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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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).

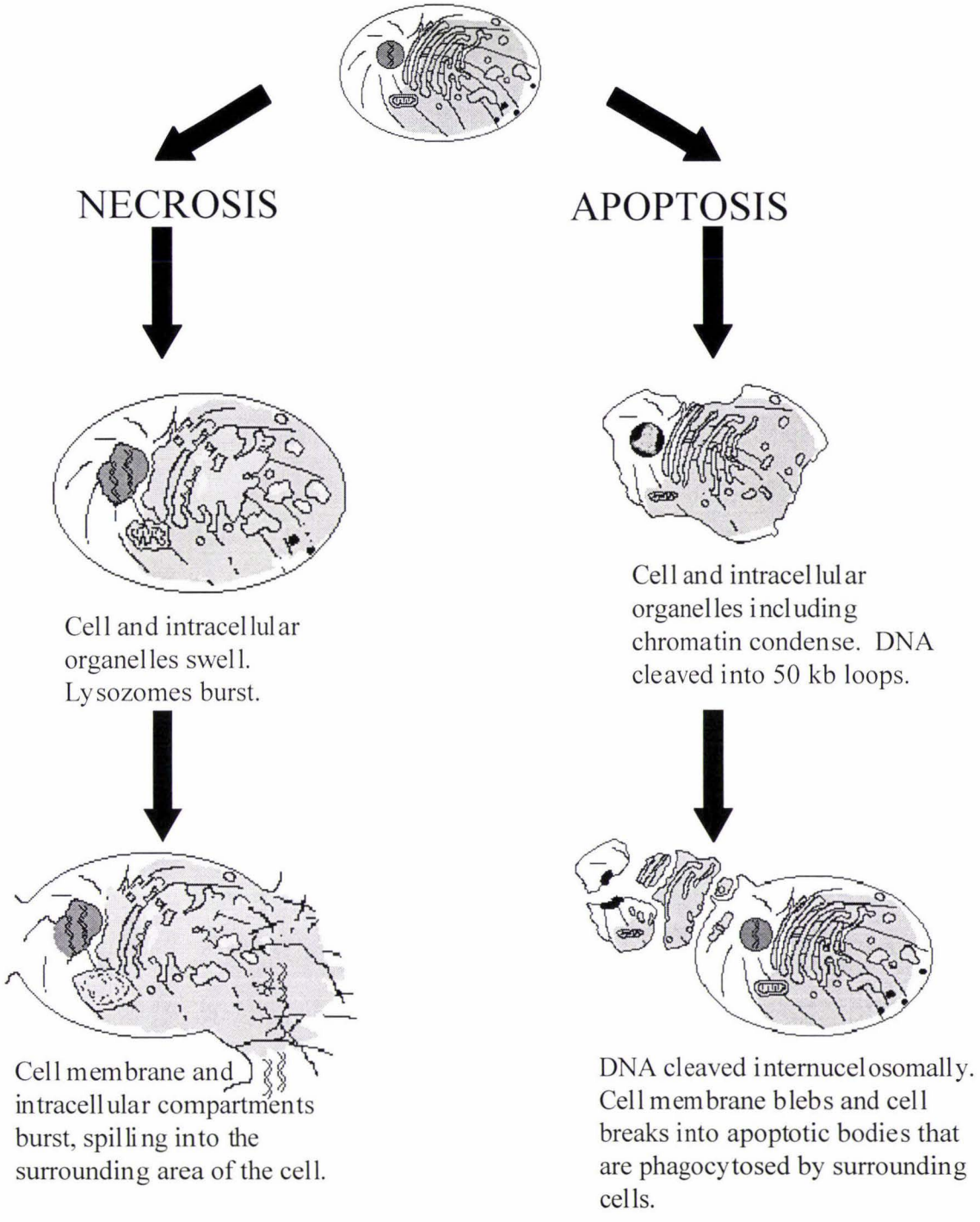


Figure 1-1: Comparison of apoptotic and necrotic cell death.
Initial features of apoptotic cell death are the shrinkage of the cell and internal organelles, followed by the cleavage of the genomic DNA and break up of the cell into apoptotic bodies, which are engulfed by the surrounding cells. In contrast,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

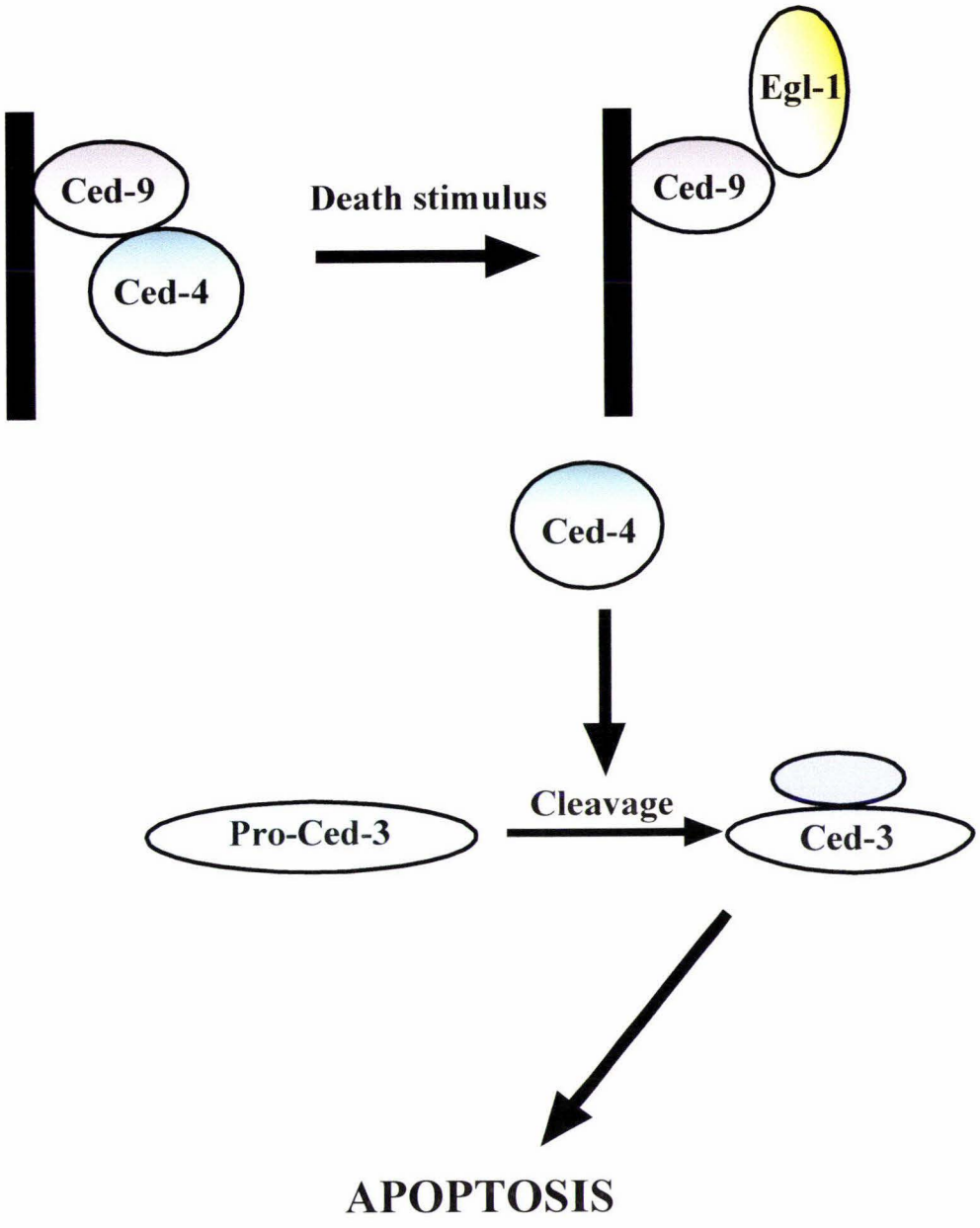


Figure 1-2: Diagrammatic representation of the interaction of the Ced proteins and Egl-1.

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.

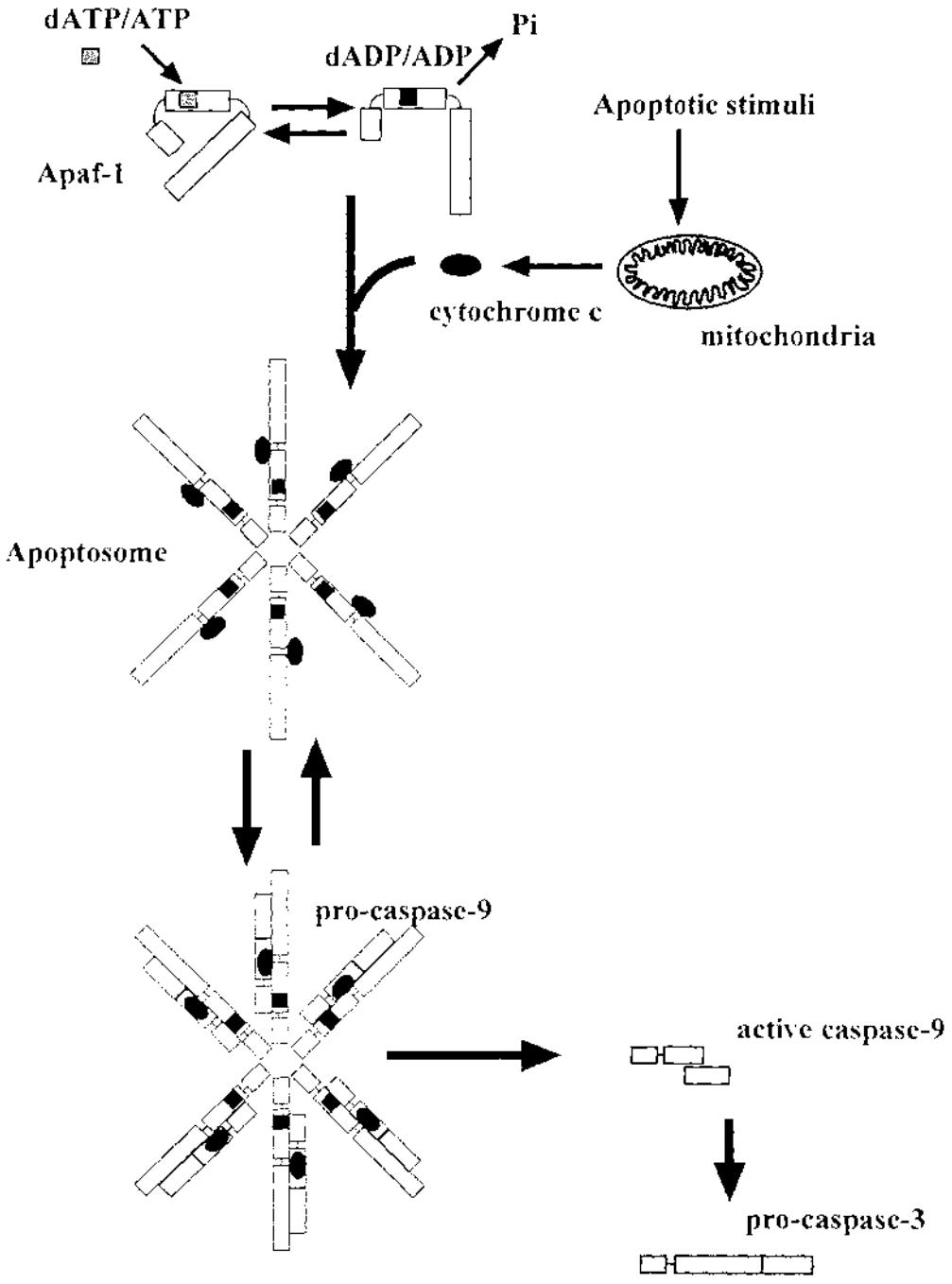


Figure 1-3: Schematic model of the activation of Caspase-9.
Apaf-1-mediated hydrolysis of ATP causes a conformational change in Apaf-1. Cyt. c binds this form of Apaf-1 and promotes oligomerisation into a multimeric complex, which recruits and activates Caspase-9. Active Caspase-9 promotes downstream 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).

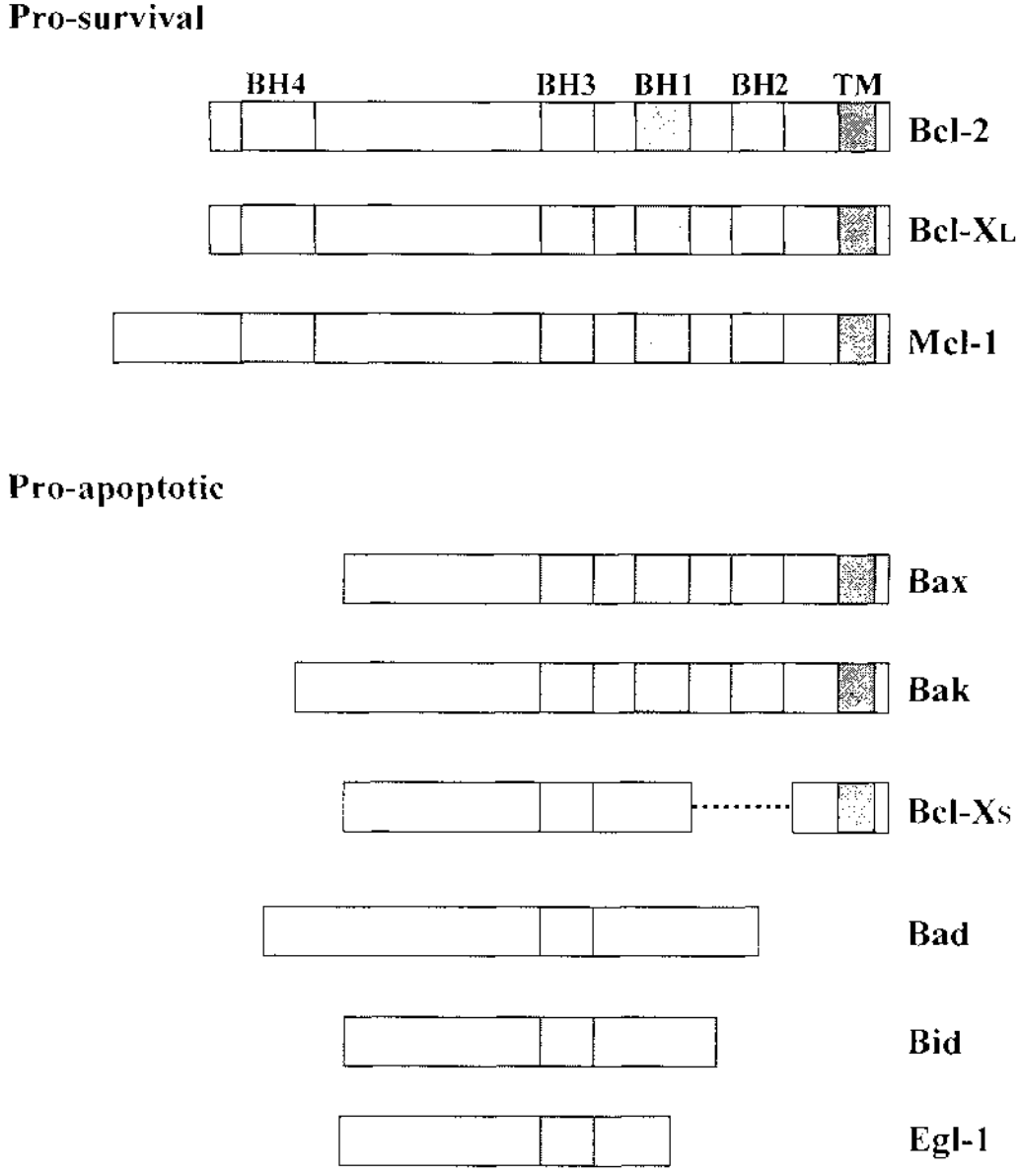


Figure 1-4: Schematic representation of the Bcl-2 family members and Egl-1.
The Bcl-2 homology (BH) domains and hydrophobic membrane-targeting domain (TM) are indicated. The deletion in Bcl-X_S resulting from differential splicing is indicated by dashed lines. The proteins are not drawn to scale. This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

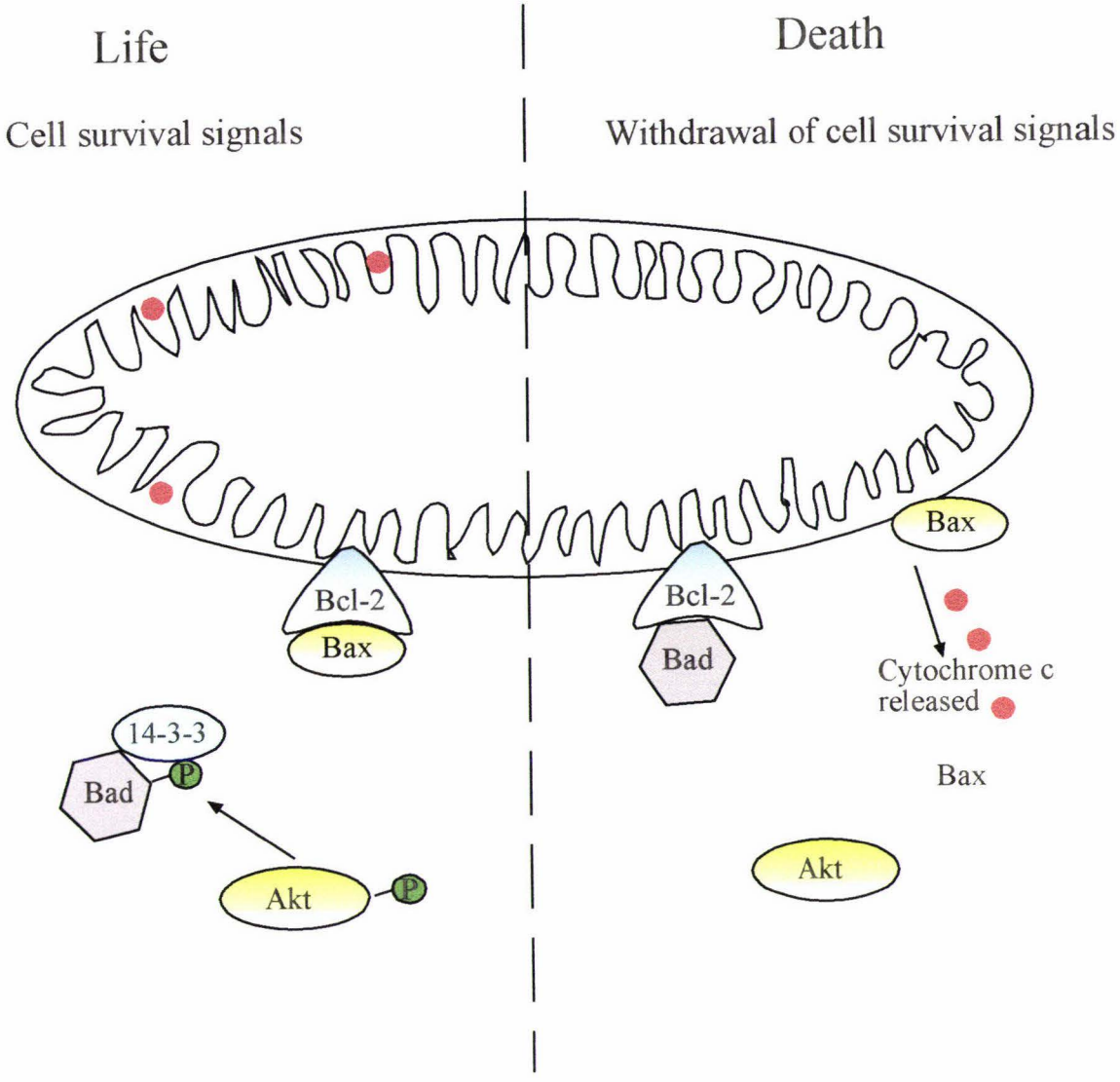


Figure 1-5: Phosphorylation of Bad prevents mitochondria-mediated apoptosis.
In the presence of cell survival signals, such as phospho-Akt, Bad is phosphorylated, and then sequestered in the cytosol by 14-3-3. In the absence of these survival signals, Bad is not phosphorylated, and heterodimerises with 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

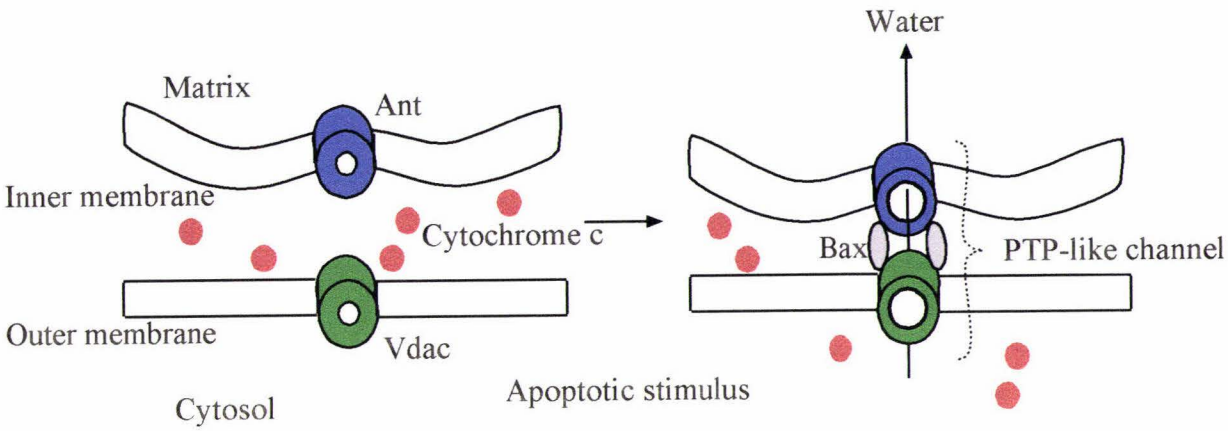
When a permeability transition is induced in isolated mitochondria, they swell and release apoptogenic proteins (Scarlett and Murphy, 1997). A megachannel called the permeability transition pore (PTP) is thought to open during apoptosis allowing H_2O and other solutes to enter mitochondria and the release 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

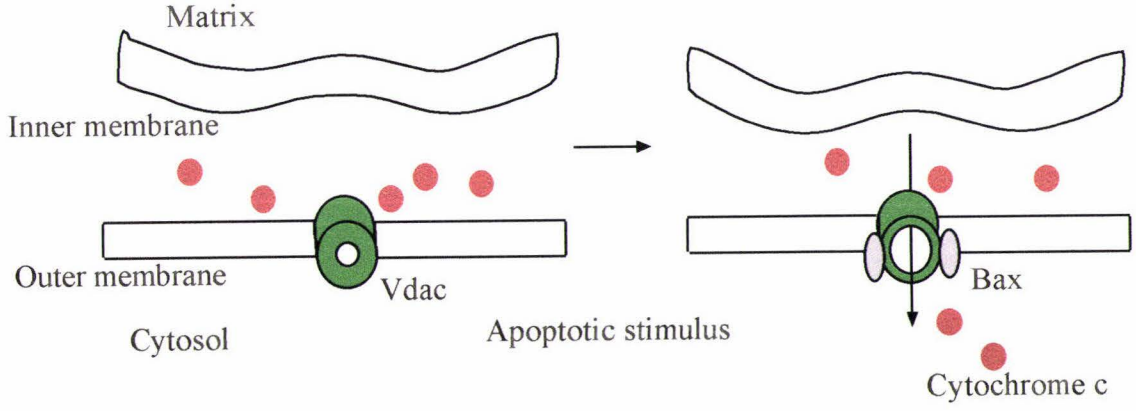


Figure 1-6: Models for the release of cyt. c from mitochondria.
Model I – Bax interacts with both Ant and Vdac to form a permeability transition pore (PTP), which allows water and solutes to enter the mitochondrion causing the matrix to swell and its outer membrane to rupture. The result is the release 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).

