IN VITRO SYSTEMS TO STUDY THE RELATIONSHIP BETWEEN APOPTOSIS IN MULTICELLULAR ORGANISMS AND YEAST

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ABSTRACT

Apoptosis is a distinct form of cell death that is characterised by specific morphological and biochemical markers, such as chromatin condensation and internucleosomal DNA cleavage. This type of cell death is evolutionarily conserved in higher eukaryotes. Homologues of the main apoptosis regulators, such as the Bcl-2 family of proteins and caspases, have been found in multicellular organisms. However, homologues of these proteins have not been found in the unicellular organism *Saccharomyces cerevisiae*, although in certain circumstances *S. cerevisiae* will exhibit features of apoptosis. In this project, we developed *in vitro* systems to explore the relationship between mammalian apoptosis and any similar mechanism that may be present in yeast.

Components derived from yeast and mammalian cells were incubated together *in vitro* and assessed for the activation of apoptosis. Rat cytochrome c activates apoptosis in mammalian cell-free extracts (human neuroblastoma SY5Y cells). Internucleosomal DNA cleavage was observed in *S. cerevisiae* spheroplasts when they were incubated in mammalian cell-free extracts activated by rat cytochrome c. Although yeast cytochrome c is similar to rat cytochrome c, it failed to induce apoptosis in mammalian cell-free extracts. Yeast cytosol caused internucleosomal DNA cleavage in PC12 nuclei. This cleavage was enhanced by rat cytochrome c and was mostly inhibited by the caspase inhibitor DEVD-CHO, but only in the presence of rat cytochrome c. Yeast cytosol did not cause chromatin condensation in PC12 nuclei or cleavage of Parp (a downstream caspase substrate). Yeast cytosol was therefore unable to induce apoptosis in PC12 nuclei.

Mitochondria play a central role in most forms of mammalian apoptosis. We developed a cell-free system in which we could examine the role of mitochondria in apoptosis. We attempted to activate apoptosis in SY5Y cytosol by the addition of mitochondria subjected to rupture-inducing treatment, with limited success. However, we found that mitochondria purified from healthy SY5Y cells protected PC12 nuclei from undergoing apoptosis *in vitro*.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
AIF	Apoptosis inducing factor
AMP	Adenosine monophosphate
Ant	Adenine nucleotide translocator
Apaf-1	Apoptosis promoting factor 1
APS	Ammonium persulphate
Asn	asparagine
ATP	Adenosine triphosphate
Atr	Atractyloside
Atr-Mitochondria	Mitochondria pre-treated with Atr
Bad	Bcl-X_L/Bcl-2 associated death promoter
BBIII	Bud Buffer III
BH	Bcl-2 homology
BSA	Bovine serum albumin
Cad	Caspase-activated DNase
CARD	Caspase recruitment domain
Caspase	Cysteine aspartic acid protease
Ced	Cell death abnormal
Ces	Cell death specification
CrK	Creatine kinase
CrP	Creatine phosphate
Cyt. c	Cytochrome c
dADP	Deoxyadenosine diphosphate
ddATP	Dideoxy-adensoine triphosphate
ddH_2O	Double distilled water
DED	Death effector domain
DEVD-CHO	acetyl-Asp-Glu-Val-Ala-aldehyde
Diap1	*Drosophila* inhibitor of apoptosis 1
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	DNA endonuclease
DR	Death receptor
DTT	Dithiothretiol
ECL	Enhanced chemluminescence
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
Egl	Egg laying defective
Erk1/2	Extracellular-signal regulated kinase (Erk1 = p44Mapk, Erk2 = p42Mapk)
Fadd	Fas-associated death domain
FasL	Fas ligand
GSH	Glutathione
GFP	Green Fluorescent Protein
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid

Hid	head involution defective
HSNs	Hermaphrodite specific neurons
Iap	Inhibitor of apoptosis
Icad	Inhibitor of caspase-activated DNase
IL-3	Interleukin-3
JC-1	5,5',6,6'-tetrachloro-1,1'3,3'-tetraethylbenzimidazolylcarbocyanine iodide
kB	kilobases
kDa	kilodalton
Lys	lysine
Mapk	Mitogen-activated protein kinase
MOPS	3-(N-Morpholino) propanesulphonic acid
NGF	Nerve growth factor
NRK	Normal rat kidney
NSMs	Neurosecretory motor neurons
P2	isolated mitochondria
Parp	Poly(ADP)-ribose polymerase
PBS	Phosphate-buffered saline
PC12	Adrenal Pheochromocytoma rat cell line
PCD	Programmed Cell Death
PEF	Permeability enhancing factor
PI3K	Phosphotidyl inositol 3-kinase
PK	Proteinase K
PKA	Protein kinase A
PMSF	Phenylmethylsulfonyl fluoride
PTP	Permeability transition pore
ROS	Reactive oxygen species
Rpr	reaper
S3	cytosol
SDS-PAGE	Sodium dodecyl suflate polyacrylamide gel electrophoresis
STS	Staurosporine
SY5Y	Human neuroblastoma cell line
TAE	Tris-Glacial acetic acid-EDTA
TBS	Tris-buffered saline
TdT	Terminal deoxynucleotideal transferase
TE	Tris-EDTA
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
tRNA	Transfer ribonucleic acid
Tween-20	Polyoxyethylenesorbitan monolaurate
v/v	volume/volume
Vdac	Voltage-dependent anion channel
w/v	weight/volume

Note on genetic nomenclature:

The conventions used for writing the names of genes and gene products are according to Murray. (1993). Gene names are always written in lower case letters and are italicised. Gene products are written with the first letter capitalised and are not italicised.

CHAPTER 1: LITERATURE REVIEW

1.1 WHAT IS APOPTOSIS?

Apoptosis, or programmed cell death (PCD), is a distinct form of cell death determined by a genetically encoded cell suicide program (Kerr *et al.*, 1972). It allows the rapid, controlled and complete removal of unnecessary or damaged cells without disruption to the surrounding cells and this process is conserved in all multicellular organisms. Apoptosis is characterised by specific morphological and biochemical changes that require the cell to be metabolically active and depends on RNA and protein synthesis in most, but not all, cell types (Wyllie *et al.*, 1984). During apoptosis, DNA is cleaved first into large 50 kB loops and then cleaved internucleosomally (Wyllie *et al.*, 1980b), while concurrently chromatin condenses (Kerr *et al.*, 1972). Finally, the membrane starts to bleb (bud) and the cell breaks into small apoptotic bodies, which are rapidly engulfed by nearby cells (Figure 1-1) (Ellis *et al.*, 1991a; Ellis *et al.*, 1991b). Assays for these changes can be used as a basis for determining whether a cell is undergoing apoptosis or necrosis.

In contrast, cell death by necrosis, is largely uncontrolled and results in the release of oxidative enzymes and free radicals that inflame the surrounding healthy tissue. Necrotic cell death is characterised by the early dilation of cytoplasmic organelles, followed by the rupture of cell membranes causing an inflammatory response (Figure 1-1) (Wyllie *et al.*, 1980a). Necrotic DNA degradation is a late phenomenon, detected several hours after cellular degeneration is observed. It is thought that chromatin digestion by the cell's nucleases and proteases is a consequence rather than the cause of cell death (Duvall and Wyllie, 1986).

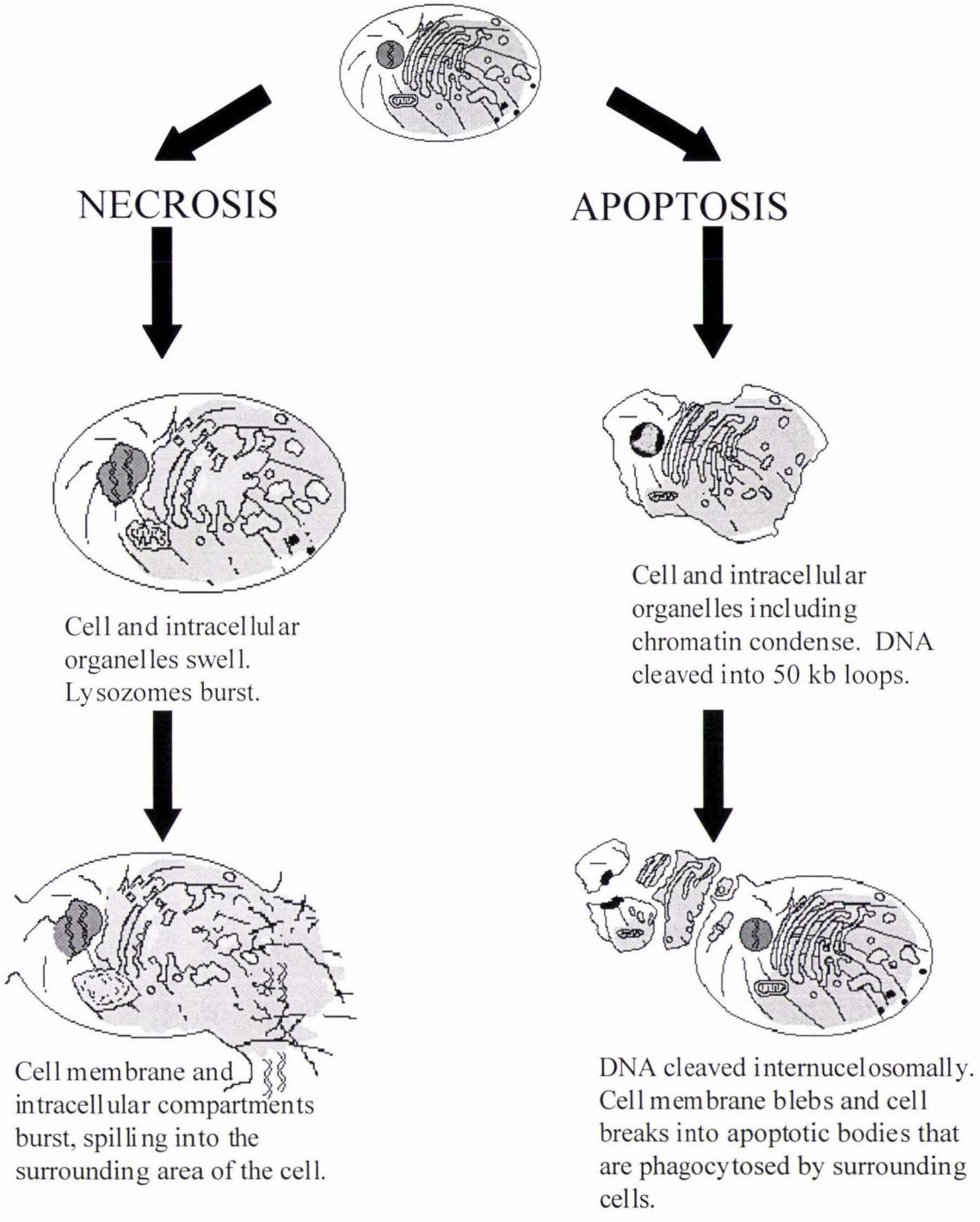


Figure 1-1: Comparison of apoptotic and necrotic cell death.
Initial features of apoptotic cell death are the shrinkage of the cell and internal organelles, followed by the cleavage of the genomic DNA and break up of the cell into apoptotic bodies, which are engulfed by the surrounding cells. In contrast, during necrotic cell death the cell swells and internal organelles burst, spilling the cells' contents into the surrounding tissue.

