Regulation of Apoptosis in Neural Cells: Two methods for overcoming asynchrony

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Abstract

Programmed cell death, or apoptosis, plays a major role in the development of the nervous system and in the pathogenesis of neurodegenerative diseases. Although many proteins that play a key role in apoptosis in other systems also appear to function in neurons, the mechanism that triggers apoptosis in neurons is unknown. Apoptosis occurs asynchronously in neural and differentiated neuronal cells, which makes biochemical studies difficult because a small number of cells are at a particular stage at any one time. Two strategies were devised to overcome asynchrony during neural cell death.

The first strategy was to separate rat pheochromocytoma (PC12) cells at different stages of commitment to cell death on the basis of cell density using equilibrium density gradient centrifugation. Three populations were defined. Cells in population 1 were the most dense and committed to cell death. They showed extensive loss of mitochondrial cytochrome c, DNA fragmentation, and chromatin condensation. Population 3 contained live cells that floated to the top of density gradients. Population 2 displayed some chromatin condensation, yet little DNA fragmentation and loss of cytochrome c. This population showed upregulation of the pro-death factor, c-Jun, and downregulation of pro-survival kinase, Akt. Importantly, these cells could be rescued from death by nerve growth factor (NGF) and thus represent an intermediate stage of apoptosis, upstream of irreversible commitment.

The second strategy was to create a cell-free system to reconstitute apoptosis. The addition of cytochrome c to human neuroblastoma (SY5Y) cell extracts activated caspase-9 and –3, and nucleolytic events in PC12 nuclei. Using this system, requirements for ATP and phosphatase activity for caspase activation and nuclear apoptosis were characterised. In addition, pro-survival molecules Akt and Creb were identified as caspase substrates during apoptosis *in vitro*.

To assess whether these events occurred *in vivo*, the kinase inhibitor staurosporine and the topoisomerase inhibitor camptothecin were used to induce apoptosis in intact SY5Y cells. The pro-survival signalling kinase Raf-1 was downregulated during both staurosporine- and camptothecin-induced apoptosis, but Akt was only downregulated by camptothecin. These studies illustrate the complex interactions of apoptosis and signalling mechanisms in neural cells.

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AD	Alzheimer's Disease
AIDS	Acquired Immune Deficiency Syndrome
Aif	Apoptosis inducing factor
Akap	A-kinase anchoring protein
ALPS	Autoimmune lymphoproliferative syndrome
ALS	Amyotrophic lateral sclerosis
Ant	Adenine nucleotide translocator
Apaf-1	Apoptosis promoting factor 1
App	Amyloid precursor protein
ATP	Adenosine triphosphate
Bad	Bcl-X_L/Bcl-2 associated death promoter
BDNF	Brain-derived neurotrophic factor
BH	Bcl-2 homology
BIR	Baculovirus IAP repeat
BSA	Bovine serum albumin
Cad/Dff40	Caspase-activated DNase/DNA fragmentation factor 40
CamkII or IV	Ca^{2+}/calmodulin dependent kinase II or IV
cAMP	cyclic adenosine monophosphate
CARD	Caspase recruitment domain
Caspase	Cysteine aspartic acid protease
Ced	Cell death abnormal
Ces	Cell death specification
CHEF	Clamped homogeneous electrical field
CNS	Central nervous system
CPT	Camptothecin
Creb	cAMP response element binding protein
CuZnSOD	Copper/zinc superoxide dismutase
Cyt. c	Cytochrome c