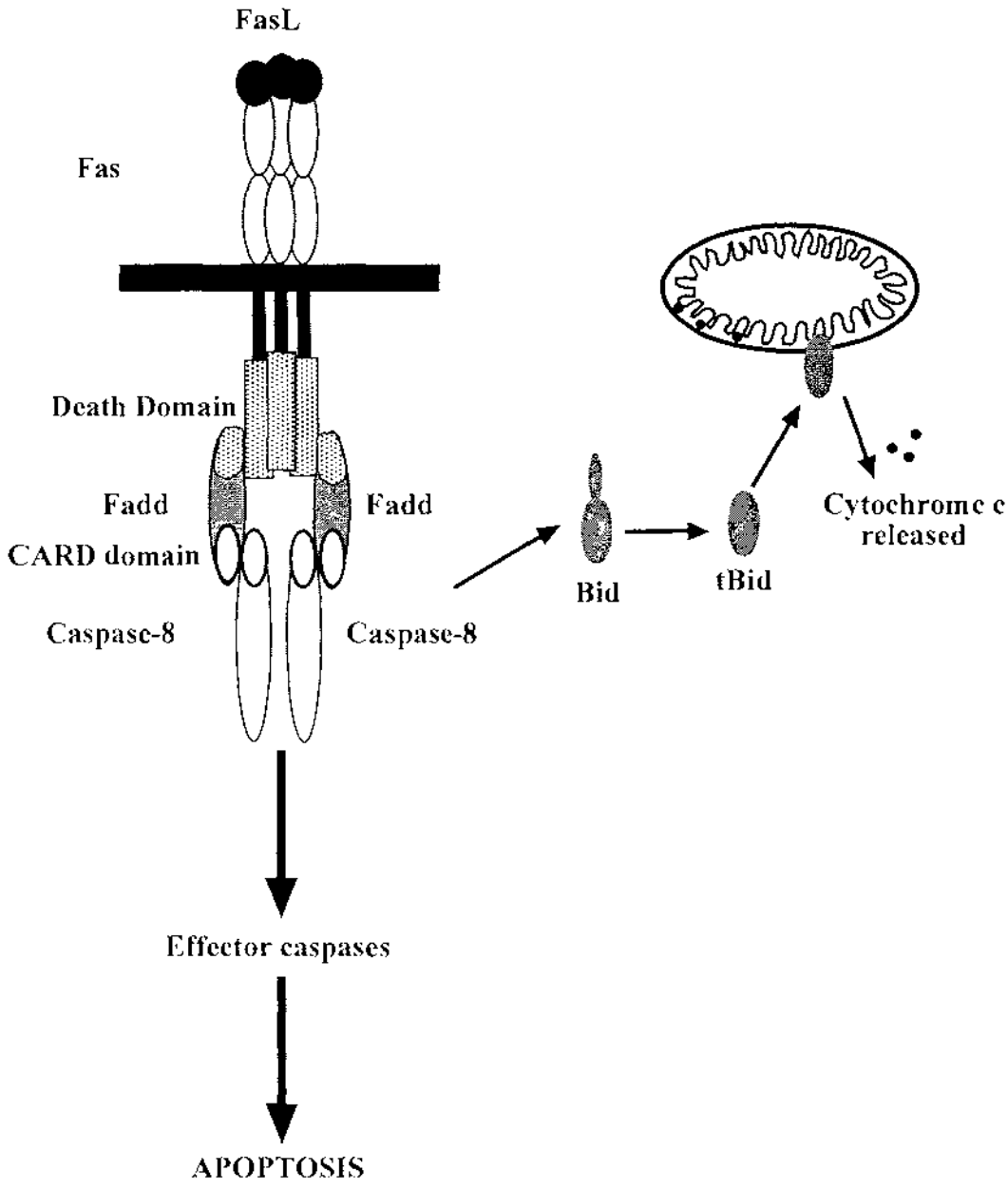


Figure 1-7: Death receptor-mediated apoptosis.
Fas receptor molecules trimerise following ligand binding, which allows the recruitment of Fadd, then Caspase-8, which is subsequently activated. Active Caspase-8 triggers a caspase cascade and cleaves Bid. The COOH-terminal part of Bid (tBid) translocates 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).

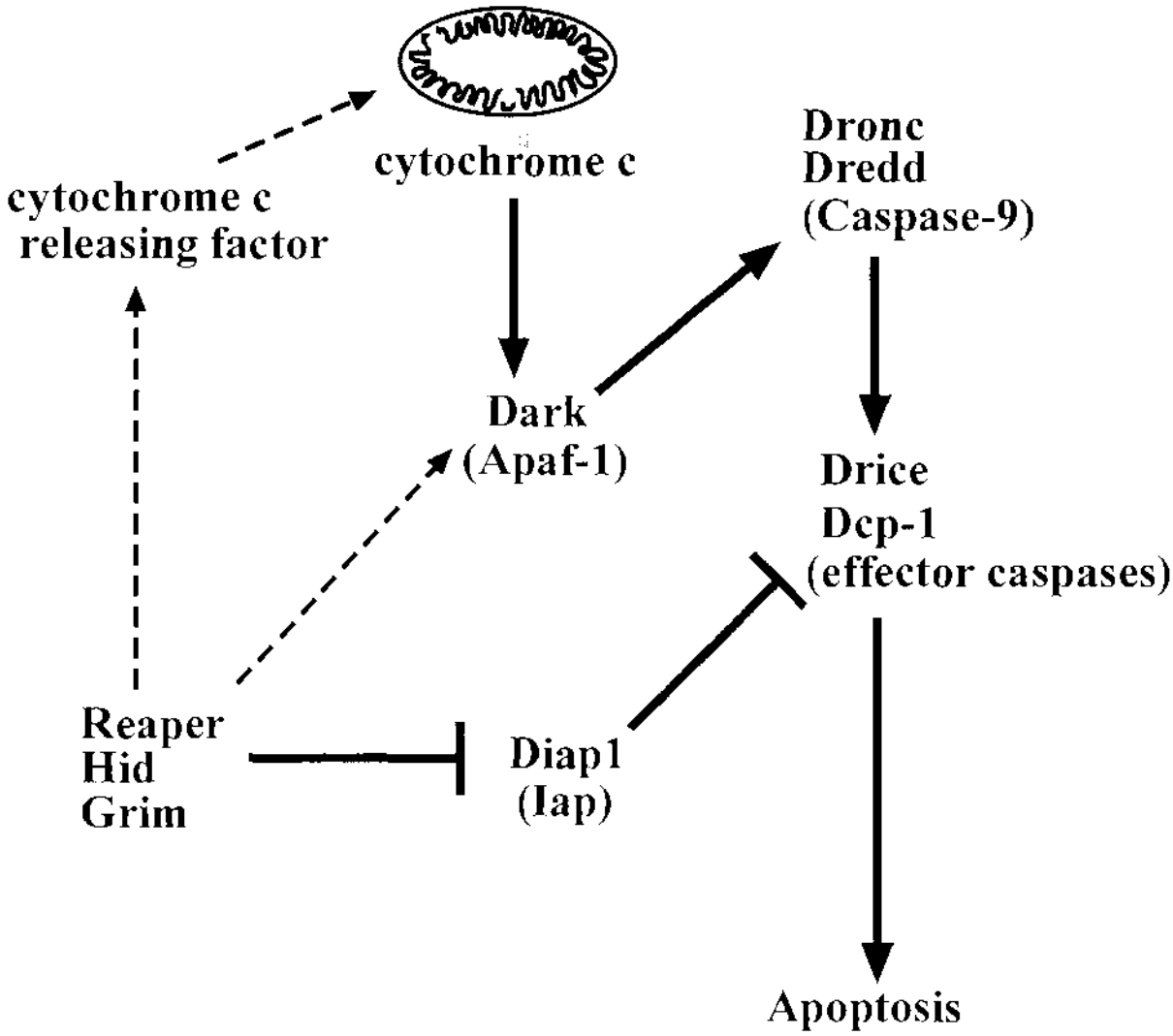


Figure 1-8: Model of apoptosis in *Drosophila*.
Grim, Rpr and Hid are believed to promote apoptosis by disrupting interactions between Iaps and Caspases. Rpr, Hid and Grim may cause cyt. c release through an unknown factor. Released cyt. c binds Dark, which can then activate the activator caspases, Dronc 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*?

CHAPTER 2: MATERIALS AND METHODS

2.1 CHEMICALS AND SOLUTIONS

Most general chemicals were purchased from Sigma (St. Louis, MO) including RPMI 1640 media. Media for yeast cultures, Horse serum, Foetal Calf serum and BenchMark^{TM} Prestained Protein Ladder were from Life Technologies (Gathersberg, MD). [α-^{32}P]ddATP was purchased from Amersham Life Science (Buckinghamshire, England). Anti-Caspase-3, anti-Caspase 9, anti-Parp and anti-Erk antibodies were purchased from Transduction Laboratories (Lexington, KY), PharMingen (San Diego, CA), Boehringer Mannheim (Roche Molecular Biochemicals, Auckland) and Santa Cruz Biotechnology Inc. (Santa Cruz, CA) respectively. Solutions were made using double distilled water (ddH_2O) unless otherwise stated.

2.2 CELL CULTURE

2.2.1 PC12 tissue culture

Dr. M. Grimes (Massey University, NZ) obtained rat pheochromocytoma (PC12) cells from Lloyd Greene (Columbia University, NY). The cells were grown on rat-tail-collagen coated plates in RPMI 1640 medium containing 10% horse serum (Gibco BRL), 5% Foetal Calf Serum (Gibco BRL) and 26.2 mM $NaHCO_3$, with 5% CO_2 at 37°C. Fresh media was supplied three times a week, and replaced completely when required. Cells were harvested and used, or passaged onto new plates (1 plate onto 4 plates) every 10-14 days.

2.2.2 SY5Y tissue culture

Human neuroblastoma (SY5Y) cells obtained from Dr. Mark Israel (University of California, San Francisco, CA) were grown in RPMI 1640 medium containing 10% Foetal Calf Serum (Gibco BRL) and 26.2 mM $NaHCO_3$, with 5% CO_2 at 37°C. Fresh media was supplied three to four times a week, and replaced completely when required. Cells were harvested and used, or passaged onto new plates (1 plate on to 5 plates) within 7 days.

2.2.3 Yeast cell culture

Saccharomyces cerevisiae MATα lys1- stocks obtained from Dr. Ira Herskowitz (University of California, San Francisco, CA) were maintained on YPD agar (1% select yeast extract, 2% select peptone 140, 2% select agar, Gibco BRL; 2% dextrose) plates at 4°C. New stocks of yeast were prepared every 4-6 weeks by streaking yeast on to two sterile YPD agar plates. These plates were sealed with parafilm and incubated at 30°C for three days and then stored at 4°C until required.

2.3 PREPARATION OF THE MAJOR COMPONENTS OF *IN VITRO* REACTIONS

2.3.1 Cell cracking

Cell permeabilisation or cracking forms an integral part of many of the protocols listed in this section. Cell cracking was performed using a stainless-steel ball homogeniser obtained from the European Molecular Biology Laboratory (Heidelberg, Germany). Tungsten carbide balls were obtained from Industrial Tectronics (Ann Arbor, MI). The ball homogeniser was assembled in a ddH_20 water bath, where air bubbles were removed from individual components and the assembled cracker was flushed out with a syringe. The cell cracker was left on ice to chill. 2-3 mL chilled Bud Buffer III, a cytosolic like buffer, (BBIII - 38 mM aspartic acid, 38 mM gluconic acid, 38 mM glutamic acid, 20 mM MOPS, 10 mM potassium bicarbonate, 0.5 mM magnesium carbonate, 1 mM EDTA, 1 mM EGTA adjusted to pH 7.4, and 5 mM glutathione (GSH) was added prior to use) was passed through the homogeniser.

2.3.2 Preparation of SY5Y or PC12 cytosol

Media was removed from 8-10 plates of confluent SY5Y or PC12 cells and each plate washed with 10 mL of warm PBS (phosphate buffered saline: 2.68 mM KCl, 136.9 mM NaCl, 1.47 mM $KHPO_4$, 7.97 mM Na_2HPO_4, adjusted to pH 7.4). The cells were harvested in 10 mL of warm PBS and a sample removed to determine the number of apoptotic cells. The remaining cells were centrifuged at 100×g for 3 minutes, the

supernatant removed, and the cell pellet washed with 10 mL cold PEE (PBS with 1 mM EDTA and 1 mM EGTA). Cells were washed on ice with BBIII followed by recentrifugation and then resuspended in 0.5 mL BBIII with protease inhibitors (17.4 mg/ml PMSF, 0.1 mg/mL o-phenathroline, in anhydrous ethanol; and 10 µg/mL pepstatin, 10 µg/mL chymotrypsin, 10 µg/mL leupeptin, 10 µg/mL aprotinin in DMSO). Cells were cracked open at 4° C by passing the cell suspension through the ball homogeniser using a ball with a diameter of 8.016 mm with a cylinder size of 8.020 mm. The cell lysate was centrifuged at 1,000×g, for 10 minutes at 4°C to remove cell ghosts and nuclei (Figure 2-1). The resulting supernatant was then centrifuged at 100,000×g, for 60 minutes at 4°C (Figure 2-1). The 100,000×g supernatant containing cytosolic proteins was carefully removed and stored at –70°C in 40 µL aliquots for *in vitro* reactions.

2.3.3 Protein concentration determination

The protein concentration of cytosol was determined based on the method described by Lowry *et al.*, (1951). Briefly, bovine serum albumin (BSA) protein standards were made up to 1 mL, as were the unknown samples. The reaction mix was made up daily by mixing 1 part Solution C (2% w/v NaK tartrate in ddH_2O) with 100 parts Solution A (2% w/v Na_2CO_3 in 0.1M NaOH), and adding 1 part Solution B (1% w/v $CuSO_2.5H_2O$ in ddH_2O). Equal volumes of the reaction mix and samples were mixed and allowed to stand at room temperature for 10 minutes. 100 µl of Folin-Ciocalteau reagent, diluted 4-fold with ddH_2O, was added to the samples and the colour was allowed to develop for 30 minutes at room temperature. The absorbance of these samples was read at 550 nm.

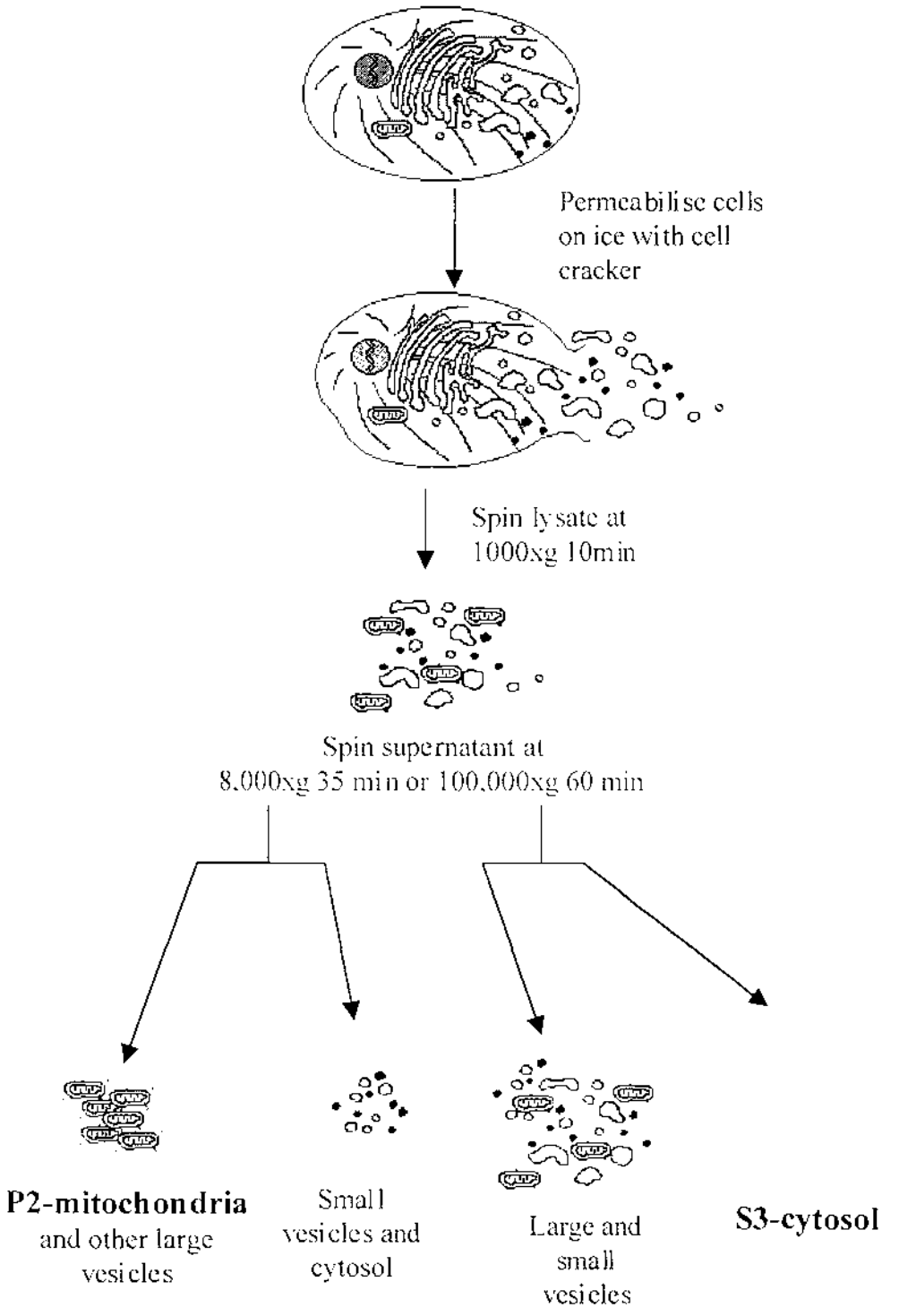


Figure 2-1: Overview of the cell fractionation procedure to isolate mitochondria and cytosol.

Cells were permeabilised then the lysate spun at 1000g for 10 minutes o pellet cell ghosts and nuclei. The resulting supernatant from the 1000×g spin is spun at either 8,000×g for 35 minutes or 100,000×g for 60 minutes to obtain the Pellet 2 (**P2**) – **Mitochondria**/and other large vesicles or Supernatant 3 (**S3**) – **Cytosol**, respectively.

2.3.4 Preparation of apoptotic SY5Y cytosol

Apoptotic human cytosol from SY5Y cells was prepared in the same manner as control cytosol, however the SY5Y cells were first treated with staurosporine to induce apoptosis. 8-10 plates of confluent SY5Y cells were rinsed with 10 mL of warm PBS and harvested as previously described (section 2.3.2). The cells were washed three times with serum free media, and resuspended in serum free media containing 0.5 mM staurosporine, and incubated for 24 hours. Cells were harvested and cytosol prepared as in section 2.3.2.

2.3.5 Isolation of PC12 nuclei

10 plates of confluent PC12 cells were washed and harvested in 10 mL of warm PBS. Cells were pelleted by centrifugation at 200×g for 3 minutes at 4°C. The cells were washed in 10 mL of cold PBS with a final wash in cold Nuclear Buffer (10 mM PIPES pH 7.4, 10 mM KCl, 2 mM $MgCl_2$, and freshly added 1 mM DTT with 10 µM cytochalsin B and 1 mM PMSF). The volume of the cell pellet was recorded, the cells were resuspended in 10 volumes of Nuclear Buffer and allowed to swell on ice for 20 minutes. The cells were passed through the cell cracker 8 times using a ball with a diameter of 8.008 mm with a cylinder size of 8.020 mm. The collected cell lysate was centrifuged at 1000×g for 10 minutes at 4°C. The nuclei pellet was resuspended in 2.5 mL of nuclear buffer and a 1 µL aliquot was diluted 100-fold with nuclear buffer. 5 µL of this dilution was stained with an equal volume of Trypan Blue and loaded onto a cell counter (Neubauer, Planoptik) to assess the number of nuclei in the 2.5 mL suspension and check for debris of which there was little. The nuclear suspension was centrifuged at 1000xg for 10 minutes at 4°C and the nuclei resuspended to a concentration of ~4.5 x 10^8 nuclei/mL in Nuclear Storage Buffer (0.99 mM DTT, 0.2 mM spermine, 0.5 mM spermidine, 50% Glycerol, 10 mM PIPES pH 7.4, 80 mM KCl, 20mM NaCl, 250 mM sucrose, 5 mM EGTA). The nuclear suspension was stored in 100 µL aliquots at -70° C. On the day of use nuclei were washed twice with 400 µL BBIII before being resuspended in 20 µL of BBIII.

2.3.6 Spheroplast preparation

For spheroplast preparation, 5 mL of YPD broth (1% select yeast extract, 2% select peptone, 2% dextrose) was inoculated with a sterile loop of *S. cerevisiae* from stock plates. Yeast was grown overnight on an orbital shaker at 200 rpm at 30°C and collected by centrifugation at 1,000×g for 5 minutes. The cell pellet was washed with 1 M Sorbital, and resuspended in 1 mL of lyticase buffer, (1 M Sorbital, 50 mM KPO_4, and 14.3 mM (~2%) ß-mercaptoethanol, freshly added) then 100-200 units lyticase was added and quickly mixed. Cells were incubated rotating slowly at 30°C for ~1 1/2 hours. To test whether spheroplast formation was complete 10 µL of cell suspension was placed on a microscope slide, covered with a coverslip, and water added to the side of the coverslip. The cells are observed under a light microscope to detect lysis due to osmotic pressure on the cell membrane. If the majority of the cells lysed then spheroplast formation was deemed complete. Spheroplasts were collected by centrifugation at 1,500×g for 5 minutes at 4°C and the cell pellet was washed gently with 1 M Sorbital 3 to 4 times collecting the cells at 1,500×g between washes. The final pellet was resuspend in ~100 µL cold BBIII, with protease inhibitors and kept on ice.

2.3.7 Isolation of Yeast nuclei

Nuclei from *S. cerevisiae* were isolated according to Nelson *et al.*, (1977) with modifications as follows. Cells were grown in YPD broth until late exponential growth phase. The cells were harvested and spheroplasts formed according to the protocol in section 2.3.5 except the final resuspension solution used was 1 M Sorbital before nuclei were isolated.

To isolate nuclei from spheroplasts cells were gently broken open. The spheroplasts were collected and gently resuspended in 5-10 volumes of cold Ficoll buffer (18% w/v Ficoll-400, 10 mM Tris-HCl, pH 7.5; 20 mM KCl, 5 mM $MgCl_2$, 3 mM DTT, 1 mM EDTA, and protease inhibitors). The cells were incubated on ice for 20 minutes, then passed through the cell cracker 6 times using a ball with a diameter of 8.016 mm and the collected cell lysate was centrifuged at 3,000×g for 5 minutes at 4°C. The resulting supernatant was

centrifuged at 20,000×g for 20 minutes at 4°C to pellet the nuclei. The nuclear pellet was resuspended in yeast nuclei storage buffer (10% glycerol, 20 mM Tris-HCl, pH 7.5; 100 mM KCl, 1 mM DTT, 0.1 mM EDTA, 1 mM PMSF and protease inhibitors). A 1 µL aliquot was diluted 100-fold, stained with Trypan blue and the number of nuclei in the suspension, assessed as described in section 2.3.5. The suspension was then stored at -70° C in 100 µL aliquots.

2.3.8 Preparation of Yeast cytosol

The protocol for preparing cytosol from *S. cerevisiae* was adapted from Rieder and Emr, (1997) with modifications as follows. Cells were grown in YPD broth until mid exponential growth phase. The cells were harvested and spheroplasts formed as previously described (Section 2.3.5), except that the final resuspension solution used was 0.5 volume of lysis buffer (0.2 M Sorbital, 50 mM K acetate, 1 mM EDTA, 20 mM HEPES pH 6.8 with protease inhibitors and PMSF) to total volume of wet packed spheroplasts. The resuspended spheroplasts were passed through the cell cracker 6-8 times using a ball with a diameter of 8.0186 mm and the collected cell lysate was centrifuged at 300×g for 5 minutes at 4°C to pellet unlysed spheroplasts. The cleared lysate was centrifuged at 13,000×g for 15 minutes at 4°C to pellet membranes and other large organelles. The resulting supernatant was centrifuged at 100,000×g for 45 minutes at 4°C to generate the clear cytosolic fraction of yeast proteins. The 100,000×g supernatant was carefully removed and stored at –70°C in 40 µL aliquots for *in vitro* reactions. The protein concentration of the yeast cytosol was determined using the Lowry method (previously described in section 2.3.21).

2.3.9 Preparation and pre-incubation of mitochondria with JC-1 and Atractyloside

8-10 plates of SY5Y cells were harvested and resuspended in 15 mL of RPMI 1640 media with 10% Foetal Calf Serum, 26.2 mM $NaHCO_3$ and 3 µM JC-1 (5,5',6,6'-tetrachloro-1,1'3,3'-tetraethylbenzimidazolylcarbocyanine iodide), and recultured for 30 minutes at 37°C. Cells were then harvested and cracked open as previously described in section 2.3.2.

The resulting cell lysate was centrifuged at 1,000×g for 10 minutes at 4°C to pellet the nuclei and cell ghosts (Figure 2-1). The resulting supernatant was centrifuged at 8,000×g for 35 minutes at 4°C to pellet mitochondria and other organelles of a similar size (Figure 2-1). The resulting pellet was measured and then resuspended in 2 volumes of BBIII and named pellet 2 (P2). 20 µL aliquots of P2 were incubated with, or without, either 0.24 mM Atractyloside (Atr) or 1.1 mM Atr, for varying times at 37°C, before addition to *in vitro* reactions. Healthy mitochondria and Atr-treated mitochondria were examined using a fluorescent microscope (Zeiss Axioskop, Germany) using filter set 00 with an excitation wavelength of 530-585 nm and emission >615 nm at 630× magnification.

2.4 *IN* VITRO REACTION PROTOCOL

The *in vitro* reactions were all set up on ice with all components prepared from freezer stocks, or in the case of spheroplasts and P2 fractions prepared on the day of use as described in sections 2.3.5 and 2.3.7 respectively. The components of the ATP regenerating (1 mM ATP, 17.5 U/mL creatine kinase, and 8 mM creatine phosphate) and depleting (1.5 mM glucose and 14.3 U/mL hexokinase) systems were thawed and diluted with BBIII as required. One or two aliquots of PC12 and/or yeast nuclei were thawed and washed twice in ice cold BBIII and finally resuspended in 20 µL of BBIII. Once the *in vitro* reaction components were put together, the tubes were incubated at 37°C for 4 hours then the tubes were cooled on ice to end the reactions.