1.2 APOPTOSIS AND ITS CONTROL

Apoptosis plays many important roles in shaping the final form of an organism during development, for example, the removal of inter-digital cells to shape fingers. Multicellular organisms also use apoptosis as a defence mechanism. For example, cells will commit suicide when exposed to viruses, which eventually leads to the shutdown of DNA and RNA synthesis so new viruses cannot be made. The DNA endonucleases activated during PCD will cleave both the host and viral DNA eliminating the virus completely and therefore protecting the whole organism (Vaux *et al.*, 1994).

Apoptosis plays a prominent role in the development of the mammalian nervous system (Oppenheim, 1991), and it is vital that strict control is maintained over its initiation. When mammalian neurons are formed they attempt to make connections with other neurons, and those that do not make the correct connections undergo apoptosis. As many as 85% of certain populations of developing neurons will undergo PCD (Ellis *et al.*, 1991b). The failure of neurons to undergo apoptosis during development may cause the formation of tumours.

Understanding the molecular mechanisms of apoptosis and identifying its mechanism of activation will provide benefits for human health. Abnormal control of apoptosis may play a role in neurodegenerative disorders such as Parkinson's disease and Alzheimer's disease. In fact, in Alzheimer diseased brains, levels of pro-apoptotic proteins such as Bad (Bcl-X_L/Bcl-2 associated death promoter) and Bak were elevated when compared with normal brains (Kitamura *et al.*, 1998).

1.3 THE NEMATODE MODEL FOR APOPTOSIS

The nematode *Caenorhabditis elegans* has been extensively studied and provides a genetic model for PCD (Hengartner and Horvitz, 1994c). *C. elegans* requires the birth of 1090 somatic cells to produce an adult nematode, but 131 of these cells die during development. The specific 131 cells that die are always the same in all developing nematodes (Ellis *et al.*,

1991b), and their fate is predetermined. The removal or death of surrounding cells does not affect the timing or manner of the death of these 131 cells, indicating that the death process is cell autonomous (Hedgecock *et al.*, 1983). Three genes, *ced-3, ced-4 and ced-9* (cell death abnormal) were identified in which mutations affected these programmed cell deaths (Ellis and Horvitz, 1986; Hengartner *et al.*, 1992). Blocking apoptosis in nematodes is not lethal, and produces an organism with only a few minor functional problems making them an ideal organism to study (Ellis *et al.*, 1991b; Jacobson *et al.*, 1997).

The central cell death machinery involving the *ced-3, ced-4 and ced-9* genes has been well characterised. Proteins encoded by *ced-3* and *ced-4* (Ced-3 and Ced-4, respectively) cause cells to undergo programmed cell death, while the Ced-9 protein antagonises their actions and protects cells from death (Horvitz *et al.*, 1994). Overexpression of either the *ced-3* or *ced-4* genes induces apoptosis, but a functional Ced-3 protein is required for Ced-4 induced death (Shaham and Horvitz, 1996). *Ced-3*, *ced-4* and *ced-9* are constitutively expressed, suggesting that expression of all three genes is required not only for PCD but also for survival of the cell (Shaham and Horvitz, 1996).

The mechanism by which these proteins act to induce or prevent apoptosis has been established through both genetic and biochemical analysis and is diagrammatically represented in Figure 1-2. Ced-9, a mitochondria membrane-associated protein, normally sequesters Ced-4 (Chen *et al.*, 2000; Wu *et al.*, 1997). Following an apoptotic signal, Ced-9 releases Ced-4 into the cytosol, where it can bind and activate the cysteine aspartic acid protease (caspase), Ced-3 (Wu *et al.*, 1997). Ced-4 binds Ced-3 through a caspase recruitment domain (CARD domain) found on both proteins, an interaction that requires ATP and results in a conformational change in Ced-3 (Zou *et al.*, 1997). Caspases are present in the cell in an inactive precursor form, which is activated by the cleavage of the pro-domain and the formation of a heterotetramer of the two domains of the protein. Following processing and activation, Ced-3 cleaves proteins at aspartate residues at a specific sequence, DEVD (Yuan *et al.*, 1993).

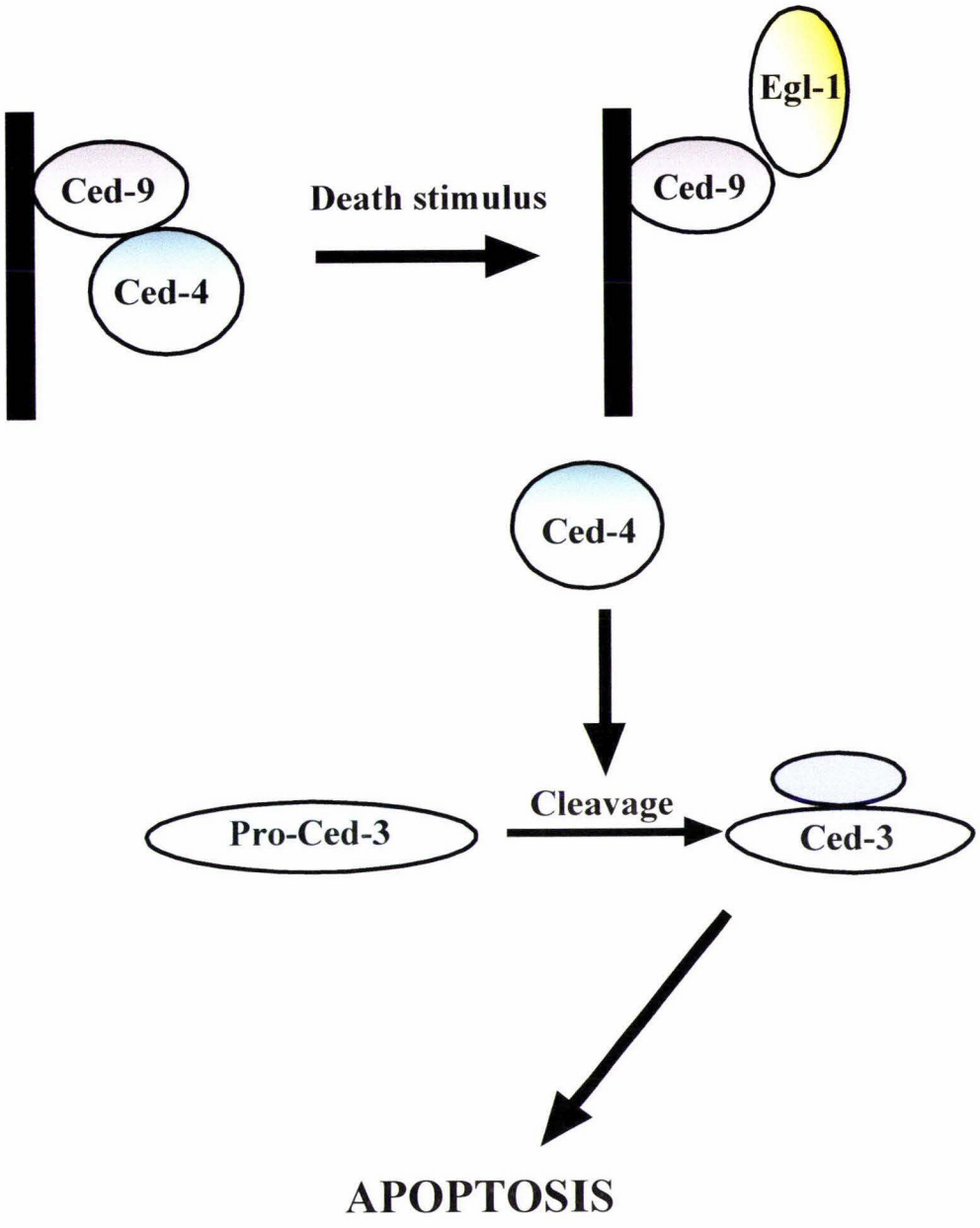


Figure 1-2: Diagrammatic representation of the interaction of the Ced proteins and Egl-1.

Ced-4 is normally sequestered by Ced-9 at the mitochondria membrane in live cells. Egl-1 is produced in response to upstream signals and initiates cell death by displacing Ced-4, which then binds Ced-3 causing it to autoactivate and carry out the execution of the cell. This figure is adapted from Metztein, *et al.*, (1998) and de Peso, *et al.*, (1998).

A fourth gene in the nematode apoptosis model, *egl-1* (egl, egg laying defective gene), was recently cloned. *Egl-1* encodes a protein that disrupts the interaction of Ced-9 with Ced-4 (Conradt and Horvitz, 1998; del Peso *et al.*, 1998). The currently accepted model of the interaction between these four proteins Egl-1, Ced-3, Ced-4 and Ced-9 has been depicted in Figure 1-2. Egl-1 is produced in response to a death signal and initiates apoptosis by binding directly to Ced-9, displacing Ced-4 (Conradt and Horvitz, 1998; del Peso *et al.*, 1998), which can then bind Ced-3 and mediate the initiation of cell death.