dADP	Deoxyadenosine diphosphate
ddATP	Dideoxyadenosine triphosphate
DcR	Decoy receptor
ddH_2O	Double distilled water
DEVD-CHO	acetyl-Asp-Glu-Val-Ala-aldehyde
Diap1	*Drosophila* inhibitor of apoptosis 1
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	DNA endonuclease
DR	Death receptor
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether N,N,N',N'-tetraacetic acid)
eNOS	Nitric oxide synthase enzyme
ER	Endoplasmic reticulum
Erk	Extracellular-signal regulated kinase (Erk1 = p44Mapk, Erk2 = p42Mapk)
Fadd/Mort	Fas-associated death domain
FasL	Fas ligand
FGF	Fibroblast growth factor
GAP	GTPase-activating protein
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
Gsk-3	Glycogen synthase kinase-3
GST	Glutathione S-transferase
GTP	Guanosine triphosphate
HD	Huntington's Disease
HSNs	Hermaphrodite specific neurons
Iap	Inhibitor of apoptosis protein
Icad	Inhibitor of caspase-activated DNase
Ice	Interleukin-1β-converting enzyme

IGF	Insulin-like growth factor
Ikk	IκB-kinase
Jnk	c-Jun amino-terminal kinase (Sapk)
kb	kilobase
kD	kilodalton
Mapk	Mitogen-activated protein kinase
Mek	Mapk/Erk kinase (Mek1 = Mkk1, Mek2 = Mkk2)
Mekk	Mapk/Erk kinase kinase
Mkk	Mapk kinase
Mkkk	Mkk kinase
mRNA	Messenger ribonucleic acid
Na_3VO_4	Sodium orthovanadate
NF-κB	Nuclear factor-κB
NGF	Nerve growth factor
Nik	NF-κB -inducing kinase
NO	Nitric oxide
NSMs	Neurosecretory motor neurons
NT	Neurotrophin
OKA	Okadaic acid
$p75^{NTR}$	p75 neurotrophin receptor
Pak	p21-activated kinase
Parp	Poly(ADP)-ribose polymerase
PBS	Phosphate-buffered saline
PC12	Rat adrenal pheochromocytoma cell line
PCD	Programmed cell death
PD	Parkinson's disease
PDGF	Platelet-derived growth factor
Pdk	Phosphoinositide-dependent kinase
PFGE	Pulse-field gel electrophoresis
PGB	PBS with glucose and BSA
PH	Pleckstrin homology domain

PI3-K	Phosphatidyl inositol 3-kinase
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
PMSF	Phenylmethylsulfonyl fluoride
PP2A	Protein phosphatase 2A
PT	Permeability transition
PtdIns	Phosphatidyl inositol
Rip	Receptor interacting protein
ROS	Reactive oxygen species
Rsks	pp90 ribosomal S6 kinases
Sapk	Stress-activated protein kinase
SAR	Scaffold attachment region
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sek1	Sapk/Erk kinase 1 (Mkk4, Jnkk)
SH	Src homology
SODD	Silencer of death domains
STS	Staurosporine
SY5Y	SK-N-SH-SY5Y human neuroblastoma cell line
TBS	Tris-buffered saline
TCA	Trichloroacetic acid
TE	Tris-EDTA
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
Tradd	TNFR-associated death domain
Traf-2	TNFR-associated factor 2
Trk	Tyrosine receptor kinase
tRNA	Transfer RNA
Tween-20	Polyoxyethylenesorbitan monolaurate
Vdac	Voltage-dependent anion channel

v/v	volume/volume
w/v	weight/volume
z-VAD-fmk	benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone

Note on genetic nomenclature:

The conventions used for writing the names of genes and gene products is according to Murray & Hunt (1993). Gene names are always written in lower case letters and are italicised. Gene products are written with the first letter capitalised and without italics.

C. elegans	*Caenorhabditis elegans*
E. coli	*Escherichia coli*
S. cerevisiae	*Saccharomyces cerevisiae*
S. pombe	*Schizosaccharomyces pombe*

Abbreviations for amino acids

Amino acid	*Three-letter abbreviation*	*One-letter symbol*
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Asparagine or aspartic acid	Asx	B
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glutamine or glutamic acid	Glx	Z
Glycine	Gly	G
Histidine	His	H
Isoleucine	Iso	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

(from Stryer, 1988)

List of publications arising from this thesis

François, F. and Grimes, M.L. (1998) Stages in apoptotic commitment in PC12 cells. *Mol. Biol. Cell* **9,** 368a [abstract].