2.5 SDS-PAGE OF *IN VITRO* REACTIONS

SDS-PAGE was based the protocol by Laemmli, (1970), with the following modifications. Stacking gels were made with 5% w/v (50:1) acrylamide: bisacrylamide, 0.125 M Tris HCl pH 6.95, 1 mM EDTA, 0.1% w/v SDS, 0.06% w/v APS, and 0.06% v/v TEMED. Resolving gels were made with 8-12% w/v (37:1 or 50:1) acrylamide: bisacrylamide, 0.375 M Tris HCl pH 8.95, 0.1% w/v SDS, 0.06% w/v APS, and 0.06% v/v TEMED. The SDS electrophoresis buffer contained 40 mM Tris base, 200 mM glycine, 1 mM EDTA and 0.1% w/v SDS.

To prepare samples for SDS-PAGE 20 µL of 3×SDS sample buffer (187.5 mM Tris HCl, 6% w/v SDS, 30% glycerol, 0.3% w/v bromophenol blue, and 100 mM DTT added prior to use) was added to the *in vitro* reactions. The samples were boiled for 3 minutes and then passed through an 18 gauge needle several times to shear genomic DNA.

Samples were diluted to approximately 100 mg total protein in a final volume of 30 µL with 1×SDS sample buffer (62.5 mM Tris HCl, 2% w/v SDS, 10% glycerol, 0.1% w/v bromophenol blue). Samples and 6 µL of BenchMark™ Prestained Protein Ladder were loaded and gels were run at 20 mA.

2.6 IMMUNOBLOTTING OF *IN VITRO* REACTIONS

Proteins from the SDS-PAGE gel were transferred onto nylon-reinforced nitrocellulose (Optitran™, Schleicher and Schuell, Dassel Germany) in transfer buffer (20% v/v methanol, 150 mM glycine, 20 mM Tris base) at 100 mA for 16-20 hours. The membrane was washed in ddH_2O, and stained with Ponceau S protein stain (0.2% Ponceau S in 3% TCA) for 10 minutes. Excess stain was removed by rinsing the membrane for 5 minutes in ddH_2O. The membrane was then photocopied to locate the position of the molecular weight markers and ensure correct transfer of proteins. To prevent non-specific binding the membrane was blocked for 60 minutes in blocking buffer (TBS (tris buffered saline: 10 mM Tris, 150 mM NaCl to pH 7.7 with HCl), with 0.1% v/v Tween 20, and 5% w/v non-fat milk powder).

2.6.1 Probing for Caspase-3

After blocking, membranes were probed with 1:1,000 anti-Caspase-3 primary antibody in blocking buffer with gentle agitation overnight at 4°C. The membrane was then washed 3 times for 15 minutes in TBS with 0.1% v/v Tween 20, then incubated with 1:5,000 horseradish peroxidase-conjugated anti-mouse secondary antibody (Amersham) in blocking buffer for 1 hour at room temperature. The membrane was washed 3 times for 15 minutes using TBS with 0.1% v/v Tween 20) before an enhanced chemiluminescent reaction was

performed on the membrane using an Amersham ECL™ western blotting detection kit. The blot was then exposed to x-ray film for an exposure time determined by the band intensity achieved.

2.6.2 Probing for Caspase-9

Membranes were stripped with TBS pH 2.0 for exactly 10 minutes to remove the antibodies but not proteins. The membrane was then washed 3 times for 5 minutes each time with ddH_2O, to remove loose antibodies, and then soaked in TBS pH 8.0 to restore the pH of the membrane. The membrane was reprobed using the method described in 2.6.1 except using 1:2,000 anti-Caspase-9 primary antibody and 1:5,000 horseradish peroxidase-conjugated anti-rabbit secondary antibody (Amersham).

2.6.3 Probing for Parp

Membranes were stripped and reprobed as described for section 2.6.2 except using 1:2,000 anti-Parp primary antibody and 1:5,000 horseradish peroxidase-conjugated anti-rabbit secondary antibody (Amersham).

2.6.4 Probing for Erk

Membranes were stripped and reprobed as described for section 2.6.2 except using 1:2,000 anti-Erk primary antibody and 1:5,000 horseradish peroxidase-conjugated anti-rabbit secondary antibody (Amersham).

2.7 DNA EXTRACTION FROM *IN VITRO* REACTIONS

The genomic DNA extraction protocol used was modified from Newmeyer *et al.*, (1994). 500 µL of DNA extraction buffer (10 mM Tris HCl, pH 8.0, 100 mM EDTA, 0.5% SDS) was added to *in vitro* reaction samples on ice. 20 µL of 10 mg/mL Proteinase K (Gibco BRL) in 50 mM Tris, pH 8.0, 5 mM Ca actetate was added to each sample and incubated for 2 hours at 50°C. Samples were subjected to phenol/chloroform extraction to remove protein fragments, where an equal volume of phenol/chloroform (Gibco BRL) was added to each tube, vortexed, and then centrifuged at 10,000×g for 3 minutes at 4°C. The top layer

of DNA was carefully removed into a fresh tube. An equal volume of chloroform was added to each sample, vortexed, and centrifuged at 10,000×g for 3 minutes and the top layer of DNA removed to a clean tube. DNA was precipitated by the addition of 1 mL of 100% ice-cold ethanol and subsequent incubation at-70°C for 1 hour. After incubation, samples were centrifuged for 30 minutes at 15,000×g at 4°C, the supernatant carefully removed and the DNA pellet dried by SpeedVac for 15 minutes. The DNA pellet was resuspended in 100 µL TE buffer (10 mM Tris HCl, 1 mM EDTA adjusted to pH 8.0) and 5 µL 1 mg/mL DNase-free RNase added. Samples were incubated for 1 hour at 37°C to digest contaminating RNA and then were subjected to phenol/chloroform extraction as described above. 35 µL 3 M Na acetate and 250 µL 100% ice-cold ethanol was added to precipitate the DNA and the samples incubated for 1 hour at -70°C. The DNA was pelleted by centrifugation at 15,000×g for 30 minutes and washed with 80% ethanol before centrifugation at 15,000×g for 20 minutes at 4°C. The DNA pellet was dried in the SpeedVac as described, resuspended in 30 µL of sterile water, and stored at 4°C until required. The DNA concentration and purity was assayed.

2.7.1 DNA concentration determination

DNA concentration and purity were determined by UV absorbance at 260 nm using a Ultrospec 3000 UV/Visible Spectrophotometer (Pharmacia Biotech). 5 µL DNA sample in 995 µL sterile water were measured against 1 mL of sterile water. The concentration was calculated as DNA in µg/mL.

2.8 RADIOACTIVE END LABELLING AND SEPARATION OF GENOMIC DNA

DNA extracted from the *in vitro* reactions was radioactively labelled as described by Tilly and Hsueh (1993). Briefly, DNA volumes and 1 µL of 1 µg/µL 1kb ladder (Gibco BRL) were adjusted to a total volume of 30 µL with sterile water. A master mix (8 µL sterile water, 1 µL [α-^{32}P] ddATP, 1 µL (25 u) terminal deoxynucleotideal transferase (TdT) enzyme, and 10 µL 5×TdT reaction buffer, per tube) was made and dispensed in 20 µL

aliquots per tube of DNA on ice. Samples were mixed and incubated at 37°C for 60 minutes. A master stop mix (3 µL sterile water, 5µL 0.25 mM EDTA (pH 8.0) and 2 µL tRNA 25 mg/mL stock per reaction tube) was prepared and dispensed in 10 µL aliquots after incubation to stop the labelling reaction. The DNA was subjected to repeat washing steps to remove un-incorporated radioactive label. The DNA from each reaction tube was precipitated by adding 10 µL of 3 M Na acetate with 200 µL ice-cold 100% ethanol followed by a 1 hour incubation at -70°C. The DNA was pelleted by centrifugation at 15,000×g for 30 minutes and resuspended in 100 µL of TE buffer, 20 µL 3 M sodium acetate and 400 µL ice-cold 100% ethanol before incubation at -70°C for 60 minutes. The samples were then centrifuged at 15,000×g for 30 minutes, the supernatant removed, and the DNA pellet washed with 1 mL of ice-cold 80% ethanol. The samples were again centrifuged at 15,000xg for 20 minutes and the DNA pellets were dried in heating block at 50°C for 10-15 minutes to avoid contaminating the SpeedVac. Finally, the DNA was resuspended in 10 µL TE buffer and 1.5µL 6x DNA loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 40% sucrose in water) before loading onto a 2% agarose (Sigma) gel and subjected to electrophoresis at 150V for 90 minutes using 1×TAE buffer (40 mM Tris, 0.11% Glacial Acetic Acid, 1 mM EDTA). After electrophoresis the bottom of the gel was cut off below the lower dye front and discarded to remove DNA of <20-50 base pairs and unincorporated radioactive label. The gel was washed twice for 1 hour in TdT wash buffer (10% acetic acid, 10% isopropanol), and then overnight (or an extra hour wash), to remove free radioactive label. The gel was drained and placed onto 3 large pieces of Whatman filter, covered with Saran-wrap and dried in gel dryer without heat for two hours only. The partially dried gel was placed on a piece of Whatman paper cut to size, covered with Saran wrap and the gel was exposed to Kodak X-Omat film under intensifying screens. Several exposures were made and the film was developed using an automatic x-ray film processor.

2.8.1 Determination of equal loading of DNA

To determine if there was the same amount of DNA in each lane of the agarose gel, the dried gel above was stained with SYBRI Green (Molecular Probes) in 1xTAE at a 1:10,000 dilution for one hour. The DNA was visualised by UV light and photographed.

2.9 FLUORESCENT LABELLING OF PC12 NUCLEI, YEAST NUCLEI AND SPHEROPLASTS

Nuclei/spheroplasts from *in vitro* incubations were subjected to morphological examinations with a fluorescent microscope (Zeiss Axioskop, Germany) using filter set 02 with an excitation wavelength of 365 nm and emission 420 nm. The nuclei/spheroplasts were isolated from the *in vitro* reactions by centrifugation at 1,000×g for 10 minutes and were fixed in 50 µL 4% parafomaldehyde on ice for 20 minutes. The nuclei/spheroplasts were washed with 400 µL of PBS and collected by centrifugation at 1,000×g for 10 minutes at 4°C. The nuclei/spheroplasts were resuspended in 10 µL 1% Hoechst 33342 and incubated for 30 minutes at room temperature in the dark. The SLOWFADE™ Antifade Kit (Molecular Probes) was utilised to enable the samples to be assessed for a greater length of time under the fluorescent microscope without the fluorescent dye fading.

CHAPTER 3: YEAST *IN VITRO* MODEL OF APOPTOSIS - I

3.1 INTRODUCTION

The mechanisms and proteins involved in apoptosis are conserved in multicellular organisms. However, no homologues of the genes encoding the main proteins involved in apoptosis have been identified in unicellular organisms such as *S. cerevisiae*. Apoptotic features have been observed in yeast in certain circumstances (outlined in Section 1.8.2), and whether yeast undergo apoptosis is a hotly discussed debate. We attempted to reconstitute features of apoptosis using yeast cell components. If features of apoptosis could be reconstituted *in vitro* we could use this protocol to identify yeast proteins involved in a type of apoptosis found in yeast.

We designed a system to reconstitute apoptosis using yeast and mammalian cell components (Figure 3-1A). This system was based on an *in vitro* protocol designed by Francois and Grimes (1999) (Figure 3-1B). In their protocol, the addition of exogenous rat cyt. c to SY5Y cytosol mimics cyt. c release from mitochondria into the cytosol during apoptosis. PC12 nuclei undergo DNA internucleosomal cleavage and show chromatin condensation when incubated with rat cyt. c-activated SY5Y cytosol, and this paradigm served as a control for our experiments (Francois and Grimes, 1999). A variation of this protocol used cytosol derived from staurosporine (STS – a kinase inhibitor) treated SY5Y cells. These cells undergo apoptosis after STS treatment and accordingly cytosol derived from these cells (apoptotic SY5Y cytosol) will cause apoptosis in PC12 nuclei (Bertrand *et al.*, 1994; Francois and Grimes, 1999).

Using this *in vitro* system we asked several questions. We asked if apoptotic or rat cyt. c-activated SY5Y cytosol would induce features of apoptosis, such as chromatin condensation or DNA fragmentation, in yeast nuclei or spheroplasts (Figure 3-1).

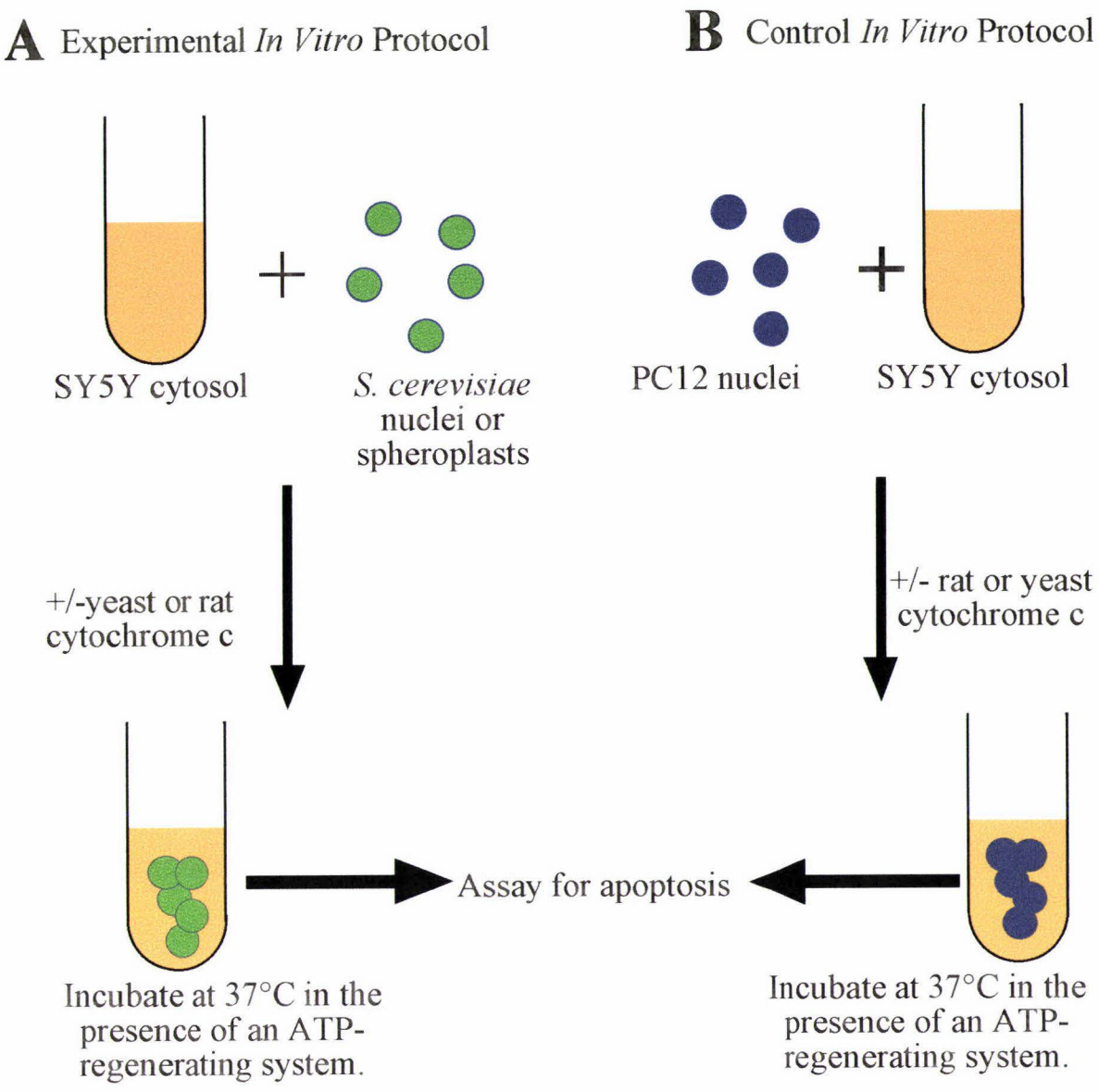


Figure 3-1: Schematic diagram for *in vitro* reactions using yeast nuclei or spheroplasts.

A - Experimental *in vitro* protocol. Cytosol harvested from SY5Y cells was incubated with either *S. cerevisiae* nuclei, or spheroplasts, in the presence or absence of an ATP-regenerating system. Rat or yeast cyt. c was added to the reactions to induce apoptosis. **B** - Control *in vitro* protocol (Francois and Grimes, 1999). Cytosol harvested from SY5Y cells was incubated with PC12 nuclei in the presence of an ATP-regenerating system. Rat or yeast cyt. c was added to the reactions to activate caspases. After incubation, the reactions were assayed for apoptosis by: 1) proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, which were probed for caspases; 2) nuclei were isolated, stained with Hoechst 33342, and subjected to morphological examination; 3) DNA was extracted, end-labelled with [α-^{32}P]ddATP and separated by gel electrophoresis, the gel was dried and exposed to film.

Spheroplasts are whole yeast cells that have had their hard outer cell wall degraded by lytic enzymes, such as lyticase. The cell membrane remains mostly intact, but is permeable to water and possibly other proteins. The hope was that we could use spheroplasts to examine the role of other yeast proteins in the induction of apoptotic features in yeast. Inhibition of mammalian caspase activation with DEVD-CHO (the aldehyde caspase inhibitor), might allow other yeast proteins to induce apoptotic features in spheroplast nuclei, which could lead to the identification of yeast proteins involved in mediating features of apoptosis in yeast.

The next question we wanted to address was whether yeast cyt. c could induce apoptosis or features of apoptosis. The induction of apoptosis in cell free systems using either rat or bovine cyt. c has been well documented (Francois and Grimes, 1999; Li *et al.*, 1997b; Liu *et al.*, 1996). However, some researchers have found that yeast cyt. c failed to activate mammalian mechanisms of apoptosis. For instance, *S. cerevisiae* cyt. c does not induce apoptosis in *Xenopus* extracts, even at 100 times the concentration of bovine cyt. c (Kluck *et al.*, 1997b). Electroporation of *S. cerevisiae* cyt. c into murine IL-3-dependent cells does not induce apoptosis, whereas bovine cyt. c does (Garland and Rudin, 1998). Interestingly, isolated *S. cerevisiae* mitochondria release their cyt. c when incubated with apoptotic *Xenopus* extracts, but they do not induce apoptotic changes in sperm or rat kidney nuclei (D. Newmeyer, personal communication). Cyt. c is highly conserved in terms of its sequence and respiratory function, evidenced by the fact that cyt. c of any eukaryote reacts *in vitro* with the cytochrome oxidase of any other species (Kang *et al.*, 1977; Stryer, 1995). The structures of cyt. c from many different species, including some prokaryotic species, are very similar but there are some differences in side chain structures and post-translation modification (Koshy *et al.*, 1994; Paik *et al.*, 1989; Stryer, 1995). We analysed whether yeast cyt. c was able to induce features of apoptosis in spheroplasts or PC12 nuclei.

3.2 RESULTS

3.2.1 Yeast nuclei undergo random DNA cleavage

Nuclei isolated from *S. cerevisiae* (prepared according to the protocol outlined in Section 2.3.7) were incubated with SY5Y cytosol (prepared according to the protocol outlined in Section 2.3.2), in the presence or absence of rat cyt. c, and then assayed for DNA cleavage by end-labelling DNA followed by gel electrophoresis (Figure 3-2A). This method of detecting cleaved DNA is at least a 100 times more sensitive in the detection of apoptotic DNA, compared with ethidium bromide staining (Tilly and Hsueh, 1993). When isolated yeast nuclei were incubated with SY5Y cytosol, a smear was observed, indicative of random DNA cleavage (Figure 3-2A, lane 3). Yeast nuclei incubated with either rat cyt. c-activated SY5Y cytosol or apoptotic cytosol, derived from STS treated SY5Y cells (lanes 4 & 6, respectively), showed a slight increase in the amount of randomly cleaved DNA than was observed with control SY5Y cytosol (lane 3). However, there was no control to see if equal amounts of DNA were being compared with this set of data. These results were repeated twice and summarised in Table 3-1 and in both experiments the same random DNA smear was observed (Table 3-1, rows 2-5). The type of DNA cleavage found in yeast nuclear DNA was compared with cleavage observed in PC12 nuclei induced to undergo apoptosis (Figure 3-2A, compare lane 3 with 2). When PC12 nuclei were incubated with SY5Y cytosol and rat cyt. c, a distinct internucleosomal DNA ladder was observed (lane 2). This was due to rat cyt. c activation of downstream caspases and apoptotic DNases because no DNA cleavage was observed in the reaction in the absence of rat cyt. c (lane 1). The DNA from isolated yeast nuclei was end-labelled to see if it was randomly cleaved before *in vitro* incubations. No cleaved DNA was visible (data not shown, but tabulated in Table 3-1, row 1). A further control, to see if incubating isolated yeast nuclei at 37°C for four hours induced random DNA cleavage, has not been tested yet.

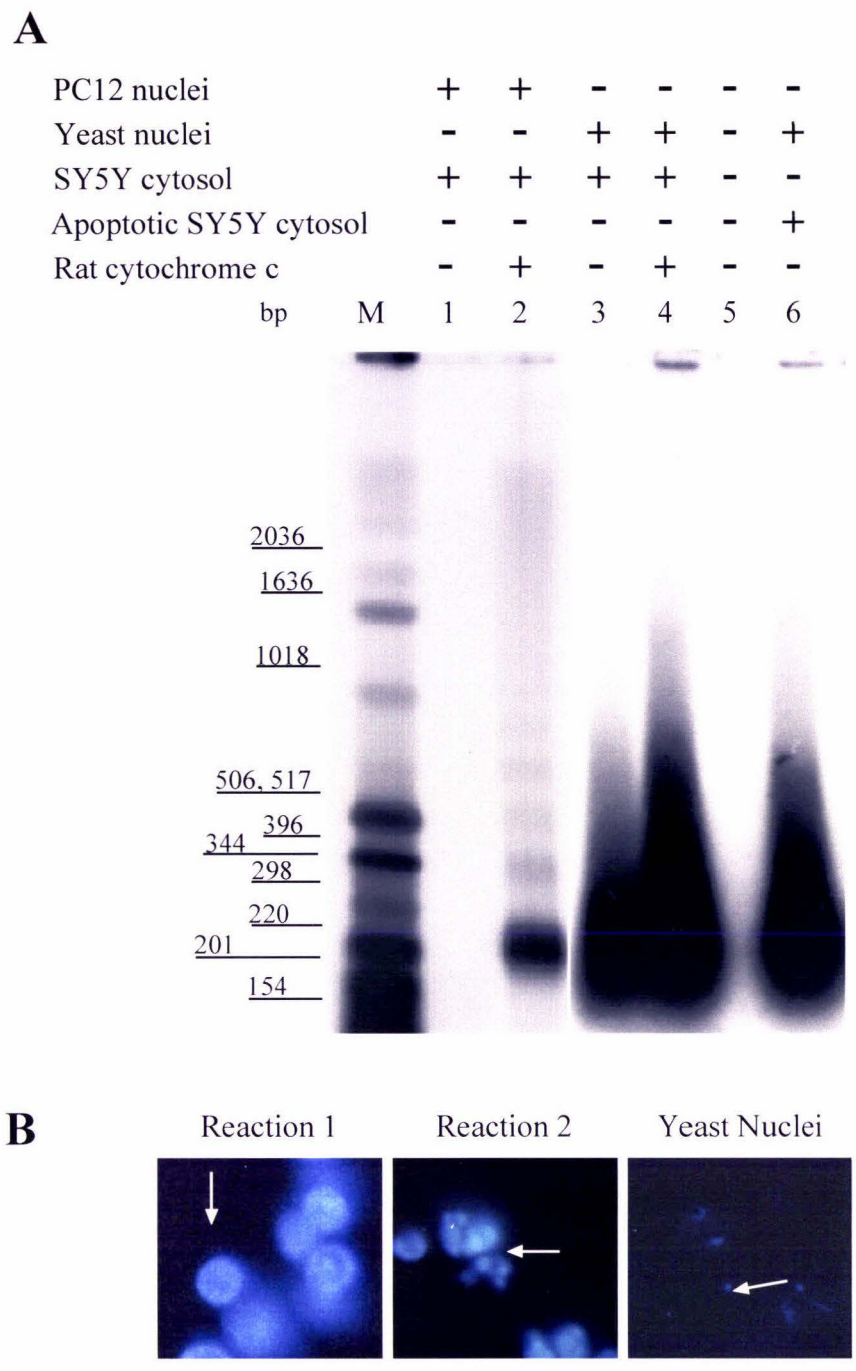


Figure 3-2: DNA cleavage and morphology of nuclei after *in vitro* reactions.
A - SY5Y cytosol incubated with either PC12 nuclei (lanes 1 & 2) or yeast nuclei (lanes 3, 4 & 6), and 3 μM rat cyt. c (lane 2 & 4) for 4 hours at 37°C. Cytosol derived from staurosporine (STS) treated SY5Y cells (apoptotic cytosol) incubated with yeast nuclei (lane 6). All reactions occurred in the presence of an ATP regenerating system. DNA was extracted and visualised as described in Figure 3-1. Note that lanes 1 and 2 are from a 12 hour exposure, while lanes 3-6 are from a 24 hour exposure at –70°C. Note also that lane 5 contains no DNA. M- Radioactively end-labelled 1 kB ladder Marker. **B** - PC12 nuclei from reactions 1 & 2 in **A** and isolated yeast nuclei were stained with Hoechst 33342 as described in Figure 3-1 and observed at 630× magnification. Data are representative of 2 independent experiments. Note that there is possibly unequal loading of DNA. White arrow indicates single nucleus in Reaction 1 and Yeast Nuclei and indicates shrunken fragmented chromatin in Reaction 2.