Some programmed cell death events in the nematode, including those in the sister cells of the serotoninergic neurosecretory motor (NSM) neurons, are controlled by the expression of *ces-1* and *ces-2* (cell death specification genes) (Ellis and Horvitz, 1991). *Ces-2* encodes a transcription factor, which regulates the expression of *egl-1* in the pharynx (Metzstein *et al.*, 1996; Metzstein *et al.*, 1998), and also acts as a negative regulator of *ces-1*. *Egl-1* expression is also repressed by Tra-1A, a protein involved in sex-determination of the nematode, which dictates whether the hermaphrodite sister neurons (HSN) die (Conradt and Horvitz, 1999).

Genetic analysis has identified a set of genes encoding proteins that act downstream of Ced-3. These proteins (Ced-1, Ced-2, Ced-5, Ced-6, Ced-7, Ced-10, and Nuc-1), are involved in the phagocytosis and degradation of the cell corpses (Ellis *et al.*, 1991a; Hevelone and Hartman, 1988; Sulston, 1976).

The regulation of apoptosis in *C. elegans* has provided a framework with which to identify the components involved in the programmed cell death mechanism. The limitation of the *C. elegans* model is the timing of these apoptotic cell deaths is only during the development of the nematode.

1.4 MAMMALIAN MODELS OF APOPTOSIS

The mammalian apoptotic process is more complex than the nematode model. Two methods of programmed cell death in mammals have been identified, cytochrome c

(cyt. c)-mediated and death receptor-mediated cell death. In the former case, cells receive survival signals that tell them to turn off the PCD mechanism. Mammalian cell survival is thus an active process, which requires signalling pathways to be constantly activated. Extracellular growth factors, for example NGF (nerve growth factor) in neurons, operate to turn on cell survival signalling pathways. In cases where the cell fails to bind/recognise these factors, PCD (cyt. c-mediated apoptosis) is activated, which involves the release of apoptogenic proteins from mitochondria (Raff, 1992). In death receptor-mediated apoptosis, ligands such as Fas ligand (a cytokine from the Tumour Necrosis Factor family), bind to their respective cell receptors, and activate PCD directly.

1.4.1 Mammalian cell death decisions involve Apaf-1, 2, 3 and Bcl-2

The central players in mammalian apoptosis have been identified as mammalian homologues of *ced-3, ced-4 and ced-9*. *Bcl-2*, a proto-oncogene, was found to encode the functional homologue of *ced-9* (Hengartner and Horvitz, 1994b), and has been shown to delay or prevent programmed cell death of many kinds of cells exposed to a variety of stimuli. In fact, Bcl-2 and Ced-9 proteins are able to substitute for each other in nematodes and mammalian cells (Hengartner and Horvitz, 1994b). Bcl-2, like Ced-9, is found associated with the mitochondrial membrane (Hockenbery *et al.*, 1990), however this membrane attachment is not necessary for the cell survival activity of this protein (Borner *et al.*, 1994). Bcl-2 prevents both the release of cyt. c (a pro-apoptotic protein in mammals) from mitochondria and activation of caspases by cyt. c-activated Apaf-1 (Apoptosis promoting factor 1) (Kluck *et al.*, 1997a; Rosse *et al.*, 1998).

Apaf-1 was purified from cytosolic cell extracts used in an *in vitro* system designed to mimic apoptosis in the test tube. This protein is encoded by the mammalian homologue of *ced-4* (Zou *et al.*, 1997). Three other factors that are required to induce apoptosis, cyt. c (also known as Apaf-2), ATP, and Apaf-3, were also identified in the same manner (Li *et al.*, 1997b; Liu *et al.*, 1996). Apaf-3, the third apoptosis-activating factor, is a caspase and is identical to, and now called, Caspase-9 (Alnemri, 1997; Li *et al.*, 1997b). Apaf-1 binds

to cyt. c, and concomitantly binds and hydrolyses ATP to ADP in a reaction that appears to promote Apaf-1 oligomerisation (Figure 1-3) (Zou *et al.*, 1999). The multimeric complex containing Apaf-1 and cyt. c is termed an apoptosome, which recruits and activates Caspase-9 (Figure 1-3) (Li *et al.*, 1997b; Zou *et al.*, 1999). Active Caspase-9 activates Caspase-3 by cleaving off the pro-domain of the protein, initiating a caspase cascade (Li *et al.*, 1997b; Zou *et al.*, 1997).

1.4.2 Execution and degradation of the cell

Once the caspases are active, the death of the cell is irreversible. The initiator caspases - Caspase-9, in the cyt. c-mediated caspase cascade, and Caspase-8, in death receptor-activated apoptosis, activates effector caspases, such as Caspase-3 and Caspase-7. The effector caspases have the job of activating all the downstream proteins involved in the destruction and packaging of the cell into small apoptotic bodies ready for phagocytosis by the neighbouring cells.

So far twelve caspases have been identified, including the initiator caspases -8 and -9. Some of these caspases have been placed into subgroups according to their substrate preference. Group I (Caspase-1, -4, and -5) prefer the tetrapeptide sequence WEHD. Group II (Caspase-3, -7, -2) have a preference for the DEXD sequence, and Group III (Caspase-6, -8, -9) will cleave proteins at a (I/V/L)EXD consensus sequence (Rano *et al.*, 1997; Thornberry *et al.*, 1997). Group III caspases can cleave group II caspases, caspases within their own group, and other proteins such as poly-(ADP)-ribose polymerase (Parp), a DNA repair enzyme.

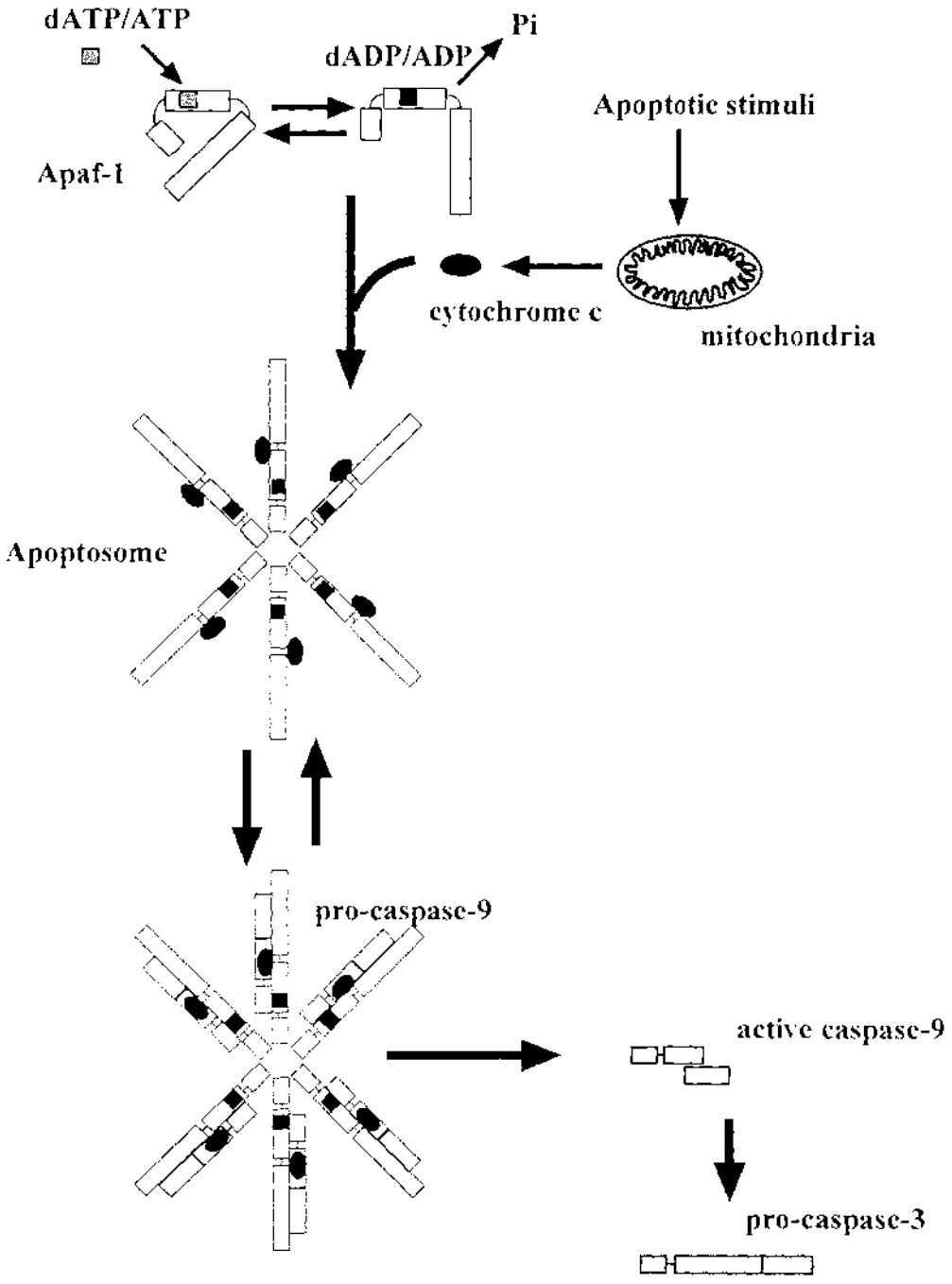


Figure 1-3: Schematic model of the activation of Caspase-9.
Apaf-1-mediated hydrolysis of ATP causes a conformational change in Apaf-1. Cyt. c binds this form of Apaf-1 and promotes oligomerisation into a multimeric complex, which recruits and activates Caspase-9. Active Caspase-9 promotes downstream processing of other caspases. This figure is adapted from Zou, *et al.*, (1999).

