In addition, a frameshift mutation and a premature stop codon in the human *CASPASE-12* gene results in a lack of expression of a functional enzyme in the majority of individuals (Fischer *et al.*, 2002). Therefore, further research is needed to properly define this particular caspase-activating cascade and to deduce the order of this cascade in cells lacking caspase-12.

Caspase-2, as previously discussed, has been proposed to act upstream of apoptosome formation to promote cytochrome *c* release from mitochondria (Guo *et al.*, 2002; Lassus *et al.*, 2002; Robertson *et al.*, 2002). These reports are hard to reconcile with various other studies however, such as the normal *CASPASE-2* knockout phenotype (Bergeron *et al.*, 1998; O'Reilly *et al.*, 2002) and with our finding that even in the absence of this protease, apoptosome formation occurs as normal in primary human neutrophils. Caspase-2 contains a CARD within its prodomain (Kumar *et al.*, 1994; Wang *et al.*, 1994), and has been reported to interact with the CARD of the adaptor protein RAIDD (Duan and Dixit, 1997; Chou *et al.*, 1998). The death domain (DD) of RAIDD can also bind the DD of RIP (Grimm *et al.*, 1996). RIP is a serine/threonine kinase that acts as a signalling component of the TNF receptor pathway (Grimm *et al.*, 1996). Thus, it has been proposed that caspase-2 may play a role in the TNF pathway (Grimm *et al.*, 1996). Again however, this putative role for caspase-2 as an initiator caspase is undermined by the fact that *CASPASE-2*^{-/-} embryonic fibroblasts remain sensitive to TNF- α -induced apoptosis (Bergeron *et al.*, 1998).

Interestingly, the apoptotic caspases are not the only members of the caspase family reported to be activated in such a manner. The inflammatory caspases, caspases -1, -4, -5 and -11, may also be activated in cascades. Caspase-1 plays a role in inflammation where it is involved in the processing of inflammatory cytokines such as proIL-1 β (Thornberry *et al.*, 1992). Caspase-1 possesses a long prodomain, suggesting it is fact an “initiator” type caspase, but the actual mechanisms leading to caspase-1 activation remain unclear (Earnshaw *et al.*, 1999). It has been suggested that perhaps caspase-11 is involved upstream of caspase-1 processing as *CASPASE-11*-null mice have a phenotype very similar to *CASPASE-1*-null mice and the

processing of caspase-1 is inhibited in *CASPASE-11*-null mice (Li *et al.*, 1995; Wang *et al.*, 1998). The human homolog of caspase-11 is unknown but caspases-4 and -5 are proposed to be the duplicated counterparts of this murine caspase (Faucheu *et al.*, 1995; Kamens *et al.*, 1995; Munday *et al.*, 1995; Faucheu *et al.*, 1996). Tschopp and colleagues have suggested that caspase-5 is involved in caspase-1 processing (Martinon *et al.*, 2002). These authors describe the formation of a multi-protein complex, similar to the apoptosome, termed the inflammasome. In this complex, NALP-1 is the scaffolding protein recruiting both caspase-1 and caspase-5. Caspase-1 recruitment is mediated by CARD-CARD interactions between this protease and ASC. Caspase-5 is believed to interact with the CARD domain present within the C-terminus of NALP-1. The formation of such a multimeric complex is believed to facilitate the activation of caspase-1 and caspase-5 (Martinon *et al.*, 2002). This model awaits verification *in vivo* however, as the inflammasome complex was only detected *in vitro* by this analysis.

It is evident that while much is already known about certain cascades, such as the apoptosome cascade, others, such as the putative caspase-1 activating cascade, remain to be properly deciphered. It will be interesting to note the exact mechanisms leading to caspase-1 activation, in particular. This caspase was discovered ten years ago, yet it is still unclear whether this protease even requires processing to achieve a catalytically favourable configuration. Our knowledge concerning caspases-4 and -5 is severely limited. We do not know exactly how these proteases are activated, nor have we identified any substrates that are targeted by these caspases, although Tschopp and colleagues suggest that caspase-5 may be involved in proIL-1 β processing (Martinon *et al.*, 2002). Furthermore, it must be noted that caspase-2 is highly expressed during embryogenesis, suggesting an important role for this protease in development. A definitive upstream role for this caspase has yet to be identified however. Therefore, further research is most certainly required to increase our knowledge of how all caspases are activated and the exact sequence of events occurring both upstream and downstream of such activating cascades.