Row	Experiment No. / Reaction	1	2	3*	4	5	6	Number of significant changes
1	Yeast nuclei alone			-				
2	Yeast nuclei, cytosol, ATP	R ++	R ++					Control
3	Yeast nuclei, cytosol, ATP, rat cyt. c	R +++	R ++					0/2 change
4	Yeast Nuclei, cytosol, rat cyt. c	?	R +++					Not enough data
5	Yeast nuclei, apoptotic cytosol, ATP, rat cyt. c	R ++	?					Not enough data
6	Spheroplasts, cytosol, ATP	-	I +	I +	-	I +	I +	Control
7	Spheroplasts, cytosol, ATP, rat cyt. c	I +++	?	I +	I ++++	I ++++	I +++	4/5 increase
8	Spheroplasts, cytosol, rat cyt. c	I +++	I ++++	I +	I +++			3/4 increase
9	Spheroplasts, cytosol, deplete ATP, rat cyt. c					I +	I +	0/2 change
10	Spheroplasts, cytosol, ATP, rat cyt. c, DEVD-CHO			-	I +	I +	-	0/4 change
11	Spheroplasts, apoptotic cytosol, ATP	I ++++	I ++++	I ++				2/3 increase
12	Spheroplasts, cytosol, ATP, yeast cyt. c					I +	I +	0/2 change
13	Spheroplasts, cytosol, ATP, rat cyt. c, DEVD-CHO					I +	I +	0/2 change
14	Spheroplasts, cytosol, deplete ATP, yeast cyt. c					I +	I +	0/2 change

Table 3-1: Pooled data from Yeast nuclei and spheroplast *in vitro* reactions.
6 experiments are summarised. Yeast nuclei or spheroplasts were incubated with SY5Y cytosol and DNA fragmentation analysis as in Figures 3-2 and 3-3. The type and amount of DNA cleavage was tabulated and scored to the following: R - random DNA cleavage, I - internucleosomal cleavage, with - to ++++ indicating light to dark intensity of DNA cleavage signal. Amounts of DNA in each lane were taken into account when classifying types of DNA cleavage. ?=DNA lost during sample preparation. Summary is defined as the number of experiments that showed increase or decrease of DNA cleavage of two or more changes in intensity, compared to the control, over the total number of experiments. Data shown are: experiment 1 (Figure 3-2), experiment 4 (Figure 3-3) and experiment 5 (Figure 3-7). *-Data from this experiment was very faint and difficult to analyse the results as the radioactive label used had gone through two half lives prior to use.

3.2.2 Comparison of Yeast nuclei and PC12 nuclei

Non-apoptotic PC12 nuclei, when stained with Hoechst 33342, have a diffuse chromatin staining pattern (Figure 3-2B, Reaction 1). In contrast, PC12 nuclei undergoing apoptosis had a shrunken condensed half moon type of morphology (Reaction 2). We examined yeast nuclei from *in vitro* reactions for the appearance of apoptotic morphology (data not shown), by staining with Hoechst 33342. However, we were unable to ascertain whether yeast nuclei had condensed apoptotic chromatin, because they are about one tenth of the size of PC12 nuclei and too small to analyse. Yeast nuclei that had not been subjected to *in vitro* manipulation were stained and shown beside PC12 nuclei to show their relative sizes (Figure 3-2B, Yeast Nuclei and Reaction 1, respectively).

3.2.3 DNA cleavage in Yeast spheroplasts was induced by apoptotic SY5Y cytosol

Since yeast nuclei were difficult to isolate and susceptible to random DNA cleavage when incubated with SY5Y cytosol, we decided to use spheroplasts in our *in vitro* reactions. Freshly made spheroplasts (prepared as described in Section 2.3.6) were incubated in either healthy SY5Y cytosol, apoptotic SY5Y cytosol, or rat cyt. c-activated SY5Y cytosol. We examined the spheroplasts by several different methods to look for features of apoptosis. Figure 3-3 shows radioactively end-labelled DNA extracted from spheroplasts after *in vitro* incubations. When spheroplasts were incubated with SY5Y cytosol alone, no DNA cleavage was reproducibly observed (Figure 3-3, lane 1; Table 3-1, row 6), but the addition of rat cyt. c caused the appearance of a DNA ladder (Figure 3-3, lane 2), similar to that observed in apoptotic PC12 nuclei (lane 8). Cytosolic extracts made from STS treated SY5Y cells (apoptotic cytosol) also caused some DNA cleavage in spheroplasts (lane 5, cleavage fragments observed on longer exposures, not shown). Repetitions of these experiments showed an increase in DNA cleavage due to the presence of rat cyt. c (Table 3-1, row 7) or apoptotic cytosol (row 11).

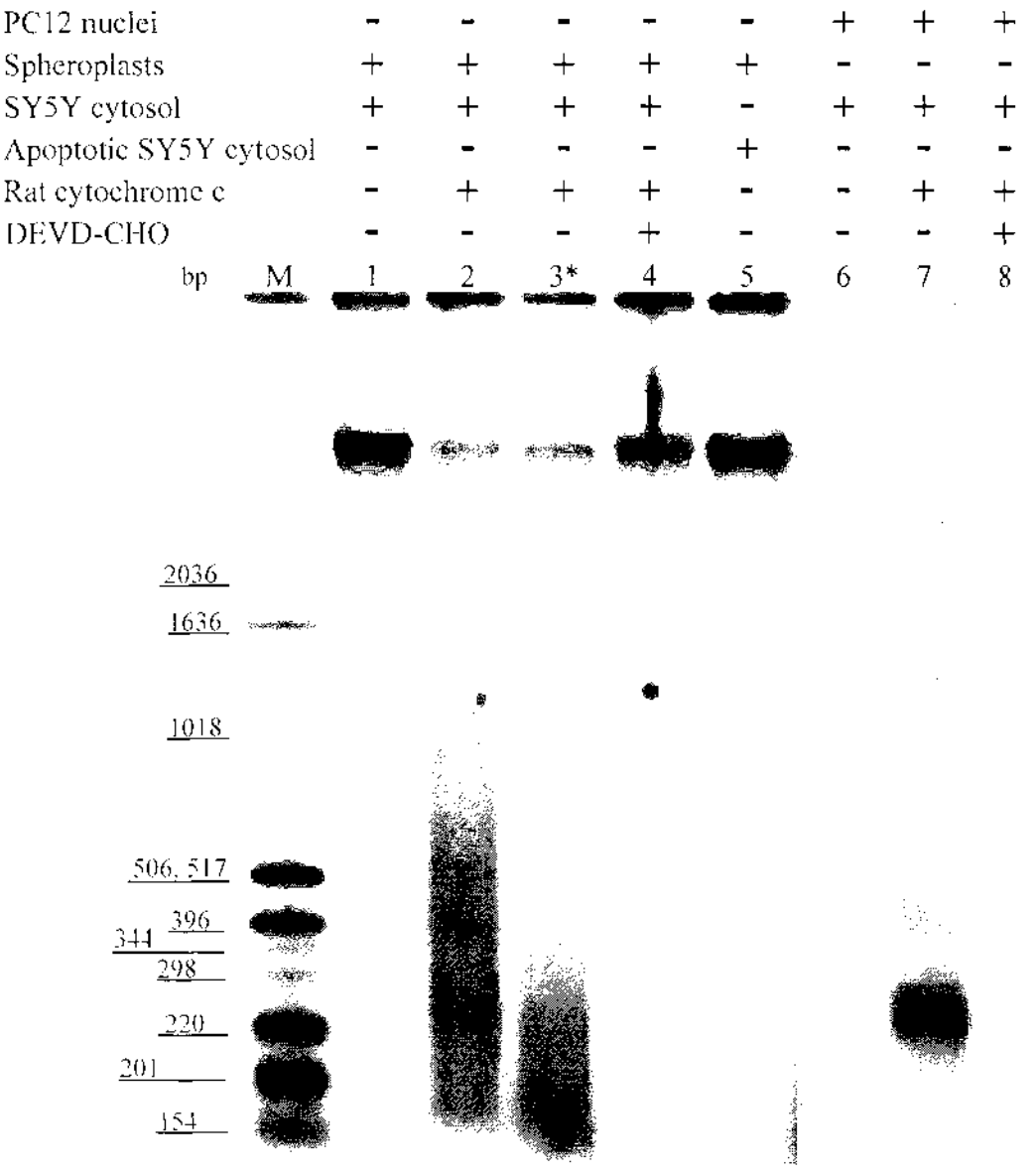


Figure 3-3: Spheroplast DNA is cleaved by apoptotic DNases in SY5Y cytosol.
SY5Y cytosol incubated with spheroplasts (lanes 1-5) or PC12 nuclei (lanes 6, 7 & 8) with the addition of 3 μM rat cyt. c (lanes 2-4 & 7-8) and 2 μM DEVD-CHO (lanes 4 & 8). Apoptotic SY5Y cytosol incubated with spheroplasts (lane 5). *All reactions were performed in the presence of an ATP-regenerating system except lane 3, where it was omitted. DNA was extracted and visualised as described in Figure 3-1. Lanes 6-8 are from a 12 hour exposure, while lanes M, 1-5 are from a 24 hour exposure. Data are representative of 3 or more independent experiments.

In vitro systems require the addition of exogenous ATP to efficiently cleave nuclear DNA (Liu *et al.*, 1996). When we omitted the ATP-regenerating system from *in vitro* reactions the amount of cleaved DNA decreased slightly (Figure 3-3, lane 3 compared with lane 2; Table 3-1, row 8). However, there was not a complete absence of DNA cleavage in this reaction possibly due to the endogenous ATP present in SY5Y cytosolic extracts and in spheroplasts. In contrast, functional depletion of ATP from *in vitro* reactions reduced spheroplast DNA cleavage to the level found in the control experiment (Figure 3-4, compare lanes 4, 5 and 7). These results are summarised in Table 3-1 (row 9).

3.2.4 Caspase activation in spheroplast *in vitro* reactions

To test whether caspases were activated in reactions with spheroplasts and SY5Y cytosol, the cytosolic proteins were separated by SDS-PAGE gel electrophoresis and immunoblotted for mammalian caspases (Figure 3-5A). During apoptosis, both inactive 48 kDa Caspase-9 and 32 kDa Caspase-3 are activated by proteolytic cleavage to yield a fragment of 37 kDa and 17 kDa, respectively and the inactive form of the protein is depleted (Darmon *et al.*, 1995; Fernandes-Alnemri *et al.*, 1994; Pellegrini *et al.*, 1999; Srinivasula *et al.*, 1998). Using antibodies to human Caspase-3 and human Caspase-9 to probe membranes, we found, as expected, inactive full-length Caspase-3 and Caspase-9 in the non-apoptotic control reaction (Figure 3-5A, lane 1) and the active forms of both Caspase-3 and Caspase-9 in the reactions containing rat cyt. c (lanes 2 & 3). No cleaved Caspase-3 was observed in reactions that contained spheroplasts (lanes 4-7). However, the addition of rat cyt. c to spheroplasts incubated in SY5Y cytosol slightly decreased the amount of unprocessed 32 kDa Caspase-3, compared to the control reaction without rat cyt. c (Figure 3-5A, compare lane 5 with 4). Erk protein is not degraded during apoptosis and can be used to show equal protein loading (Francois and Grimes, 1999). Erk protein was relatively constant in lanes 4-10, indicating that the depletion of Caspase-3 in lane 5 was due to the addition of rat cyt. c and not unequal protein loading (Figure 3-5A, lower panel). Interestingly, Erk protein in the lanes containing spheroplasts (lanes 4-10) appears to be less than in lanes 1-3. This discrepancy may be due to a protease activity in the spheroplasts.

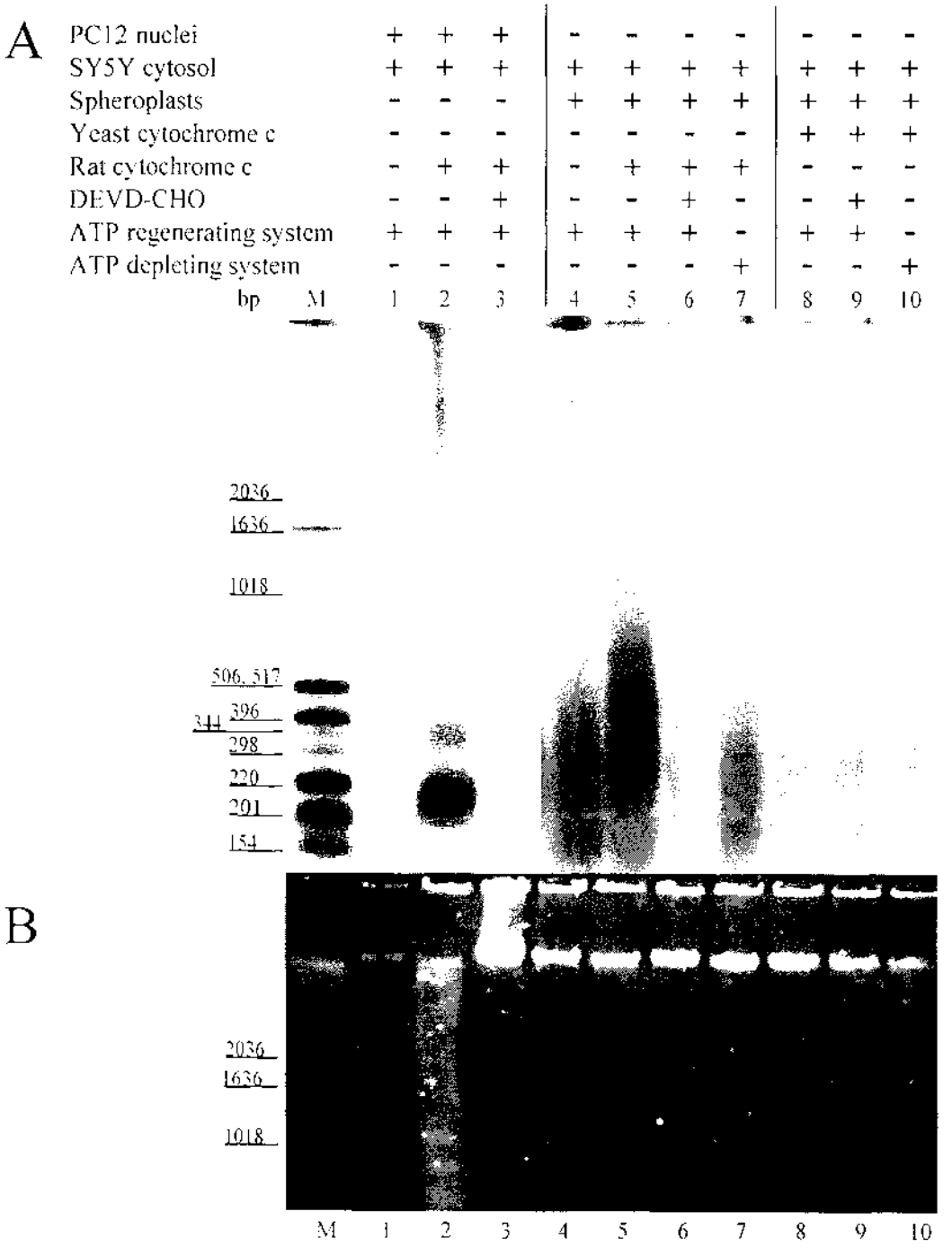


Figure 3-4: Yeast spheroplast DNA cleavage after *in vitro* reactions with activated SY5Y cytosol.

A - SY5Y cytosol incubated with PC12 nuclei (lanes 1-3) or spheroplasts (lanes 4-10) with 3 μM rat cyt. c (lane 2, 3 & 5-7) or 3 μM yeast cyt. c (lane 8-10) and 2 μM DEVD-CHO (lanes 3, 6 & 9) in the presence of an ATP-regenerating system (lanes 1-6, 8-9) or an ATP-depleting system (lanes 7 & 10). DNA was extracted and treated as described in Figure 3-1. Lanes 1-3 were from a 12 hour exposure, while lanes 4-10 are from a 24 hour exposure at 70°C. **B** - Gel from A was incubated with 1×SYBRI Green in 1×TAE for 1 hour and exposed to UV light. The lanes correspond to the gel above in A. Data are representative of 2 or more independent experiments.

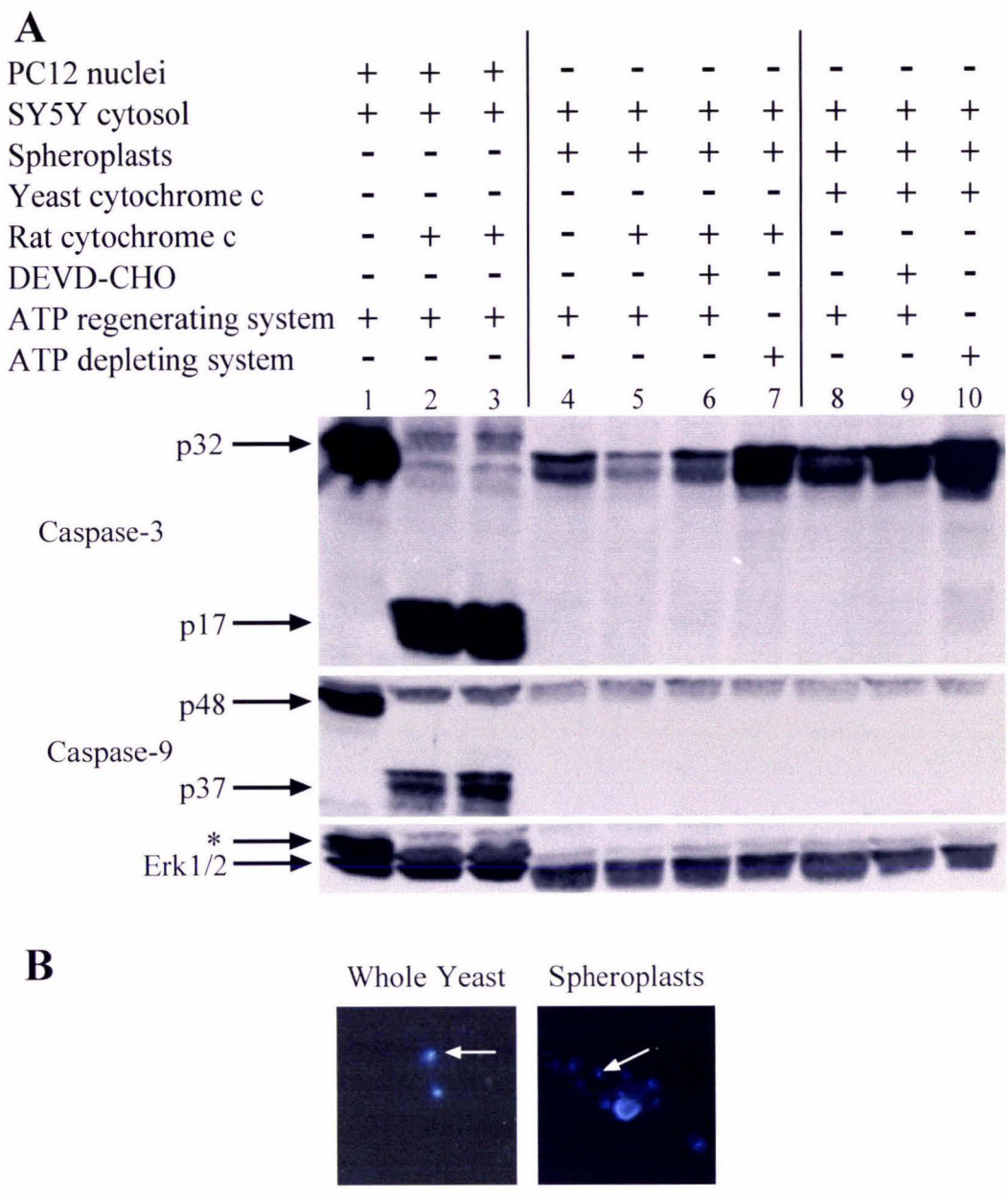


Figure 3-5: Caspase activation in SY5Y cytosol - Morphological comparison of spheroplast and whole yeast cells.

A - Control SY5Y cytosol incubated with PC12 nuclei (lanes 1-3) or spheroplasts (lanes 4-10), with the addition of 3 μM rat cyt. c (lanes 2, 3 & 5-7), or 3 μM yeast cyt. c (lanes 8-10) and 2 μM DEVD-CHO (lanes 6 & 9) in the presence of an ATP-regenerating system (lanes 1-6, 8, & 9) or ATP-depleting system (lanes 7 & 10). Proteins from each reaction were treated as described in Figure 3-1. Membranes were probed for the proteins indicated on the left of the figure using human specific anti-Caspase-3 and Caspase-9 antibodies. Note the uppermost band on the Erk blot marked with *, was signal remaining from previous Caspase-9 immunoblot analysis of the same membrane. Data are representative of 2 experiments. **B** - Spheroplasts incubated with healthy SY5Y cytosol (spheroplasts) are compared with whole yeast cells (whole yeast). Cells were treated as described in Figure 3-1 and observed at 630× magnification. The white arrows indicate a single spheroplast or whole yeast cell. Data are representative of 3 independent experiments.

The decrease in full-length Caspase-3 was ATP dependent, since functional depletion of ATP prevented this in the spheroplast reactions (Figure 3-5A, lane 7). Caspase activation is ATP-dependent and these results are consistent with prevention of caspase activation by ATP depletion (Chou *et al.*, 1995; Francois and Grimes, 1999). However, these results could also indicate that an ATP dependent protease was active in these reactions shown by the marked increase in full-length Caspase-3, over that observed in the control, when reactions were depleted of ATP (Figure 3-5A, compare lane 7 with 4 & 5).

In contrast to Caspase-3, Caspase-9 showed no differences between reactions that contained control SY5Y cytosol, rat cyt. c activated SY5Y cytosol or an ATP depleting system (Figure 3-5A, lanes 4, 5, & 7 respectively). Although, there is less Caspase-9 signal in reactions that contain spheroplasts compared to PC12 nuclei, which may indicate overall protein degradation in spheroplast reactions (compare lanes 4-7 with 1).

Whole yeast and spheroplasts were stained with Hoechst 33342 and compared (Figure 3-5B). While they appeared similar, we could not ascertain whether any morphological changes occurred because yeast nuclei were too small as noted above.

3.2.5 Spheroplast DNA cleavage requires mammalian caspases

The caspase inhibitor DEVD-CHO, a non-cleavable substrate for Caspase-3, was added to *in vitro* reactions to prevent activation of mammalian caspases and their downstream targets. Should mammalian apoptotic proteins upstream of Caspase-3 activate any yeast apoptotic proteins, then apoptotic features would be observed in the spheroplasts independent of the mammalian caspases.

When spheroplasts or PC12 nuclei were incubated with SY5Y cytosol, rat cyt. c and DEVD-CHO, no DNA cleavage was observed (Figure 3-3, lane 4 and 8; summarised in Table 3-1, row 10). This suggests that activation of mammalian caspases is responsible for activating the DNase that cleaves spheroplast DNA.

Immunoblot analysis of Caspase-3 showed that the addition of DEVD-CHO to rat cyt. c-activated reactions prevented the decrease of full-length Caspase-3 (Figure 3-5, compare lane 6 & 5). While we cannot detect the cleaved active form of Caspase-3 in our spheroplast *in vitro* reactions, results point to its activation, i.e. the addition of the caspase inhibitor, DEVD-CHO, inhibits both spheroplast DNA cleavage and depletion of the full-length form of Caspase-3 in reactions with rat cyt. c.

3.2.6 Yeast cyt. c did not induce apoptosis in spheroplasts

We have shown that spheroplast DNA is cleaved by SY5Y cytosol with rat cyt. c. To determine if this cleavage could be induced by yeast cyt. c we incubated spheroplasts with SY5Y cytosol and yeast cyt. c, and assayed for DNA cleavage (Figure 3-4). Yeast cyt. c did not induce DNA cleavage (compare lanes 4 & 8, summarised in Table 3-1, row 12). The addition of DEVD-CHO or an ATP-depleting system had no affect on spheroplast DNA cleavage in yeast cyt. c containing reactions (Figure 3-4A, lanes 9 & 10; summarised in Table 3-1, rows 13 &14). SYBRI Green staining of the gel revealed approximately equal loading of DNA between lanes 4-9, lane 10 had a little less DNA (Figure 3-4B). From these results we conclude that yeast cyt. c did not activate any yeast or mammalian proteins to cleave spheroplast DNA.

Cytosolic proteins from these *in vitro* reactions were immunoblotted for Caspase-3 and Caspase-9 (Figure 3-5). In contrast to rat cyt. c, yeast cyt. c did not reduce the amount of inactive full length Caspase-3 and Caspase-9, nor were the cleaved active forms of both Caspase-3 and Caspase-9 observed in reactions containing yeast cyt. c (Figure 3-5, lane 8 compare with 4). These results indicated that yeast cyt. c did not interact with any proteins, yeast or mammalian, to cause caspase activation. Addition of DEVD-CHO or an ATP-depleting system to yeast cyt. c containing reactions appeared to slightly increase the amount of full length Caspase-3 (compare lane 8 with 9 & 10). The most notable increase was with the ATP-depleting system (lane 10), which as stated above suggests the presence of an ATP-dependent protease.