Most of the caspases have specific downstream targets *in vivo*, and perhaps the Caspase-3 targets are understood the best. Parp cleavage by Caspase-3 and Caspase-7 is a hallmark for apoptosis (Casciola-Rosen *et al.*, 1996; Salvesen and Dixit, 1997). Gelsolin, thought to sever actin filaments, is activated by Caspase-3 cleavage, and the active form is involved in cell blebbing (Kothakota *et al.*, 1997). Caspase-3-activated Acinus, a recently identified apoptotic factor, is required for chromatin condensation (Sahara *et al.*, 1999). Caspase-3 disables several other DNA repair machines and mRNA splicing machinery (Casciola-Rosen *et al.*, 1996; Casciola-Rosen *et al.*, 1995; Casciola-Rosen *et al.*, 1994). It also cleaves sterol producing proteins and activates cyclin dependent kinases (cdk) by cleavage of cdk inhibitors (Goldberg *et al.*, 1996; Kaufmann *et al.*, 1993; Levkau *et al.*, 1998; Wang *et al.*, 1995; Waterhouse *et al.*, 1996; Zhou *et al.*, 1998).

The caspases are also involved in the cleavage of cell survival signalling molecules during apoptosis, such as Ras GTPase-activating protein Raf-1, and Akt-1, which turns off cell survival (Francois and Grimes, 1999; Widmann *et al.*, 1998).

1.4.3 DNA fragmentation

Caspase-activated deoxyribonuclease (Cad) and its inhibitor (Icad) have both been isolated and characterised from a mouse cell line (Enari *et al.*, 1998; Sakahira *et al.*, 1998). During apoptosis, Icad is cleaved by Caspase-3, releasing Cad and allowing it to translocate to the nucleus (Sakahira *et al.*, 1998). The COOH terminus of Cad has a sequence of 15 amino acid residues which has the features of a nuclear localisation signal (Dingwall and Laskey, 1991). This sequence is masked by Icad and is revealed when the inhibitor is cleaved. Removal of Icad activates and allows nuclear targeting of Cad. Icad is expressed in two alternate forms called Icad-s and Icad-l, the long form is mostly found complexed with Cad and works as a specific chaperone for CAD facilitating its correct folding during synthesis (Sakahira *et al.*, 1999).

1.4.4 Bcl-2 family of proteins

Following the identification of Bcl-2, similar proteins were discovered with regions of high homology, termed Bcl-2 homology (BH) domains. These proteins are now known to be

part of a growing family of proteins called the Bcl-2 family of proteins. The members of the Bcl-2 family of proteins are either anti-apoptotic i.e. Bcl-2 and Bcl-X_L, or pro-apoptotic i.e. Bax, Bad, and Bid, and are generally found associated with intercellular membranes (Adams and Cory, 1998). Certain members of the family, such as Bax or Bad, remain in the cytosol where they await a death signal that causes their translocation to the outer mitochondrial membrane (Adams and Cory, 1998; Li *et al.*, 1998; Luo *et al.*, 1998; Puthalakath *et al.*, 1999; Wolter *et al.*, 1997). Most of the Bcl-2 proteins contain a carboxyl terminal transmembrane tail, which targets these proteins to intracellular membranes (see Figure 1-4) (Nguyen *et al.*, 1993). In most instances, localisation of the Bcl-2 family of proteins to the mitochondria is important for the promotion or inhibition of cell death. For example, the translocation of the pro-apoptotic protein Bax from the cytosol to the mitochondria is necessary for Bax to induce cell death (Gross *et al.*, 1998; Hsu *et al.*, 1997; Nguyen *et al.*, 1994; Putcha *et al.*, 1999; Wolter *et al.*, 1997).

The Bcl-2 family of proteins share structural and functional similarity with Ced-9 (Adams and Cory, 1998; Hengartner and Horvitz, 1994a; Hengartner and Horvitz, 1994b). Sequence alignment of these proteins has identified four main regions of homology, which have been designated Bcl-2 homology (BH) domains - BH1, BH2, BH3 and BH4 (Figure 1-4) (Chittenden *et al.*, 1995; Yin *et al.*, 1994; Zha *et al.*, 1996a). The BH3 domain is sufficient for the pro-apoptotic proteins of this family to act as death stimuli, and for some proteins, such as Bad, this is the only region of homology with the rest of the family (Ottilie *et al.*, 1997). The BH1 and BH2 domains are crucial for the anti-apoptotic properties of Bcl-2 enabling it to suppress cell death by heterodimerising with Bax (Yin *et al.*, 1994).

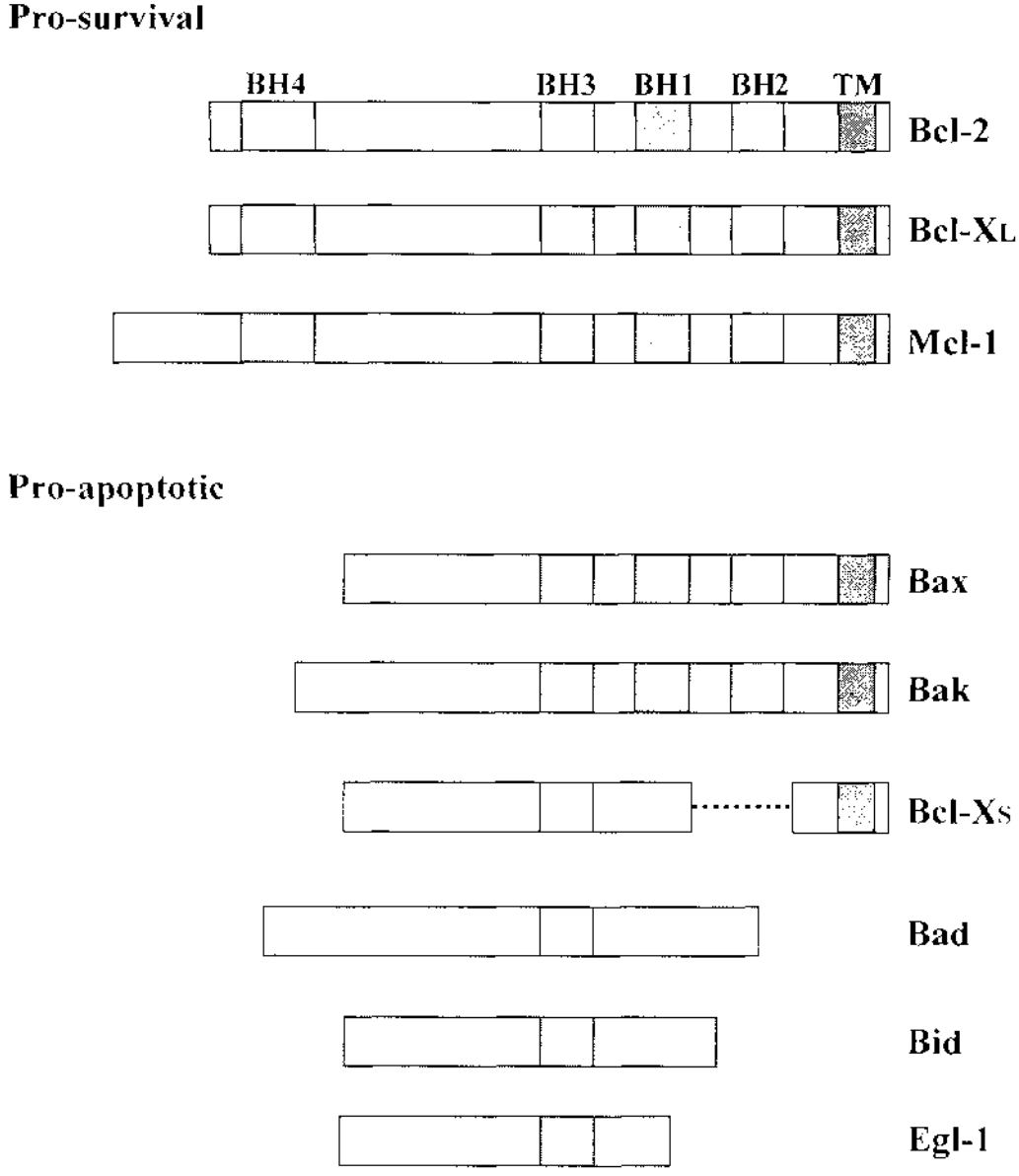


Figure 1-4: Schematic representation of the Bcl-2 family members and Egl-1.
The Bcl-2 homology (BH) domains and hydrophobic membrane-targeting domain (TM) are indicated. The deletion in Bcl-X_S resulting from differential splicing is indicated by dashed lines. The proteins are not drawn to scale. This figure is adapted from Rao and White, (1997) and Adams and Cory, (1998).

1.4.5 Phosphorylation of Bad promotes cell survival

Bcl-X_S and Bad are involved in the indirect promotion of mammalian cell death by sequestering anti-apoptotic proteins like Bcl-2 (Boise *et al.*, 1993; Yang *et al.*, 1995). Bad is phosphorylated in the presence of several cell survival factors such as interleukin-3 (IL-3) or through the activation of a cell survival pathway such as the phosphatidyl inositol-3 kinase (PI-3 kinase) pathway. Phosphorylated Bad is unable to dimerise with Bcl-2 and therefore promotes cell survival shown in Figure 1-5 (Datta *et al.*, 1997; Zha *et al.*, 1996b). Akt, also called protein kinase B (PKB), is activated by the PI-3-kinase pathway and is capable of phosphorylating Bad, as shown in Figure 1-5 (Dudek *et al.*, 1997; Kauffmann-Zeh *et al.*, 1997). Mitochondrial-anchored Protein Kinase A, which is activated in the presence of the survival factor IL-3, is also known to phosphorylate Bad (Harada *et al.*, 1999). Phosphorylated Bad is sequestered in the cytosol by 14-3-3, which binds phospho-serine residues in proteins, inhibiting mitochondria localisation (Figure 1-5) (Zha *et al.*, 1996b).

In the absence of cell survival signals such as, phospho-Akt, Bad is not phosphorylated and is able to heterodimerise with Bcl-2 and Bcl-X_L (Ottilie *et al.*, 1997). This interaction prevents the cell survival activities of Bcl-2 by displacing pro-apoptotic proteins, like Bax (Figure 1-5). Displaced Bax is then able to translocate to the mitochondria and induce cyt. c release.