An interesting observation that can be noted from our current understanding of caspase activating cascades is that the description of caspases as either “initiator” or “executioner” is somewhat misleading. Caspase-9 knockout studies have clearly established this protease as the initiator caspase in the apoptosome cascade (Hakem

et al., 1998; Kuida *et al.*, 1998). In contrast, in the granzyme B cascade, caspase-9 processing is dependent on caspase-3. Likewise the activation of caspase-8 is in the final tier of caspase activating events in the mitochondrial pathway (Slee *et al.*, 1999b), yet this protease is the critical initiator caspase in the death receptor pathway (Boldin *et al.*, 1996; Muzio *et al.*, 1996). Therefore, while it is clear that caspases bearing long prodomains may be activated as part of oligomerizing complexes, this statement oversimplifies the flexibility of the caspase family that may be activated via different mechanisms in response to various death-inducing stimuli.

Another distinction that warrants further scrutiny is the classification of caspases as either “apoptotic” or “inflammatory”. The role of caspase-8 as an apoptotic caspase has been well established. This protease is critical for death-receptor mediated apoptosis, as demonstrated by the resistance of *CASPASE-8*-null embryonic fibroblasts to such death-inducing stimuli (Varfolomeev *et al.*, 1998). In addition caspase-8 is also known to be involved in granzyme B-mediated cell death (Fig 4.9; Medema *et al.*, 1997b; Sutton *et al.*, 2000). Recent evidence suggests that, in contrast to this role as a death-inducing caspase, caspase-8 may also be involved in lymphocyte proliferation (Chun *et al.*, 2002; Salmena *et al.* 2003). The human disorder, autoimmune lymphoproliferative syndrome (ALPS), is characterised by defective lymphocyte apoptosis, lymphadenopathy, splenomegaly and autoimmunity. Mutations within the death receptor Fas/CD95 (Martin *et al.*, 1999; Siegel *et al.*, 2000), its ligand FasL/CD95L (Jackson and Puck 1999) and caspase-10 (Wang *et al.*, 1999) have all been identified as the genetic defects associated with various forms of this disease. Two individuals have been identified with ALPS-like disorders but do not have mutations within Fas/CD95, FasL/CD95L or caspase-10. These patients actually harbour a C-T mutation within the p18 protease subunit of the *CASPASE-8* gene that results in the formation of an enzymatically inactive protease (Chun *et al.*, 2002). These patients also suffer from immunodeficiency and analysis of the lymphocytes of these individuals suggests that the immunodeficiency is caused by the caspase-8 mutation interfering with the activation of T, B and NK cells. To verify a role for caspase-8 in immune cell activation, Lenardo and colleagues used RNAi and antisense techniques to ablate caspase-8 expression in normal human peripheral blood lymphocytes (PBLs) and suggested that interfering with the expression of

caspase-8 reduced the response of these cells to TCR stimulation (Chun *et al.*, 2002). However, the level of caspase-8 message ablation achieved was not shown and the previously discussed complications associated with the use of RNAi were not accounted for in this study. In addition, these authors claimed that transfection of a caspase-8 construct into the PBLs of one of the patients resulted in the restoration of CD25 upregulation upon anti-CD3/anti-CD28 stimulation (Chun *et al.*, 2002). However, this increase was only very modest. More convincing evidence for the role of caspase-8 in T-cell proliferation came from the generation of T-cell-specific caspase-8 deficient mice (Salmena *et al.*, 2003). It was necessary to generate such an animal as the *CASPASE-8* knockout phenotype in mice is lethal *in utero* (Varfolomeev *et al.*, 1998). The T-cell caspase-8 deficient mice had significantly fewer mature T-cells, in particular CD8⁺ T-cells, in comparison to wild-type siblings. In addition, the T-cell caspase-8 deficient mice were unable to mount an effective cytotoxic T-cell response upon infection with lymphocytic choriomeningitis virus (LCMV) and purified *CASPASE-8*^{-/-} T-cells did not proliferate as well as wild-type cells upon activation by anti-CD3 antibody *in vitro* (Salmena *et al.*, 2003). These data suggest an important role for caspase-8 in the maintenance of T-cell homeostasis and in preserving T-cell-mediated immunity. Of course, much has still to be learned about this pro-survival function of the mammalian caspases. It will be interesting to decipher exactly how such a dual personality is regulated. Is it the manner in which the protease is activated or is it regulated by signals from other cellular compartments? What are the differences between the substrates targeted by the differently functioning sides of the protease? It will also be interesting to learn if any other caspase also harbours a similar split personality.