François, F. and Grimes, M.L. (1999) Phosphorylation-dependent Akt cleavage in neural cell *in vitro* reconstitution of apoptosis. *J. Neurochem.* **73,** 1773-1776.

Chapter 1

General Introduction

Chapter 1 General Introduction

Programmed cell death (PCD), or apoptosis, the mechanism by which unwanted cells are removed is essential for the proper development of multicellular organisms. For example, formation of digits by removal of interdigital tissue, resorption of the tadpole tail, and negative selection of auto-reactive T cells within the immune system occur by PCD (reviewed in Jacobson, et al., 1997). Apoptosis also plays a vital role in maintaining normal tissue homeostasis, removing damaged cells, and host defense mechanisms (Vaux & Korsmeyer, 1999).

Apoptosis is an energy-requiring, controlled form of cell death. During apoptosis, cells exhibit a distinct set of morphological and biochemical features, which include membrane blebbing, DNA fragmentation, chromatin condensation and fragmentation of the cell into membrane-enclosed vesicles (apoptotic bodies) (Wyllie, et al., 1980). The cell shrinks and condenses, yet membrane integrity is maintained. Finally the cell's cytoplasmic and nuclear contents are repackaged into apoptotic bodies, which can be phagocytosed by neighbouring cells. Apoptosis is distinct from necrosis, cell death due to injury. During necrosis, cells die in an uncontrolled manner, by swelling and plasma membrane disruption, resulting in the inflammation of surrounding tissue.

Apoptosis is highly regulated and the deregulation of apoptosis underlies the pathogenesis of many diseases, such as cancer, neurodegenerative disorders and autoimmune disease (Thompson, 1995). Therefore, understanding the biochemical mechanisms of apoptosis is a major goal of current biological research. A balance must exist between the rate of cell proliferation and the rate of cell death to maintain normal tissue homeostasis (Figure 1). If the rate of cell proliferation exceeds that of cell death, disorders of cell accumulation may result, allowing the growth of cancerous tumours. In contrast, if the rate of cell death is excessive, disorders of cell loss occur, such as neuron loss in neurodegenerative diseases. Examples where deregulation of cell death plays a