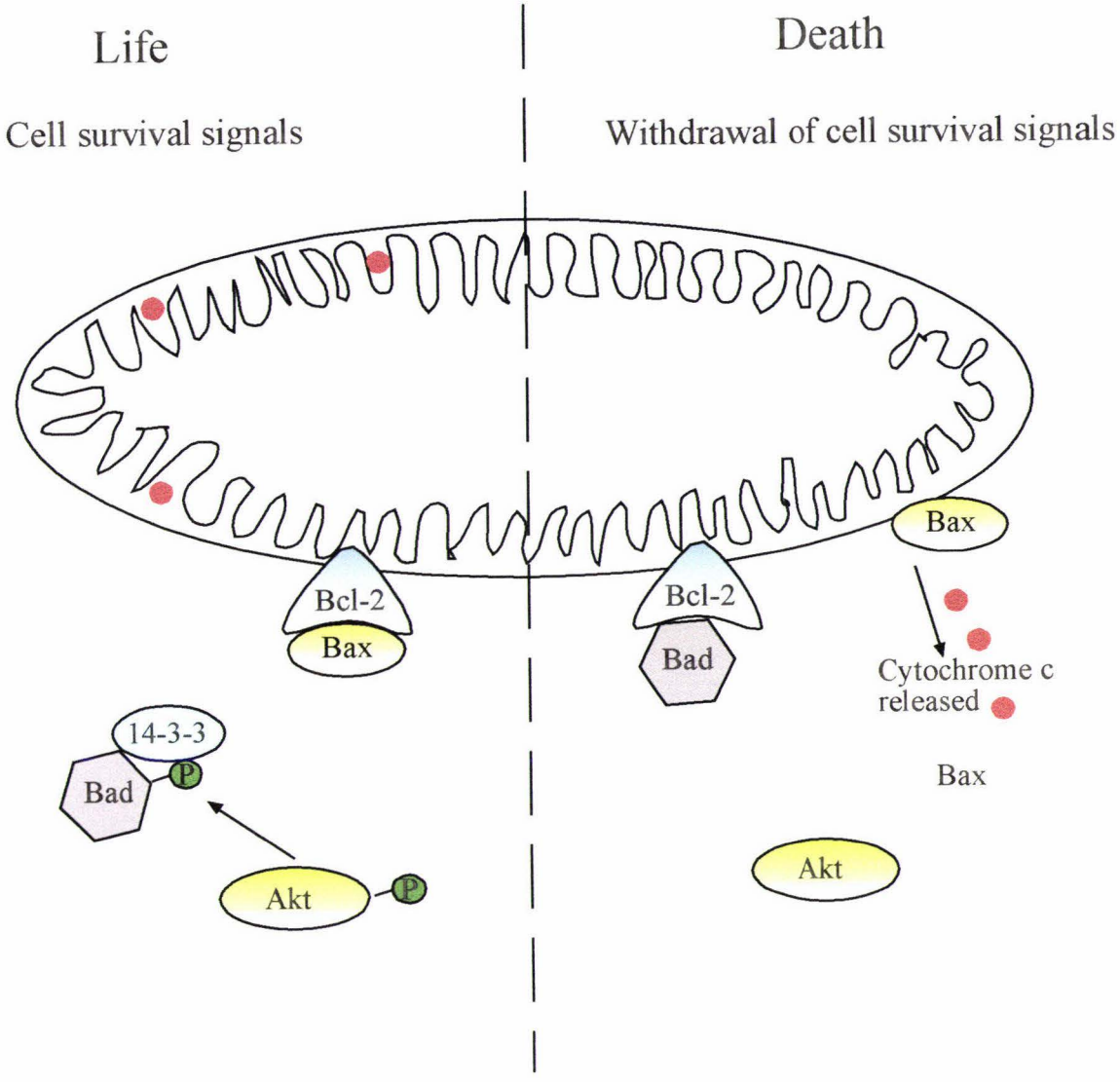


Figure 1-5: Phosphorylation of Bad prevents mitochondria-mediated apoptosis.
In the presence of cell survival signals, such as phospho-Akt, Bad is phosphorylated, and then sequestered in the cytosol by 14-3-3. In the absence of these survival signals, Bad is not phosphorylated, and heterodimerises with Bcl-2, displacing pro-apoptotic proteins, like Bax, which can then translocate to the mitochondrial membrane causing the release of cyt. c.

1.5 MITOCHONDRIA-MEDIATED APOPTOSIS

Interactions of proteins on the mitochondrial surface dictate the cells' fate in most instances of programmed cell death. Indeed, when cell-free systems were developed it was discovered that extracts enriched in mitochondria were required for the induction of apoptosis (Newmeyer *et al.*, 1994). While mitochondria produce metabolic energy for the cell in the form of ATP, they also harbour proteins that are lethal to the cell such as cyt. c and apoptosis inducing factor (AIF) (Susin *et al.*, 1999). Cyt. c is an essential protein in the electron transport chain and is also required for the induction of *in vitro* reconstitution of apoptosis. Cyt. c has been described as a double-edged sword, because without it the cell would halt metabolic activity, yet if released into the cytoplasm it can initiate apoptosis in conjunction with Apaf-1.

Cyt. c is released through the outer mitochondrial membrane during apoptosis (Newmeyer and Green, 1998; Reed, 1997). Along with ATP or dATP, cyt. c is required for the induction of apoptosis in cell-free systems (Francois and Grimes, 1999; Liu *et al.*, 1996). Microinjection of mammalian cyt. c into human kidney 293 cells, mouse embryonic Swiss 3T3 fibroblasts, normal rat kidney (NRK) epithelial cells, or rat promyelocytic IPC-81 leukaemia cells induces apoptosis, which is inhibited by overexpression of Bcl-X_L or Bcl-2 (Li *et al.*, 1997a; Zhivotovsky *et al.*, 1998). Interestingly, microinjection of higher concentrations of cyt. c was required to induce apoptosis in whole cells, compared with the amount required in cell-free systems, which indicates the involvement of other survival factors within the cell (Li *et al.*, 1997a; Zhivotovsky *et al.*, 1998). Indeed, neurons maintained with NGF and microinjected with cyt. c did not undergo apoptosis indicating that another event was required before the complete induction of apoptosis (Deshmukh and Johnson, 1998).

During apoptosis *in vivo*, cyt. c is redistributed from mitochondria to the cytoplasm (Goldstein *et al.*, 2000; Martinou *et al.*, 2000; Neame *et al.*, 1998). A major unresolved question is, how is cyt. c released? Alteration of the mitochondrial structure, its function, and membrane depolarisation, have been reported during apoptosis (Mancini *et al.*, 1997;

Petit *et al.*, 1995). Members of the Bcl-2 family have been implicated in the changes reported in mitochondria during apoptosis. In whole cells, overexpression of Bax induces the early release of cyt. c, which is inhibited by the expression of Bcl-X_L (Finucane *et al.*, 1999). Small amounts of recombinant Bax can induce the release of cyt. c from isolated mitochondria, which is prevented by the addition of recombinant Bcl-X_L (Jurgensmeier *et al.*, 1998). The pro-apoptotic Bcl-2 family members, Bid and Bax, caused the release of cyt. c from isolated mitochondria, but required the presence of a macromolecular cytosolic factor called permeability enhancing factor (PEF) to complete the permeabilisation of the mitochondrial outer membrane (Kluck *et al.*, 1997a; Kluck *et al.*, 1999).

Two models have been proposed to explain how factors such as cyt. c is released from the mitochondria. The first model requires a permeability transition resulting in the swelling and subsequent rupture of mitochondrial membranes, while the second model proposes the opening of a specific pore allowing the release of apoptogenic factors without disruption of the mitochondrial membranes (Figure 1-6) (Green and Reed, 1998; Martinou, 1999). These models are presented below.

1.5.1 Cyt. c release from mitochondria – Model I

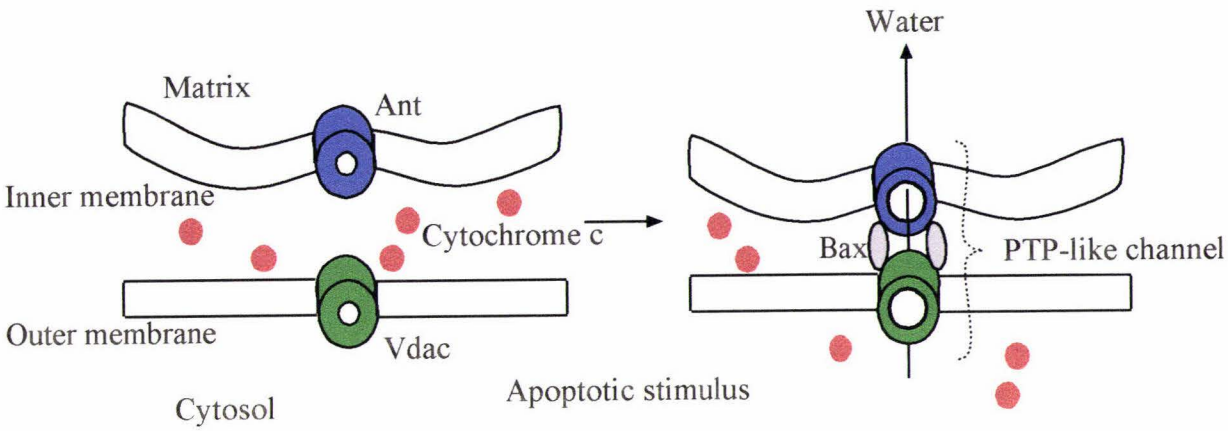
When a permeability transition is induced in isolated mitochondria, they swell and release apoptogenic proteins (Scarlett and Murphy, 1997). A megachannel called the permeability transition pore (PTP) is thought to open during apoptosis allowing H_2O and other solutes to enter mitochondria and the release of proteins such as cyt. c. The PTP is made up of several proteins including the adenine nucleotide translocator (Ant) and porin (voltage dependent anion channel; Vdac), located on the inner and outer mitochondrial membranes, respectively, which act in concert to create the PTP (Figure 1-6) (Green and Reed, 1998). Vdac/Ant also act to allow ATP/ADP exchange, and an early event in apoptosis has been found to be a defect in mitochondrial ATP/ADP exchange (Vander Heiden *et al.*, 1999). Ant and Bax were found to interact directly with one another, and ectopic expression of Bax induced cell death in wild type yeast but not Ant-deficient yeast, indicating that the interaction of these two proteins is important for apoptosis (Marzo *et al.*, 1998).