3.2.7 Yeast cyt. c did not induce apoptosis in PC12 nuclei

We examined the ability of yeast cyt. c to induce apoptosis in *in vitro* reactions with mammalian nuclei (Figure 3-6). Proteins from these reactions were immunoblotted for Caspase-3, Caspase-9, and a PC12 nuclear protein, Parp (Figure 3-6). Neither Caspase-3, Caspase-9 or Parp were cleaved in reactions with yeast cyt. c (lane 4). In contrast, the cleaved fragments of Caspase-3, Caspase-9 and Parp were visible in rat cyt. c-activated reactions (lane 2). Interestingly, in the presence of rat cyt. c and DEVD-CHO, partial processing of Caspase-3 occurred. However, this form was probably inactive as Parp, a downstream cleavage target, was uncleaved (lane 3). Caspase-3, Caspase-9 and Parp were uncleaved in negative control reactions, where PC12 nuclei were incubated with control SY5Y cytosol only (lane 1). Immunoblot analysis of Erk demonstrates that there was equal protein loading in each lane (lower panel). These data suggest that yeast cyt. c was unable to activate the Apaf-1/Caspase-9 pathway in SY5Y extracts because it failed to activate Caspase-9, Caspase-3 and Parp cleavage.

PC12 nuclei from *in vitro* reactions with yeast cyt. c were examined for apoptotic chromatin condensation (Figure 3-7A). Rat cyt. c induced apoptotic chromatin condensation in PC12 nuclei (photo 2), but reactions containing yeast cyt. c did not show apoptotic nuclear morphology (photo 4). This assay is not particularly sensitive, as there must be high caspase activity before apoptotic nuclei can be observed.

We examined PC12 nuclei for internucleosomal DNA cleavage by radioactive end-labelling (Figure 3-7B). Yeast cyt. c was unable to induce DNA cleavage *in vitro*, as rat cyt. c did (lane 4 compared with lane 2). DEVD-CHO prevented both DNA cleavage (lane 3) and chromatin condensation (photo 3) induced by rat cyt. c *in vitro*, which is consistent with both of these events lying downstream of caspase activation.

This data is consistent with evidence from other researchers showing that, unlike rat cyt. c, yeast cyt. c does not induce apoptosis in cell-free extracts from other multicellular organisms (Garland and Rudin, 1998; Kluck *et al.*, 1997b).

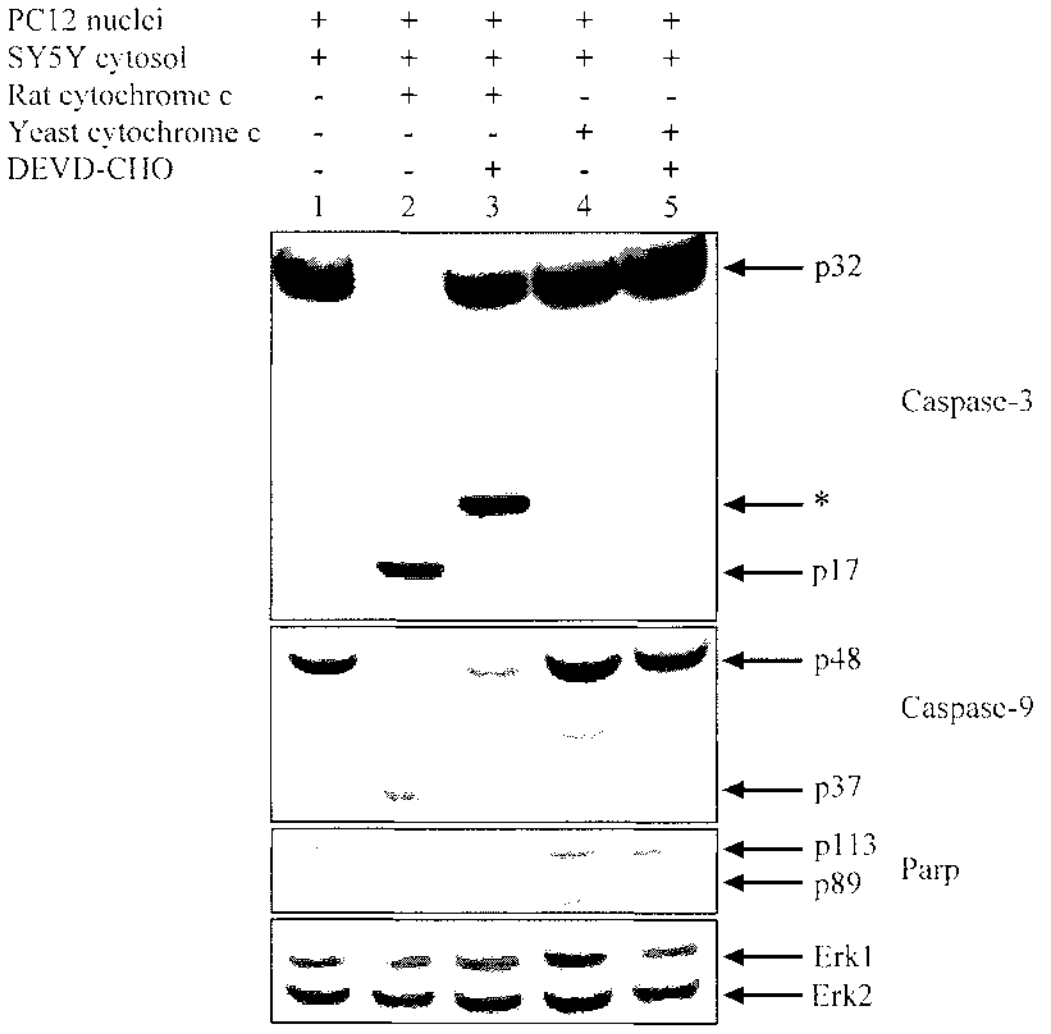


Figure 3-6: Yeast cyt. c does not activate SY5Y caspases.
PC12 nuclei incubated with SY5Y cytosol (lane 1-5) and 3 μM rat cyt. c (lanes 2 & 3) or 3 μM yeast cyt. c (lanes 4 & 5) and 2 μM DEVD-CHO (lanes 3 & 5) in the presence of an ATP regenerating system. Samples were treated as described in Figure 3-1 and probed for proteins indicated. * - Partially processed but presumably inactive form of Caspase-3 as no downstream processing of Parp occurred. This is possibly due to freeze/thawing the inhibitor or saturation of the inhibitor due to high protein levels in the cytosol. Anti-Parp antibodies also react with non-Parp proteins, shown directly below the 89 kDa mark. Data are representative of 3 independent experiments.

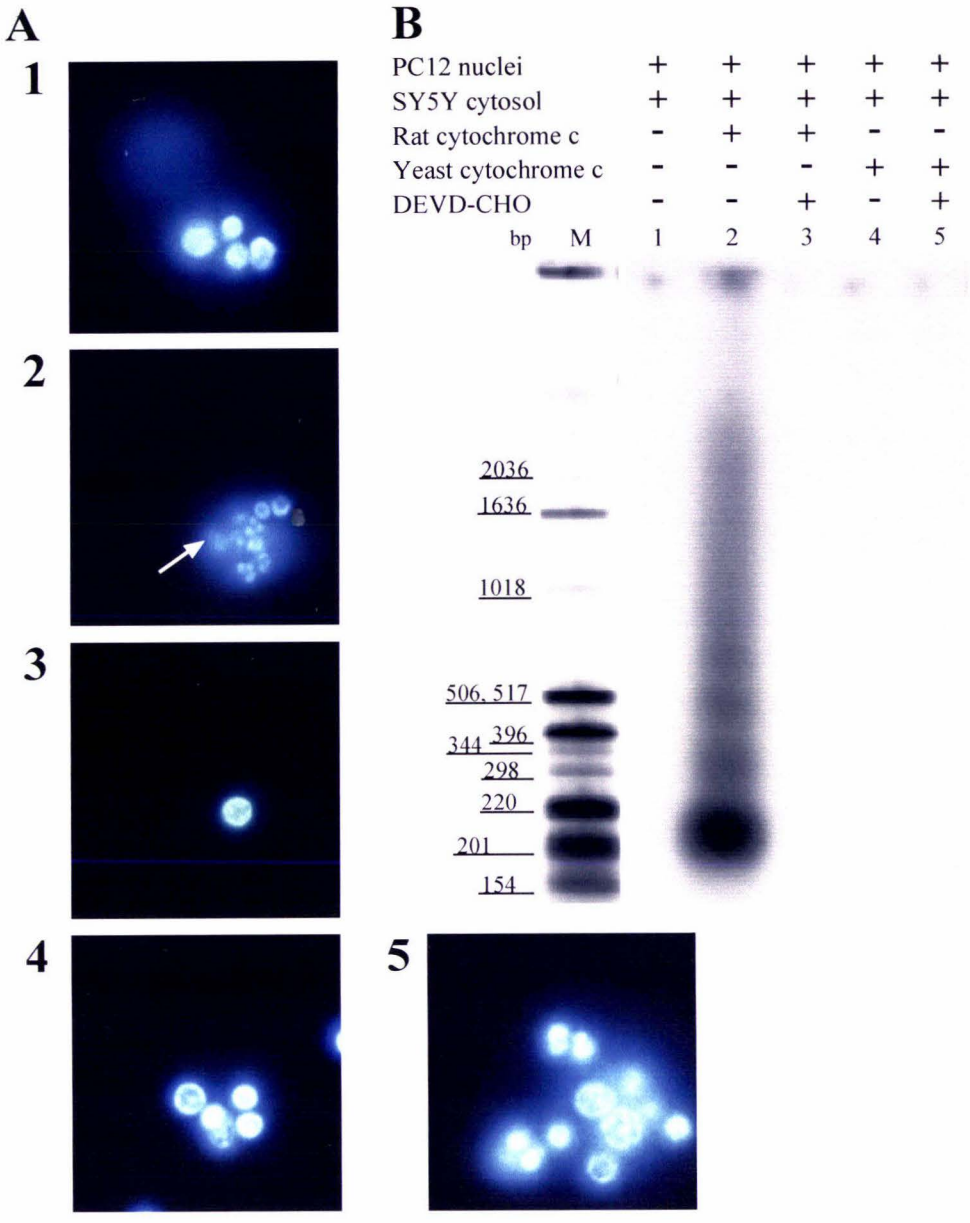


Figure 3-7: Yeast cyt. c did not cause chromatin condensation and DNA cleavage in PC12 nuclei.

A - PC12 nuclei incubated with SY5Y cytosol (Photos 1-5) and 3 μM rat cyt. c (Photos 2 & 3) or 3 μM yeast cyt. c (Photos 4 & 5) and 2 μM DEVD-CHO (Photos 3 & 5), in the presence of an ATP-regenerating system. PC12 nuclei were treated as described in Figure 3-1 and observed at 630× magnification. White arrow indicates fragmented and condensed PC12 nuclei. **B** - PC12 nuclei incubated with SY5Y cytosol (lanes 1-5) and 3 μM rat cyt. c (lanes 2 & 3) or 3 μM yeast cyt. c (lanes 4 & 5) and 2 μM DEVD-CHO (lanes 3 & 5), in the presence of an ATP-regenerating system. DNA was extracted and treated as described in Figure 3-1. Lanes 1-5 were from a 6 hour exposure at room temperature, while M lane was from a 12 hour exposure at –70°C. Data are representative of 2 independent experiments. Approximately 100 nuclei were examined for each condition in each experiment.

3.3 DISCUSSION AND FUTURE WORK

3.3.1 Yeast nuclei are fragile and small

Isolation of yeast nuclei was a difficult procedure that yielded only a small amount of nuclei. DNA isolated from yeast nuclei, after *in vitro* incubation with SY5Y cytosol, was randomly cleaved. However, in one assay yeast nuclear DNA was not cleaved before any *in vitro* manipulation. This suggests that either incubation of yeast nuclei at 37°C, or incubation with SY5Y cytosol caused DNA cleavage. To analyse whether yeast nuclei underwent any morphological changes such as chromatin condensation, electron microscopy should be used as they are too small to analyse by light microscopy.

3.3.2 Spheroplasts undergo caspase dependent apoptosis

We designed a system in which we could reconstitute some features of apoptosis *in vitro* using yeast cell components. In this simple system, spheroplasts incubated with apoptotic SY5Y cytosol showed DNA cleavage, a hallmark feature of apoptosis. While no other apoptotic features were able to be analysed, we discovered that the DNA cleavage observed was dependent on mammalian caspases. Interestingly, the DNA cleavage pattern appeared to be internucleosomal, although the ladder was less distinct than that seen with PC12 nuclei. This discrepancy may be explained by differences in the way DNA is packaged into yeast nucleosomes compared with mammalian nucleosomes. Yeast chromatin organisation is structurally similar to that in mammals (Lohr *et al.*, 1977; Lohr and Van Holde, 1975; Nelson *et al.*, 1977). The main difference is that yeast have a smaller total repeat length of DNA, ~160 base pairs in yeast as opposed to ~185 base pairs in higher eukaryotes (Nelson *et al.*, 1977; Noll, 1974; Shaw *et al.*, 1976; Whitlock and Simpson, 1976). There is also evidence for non-uniformity of the repeating DNA/nucleosome structure in yeast, i.e. there are variances in the length of linker DNA between the nucleosomes (Lohr *et al.*, 1977). This would account for the less distinct DNA ladder seen in apoptotic cleavage of *S. cerevisiae* DNA.

DNA fragmentation in the spheroplast was dependent on the activity of mammalian effector caspases, because the caspase inhibitor, DEVD-CHO, abolished internucleosomal

DNA cleavage in spheroplasts. Apparently, no other yeast proteins took over to induce this cleavage. However, it is puzzling that cleaved forms of the caspases were not detected in spheroplasts *in vitro* reactions. The sensitivity of immunoblot analysis is not very high and cleaved Caspase-3 may not have had a strong enough signal to be observed in the reactions with spheroplasts. We also cannot discount the possibility that spheroplast proteins degraded the active caspases and perhaps the uncleaved caspase form was not as susceptible to this degradation. If there was a spheroplast protease that cleaved the caspases it would have to leach out of the spheroplasts to cleave the caspases, or alternatively, the caspases could be transported into the spheroplasts and be cleaved there. The protease involved in this degradation of the caspases was ATP-dependent since ATP depletion from the spheroplast *in vitro* reactions substantially prevented disappearance of inactive Caspase-3.

During apoptosis in mammals, Icad is cleaved by Caspase-3, releasing Cad and allowing it to translocate to the nucleus (Figure 3-8) (Sakahira *et al.*, 1998). In our *in vitro* system we hypothesise that Caspase-3 is active (shown by the disappearance of the inactive form of Caspase-3) and activates a DNase, which may be mammalian Cad or yeast DNases. This DNase was able to pass through the permeable cell membrane of the spheroplast, or was already present in the spheroplast, and enters the nucleus causing DNA cleavage (Figure 3-8). The DNase that was activated appears to have a preference for accessible DNA such as that found in the linking regions between nucleosomes. To check if the activated DNase was Cad, we would need to immunoblot the *in vitro* reactions using antibodies for both Icad and Cad.

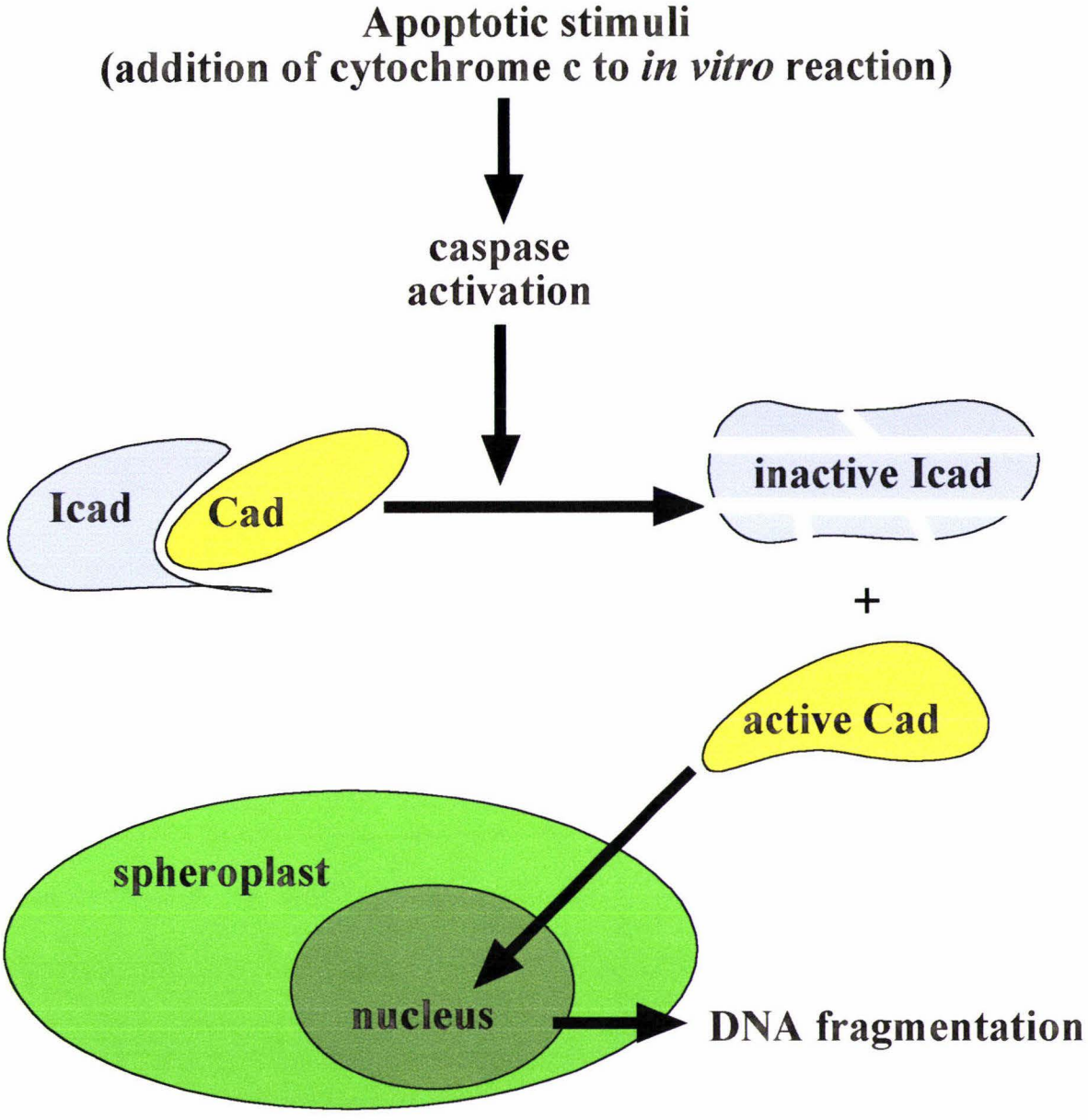


Figure 3-8: Model of Cad activation and the cleavage of spheroplast DNA.
Active Caspase-3 cleaves the inhibitory subunit of Cad (Icad) revealing a nuclear localisation signal on Cad (Enari *et al.*, 1998; Sakahira *et al.*, 1998). We hypothesise that active Cad enters the nucleus of the spheroplasts and where it carries out internucleosomal cleavage of genomic DNA.

3.3.3 Yeast cyt. c could not induce apoptosis

Although rat cyt. c induced features of apoptosis using mammalian cell-free extracts with spheroplasts (Sections 3.2.3 & 3.2.4) we found that yeast cyt. c was unable to induce these features in either spheroplast or mammalian *in vitro* systems (Sections 3.2.6 & 3.2.7). While yeast and rat cyt. c are structurally and functionally very similar, there are differences between these two proteins, which may explain differences in their ability to induce apoptosis *in vitro*.

Yeast modify their cyt. c proteins post-translationally, to give rise to a trimethylated lysine (Lys 72) residue, which is in the active site of the enzyme (Paik *et al.*, 1989). This modification enhances the protein's import into mitochondria (Park *et al.*, 1987). Although cytochrome c from tuna, pigeon and horse is not normally methylated, when these proteins are expressed in yeast, they become trimethylated at Lys 72 (Hickey *et al.*, 1991; Paik *et al.*, 1989). However, methylation of yeast cyt. c does not explain its failure to induce apoptosis *in vitro* since yeast cyt. c purified from a yeast strain deficient in lysine methylation also does not induce apoptosis in mammalian cells (personal communication, D. Newmeyer).

There are some structural differences between yeast and vertebrate cytochrome c in the vicinity of the asparagine (Asn) 52 side chain, which may account for the inability of yeast cyt. c to induce apoptosis (Koshy *et al.*, 1994). Other differences in the structure and function of the protein could be responsible for the differences in their ability to activate the apoptotic mechanism (personal communication, D. Newmeyer).

In summary, we have designed a system in which we could reconstitute some features of apoptosis using yeast and mammalian cell components. Induction of apoptotic features in the spheroplasts was dependent on mammalian caspase proteins. We could not identify any novel yeast proteins that may be involved in the induction of apoptotic features in yeast.

CHAPTER 4: YEAST *IN VITRO* MODEL OF APOPTOSIS - II

4.1 INTRODUCTION

Rat cyt. c-activated SY5Y cytosol caused internucleosomal cleavage of spheroplast DNA (Chapter 3). However, this cleavage appeared to be dependent upon the activation of mammalian caspases. In order to determine if there were any yeast proteins capable of inducing features of apoptosis, we designed a second yeast *in vitro* system using yeast cytosol.

As previously described (Section 1.8.2), yeast show features of apoptosis in certain circumstances. We hypothesised that yeast cytosolic proteins could be activated to cause apoptotic changes in PC12 nuclei. Since SY5Y cytosol activated by rat cyt. c causes apoptosis in PC12 nuclei, we attempted to activate yeast cytosol with rat cyt. c. Although an Apaf-1 homologue has not been identified in yeast, a similar protein may exist to activate other proteins to induce apoptotic changes in PC12 nuclei.

In this system, isolated PC12 nuclei (prepared according to the protocol in Section 2.3.5) were incubated with cytosol derived from *S. cerevisiae* (prepared according to the protocol in Section 2.3.8) in the presence and absence of rat or yeast cyt. c (Figure 4-1). Following incubation *in vitro*, the reactions were assayed for DNA cleavage and morphological changes.

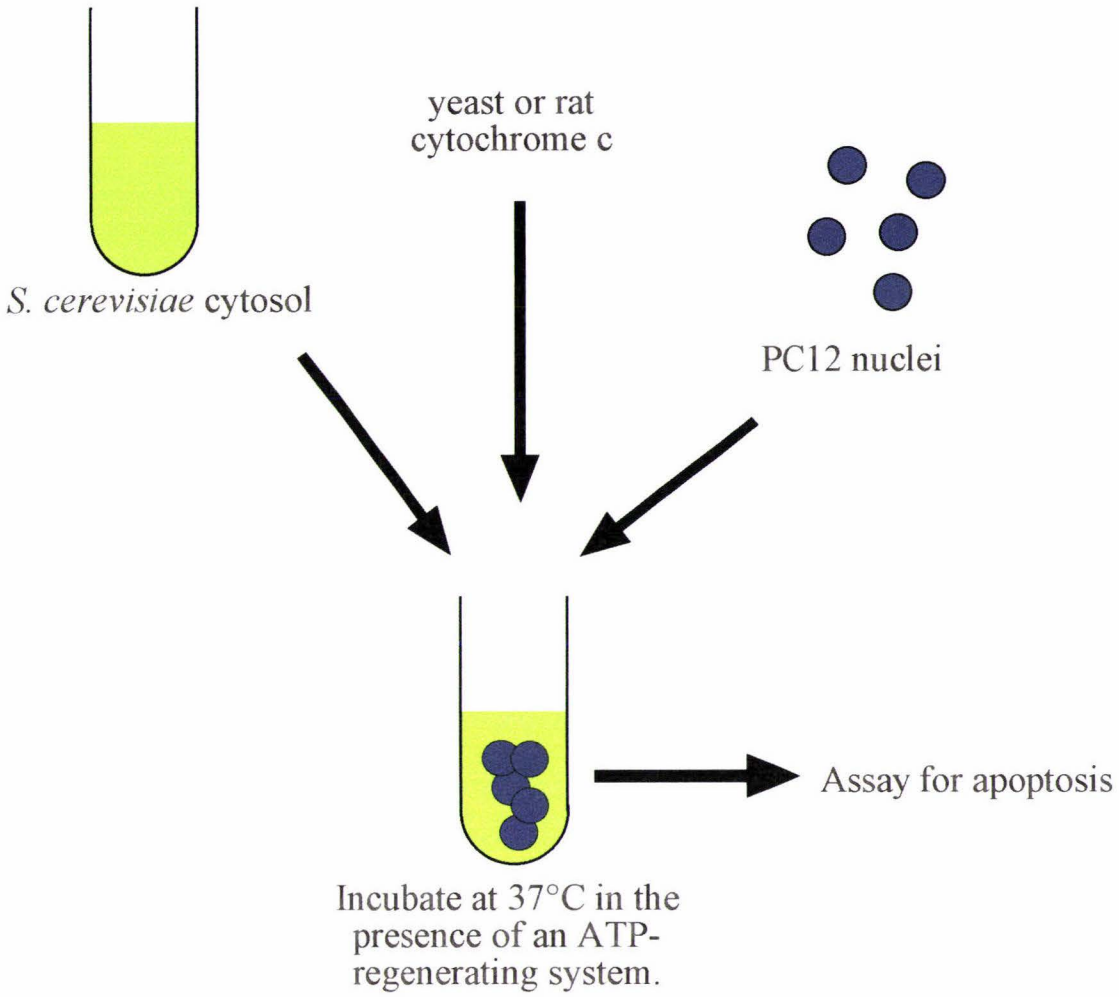


Figure 4-1: Experimental *in vitro* protocol using yeast cytosol.
Cytosol derived from *S. cerevisiae* was incubated with PC12 nuclei and yeast or rat cyt. c in the presence of an ATP-regenerating or depleting system. After incubation, the reactions were assayed for the induction of apoptosis as described in Figure 3-1.