One possible means of modulating the functions of a particular protein is the post-translational modification of the protease (Schwartz and Hochstrasser, 2003). One particular post-translational modification examined during the course of this research was ubiquitination. Ubiquitination generally targets the tagged protein for degradation by the 26S proteasome (Salvesen and Duckett, 2002). In addition to mediating such proteasomal-mediated destruction, ubiquitination and in particular monoubiquitination, may also stimulate receptor endocytosis and subsequent lysosomal-mediated degradation of the receptor (Haglund *et al.*, 2002; Haglund *et al.*,

2003). We have shown that XIAP is capable of autoubiquitination but failed to detect any of evidence of XIAP-mediated caspase polyubiquitination. XIAP-mediated caspase-7 polyubiquitination was detected by subsequent experiments in the laboratory however (Creagh *et al.*, submitted). It is interesting to speculate that in addition to mediating proteasomal degradation, monoubiquitination could also serve as an intracellular signal for targeting caspases to a lysosomal compartment for degradation. No formal results have been published to date supporting this hypothesis but it is interesting to note that c-IAP2 has been shown to monoubiquitinate caspases-3 and -7 *in vitro* (Huang *et al.*, 2000).

We also revealed that Smac inhibits XIAP autoubiquitination. The IBM (IAP-binding motif) of Smac is known to be critical for relief of XIAP-mediated caspase-9 inhibition (Srinivasula *et al.*, 2001). However, it also known that a Smac variant, missing this motif (Smac-S/Smac β) can still antagonise IAP function (Srinivasula *et al.*, 2000; Roberts *et al.*, 2001). These findings concur with the analysis that Smac has a dual personality within cells. Smac not only prevents IAPs from physically interacting with caspases but also attenuates their ability to promote caspase ubiquitination (Fig 5.13). Therefore, Smac may promote apoptosis not only by direct binding of the IAPs but also by inhibiting IAP-mediated caspase ubiquitination. It is critical that we understand all mechanisms utilised by Smac to induce apoptosis, as Smac is currently an attractive therapeutic target for the treatment of cancer (Chai *et al.*, 2000; Srinivasula *et al.*, 2001). Indeed a recent study has demonstrated that synthetic Smac peptides, consisting of either the first 6 or 8 NH₂-terminal residues of mature Smac, can enhance the effects of chemotherapeutic agents in breast cancer cell lines (Arnt *et al.*, 2002). Deciphering all mechanisms utilised by Smac to potentiate apoptosis will undoubtedly aid the development of more effective Smac peptides for cancer treatment.

Smac is not alone in its ability to influence IAP ubiquitin ligase activity. Omi/HtrA2 is a serine protease that together with Smac is released from the mitochondrial intermembrane space into the cytoplasm during apoptosis. This 36kDa protein has been shown to bind XIAP, c-IAP1 and c-IAP2. Its NH₂-terminus contains an IBM - AVPS - and this motif is critical for binding to the BIR2 and BIR3 domains of XIAP.

Binding of Omi/HtrA2 to the BIRs diminishes the inhibitory effects of XIAP and mature Omi can promote procaspase-3 activation. Over-expression of Omi/HtrA2 sensitizes cells to UV-, TRAIL- and staurosporine-induced apoptosis. (Suzuki *et al.*, 2001a; Hedge *et al.*, 2002; Martins *et al.*, 2002; van Loo *et al.*, 2002; Verhagen *et al.*, 2002). Recently, however, the serine protease activity of Omi has been shown to be capable of cleaving the IAPs, namely XIAP and c-IAP1. This cleavage event is dependent on Omi binding via its IBM. IAP cleavage by Omi/HtrA2 results in the inactivation of these proteins (Srinivasula *et al.*, 2003; Yang *et al.*, 2003). In addition Omi/HtrA2-processed c-IAP1 is proposed to have reduced ubiquitin ligase activity (Yang *et al.*, 2003). It must be noted however, that these observations were based on overexpression studies and *in vitro* assays and therefore the actual functional relevance of these properties *in vivo* warrants further investigation.

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