1.5.2 Cyt. c release from mitochondria – Model II

Model II suggests that cyt. c is released without rupture or swelling of the mitochondria. Indeed, cyt. c is released by Bax in the presence of PTP inhibitors without any detectable swelling of mitochondria (Eskes *et al.*, 1998; Jurgensmeier *et al.*, 1998; Kluck *et al.*, 1999). Pro- and anti-apoptotic members of the Bcl-2 family have been shown to bind Vdac and regulate the release of cyt. c. (Shimizu *et al.*, 1999). Interestingly, the diameter of Vdac is too small to allow cyt. c to pass through, so it is possible that Bax and Bak interact with Vdac to form a multiprotein channel that allows the passage of cyt. c (Figure 1-6).

In addition to these two models for cyt. c release from mitochondria, other models of cyt. c release have been proposed (Martinou *et al.*, 2000). The validity of any of these models may depend on cell type or apoptotic stimulus. In some cell types, certain stimuli initiate death receptor-mediated cell death, rather than using the default mitochondrial-mediated cell death pathway, which is turned on in the absence of cell survival signals. Death receptor-mediated cell death is outlined in the next section.

Model I



Model II

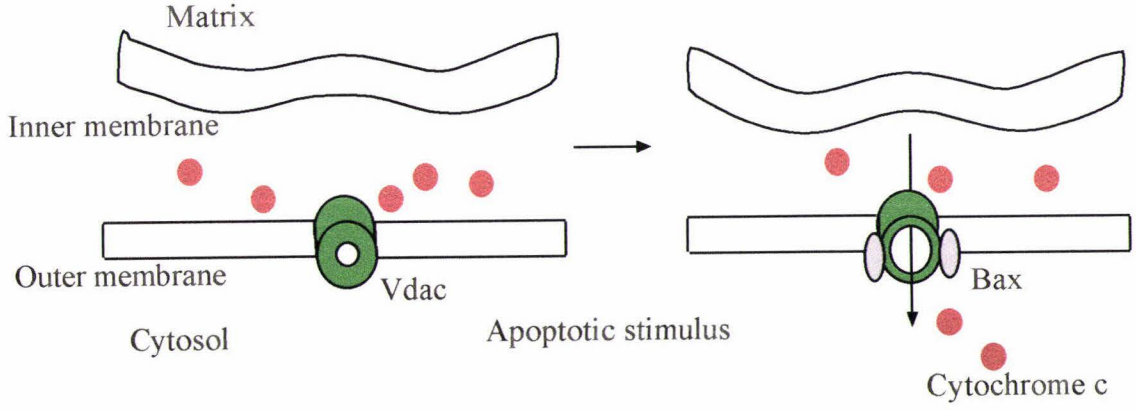


Figure 1-6: Models for the release of cyt. c from mitochondria.
Model I – Bax interacts with both Ant and Vdac to form a permeability transition pore (PTP), which allows water and solutes to enter the mitochondrion causing the matrix to swell and its outer membrane to rupture. The result is the release of cyt. c and other proteins. Model II – The conformation of Vdac changes after interaction with Bax or Bak. Vdac forms a larger channel, possibly in combination with Bax that is permeable to cyt. c, but the integrity of the outer mitochondrial membrane is maintained.

1.6 DEATH RECEPTOR-MEDIATED APOPTOSIS

An apoptotic cell death pathway that is switched on from an external source involves 'death factors' one of which is the Fas ligand (Fas L). This type of cell death is termed death receptor-mediated cell death. The Fas L is a cytokine, belonging to the tumour necrosis factor (TNF) family (Suda *et al.*, 1993), and its receptor, Fas/CD95, belongs to the TNF receptor family (Itoh *et al.*, 1991; Oehm *et al.*, 1992). Binding of Fas L to Fas induces trimerisation of the receptor and it is the trimerised cytoplasmic region of Fas, known as the death domain, that transduces the death signal (Figure 1-7) (Banner *et al.*, 1993; Itoh and Nagata, 1993; Tartaglia *et al.*, 1993). Fas-mediated apoptosis causes cell death within hours of the ligand binding its receptor. It does not require any protein synthesis, which suggests that the components required for death receptor-mediated apoptosis are already present and Fas activation simply triggers the mechanism (Itoh *et al.*, 1991; Yonehara *et al.*, 1989). Tumour cells, such as melanoma, have an elevated level of expression of the Fas ligand, which induces apoptosis in the Fas-bearing cytotoxic T lymphocytes (Hahne *et al.*, 1996). This subversion of the Fas-mediated cell death pathway is an effective survival tactic by the tumour cells.

To identify the molecules that bind to the cytoplasmic region of Fas, several groups made use of the yeast two-hybrid system. A molecule called Fadd/Mort1 (Fas-associating protein with death domain) was identified and shown to bind trimerised Fas through a death domain depicted in Figure 1-7 (Boldin *et al.*, 1995; Chinnaiyan *et al.*, 1995; Kischkel *et al.*, 1995).

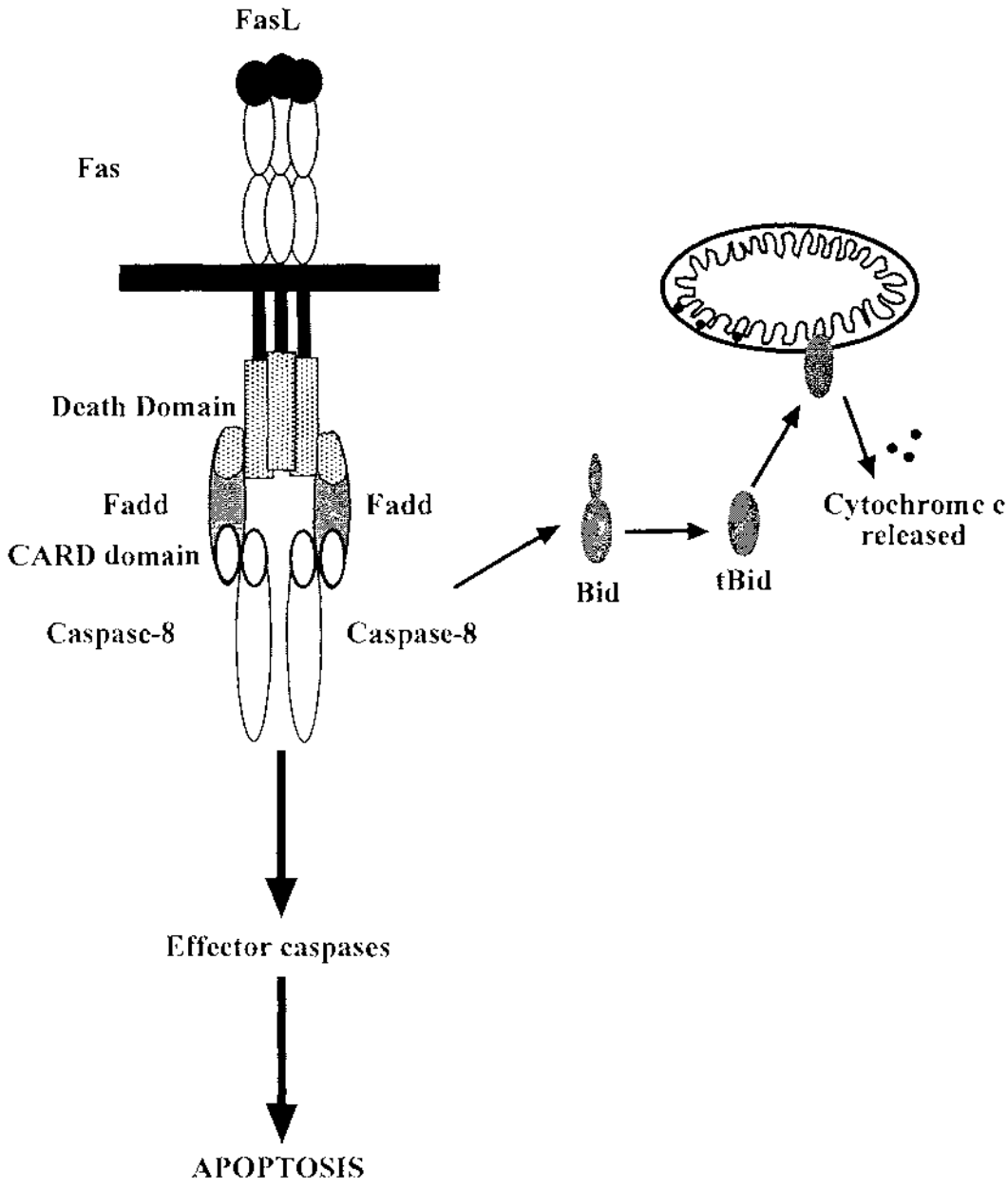


Figure 1-7: Death receptor-mediated apoptosis.
Fas receptor molecules trimerise following ligand binding, which allows the recruitment of Fadd, then Caspase-8, which is subsequently activated. Active Caspase-8 triggers a caspase cascade and cleaves Bid. The COOH-terminal part of Bid (tBid) translocates to the mitochondria and triggers the release of cyt. c possibly by a mechanism analogous to that shown in Figure 1-5. This figure is adapted from Gross *et al.* (1999).

Fadd/Mort1 also contains a CARD domain, which recruits Caspase-8 (previously called Mach or Flice), the next factor in the Fas apoptotic pathway (Alnemri *et al.*, 1996; Boldin *et al.*, 1996; Muzio *et al.*, 1996). This interaction is sufficient to induce the autoactivation of Caspase-8 in a manner similar to that of Caspase-9. Active Caspase-8 then cleaves Caspase-3, starting a caspase cascade and irreversible cell death (Figure 1-7) (Boldin *et al.*, 1996). Caspase-8 also cleaves Bid releasing the COOH-terminal part of the protein (tBid), which translocates to mitochondria triggering cyt. c release in a type of feedback amplification loop (Figure 1-7) (Gross *et al.*, 1999; Li *et al.*, 1998; Luo *et al.*, 1998).