4.2 RESULTS

4.2.1 Yeast cytosol causes cleavage of PC12 DNA

PC12 nuclei were incubated with yeast cytosol in the presence or absence of cyt. c and then assayed for DNA cleavage by end-labelling PC12 DNA followed by gel electrophoresis (Figure 4-2A). When PC12 nuclei were incubated with yeast cytosol and rat cyt. c, a ladder-type cleavage pattern was observed (lane 5). This pattern was similar to, but more smeared than, the ladder pattern seen when PC12 nuclei were incubated with apoptotic SY5Y cytosol (lane 2). Interestingly, incubation of PC12 nuclei with yeast cytosol and ATP alone also produced a very faint DNA ladder (lane 4). In fact, in all experiments conducted a DNA ladder was observed when PC12 nuclei were incubated in yeast cytosol and ATP (Table 4-1, row 1). The addition of rat cyt. c to yeast cytosol enhanced PC12 DNA cleavage (Figure 4-2A, compare lane 4 with 5). Summarising the results from six experiments, three experiments showed an increase of DNA cleavage when rat cyt. c was added to yeast cytosol (Table 4-1, compare rows 1 & 3). While these results were somewhat ambiguous, there may be an effect caused by the addition of rat cyt. c to these reactions.

No major conclusions could be made about the role of ATP in yeast cytosol-induced DNA cleavage. Functional depletion of ATP in rat cyt. c-containing reactions slightly decreased PC12 DNA cleavage (Figure 4-2A, compare lane 5 with 6). However, when the results of three experiments were analysed this pattern was not repeated, as ATP depletion increased PC12 DNA cleavage in one experiment but decreased cleavage in another (Table 4-1, compare rows 3 & 5). In the absence of cyt. c functional depletion of ATP from yeast cytosol during *in vitro* reactions was not consistent in its effect on PC12 DNA cleavage (Figure 4-2A, compare lane 4 with 11; Table 4-1, row 2).

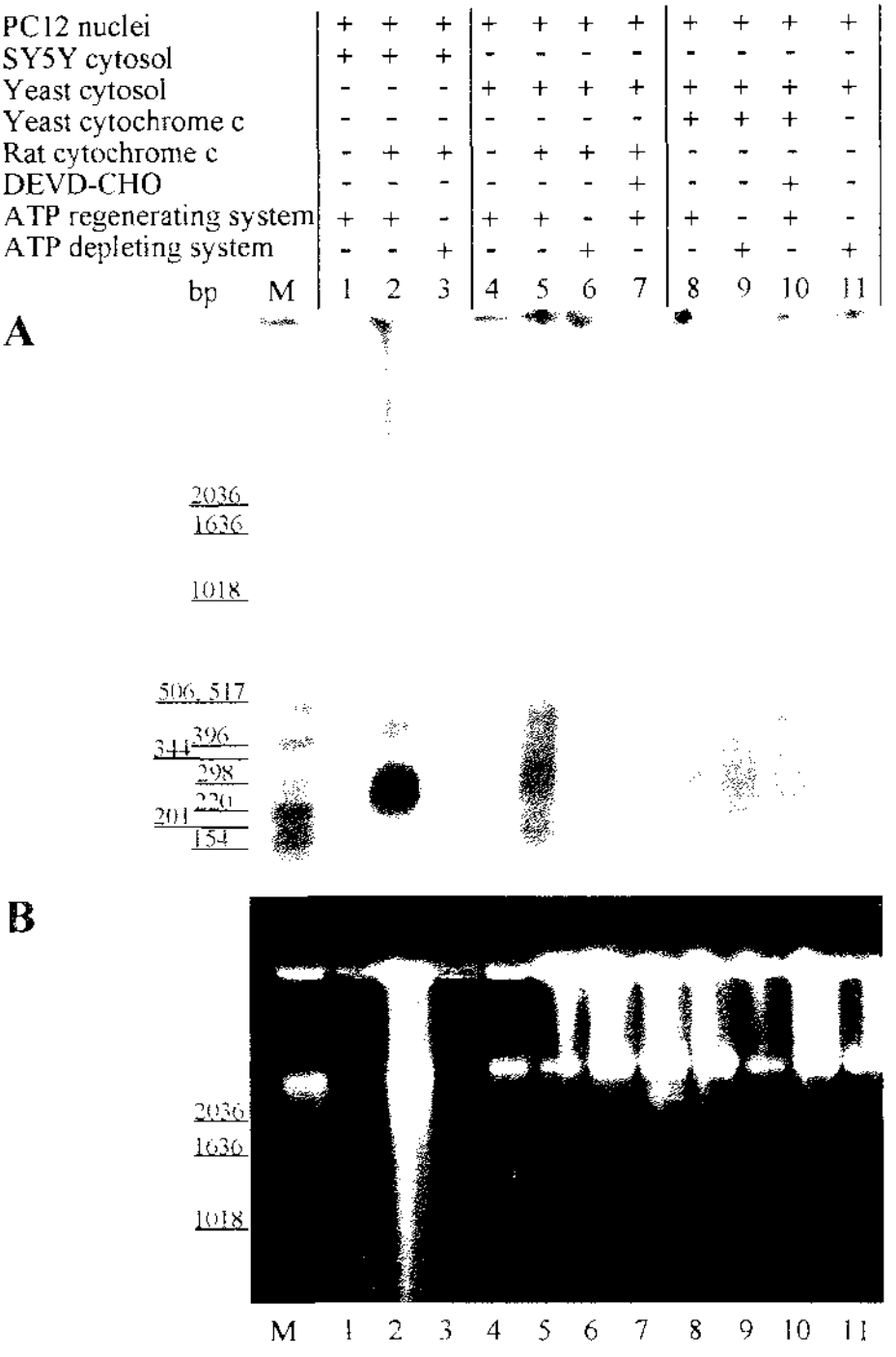


Figure 4-2: Yeast cytosol contains DNase activity.
A - PC12 nuclei incubated with SY5Y cytosol (lanes 1-3) or yeast cytosol (lanes 4-11), and 3 μM rat cyt. c (lanes 2, 3 & 5-7) or 3 μM yeast cyt. c (lanes 8-10), with 2 μM DEVD-CHO (lanes 3, 7 & 10) in the presence of an ATP-regenerating system (lanes 1, 2, 4, 5, 7, 8 & 10) or ATP-depleting system (lanes 3, 6, 9 & 11). DNA was extracted and treated as described in Figure 3-1. Lanes 1-11 were from a 12 hour exposure, M lane from a 24 hour exposure, at 70°C. **B** - The dried gel from A was stained with SYBRI Green as described in Figure 3-7. The lanes in B correspond with the lanes in A. Data are representative of 3 or more independent experiments. Note uncleaved DNA did not migrate into the gel and remains in the wells of the gel.

Row	Experiment / Reaction Components	1	2	3	4	5	6	Summary of changes
1	PC12 nuclei, yeast cytosol	++	+++	+	++	++	++	Control
2	PC12 nuclei, yeast cytosol, ATP depleted	+	+++	++	+			No overall changes
3	PC12 nuclei, yeast cytosol, rat cyt. c	+	+++	++++	+++	++	+++	3/6 increases vs. row 1
4	PC12 nuclei, yeast cytosol, rat cyt. c, DEVD-CHO	++	+	—	—	+++	+	4/6 decreases vs. row 3
5	PC12 nuclei, yeast cytosol, rat cyt. c, ATP depleted	+++	+++	+++				No overall changes vs. row 3
6	PC12 nuclei, yeast cytosol, yeast cyt. c	++	+++	+++		+++	++	2/5 increases vs. row 1
7	PC12 nuclei, yeast cytosol, yeast cyt. c, ATP depleted	++	+++	+++				No overall changes vs. row 6
8	PC12 nuclei, yeast cytosol, yeast cyt. c, DEVD-CHO	++	+++	+++		+++	++	No overall changes vs. row 6

Table 4-1: Pooled data from PC12 nuclei with yeast cytosol *in vitro* reactions.
The results of 6 independent experiments are summarised. PC12 nuclei were incubated with yeast cytosol and DNA fragmentation analysed as in Figure 4-2. The amount of DNA cleavage was tabulated and scored as — to ++++ indicating no cleavage to dark intensity of internucleosomal DNA cleavage. Summary is defined as the number of experiments over total experiments that showed an increase or decrease of DNA cleavage compared to specified experiments i.e. vs. n, where n is the row of experiments. All reactions were in the presence of an ATP-regenerating system, except where an ATP-depleting system was added. Data shown in Figure 4-2 is experiment 3.

When PC12 nuclei were incubated with yeast cytosol and rat cyt. c, the further addition of the caspase inhibitor, DEVD-CHO, inhibited PC12 DNA cleavage (Figure 4-2, lane 7). To ensure DNA was actually present in this lane, the gel was stained with SYBRI green and visualised under UV light to show total DNA (Figure 4-2B). This result was reproduced in 4 of 6 experiments conducted (Table 4-1, compare rows 3 & 4). These data, together with rat cyt. c's effects, suggest that a Caspase-3-like activity was responsible for DNA cleavage in these reactions.

We asked whether yeast cyt. c, when added to yeast cytosol, was able to induce PC12 DNA cleavage. As yeast cytosol already caused some DNA cleavage, we were looking for an increase in DNA cleavage over background. The addition of yeast cyt. c to PC12 nuclei and yeast cytosol enhanced the level of PC12 DNA cleaved in the experiment shown in Figure 4-2 (compare lane 4 to 8), but this was not reproducible. There were slight increases in DNA cleavage in two of five experiments (Table 4-1, rows 6 & 1). Neither the addition of DEVD-CHO to yeast cyt. c-activated yeast cytosol or the depletion of ATP from yeast cyt. c-activated yeast cytosol made any difference to the level of DNA cleavage observed (Figure 4-2, compare lanes 8, 9 & 10) and these results were consistently reproduced (Table 4-1, rows 7 & 8).

4.2.2 Yeast cytosol did not cause chromatin condensation

Internucleosomal cleavage of DNA is but one hallmark of apoptosis and observation of this phenomenon did not necessarily mean apoptosis was occurring. We examined the PC12 nuclei for chromatin condensation, a morphological marker of apoptosis (Figure 4-3A). PC12 nuclei incubated with yeast cytosol (Reaction 4) appeared identical to healthy nuclei, previously shown in Chapter 3 (Figure 3-2B, Reaction 1). In fact, there were no morphological changes observed with any PC12 nuclei when they were incubated with yeast cytosol alone (Reaction 4), rat cyt. c (Reaction 5), or with rat cyt. c and DEVD-CHO (Reaction 7). PC12 nuclei incubated with yeast cytosol and yeast cyt. c (Reaction 8), DEVD-CHO (Reaction 10), or an ATP-depletion system (Reaction 9) also did not display apoptotic morphology.

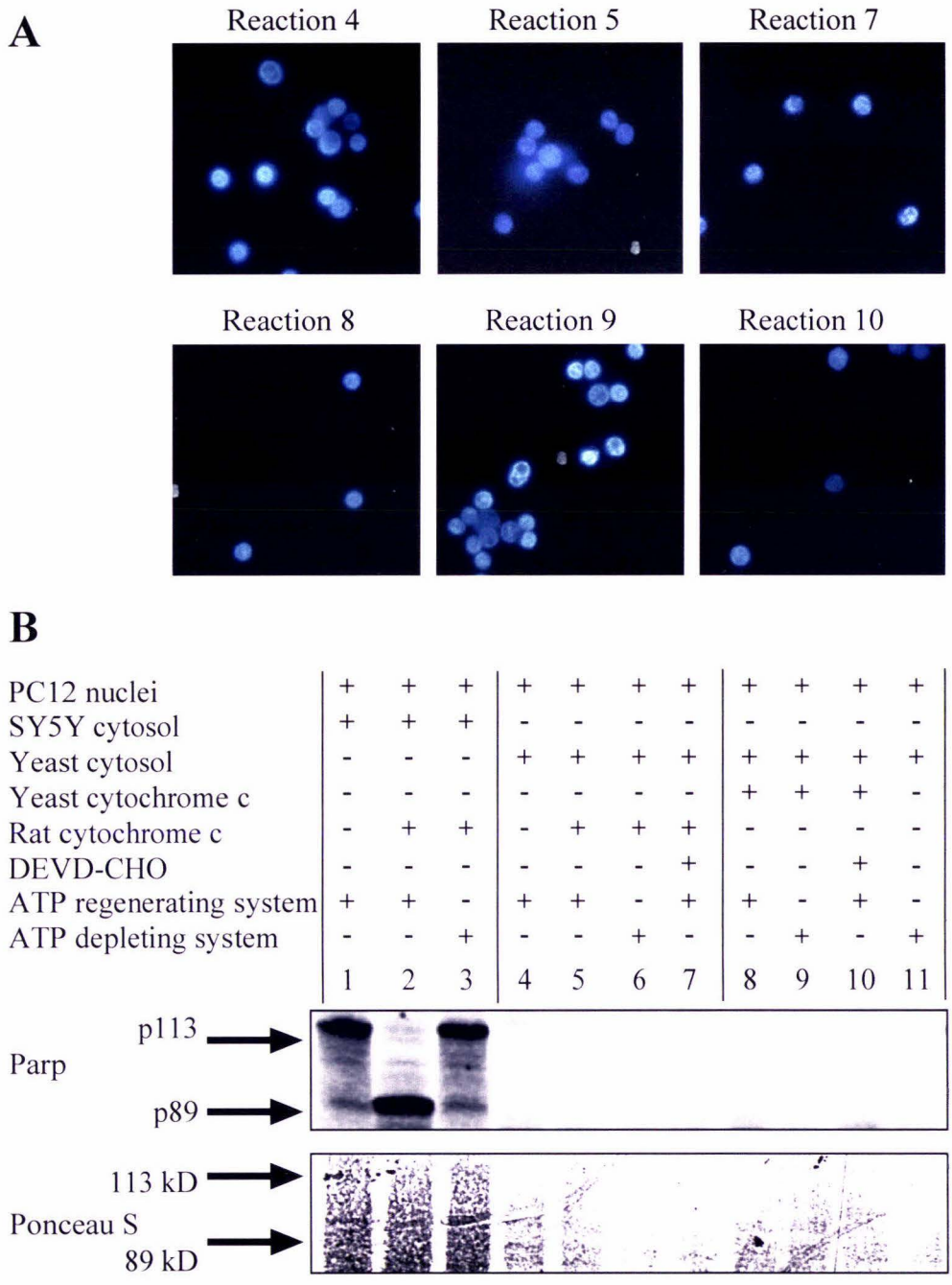


Figure 4-3: Morphological and protein examination of PC12 nuclei incubated with yeast cytosol.

A - PC12 nuclei stained treated as described in Figure 3-1 and observed at 400× magnification. Reaction numbers above the photos correspond to the reactions in B. **B** - PC12 nuclei incubated with SY5Y cytosol (lanes 1-3) or yeast cytosol (lanes 4-11), and 3 μM rat cyt. c (lanes 2, 3 & 5-7) or 3 μM yeast cyt. c (lanes 8-9), with 2 μM DEVD-CHO (lanes 7 & 10) in the presence of an ATP-regenerating system (lanes 1-5, 7, 8 & 10) or ATP-depleting system (lanes 3, 6, 9 & 11). Samples were treated as described in Figure 3-1 and membranes probed for Parp (upper panel) after being stained with a total protein dye, Ponceau S (lower panel). Data are representative of 3 or more independent experiments.

4.2.3 Yeast cytosol may degrade Parp

There are no known caspases in yeast, so we cannot examine these reactions using caspase antibodies. However, we can determine whether Parp, a PC12 nuclear protein and substrate for caspases, has been inactivated by cleavage.

In the control reactions, PC12 nuclei were incubated with SY5Y cytosol and full length 113 kDa Parp was observed (lane 1). When rat cyt. c was added to this reaction, caspases were activated, which cleaved Parp to its 89 kDa form (lane 2). Rat cyt. c-activated SY5Y cytosol was prevented from cleaving Parp by the addition of the caspase inhibitor, DEVD-CHO (lane 3). However, when PC12 nuclei were incubated with yeast cytosol, no protein bands immunoreacted with the Parp antibody (lanes 4-11). It is possible that Parp was cleaved at a different place that is not recognised by the antibodies we have. The immunoblot membrane was stained with Ponceau S, which stains total proteins, and showed that there were not many high molecular weight proteins in the reactions with yeast cytosol. This could mean that protein degradation occurred during *in vitro* reactions, or that there are not many high molecular weight proteins in yeast cytosol, which is an unlikely explanation. Note that the protein concentration of yeast cytosol in these reactions was lower than the SY5Y cytosol and, to ensure approximately equal protein loading, more sample from reactions containing yeast cytosol was loaded on the gel. Thus, approximately 1 1/2 times more PC12 nuclei were loaded from reactions containing yeast cytosol, so the lack of Parp signal is not due to a lack of PC12 nuclei in these lanes (Figure 4-3B, lanes 4-11).

4.3 DISCUSSION AND FUTURE WORK

4.3.1 Discussion

The results presented in this section show that yeast cytosol caused the formation of a DNA ladder of PC12 DNA, which was more often than not inhibited by DEVD-CHO in the presence of rat cyt. c. Yeast cytosol either cleaved Parp in a manner that we could not detect or degraded the protein completely. Apoptotic morphology in PC12 nuclei was not induced by yeast cytosol. There are several possible explanations for these results.

A DNase may be activated during yeast cytosol preparation that cleaves PC12 nuclear DNA. Mammalian cells package their DNA very tightly around nucleosomes, leaving only small linker pieces of DNA accessible to DNases. Over time, more DNA would become susceptible to cleavage as nucleosomal proteins were degraded, hence the smeared DNA ladder observed. At present we cannot distinguish whether DNA cleavage observed in PC12 nuclei was apoptotic or non-specific degradation.

The fact that DEVD-CHO inhibited DNA cleavage in the presence of rat cyt. c-activated yeast cytosol in four of six experiments suggests that the DNase may be caspase-activated rather than constitutively active. However, DEVD-CHO did not always abolish DNA cleavage when added to yeast cyt. c-activated yeast cytosol. As there are no known yeast caspases, and no other caspase-activated features of apoptosis, such as chromatin condensation were observed we could not conclude that the internucleosomal DNA observed in PC12 cells was caspase activated.

It is possible that PC12 nuclei might be contaminated with cytosolic caspases and downstream DNases, even though nuclei were washed several times during their isolation before use in the *in vitro* reactions. We cannot check for caspase contamination by immunoblot analysis as the antibodies we have are human-specific and do not react with rat caspases. We can however, measure cleavage of fluorogenic or chromogenic caspase substrates to determine if isolated PC12 nuclei contain caspases. However, if caspases were present, for rat cyt. c to induce their activation, both Apaf-1 and ATP would also need to be present. While we cannot check for the presence of Apaf-1, previous work shows that rat cyt. c incubated with PC12 nuclei and ATP does not induce DNA cleavage, therefore it is unlikely that PC12 nuclei contain caspases and Apaf-1 (Francois and Grimes, 1999). Depletion of ATP from reactions yeast cytosol, either alone or with rat or yeast cyt. c produced ambiguous results; both increases and decreases in DNA cleavage were observed (Table 4-1, compare row 1 with 2, 3 with 5, and 6 with 7). From these results we could not conclude anything about the dependence of PC12 DNA cleavage on ATP.

To explain the lack of full length or cleaved Parp in reactions containing yeast cytosol, further work will be required. Ponceau S-stained protein membranes showed a distinct lack of high molecular weight proteins in reactions with yeast cytosol, which could indicate non-specific degradation. It is also possible that Parp was cleaved by another protein such as yeast Calpain. Calpain, a cytosolic cysteine protease, is activated during both necrotic and apoptotic cell death (Wang, 2000). It is known to cleave a number of cellular proteins including Parp to yield fragments that range from 70 kDa to 40 kDa, rather than the 89 kDa and 24 kDa fragments generated by the proteolytic action of Caspase-3 (Buki *et al.*, 1997; Sallmann *et al.*, 1997; Shah *et al.*, 1996; Wang, 2000). A putative Calpain-like cysteine protease gene called *CPL1* from *S. cerevisiae* encodes for a protein that has cysteine protease activity (Futai *et al.*, 1999). This protein is required for alkaline adaptation and sporulation and may be activated during the preparation of yeast cytosol. If Calpain cleaved Parp, then it is possible that the Parp fragments were not recognised by the antibody we used.

4.3.2 Future Work

To determine if there is any caspase-like activity in the yeast cytosol we could look for caspase-like activity in yeast cytosol using assays that measure cleavage of fluorogenic or chromogenic caspase substrates. The existence of Calpain-like activity in the reactions could also be tested with a fluorogenic substrate specific for Calpain. We cannot determine if Calpain was present in yeast cytosol by immunoblot analysis as there are no commercial antibodies available. However, should Calpain activity exist in *S. cerevisiae* we could determine if Parp, a Calpain substrate, was cleaved at alternative sites by using anti-Parp antibodies that recognise the alternatively cleaved fragments of Parp.

We have been unable to cause apoptotic changes in PC12 nuclei using cytosol derived from healthy yeast treated with rat or yeast cyt. c. Other methods of activating yeast cytosol to cause apoptotic changes in PC12 nuclei could be attempted. For instance, cytosol derived from yeast that showed features of apoptosis such as the *S. cerevisiae* tsCdc48^{S565G} mutant or oxygen-stressed yeast, may induce apoptotic changes in PC12 nuclei.

CHAPTER 5: PC12 *IN VITRO* MODEL OF APOPTOSIS

5.1 INTRODUCTION

The PC12 cell line is a model cell line for undifferentiated neurons, which can be induced to differentiate when cultured in the presence of NGF, much like neurons. Our laboratory has an interest in neural disorders, hence the reason behind using this cell line. An original aim of our laboratory was to reconstitute apoptosis *in vitro* using components from undifferentiated and differentiated PC12 cells. These systems could be used to study differences, if any, between differentiated cell death and undifferentiated cell death mechanisms.

Our laboratory has attempted to reconstitute *in vitro* apoptosis using purified PC12 nuclei incubated with cytosol derived from either serum-withdrawn PC12 cells or healthy PC12 cells with rat cyt. c (personal communication, F. Francois). There were many technical difficulties with this system. One of the main problems encountered by other members of our laboratory was that cytosol derived from the PC12 cells had very low protein concentrations (~3-5 mg/ml). PC12 cells grow relatively slowly compared with SY5Y cells and they are sensitive to overcrowding. In this next chapter, we attempted to overcome some of these technical difficulties and reconstitute apoptosis using only PC12 cell components.

5.2 RESULTS

Cytosol was prepared from approximately $1.1x10^8$ PC12 cells, which were grown to 80% confluence. The protein concentration of this PC12 cytosol was ~16 mg/mL and it was used for *in vitro* reconstitution of apoptosis. We could not examine these *in vitro* reactions for Caspase-3 or Caspase-9 activation, as the antibodies we had are specific for human caspases. However we could examine PC12 nuclei from *in vitro* reactions for cleavage of Parp, a downstream caspase substrate. Parp was cleaved from its active 113 kDa form to

89 kDa during *in vitro* reconstitution of apoptosis with rat cyt. c-activated SY5Y cytosol (lane 2). Parp cleavage was dependent on caspase activation as the addition of the caspase inhibitor, DEVD-CHO, abolished Parp cleavage (lane 3) (Francois and Grimes, 1999). In contrast, rat cyt. c-activated PC12 cytosol did not induce Parp cleavage to an 89 kDa fragment (lane 5). Parp was not cleaved in the absence of rat cyt. c, as expected (lane 4). As Parp was not cleaved by rat cyt. c-activated PC12 cytosol, we cannot determine if DEVD-CHO had any effect (lane 6).

PC12 nuclei from these *in vitro* reactions were examined for the appearance of apoptotic morphology (Figure 5-1B). PC12 nuclei incubated with PC12 cytosol alone had the appearance of healthy nuclei, as expected (Reaction 4). The addition of rat cyt. c to the *in vitro* reaction did not induce chromatin condensation (Reaction 5). We could not determine whether DEVD-CHO inhibited chromatin condensation, as rat cyt. c did not activate PC12 cytosol to induce apoptotic morphology in PC12 nuclei. Chromatin condensation is a very late event during apoptosis and perhaps PC12 cytosol takes longer to induce this feature of apoptosis. As we had not observed Parp cleavage, we did not really expect to observe chromatin condensation.

Both morphological and immunoblot analysis of PC12 nuclei, from *in vitro* reactions, are not particularly sensitive assays, so internucleosomal DNA fragmentation was employed to determine whether PC12 cytosol was activated by rat cyt. c to cause apoptosis *in vitro*. PC12 DNA was end-labelled after *in vitro* incubations with rat cyt. c-activated PC12 cytosol. Initially, when PC12 nuclei were incubated with activated PC12 cytosol no internucleosomal DNA ladder was observed (data not shown).