There are other death receptors that function in a similar manner to Fas, these all belong to the TNF (tumour necrosis factor) receptor super family. Members of this family include TNFR1, Fas, death receptor (DR) 3, DR4, DR5, DR6 and $p75^{NTR}$, all of these receptors contain homologous cytoplasmic death domains. In each case, binding of the receptors respective ligands, causes oligomerisation of the receptor, which can bind adapter proteins containing death effector domains (DED) and initiate the activation of Caspase-8 (Ashkenazi and Dixit, 1998).

1.7 *DROSOPHILA MELANOGASTER* MODEL OF APOPTOSIS

Drosophila melanogaster has recently been identified as a model organism for the study of apoptosis. Since a large numbers of cells undergo apoptosis during both embryonic development and metamorphosis of *Drosophila* (Steller and Grether, 1994; Truman *et al.*, 1992), and the organism can be readily manipulated for both genetic and biochemical analysis, it is an ideal model organism in which to explore apoptosis

1.7.1 Initiation of apoptosis in *Drosophila*

Deletions in the *H99* locus block apoptosis in *Drosophila.* This region of the *Drosophila* genome contains three activators of apoptosis, *reaper (rpr)*, *hid* (head involution defective) and *grim* (Chen *et al.*, 1996; Grether *et al.*, 1995; White *et al.*, 1994). Ectopic expression of *rpr*, *hid* or *grim* induces apoptosis, and the transcriptional activation of these genes is

necessary for apoptotic cell death. Cell death in *Drosophila* is blocked by caspase inhibitors, indicating that the *H99* genes induce death via a caspase-mediated pathway (Chen *et al.*, 1996; Grether *et al.*, 1995; White *et al.*, 1994).

As with mammalian cells, it appears that the PI-3 kinase pathway plays a role in apoptosis in *Drosophila*. The *Drosophila* Akt homologue, Dakt1 is required to prevent apoptosis (Franke *et al.*, 1994; Staveley *et al.*, 1998). Apoptosis caused by loss of Dakt1 function was prevented by suppression of the caspases through ectopically expressing *p*35 (a baculoviral caspase-inhibitory protein) (Staveley *et al.*, 1998). Epistatic analysis of the *Dakt1* gene with the *grim, rpr* and *hid* genes shows that Dakt1 acts downstream and may be repressed by the *H99* locus (Staveley *et al.*, 1998).

1.7.2 *Drosophila* Apoptosome

From the emerging *Drosophila* model it appears that cyt. c is required for *Drosophila* apoptosis. However, any link between the *grim, hid* and *reaper* genes and release of cyt. c from mitochondria has not been fully established. Using *in vitro* studies Rpr was shown to bind a *Xenopus* protein called Scythe, causing the release of another protein that induces cyt. c release in purified mitochondria (Thress *et al.*, 1999; Thress *et al.*, 1998). While homologues of these two proteins have not been described in *Drosophila*, homologues of proteins in the mammalian apoptosome and downstream targets have been found.

The *Apaf-1* and *caspase-9 Drosophila* homologues were cloned and called *dark* and *dredd* respectively (Chen *et al.*, 1998; Kanuka *et al.*, 1999; Rodriguez *et al.*, 1999; Zhou *et al.*, 1999). Apoptosis induced by the ectopic expression of *grim, hid* and *rpr* is reduced in *dark* mutants indicating that Dark acts downstream of these three genes (Figure 1-8) (Rodriguez *et al.*, 1999). The Dark protein contains a CARD domain and the same WD repeat region that binds cyt. c in Apaf-1, and deletion of this WD repeat region results in a more apoptotically active molecule as a level of control is lost (Hu *et al.*, 1998; Rodriguez *et al.*, 1999; Srinivasula *et al.*, 1998).

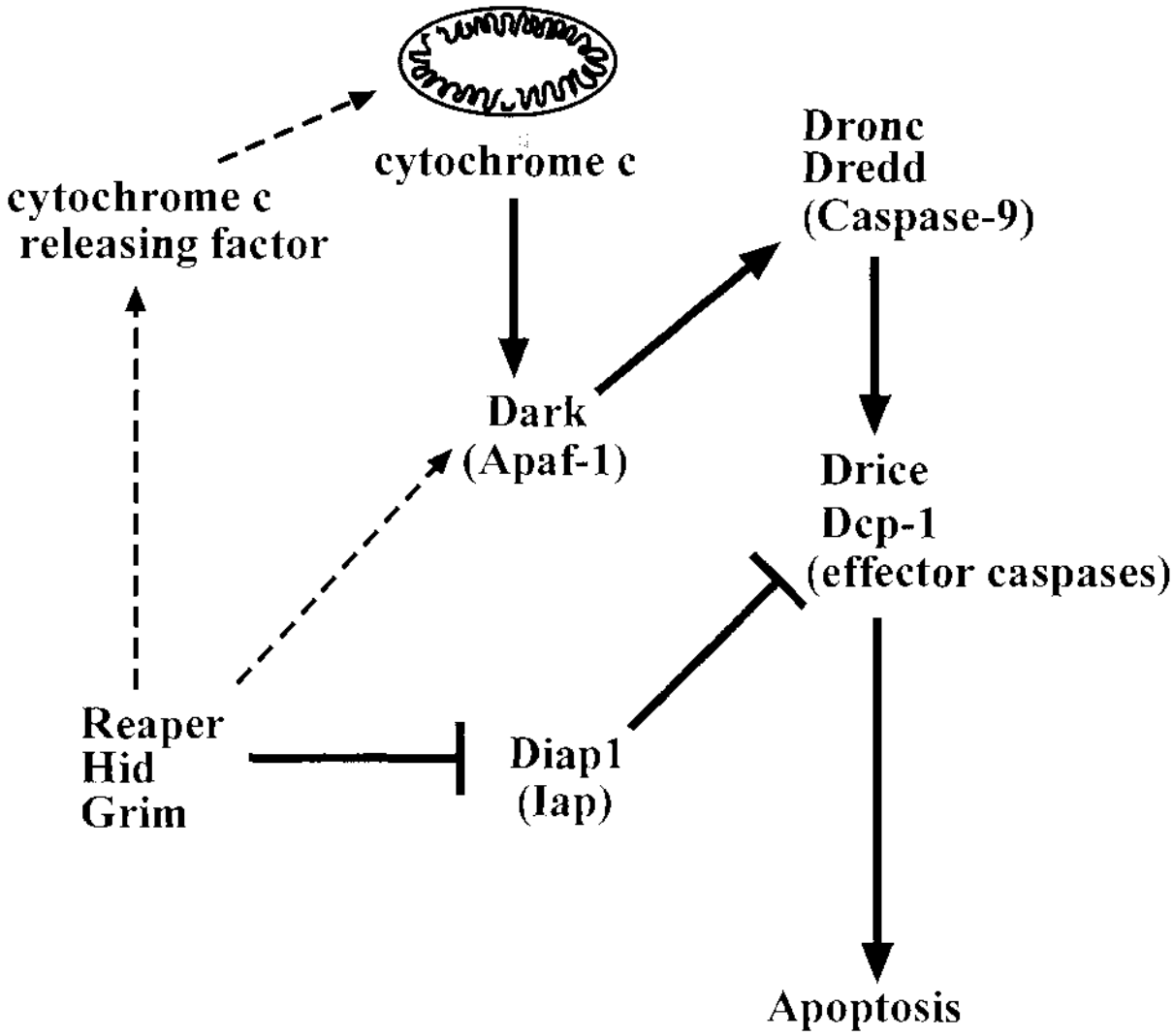


Figure 1-8: Model of apoptosis in *Drosophila*.
Grim, Rpr and Hid are believed to promote apoptosis by disrupting interactions between Iaps and Caspases. Rpr, Hid and Grim may cause cyt. c release through an unknown factor. Released cyt. c binds Dark, which can then activate the activator caspases, Dronc and Dredd. The activator caspases can then activate effector caspases, Drice and Dcp-1. It is proposed that Grim, Rpr, and Hid also bind Diap1, removing its inhibitory effect on the effector caspases, Drice and Dcp-1. Rpr, Hid and Grim have also been proposed to activate Dark directly. Known mammalian homologues are indicated in brackets.

Conformational changes were observed in cyt. c in *Drosophila* nurse cells that are known to undergo apoptosis. Cyt. c displayed an otherwise hidden epitope that is highly specific for these pre-apoptotic cells, this conformation change did not occur in cells destined to survive (Thress *et al.*, 1999; Varkey *et al.*, 1999). As more data comes to light the regulation of the release and changes in cyt. c will become clear.

While mammalian Caspase-9 requires its CARD domain for its interaction and activation by Apaf-1, it is unclear whether *Drosophila* caspases require a CARD domain for their activation. The interaction of Dredd with Dark does not appear to be mediated by CARD domains, which these proteins lack. Interestingly, another recently identified caspase Dronc does contain a CARD domain (Dorstyn *et al.*, 1999a). To date, three more caspase genes have been identified: *Decay, Dcp1* and *Drice,* and the proteins encoded by these genes seem to resemble effector-type or executioner caspases (Dorstyn *et al.*, 1999b; Fraser and Evan, 1997; Song *et al.*, 1997).

Another family of proteins found in most multicellular organisms and some unicellular organisms are called inhibitors of apoptosis (Iaps), and these proteins appear to bind and inhibit caspases (Uren *et al.*, 1998). The effector caspases, Drice and Dcp-1, which are normally sequestered by *Drosophila* inhibitors of apoptosis (Diap) are released by the action of Hid on Diap1 (Figure 1-8) (Wang *et al.*, 1999).

1.8 APOPTOSIS IN UNICELLULAR ORGANISMS

While the apoptotic mechanism has been characterised in multicellular organisms, no similar mechanism for apoptosis has been found in unicellular organisms. The genomes of several unicellular organisms have been fully sequenced i.e. *Escherichia coli*, *Saccharomyces cerevisiae* (Blattner *et al.*, 1997; Goffeau *et al.*, 1996) and no homologues of the apoptotic genes mentioned above have been found. However, in certain circumstances these unicellular organisms do show features characteristic of apoptosis.