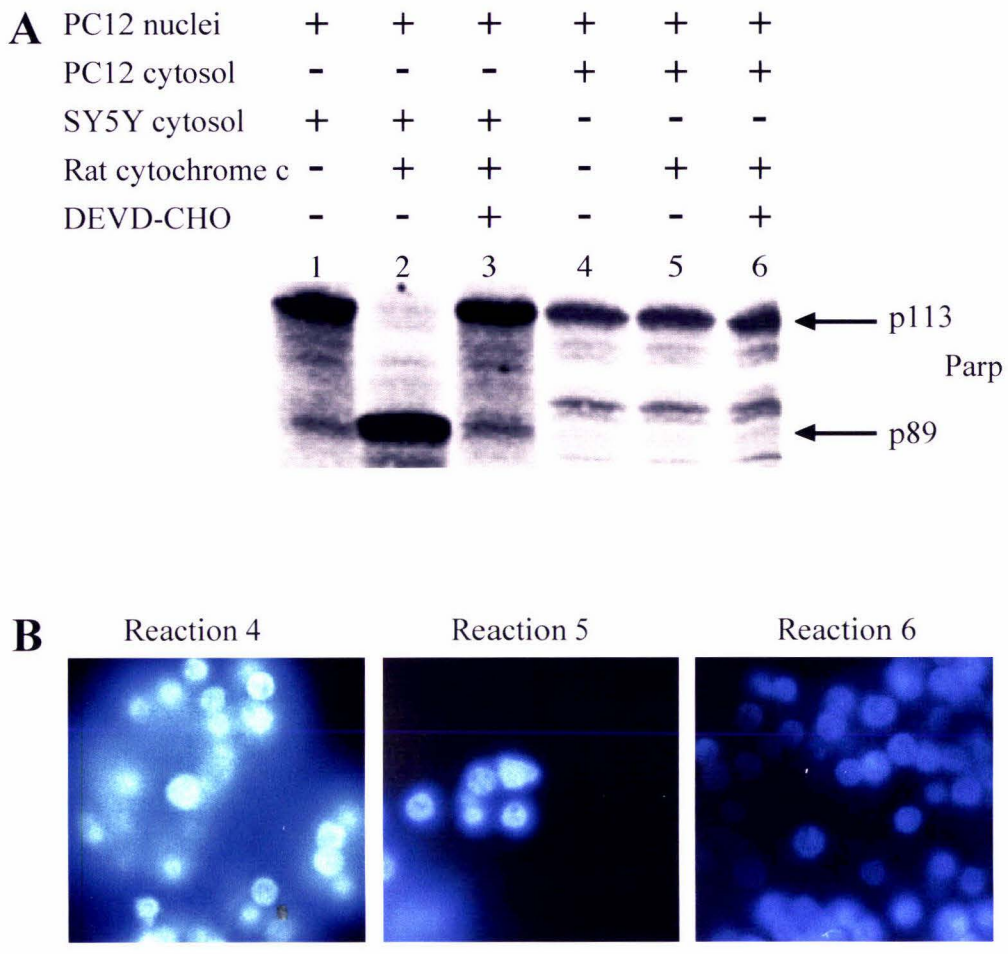


Figure 5-1: Rat cyt. c did not activate PC12 cytosol to cleave Parp or cause apoptotic morphology in PC12 nuclei.

A - PC12 nuclei incubated with SY5Y cytosol (lanes 1-3) or PC12 cytosol (lanes 4-6) with 3 μM rat cyt. c (lanes 2, 3, 5 & 6) and 2 μM DEVD-CHO (lanes 3 & 6). Samples were treated as described in Figure 3-1 and membranes probed for Parp. **B** - PC12 nuclei treated as described in Figure 3-1 and observed at 630× magnification. The reaction numbers above the photos corresponds to the reaction in A. Data are representative of 2 independent experiments.

However, a second attempt at this experiment did produce a faint DNA ladder (Figure 5-2A, lane 2), compared to that produced when PC12 nuclei were incubated with activated SY5Y cytosol (lane 5). When a longer exposure of this experiment was examined a definite DNA ladder was observed when PC12 nuclei was incubated with PC12 cytosol (lane 2'). However, the majority of total DNA from activated PC12 cytosol *in vitro* reactions was mostly uncleaved and remained near the top of the gel compared with DNA from activated SY5Y cytosol *in vitro* reactions (Figure 5-2B, compare lane 2 with 5). The DNA cleavage caused by PC12 cytosol was specifically induced by rat cyt. c because *in vitro* reactions without rat cyt. c did not produce any DNA cleavage (lane 1). DNA cleavage induced by PC12 cytosol, like that of SY5Y cytosol, was caspase dependent, because addition of the caspase inhibitor, DEVD-CHO, abolished DNA cleavage (lanes 3, 3', and 6, respectively). These data suggest that PC12 cytosol may be activated by rat cyt. c to cause internucleosomal DNA cleavage in PC12 nuclei. However, no other features of apoptosis were observed with this system, which suggests that the apoptotic mechanism was only weakly activated in PC12 cytosol by rat cyt. c.

5.3 DISCUSSION AND FUTURE WORK

Initial experiments with this PC12 *in vitro* system were not successful and it was believed that low PC12 cytosolic protein concentrations accounted for its lack of ability to induce apoptosis, when activated by rat cyt. c. We overcame this problem by preparing highly concentrated PC12 cytosol, a concentration 1 1/4 times greater than the usual concentration of SY5Y cytosol (~16 mg/mL vs. ~12 mg/mL, respectively). *In vitro* reconstitution of apoptosis using rat cyt. c-activated PC12 cytosol did not induce apoptotic features such as chromatin condensation and Parp cleavage and only weakly caused DNA fragmentation of PC12 DNA in one experiment. In contrast, rat cyt. c-activated SY5Y cytosol induced all three hallmarks of apoptosis in PC12 nuclei. Therefore, the cytosolic protein concentration was not the limiting factor in the induction of apoptosis by PC12 cytosol.

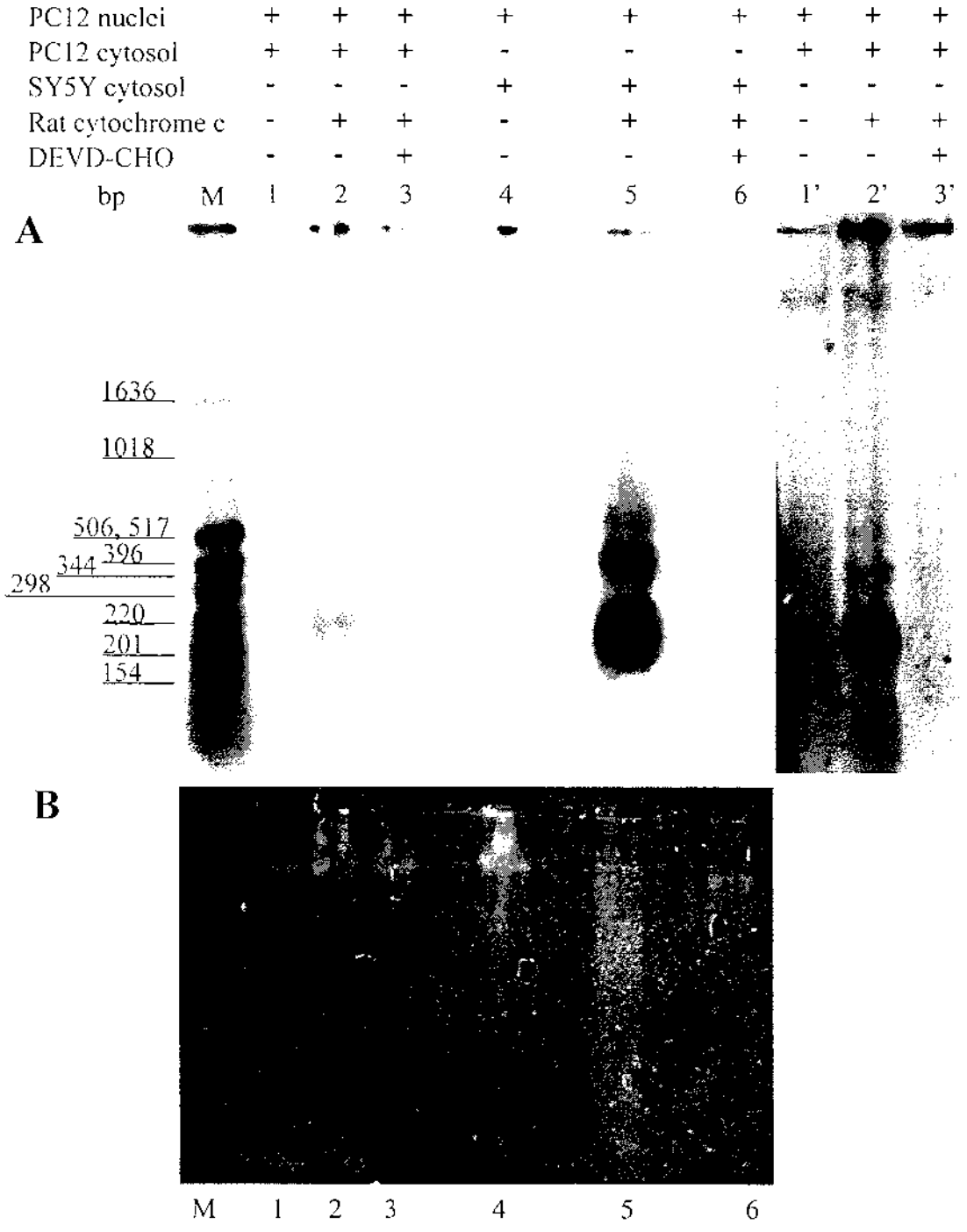


Figure 5-2: Rat cyt. c with PC12 cytosol weakly activates DNA cleavage.
A - PC12 nuclei incubated with PC12 cytosol (lanes 1-3) or SY5Y cytosol (lanes 4, 5 & 6) with 3 μM rat cyt. c (lanes 2, 3, 5 & 6) and 2 μM DEVD-CHO (lanes 3 & 6). DNA was extracted and treated as described in Figure 3-1. Longer exposure of lanes 1-3 shown as 1'-3'. **B** - The dried gel was stained with SYBRI Green as described in Figure 3-7.

Microinjections of cyt. c into human kidney 293 cells, NRK epithelial cells, mouse embryoinic Swiss 3T3 fibroblasts and rat promyelocytic IPC-81 leukaemia cells, will cause apoptosis (Li *et al.*, 1997a; Zhivotovsky *et al.*, 1998). In contrast, microinjections of cyt. c into either MCF7 breast carcinoma cells, which lack detectable levels of Caspase-3, or sympathetic neurons, cultured with NGF, does not induce apoptosis (Deshmukh and Johnson, 1998; Li *et al.*, 1997a; Neame *et al.*, 1998). However, withdrawal of NGF approximately 15 hours before microinjection, enabled the sympathetic cells to undergo apoptosis. These cells needed to acquire 'competence to die' before microinjection of mammalian cyt. c could induce cell death (Deshmukh and Johnson, 1998). PC12 cells may act in a similar manner to sympathetic neurons and treatments such as serum withdrawal may be required to induce 'competence to die' in these cells.

CHAPTER 6: MITOCHONDRIAL *IN VITRO* MODEL OF APOPTOSIS

6.1 INTRODUCTION

Mitochondria have a pivotal role in the regulation of mammalian apoptosis (Section 1.5). Interactions between proteins of the Bcl-2 family on the mitochondrial surface dictate whether mitochondria release apoptogenic proteins, such as AIF and cyt. c, and initiate cell death. The release of cyt. c from mitochondria precedes caspase activation, and we want to understand the factors involved in this release (Goldstein *et al.*, 2000). The development of an *in vitro* system to study the events leading up to cyt. c release would yield some insight into the mechanisms of mitochondrial-mediated apoptosis.

To create an *in vitro* protocol that could be used to analyse events leading to cyt. c release, we used the previously described *in vitro* protocol using PC12 nuclei and SY5Y cytosol, and also added purified mitochondria from SY5Y cells (Figure 6-1). We pre-treated some of the mitochondria with Atractyloside (Atr) to release apoptosis-inducing agents (as described in section 2.3.9). Atr is reported to be a permeability transition pore (PTP)-opening agent that acts on one of the PTP components, adenine nucleotide translocator (Ant), to induce cyt. c release. We hypothesised that Atr-treated mitochondria would release cyt. c, which would then activate the cytosolic caspases. The effect of adding mitochondria purified from healthy cells was also tested in the presence of exogenously added rat cyt. c. The experimental protocol is shown in Figure 6-1.

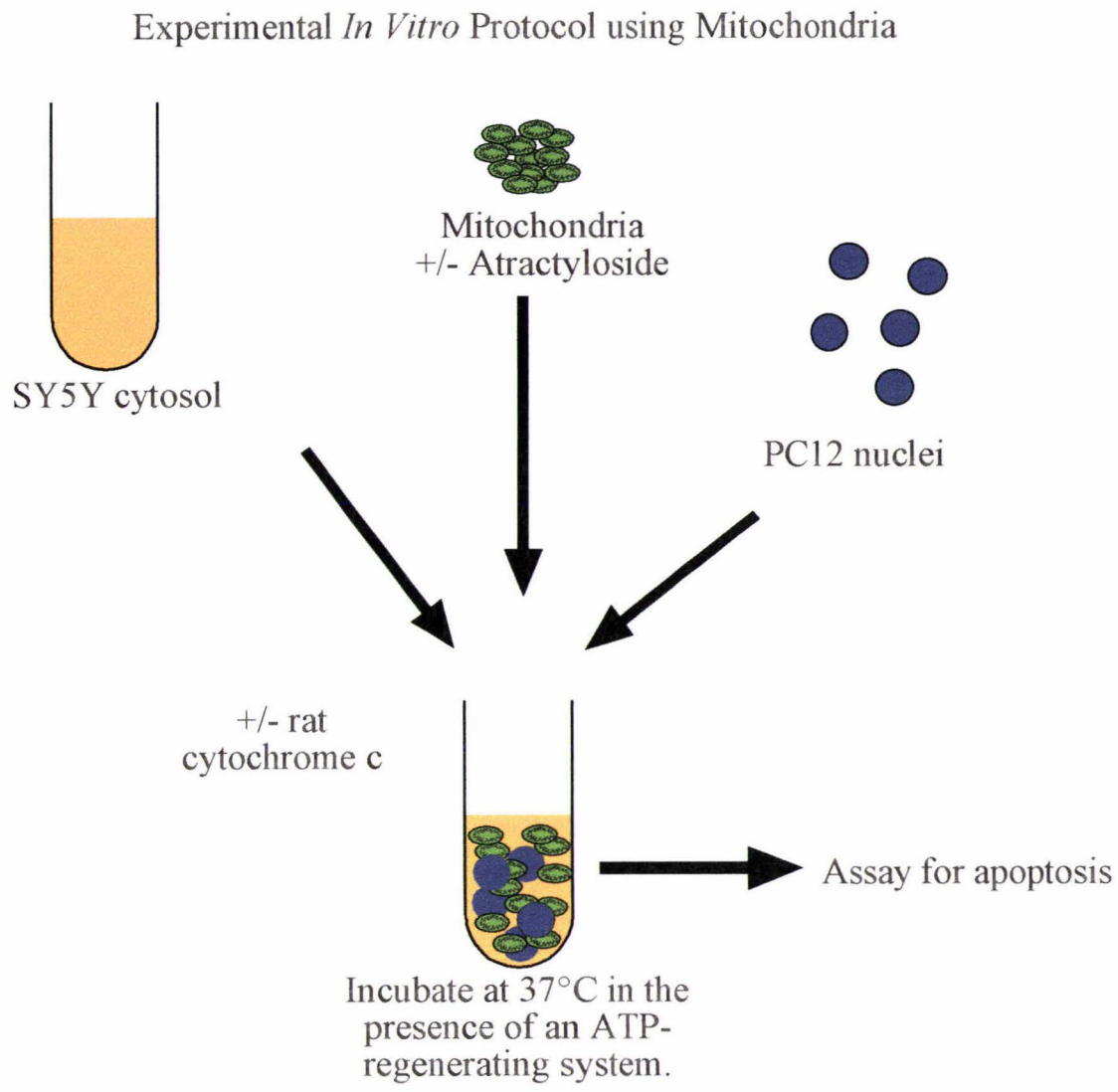


Figure 6-1: Experimental *in vitro* protocol using isolated mitochondria.
The experimental *in vitro* protocol used crudely isolated mitochondria (8,000×g pellet, P2, shown in Figure 2-1) incubated with PC12 nuclei, SY5Y cytosol and rat cyt. c in the presence of an ATP regenerating system. Mitochondria were pre-treated with Atractyloside (Atr) prior to addition to reactions. After incubation the reactions were assayed for the induction of apoptosis as previously described in Figure 3-1.

6.2 RESULTS

6.2.1 Atr-treated mitochondria did not induce apoptosis

Mitochondria pre-treated with Atr (Atr-Mitochondria) were incubated with PC12 nuclei and SY5Y cytosol, followed by immunoblot analysis of caspase activation (Figure 6-2). Incubation of SY5Y cytosol with mitochondria pre-treated with 0.24 mM Atr for 10 (lane 4) or 20 (lane 5) minutes did not cause cleavage of Caspase-3 or Caspase-9. Mitochondria pre-treated with very high concentrations of Atr (1.1 mM) for 10 (lane 6) or 20 (lane 7) minutes did not cause cleavage of Caspase-3 or Caspase-9. Interestingly, Parp was partially cleaved when Atr-Mitochondria were added to reactions (Figure 6-2, third panel, lanes 4-7). Caspase-3 and Caspase-9 were not cleaved in reactions with SY5Y cytosol alone (lane 1), but were cleaved with the addition of rat cyt. c (lane 2). Immunoblot analysis of Erk shows that equal amounts of protein from each reaction were compared.

6.2.2 Atr caused a reduction in mitochondria membrane potential

To determine if Atr was causing changes in mitochondrial membrane potential, we examined mitochondria that had been treated with Atr using a cell permeable ratiometric indicator of mitochondrial membrane potential JC-1 (5,5`6,6`-tetrachloro-1,1`3,3`-tetraethylbenzimidazolocarbocyanine iodide). JC-1 fluoresces red in the presence of an active mitochondrial membrane potential and fades to green with membrane potential loss. SY5Y cells were incubated in the presence of JC-1 prior to cell permeabilisation and mitochondrial purification (as described in Section 2.3.9).

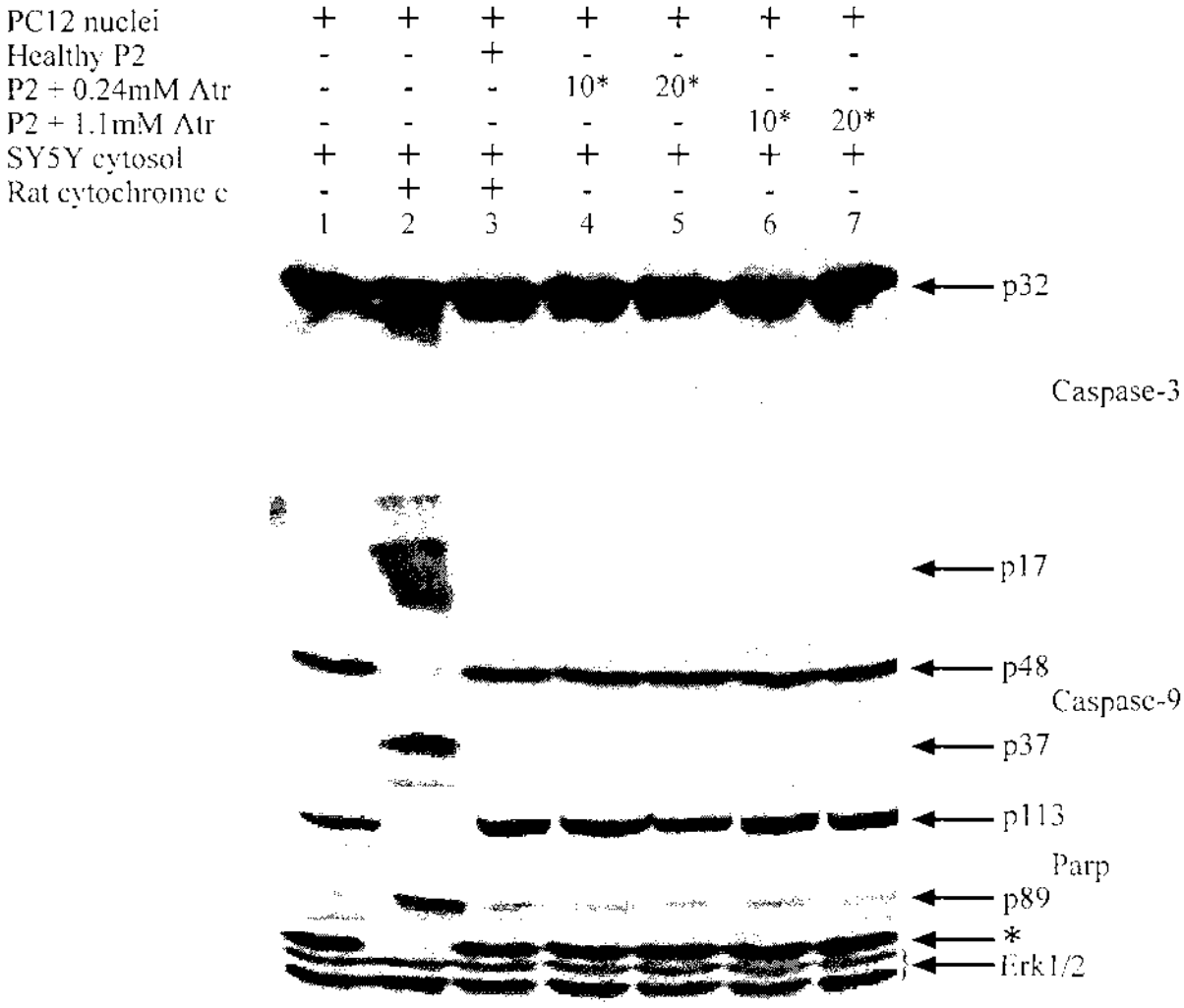


Figure 6-2: Healthy Mitochondria prevented caspase activation by rat cyt. c. Atr-Mitochondria were ineffective in promoting death.

PC12 nuclei incubated with SY5Y cytosol (lanes 1-7) and 3 μM rat cyt. c (lanes 2 & 3), with the P2 fraction containing mitochondria (lane 3) or P2 pre-treated with 0.24 mM or 1.1 mM Atr for 10 minutes (lanes 4 & 6) or 20 minutes (lanes 5 & 7). Samples were treated as described in Figure 3-1. Membranes were probed for the proteins indicated. Note the uppermost band marked with * on the Erk panel was signal remaining from previous Caspase-9 immunoblot analysis of the same membrane. Data are representative of 2 independent experiments.

There was a distinct loss of membrane potential when mitochondria were treated with 0.24 mM Atr for 20 minutes (Figure 6-3, photo B) compared with untreated mitochondria (photo A). However, there was not a complete loss of membrane potential, as some sharp red dots, representing mitochondria with an intact membrane potential, were still present. In earlier trials of this experiment, Atr was added directly to *in vitro* reactions containing mitochondria, rather than pre-incubating mitochondria with Atr. After incubation, analysis of these mitochondria showed no reduction in their membrane potential (data not shown).

We examined the morphology of PC12 nuclei incubated with SY5Y cytosol and Atr-Mitochondria (Figure 6-4, photos B-E). Atr-Mitochondria were unable to induce chromatin condensation in nuclei, in contrast to rat cyt. c-activated SY5Y cytosol (compare photo F with photos B-E). However, this assay requires high caspase activation before chromatin condensation is observed, so a more sensitive assay was used to examine PC12 nuclei for features of apoptosis.

PC12 genomic DNA isolated from *in vitro* reactions with mitochondria was radioactively end-labelled, separated by electrophoresis, and visualised by autoradiography, as described in previous chapters (Figure 6-5). When PC12 nuclei were incubated with SY5Y cytosol and Atr-Mitochondria, only faint DNA smears were observed (Figure 6-5A, lanes 5-8). The most pronounced smear was present when mitochondria were treated with 1.1 mM Atr, but only for 10 minutes (lane 7). Comparison of these DNA smears with the apoptotic DNA ladder induced by activated SY5Y cytosol (lane 2), suggests that, at best, Atr-Mitochondria caused very weak induction of apoptotic DNases in these reactions. However, it is not clear from the data that the faint smear represents internucleosomal cleavage.