1.8.1 Cell death in Bacteria

Expression of mammalian pro-apoptotic proteins in *E. coli* is lethal. *E. coli* expressing Bax were unusually elongated, and showed a 6-fold increase in monounsaturated fatty acids, along with an increased consumption of oxygen (Asoh *et al.*, 1998). The increase in oxygen consumption is correlated with an increase in the production of 'reactive oxygen species' (ROS), which consequently increases the frequency of DNA mutations bringing about cell death (Asoh *et al.*, 1998). It is not yet clear how these observations relate to the apoptotic mechanism.

1.8.2 Cell death in Unicellular Eukaryotes

Yeast, such as *Saccharomyces*, cerevisiae are simple unicellular eukaryotic organisms. At present there is a great deal of debate as to whether yeast actually undergo apoptosis.

When yeast are made to express recombinant death inducing proteins, such as Bax, very low yields of these proteins are recovered, due to the fact that the yeast die. A lethal phenotype is conferred in the fission yeast *Schizosaccharomyces pombe* by expression of the pro-apoptotic proteins Bax and Bak (Jurgensmeier *et al.*, 1997). Analysis of the dying *S. pombe* showed massive cytosolic and chromatin condensation (Ink *et al.*, 1997; Jurgensmeier *et al.*, 1997). Other groups looking at cell death induced by Bax expression in *S. pombe* and *S. cerevisiae* observed all the major hallmarks of apoptosis, including chromatin fragmentation and dissolution of the nuclear envelope (Ink *et al.*, 1997; Madeo *et al.*, 1997). Potential homologues of apoptotic proteins such as the caspases were sought, but these have not been identified even though the complete genome of *S. cerevisiae* is now known. In mammalian cells caspases are required to carry out the execution of the cell, so what proteins are activated by Bax expression to cause the changes observed in the yeast?

Both ATP and an intact F_0F_1-ATPase proton pump are required for Bax to mediate yeast cell death (Matsuyama *et al.*, 1998; Priault *et al.*, 1999). The release of cyt. c from mitochondria was observed in yeast expressing Bax. This release was independent of Vdac, which is thought to be involved in mammalian cyt. c release (Figure 1-6) (Manon *et al.*,

1997; Priault *et al.*, 1999). Co-expression of Bcl-2 in Bax-expressing yeast reduced their mortality rate, although Bcl-2 could not protect the cell from a mutant form of Bax that lacks the membrane anchor (Clow *et al.*, 1998; Greenhalf *et al.*, 1996; Manon *et al.*, 1997). In addition, expression of Bcl-X_S or Bad together with Bcl-2 suppressed Bax-induced cell death in yeast (Tao *et al.*, 1998). These data are in apparent contrast to the mammalian model where that Bcl-X_S and Bad bind Bcl-2, displacing sequestered Bax, which then causes cell death (Figure 1-5).

Overexpression of other apoptotic proteins, such as Ced-4, also causes cell death in yeast. Interestingly, a mutation that disrupts Ced-4 oligomerisation abrogates its lethality in yeast (Tao *et al.*, 1999). If Ced-4 can cause apoptosis in yeast without caspases then it may be activating some kind of distinct, evolutionarily old cell death pathway. Indeed functional screening in yeast strain (QX95001), identified a mammalian apoptosis suppresser Bax Inhibitor-1 (BI-1), which does not directly interact with Bax, but apparently acts through another, unknown, yeast protein to prevent Bax-induced cell death (Xu and Reed, 1998).

While no homologous of the major proteins involved in apoptosis, i.e. Apaf-1 or caspases, have been identified in yeast, homologues of Iap proteins have been identified in both *S. pombe* and *S. cerevisiae* (Uren *et al.*, 1998). These proteins appear to play a role in cell division but as there are no caspases in yeast, any inhibitory role in the prevention of caspase activation, can not be tested in yeast (Fraser *et al.*, 1999; Uren *et al.*, 1999).

Other native yeast proteins have been identified as potentially apoptotic. For instance *S. Pombe* Rad9, a cell cycle checkpoint protein has a human homologue, which encodes a pro-apoptotic protein containing a BH3 domain (al-Khodairy *et al.*, 1994; Komatsu *et al.*, 2000). Overexpression of human Rad9 induces apoptosis in mammalian cells, which can be blocked by Bcl-2 (Komatsu *et al.*, 2000). The *S. cerevisiae* tsCdc48^{S565G} mutant shows features of apoptosis, such as fragmented DNA, and condensed, fragmented chromatin, when cultured at non-permissive temperatures (Madeo *et al.*, 1997). Cdc48 is a cell division cycle gene that plays an important role in homotypic fusion of the endoplasmic

reticulum (Latterich *et al.*, 1995). A null mutation in *cdc48* results in cell cycle arrest, creating a large budded cell with the nucleus located in the neck between the mother and daughter cell (Frohlich *et al.*, 1991; Moir *et al.*, 1982). However, it is unclear if this protein has a role in apoptosis in multicellular organisms.

These findings suggest that some of the apoptotic machinery is present in lower eukaryotes. Whatever this machinery is, it does not appear to involve homologues of the mammalian apoptotic proteins, such as the Bcl-2 family and caspases. Is there another set of proteins that are involved in an ancient form of apoptosis that we haven't yet uncovered?

1.8.3 ROS - an evolutionarily ancient method of cell death?

ROS are generated during apoptosis in multicellular organisms and in certain circumstances ROS are generated in yeast. Indeed, Madeo *et al.* (1997) found that depletion of glutathione or the addition of low doses of H_2O_2 could induce apoptotic features, such as DNA fragmentation, in *S. cerevisiae*. These oxygen stress-induced features can be averted by hypoxia or the depletion of oxygen radicals (Madeo *et al.*, 1997). Lipid hydroperoxide has also been shown to generate ROS in *S. cerevisiae*, however the subsequent death of the yeast did not involve DNA fragmentation (Aoshima *et al.*, 1999).

1.9 *IN VITRO* MODELS OF APOPTOSIS

Apoptosis is an asynchronous event in neuronal cells (Messam and Pittman, 1998; Mills *et al.*, 1997). A cell induced to undergo apoptosis will take anywhere between 12 and 48 hours to decide to die. Then, having reached a decision, the cell can take as little as 15 minutes or as long as 2 hours to be completely engulfed by surrounding cells. Cells that have had their cell cycle synchronised still die at different times. This cell death asynchrony makes biochemical analyses difficult. One method to overcome this problem is the development of cell free systems, which are briefly described below. These systems have the advantage that reagents can be added that would not normally cross the cell membrane barrier.

One *in vitro* system employs apoptotic *Xenopus laevis* (African clawed frog) egg cytosolic extracts. Extracts, made from oocytes harvested from frogs treated with hormones to trigger egg production, incubated with nuclei isolated from either sperm or rat liver, can induce apoptosis (Newmeyer *et al.*, 1994). Removal of the mitochondrial fraction of the extracts inhibited apoptosis induction, as did the addition of the Bcl-2 protein. A second *in vitro* system is based on extracts made from chicken DU249 cells that become apoptotic after S phase/M phase synchronisation (Lazebnik *et al.*, 1993). The cytosolic extracts from these cells induced apoptosis in purified HeLa nuclei. A third *in vitro* system uses cytosolic extracts from control or apoptotic HL-60 cells, which are incubated with isolated nuclei (Solary *et al.*, 1993). Initial elucidation of many of the factors involved in apoptosis came from using these *in vitro* models of apoptosis. In fact, the requirement of both cyt. c and ATP for apoptosis was elucidated through isolation of components of HeLa cell cytosol that induce apoptosis (Liu *et al.*, 1996).

Apoptosis has been implicated in neurodegenerative disorders, and a cell-free system has also been designed to look at apoptosis in a neuron like system. The system devised by Ellerby *et al.*, (1997) can look at the induction of apoptosis at three levels: the pre-mitochondrial, mitochondrial and post-mitochondrial release of cyt. c using tamoxifen, mastoparan and cyt. c, respectively. Interestingly, Bcl-2 was only able to inhibit apoptosis at the pre-mitochondrial and mitochondrial levels of this system (Ellerby *et al.*, 1997).

In vitro models of apoptosis are a powerful way of manipulating cell components to determine the role of particular proteins in apoptosis and the identification of new factors. In this Masterate project, *in vitro* reconstitution experiments were used to dissect the components involved in apoptosis. Francois and Grimes (1999) developed an *in vitro* model of apoptosis using two neuron-like cell lines, PC12 cells (established from a rat adrenal pheochromocytoma) and SY5Y cells (thrice subcloned, nearly diploid, human neuroblastoma cell line). Incubation of nuclear and cytosolic fractions of PC12 and SY5Y cells, respectively, with rat cyt. c induces apoptosis (Francois and Grimes, 1999). Various

components of this *in vitro* system were used in this project to develop new *in vitro* systems in which to study apoptosis.

1.10 THESIS OBJECTIVES

The main objective of this thesis was to develop *in vitro* systems in which we might be able to identify novel components of apoptosis using a combination of yeast and mammalian cell fractions. The lack of obvious homologues to mammalian apoptotic proteins makes yeast an ideal naive system in which to explore the role of mammalian apoptotic proteins, and potential interaction with novel yeast proteins.

An extension of this work was the development of another *in vitro* system to examine the role of mitochondria in cell-free apoptosis. In this system, mitochondria, that have been treated with an agent that was reported to release cyt. c, were added to the cell-free system of Francois and Grimes (1999) and apoptotic events were analysed.

The following questions were addressed:

- Can apoptosis be induced in yeast nuclei with apoptotic mammalian cytosol?
- Can yeast cell components cause apoptosis in PC12 nuclei?
- Is yeast cyt. c sufficient to activate apoptosis in mammalian or yeast cytosol?
- In a cell-free system, can apoptosis be induced by the addition of isolated mitochondria induced to release cyt. c?
- Can purified mitochondria from healthy cells prevent apoptosis *in vitro*?