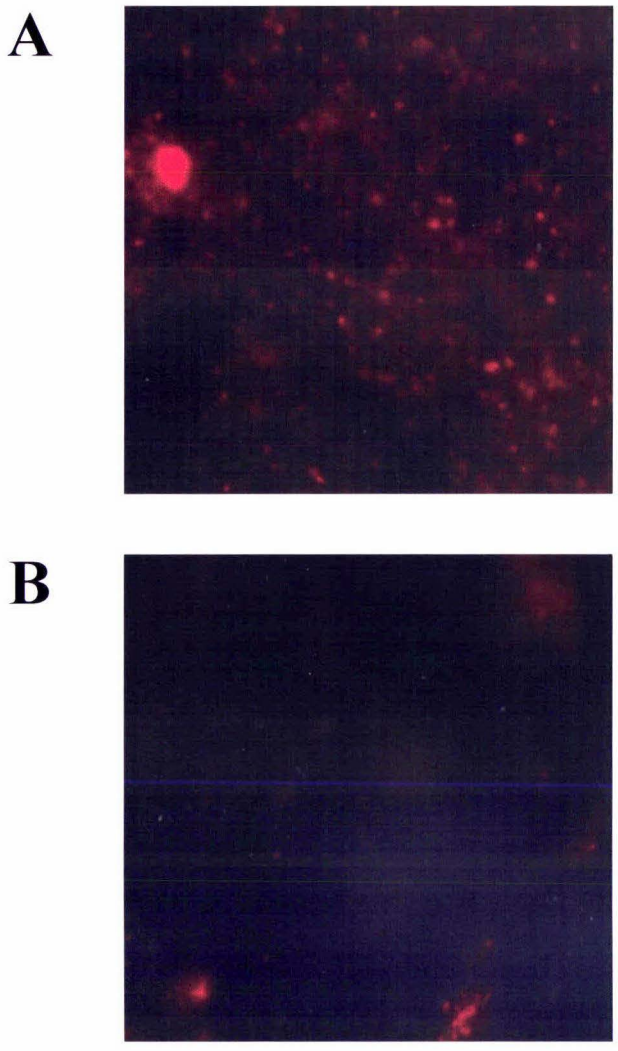


Figure 6-3: Comparison of healthy mitochondria with Atr-mitochondria.
SY5Y cells were incubated with JC-1 prior to mitochondrial isolation. After isolation mitochondria were retained on ice (healthy mitochondria), or were incubated with either 0.24 mM, or 1.1 mM Atr, for 10 or 20 minutes at 37°C (Atr-Mitochondria). **A** - Healthy mitochondria. **B** - Mitochondria treated with 0.24 mM Atr for 20 minutes. Mitochondria were observed at 630× magnification. Note that the photos shown have been enlarged by 20%. Data are representative of two independent experiments.

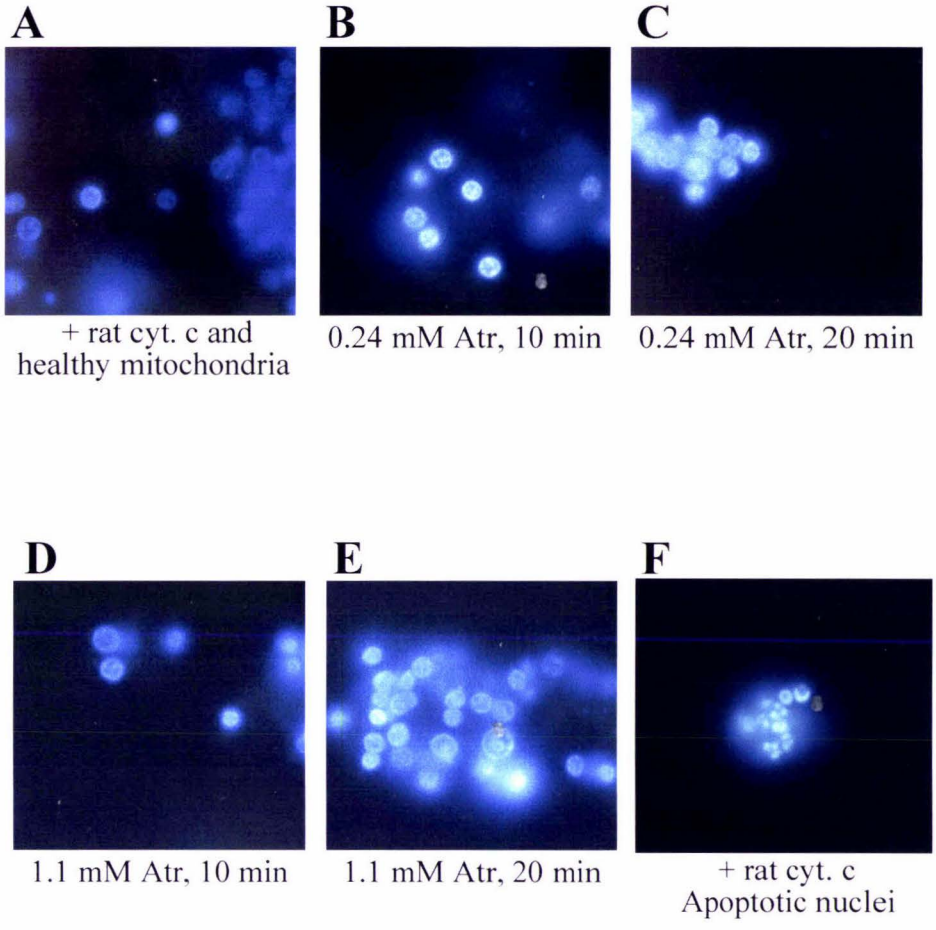


Figure 6-4: Healthy mitochondria inhibited morphological changes in PC12 nuclei. Atr-mitochondria did not induce apoptotic morphology.

PC12 nuclei incubated with SY5Y cytosol (Photos A-F) and rat cyt. c (photo A & F) with P2 (healthy mitochondria) (photo A), or P2 pre-treated with 0.24 mM Atr for 10 minutes (photo B) or 20 minutes (photo C), or P2 pre-treated with 1.1 mM Atr for 10 minutes (photo D) or 20 minutes (photo E). PC12 nuclei were treated as described in Figure 3-1 and observed at 400× magnification. Data are representative of 2 independent experiments.

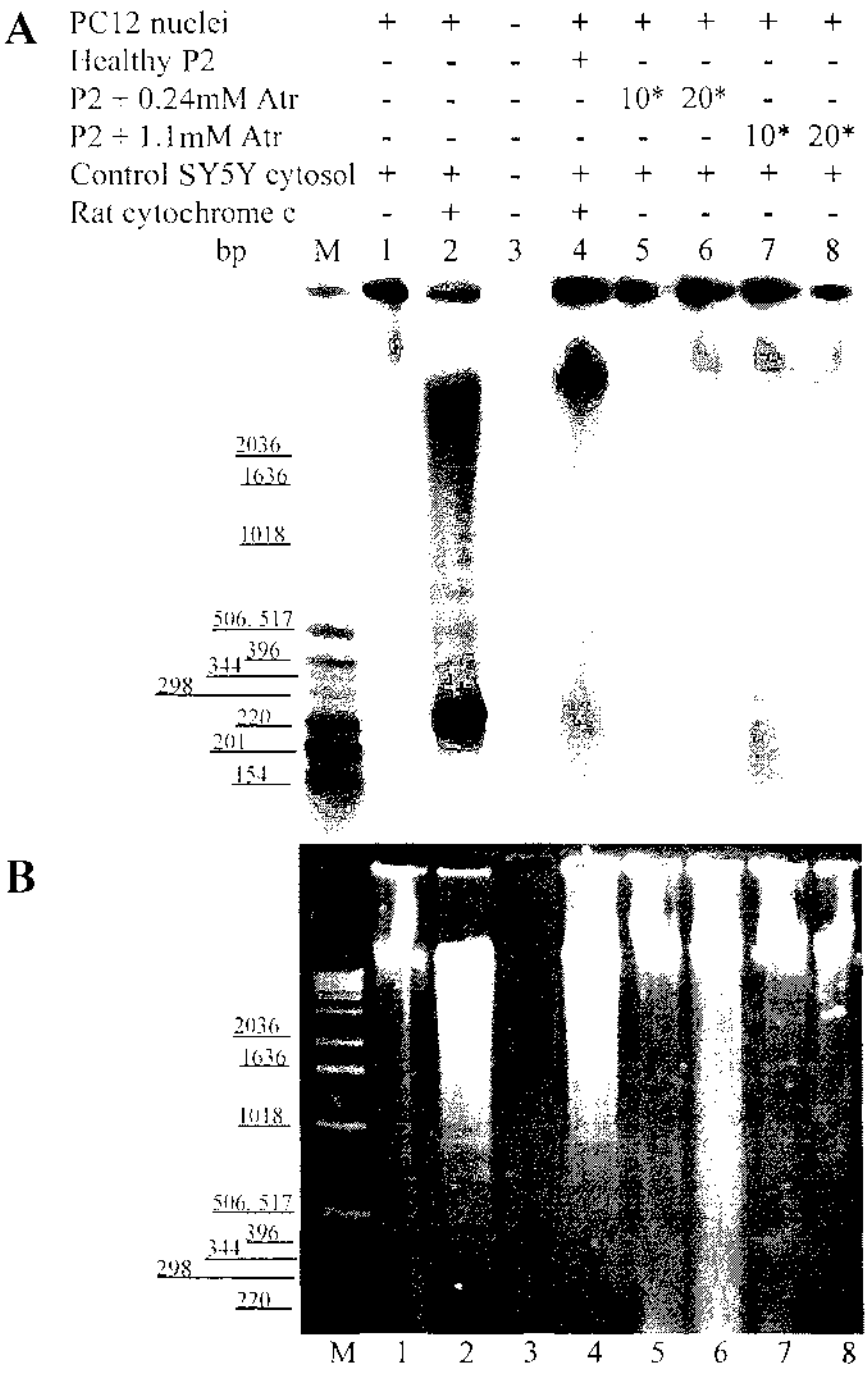


Figure 6-5: Healthy mitochondria partially protected against DNA fragmentation. Atr-mitochondria did not induce DNA cleavage.

A - PC12 nuclei incubated with SY5Y cytosol (lanes 1-7) and 3 μM rat cyt. c (lanes 2 & 4) with P2 (lane 4) or P2 pre-treated with 0.24 mM Atr for 10 minutes (lane 5) or 20 minutes (lane 6), or pre-treated with 1.1 mM Atr for 10 minutes (lane 7) or 20 minutes (lane 8). DNA was extracted from samples and as described in Figure 3-1. **B** - The dried gel was stained with 1×SYBRI Green as described in Figure 3-7. The lanes of this gel match with those in A. Data are representative of 2 independent experiments.

6.2.3 Healthy mitochondria partially inhibited rat cyt. c-activated apoptosis

When PC12 nuclei were incubated with rat cyt. c-activated SY5Y cytosol, caspases were activated, PC12 nuclei had condensed chromatin (apoptotic morphology) and a distinct internucleosomal DNA ladder was observed (Francois and Grimes, 1999) (Figure 6-2, 6-4, 6-5). However, we found that these features of apoptosis were inhibited when mitochondria were added (Figures 6-2, 6-4, & 6-5).

In vitro reactions containing PC12 nuclei incubated with rat cyt. c-activated SY5Y cytosol and healthy mitochondria were examined for caspase activation (Figure 6-2). The addition of healthy mitochondria to activated SY5Y cytosol inhibited Caspase-3 and Caspase-9 activation (compare lane 2 with 3). Parp cleavage was partially inhibited, indicating that the inhibition of caspase activation was not complete (lane 3).

Activated SY5Y cytosol caused chromatin condensation (Figure 6-4, photo F) (Francois and Grimes, 1999). However, the addition of healthy mitochondria to activated SY5Y cytosol prevented chromatin condensation in PC12 nuclei (Figure 6-4, Photo A). DNA fragmentation was also inhibited and the DNA ladder less distinct in the presence of healthy mitochondria (Figure 6-5, lane 4) compared with DNA fragmentation from activated SY5Y cytosol alone (lane 2). When total DNA was examined, more uncleaved DNA (in the well of the gel) was present in the reactions with mitochondria than without (Figure 6-5B, compare lane 2 with 4). This suggests that the protective action of healthy mitochondria was not complete, as there was still some DNA cleavage. Alternatively some of this DNA could be mitochondrial DNA.

6.3 DISCUSSION AND FUTURE WORK

The use of Atr to induce cyt. c release from mitochondria has been reported by one particular group (Kroemer *et al.*, Centre National de la Recherche Scientifique, France) but we have had difficulty repeating their work. They have reported that Atr-treated mitochondria, and microinjection of Atr into cells, causes nuclear apoptosis (Marzo *et al.*,

1998; Susin *et al.*, 1996; Zamzami *et al.*, 1996). We have spoken with other researchers (J. Scarlet, Otago, D. Newmeyer, UCSD) who have also been unable to reproduce Kroemer's work. Examination of mitochondria by fluorescent microscopy showed that ~70% of mitochondria lost membrane potential when incubated with Atr. This left a heterogeneous mixture of healthy and unhealthy mitochondria and perhaps the healthy mitochondria inhibited some features of apotosis. We need to check that Atr-Mitochondria were actually releasing cyt. c by immunoblot analysis of the mitochondrial pellet and supernatant after pre-incubation. There are many different ways of inducing the rupture of mitochondria and release of cyt. c, and we have tried only one. We could use other agents to induce the complete release of cyt. c from mitochondria. We have recently acquired a very sensitive assay for caspase activity using fluorescent caspase substrates, which could be used to determine if Atr-Mitochondria actually cause small amounts of caspase activation.

The isolation of mitochondria from SY5Y cells was a crude procedure using a method similar to that described by Grimes *et al.*, (1996). In their protocol, the 8,000xg pellet (P2) of PC12 cells was reported to contain mitochondria, dense bodies, ribosomes and uncoated vesicles with a mean diameter of 180 ± 71 nm (Grimes *et al.*, 1996). We have used this technique to isolate mitochondria from SY5Y cells, so other organelles of similar size were probably present in this fraction. The presence of these organelles may also inhibit the apoptosis-inducing abilities of Atr-Mitochondria.

We have demonstrated that isolated healthy mitochondria can partially protect against rat cyt. c-induced apoptosis, as shown by inhibition of caspase activation, DNA fragmentation and chromatin condensation. At present we don't have a method of separating healthy and non-healthy mitochondria that would enable us to add only healthy mitochondria to reactions. Not all the mitochondria in the P2 fraction had intact membrane potentials (data not shown). These mitochondria may have lost their cyt. c content and hence cause limited induction of apoptosis or they may be unable to prevent apoptosis. We do not know if healthy mitochondria have any effect on PC12 nuclei incubated in SY5Y

cytosol without rat cyt. c. A study using mouse oocytes microinjected with mitochondria does support our findings that mitochondria have protective abilities against apoptosis. Oocytes from FVB female mice have a high rate of spontaneous apoptosis *in vitro* (Morita *et al.*, 1999), but microinjection of mitochondria, purified from non-apoptotic follicular granulosa cells, prevented this high rate of apoptosis (Perez *et al.*, 2000).

Although mitochondria inhibited the apoptotic inducing action of rat cyt. c in our *in vitro* system, we do not know the mechanism by which this occurs. Are mitochondria acting like a sponge and soaking up exogenous cyt. c? NGF-deprived sympathetic neurons, protected by caspase inhibitors, recovered after re-incubation with NGF (Martinou *et al.*, 1999). Mitochondria from NGF-deprived neurons are depleted of cyt. c, but recover their cyt. c content when the neurons are re-incubated with NGF (Martinou *et al.*, 1999). One possible model of mitochondrial-mediated protection against apoptosis is presented in Figure 6-6A. This model shows exogenous cyt. c being removed from the cytosol by mitochondria reincorporating the protein. However, this model depends on enough mitochondria to be present to reincorporate cyt. c before it has a chance to activate Apaf-1. Using immunoblot analysis we can determine whether exogenous cyt. c remains in the cytosol, or if it translocates to mitochondria (isolated by centrifugation after reactions), during *in vitro* incubation. The partial activation of apoptosis could be due to some cyt. c escaping re-entry into mitochondria and activating Apaf-1 (Figure 6-6A). GFP-cyt. c has been used in whole cells to follow the release of cyt. c from mitochondria during apoptosis (Goldstein *et al.*, 2000). We could incorporate GFP-cyt. c in our *in vitro* reactions and follow, by fluorescent microscopy, exactly where cyt. c is found after *in vitro* incubations.

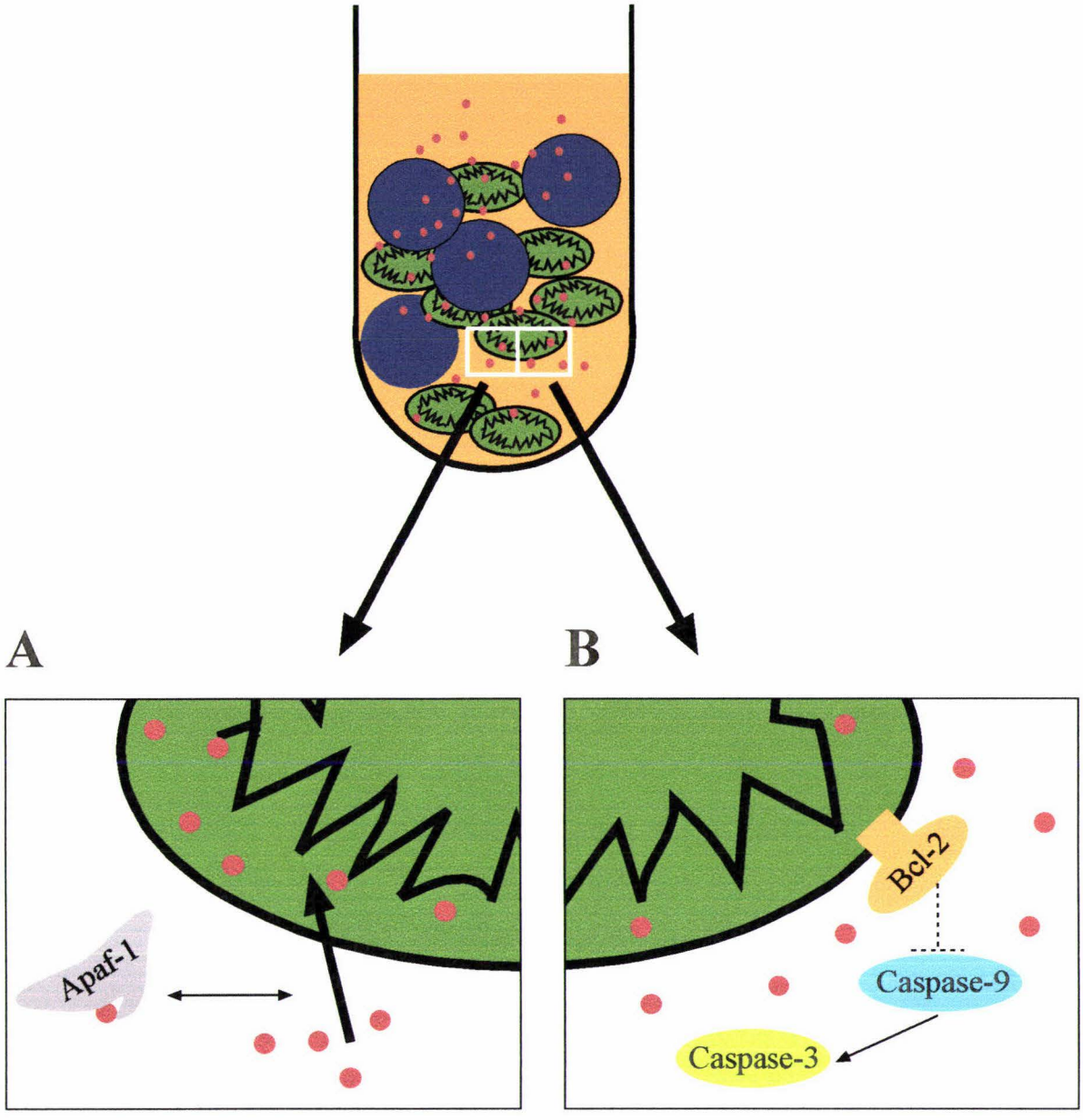


Figure 6-6: Models of mitochondria-mediated protection against apoptosis.
A - Exogenous cyt. c is removed from the cytosol by mitochondria reincorporating the protein back into the inner mitochondrial membrane before it can bind and activate Apaf-1 to cause apoptosis. **B** - Bcl-2 proteins of the surface of mitochondria indirectly prevent Caspase-9 activation, possibly through some as yet unidentified proteins. Cell components are not drawn to scale. Blue circles represent PC12 nuclei, green ovals represent mitochondria, and red dots represent cyt. c.

Another possibility is that Bcl-2 proteins on the surface of exogenous mitochondria may inhibit Caspase-9 activation in a manner that is not completely understood (Figure 6-6B). It has been proposed that Bcl-2 may inhibit a novel apoptosome complex, consisting of an unknown adapter protein and a caspase (Hausmann *et al.*, 2000). Bcl-2 may function by interacting with an as yet unidentified Ced-4 homologue, which acts upstream of Apaf-1 activation and serves to prevent the initiation of apoptosis and subsequent amplification of the apoptotic pathway by cytochrome c and Apaf-1.

CHAPTER 7: CONCLUDING COMMENTS

The main aim of this project was to develop *in vitro* systems to identify novel components that regulate apoptosis using a combination of yeast and mammalian cell fractions. The different *in vitro* systems are summarised below.

The first system used either yeast nuclei or spheroplasts and activated SY5Y cytosol to induce apoptosis (Chapter 3). Yeast nuclei exhibited random DNA cleavage when incubated *in vitro* under any conditions. Internucleosomal DNA cleavage induced in yeast spheroplasts was dependent on SY5Y caspases. We could not use this system to identify novel components of yeast cells that could cause features of apoptosis, as any apoptotic features observed were completely dependent on SY5Y caspases being active. No other yeast cell components appeared to cause the same features as activated SY5Y cytosol. Yeast cyt. c could not induce apoptosis using either yeast or mammalian components *in vitro*.

The second *in vitro* system used PC12 nuclei incubated in yeast (Chapter 4). Yeast cytosol caused DNA cleavage that was similar to apoptotic DNA fragmentation but more smeared than the typical DNA ladder observed in higher eukaryote apoptosis. However, chromatin condensation, another feature of apoptosis, was not induced by yeast cytosol. Interestingly, yeast cytosol-induced DNA cleavage was inhibited by the caspase inhibitor DEVD-CHO, in the presence of rat cyt. c and sometimes in with yeast cyt. c. We examined reactions for Parp cleavage (a hallmark of apoptosis due to activation of caspases) and found no proteins immunoreactive to the Parp antibody. This could mean that PC12 Parp was degraded by a protease or was cleaved in a manner that did not allow Parp antibodies to bind. The results in this section were ambiguous, nevertheless these results suggest that yeast cytosol contains an ATP-independent DNase that can be inhibited by DEVD-CHO, and a protease that cleaves Parp. We could not induce all the typical features of apoptosis in PC12 nuclei using yeast cytosol.

We used only PC12 cell components in a third cell free system to reconstitute apoptosis (Chapter 5). In this system PC12 cytosol was only weakly activated by rat cyt. c to induce internucleosomal DNA cleavage in PC12 nuclei. In non-neuronal cell types cyt. c release or microinjection of cyt. c is sufficient to induce apoptosis. However, neurons need to acquire a 'competence to die', which is obtained during trophic factor deprivation, before they will undergo cyt. c induced apoptosis (Deshmukh and Johnson, 1998). We believe that the PC12 cell components used in creating this *in vitro* system were not 'competent to die', hence the poor activation of apoptosis by rat cyt. c-activated PC12 cytosol.

In the final *in vitro* system we developed, we examined the role of mitochondria in apoptosis (Chapter 6). In this system, we attempted to induce apoptosis by the addition of mitochondria that had been pre-treated to release their cyt. c. We were unsuccessful at causing the dramatic induction of apoptosis observed when cyt. c was added directly to the cytosolic fraction of SY5Y cells. However, partial induction of apoptosis was observed: partial Parp cleavage and some internucleosomal cleavage of PC12 DNA. Analysis of Atr-Mitochondria showed that not all mitochondria had lost their membrane potential and hence may not have released their cyt. c to carry out the activation of caspases. We found that the addition of healthy mitochondria to rat cyt. c-activated cytosol offered protection against apoptosis. This was consistent with other data in which microinjection of healthy mitochondria prevented spontaneous *in vitro* apoptosis of FVB mouse oocytes (Perez *et al.*, 2000). However, further work is needed to determine the exact mechanism of protection.

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Errata

Page ix, line 28 - replace 'acteyl-Asp-Glu-Val-Ala-aldehyde' with 'N-acetyl-Asp-Glu-Val-Asp-aldehyde'

line 34 - replace 'Dithiothretiol' with 'Dithiotreitol'

line 35 - replace 'chemluminescence' with 'chemiluminescence'

Page x, line 30 - replace 'suflate' with 'sulphate'

Page 10, paragraph 2, line 2 - replace 'activating' with 'activated'

Page 11, line 3 - replace 'intercellular' with 'intracellular'

Page 32, line 4 - replace 'o-phenathroline' with 'o-phenanthroline'

Page 34, Section 2.3.5, line 4 - replace 'cytochalsin' with 'cytochalasin'

Page 35, Section 2.3.5, lines 4 and 13; Section 2.3.7, line 4; and Page 36, Section 2.3.8, line 5 - replace 'Sorbital' with 'Sorbitol'

Page 38, paragraph 2, line 1 - replace 'mg' with 'µg'

Page 42, Section 2.9, line 5 - replace 'parafomaldehyde' with' paraformaldehyde'

Page 52, Figure 3-4 caption, line 6 - replace '70°C' with '-70°C'

Page 65, Section 4.2.1, line 6 - replace 'apoptotic SY5Y cytosol' with 'rat cyt. c-activated SY5Y cytosol'

Page 74, line 2 - insert 'Figure 5-1A,' after opening bracket of '(lane 2)'