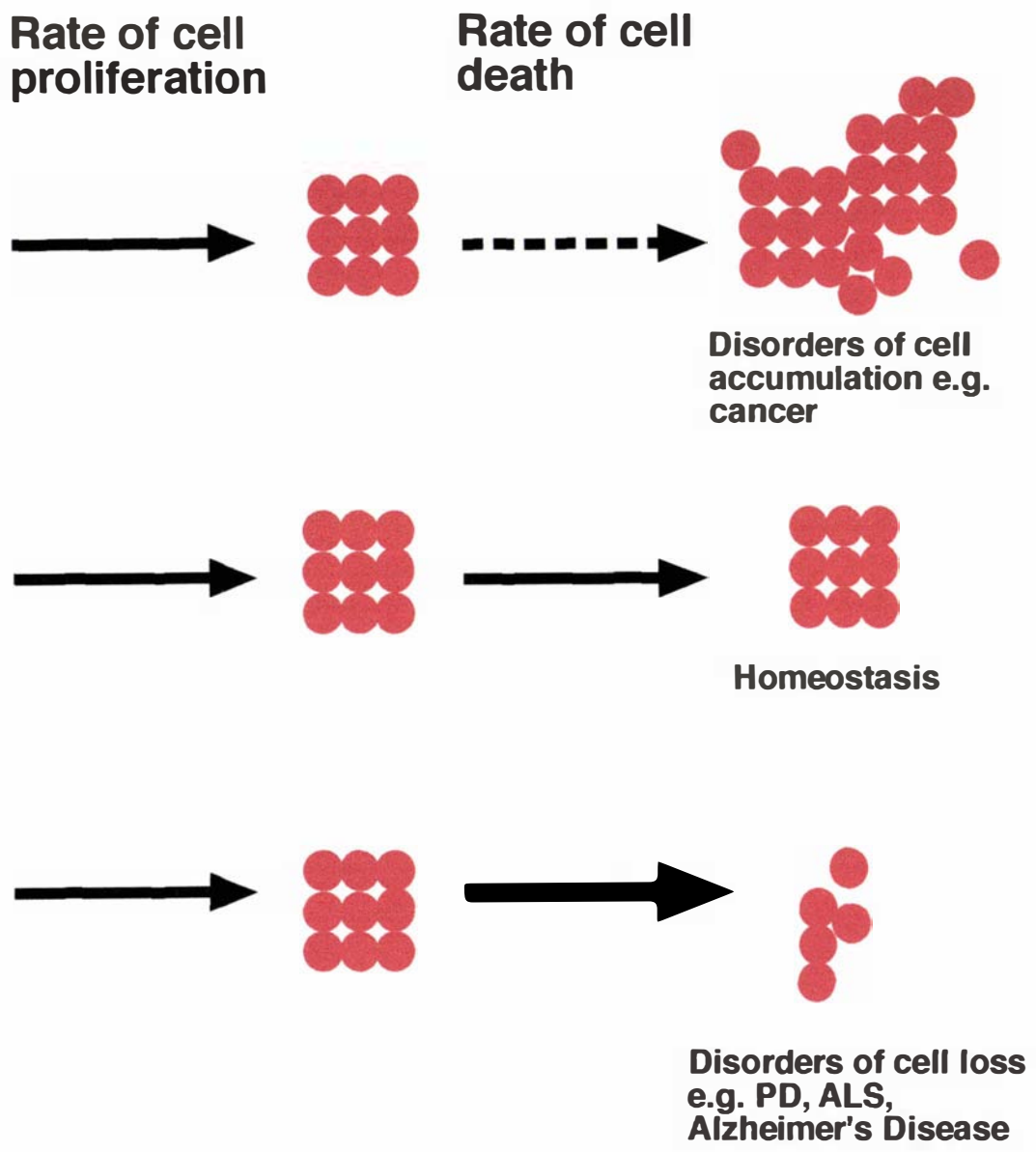

Figure 1: Deregulation of cell death underlies the pathogenesis of many disorders.
This figure is adapted from Thompson (1995).

role in human disease are presented in Table 1.

Table 1: Apoptosis in Disease

Too much cell death	Too little cell death
AIDS	Lymphoma
Neurodegenerative disease	Leukemia
Multiple Sclerosis	Canale-Smith Syndrome (ALPS)
Aplastic anemia	Autoimmune disease
Type I Diabetes mellitus	Solid tumours
Liver failure	

(Adapted from Peter, et al., 1997)

An outline of our present knowledge of the key steps that govern apoptosis is presented in Figure 2. A death signal, *e.g.* receptor activation, drug treatment, or UV irradiation, is integrated and transmitted via signal transduction pathways resulting in activation of the caspases. The caspases are cell death proteases that initiate execution of the cell. Execution results in degradation of proteins and DNA and repackaging of the cell's components for phagocytosis by neighbouring cells. Once the caspases are activated, death becomes irreversible and inevitable. Mitochondria play a role in triggering execution, adding another level of complexity to regulation. Cytochrome c released from mitochondria, triggers apoptosis. Members of the Bcl-2 family proteins can activate or inhibit apoptosis depending on their expression and subcellular localisation. Bcl-2 itself is associated with mitochondria and inhibits cell death.

In the following literature review, I demonstrate how the molecular machinery of apoptosis is conserved from the nematode to mammals. I concentrate on the additional level of complexity of control in mammals conferred by the mitochondria, inhibitor of apoptosis proteins (Iap), and death receptors. Then the role of signal transduction pathways in transmitting and integrating these death/survival responses will be discussed and the execution events that make the death decision irreversible. Finally, the particular mediators responsible for controlling apoptosis in neurons will be introduced. Experiments presented in

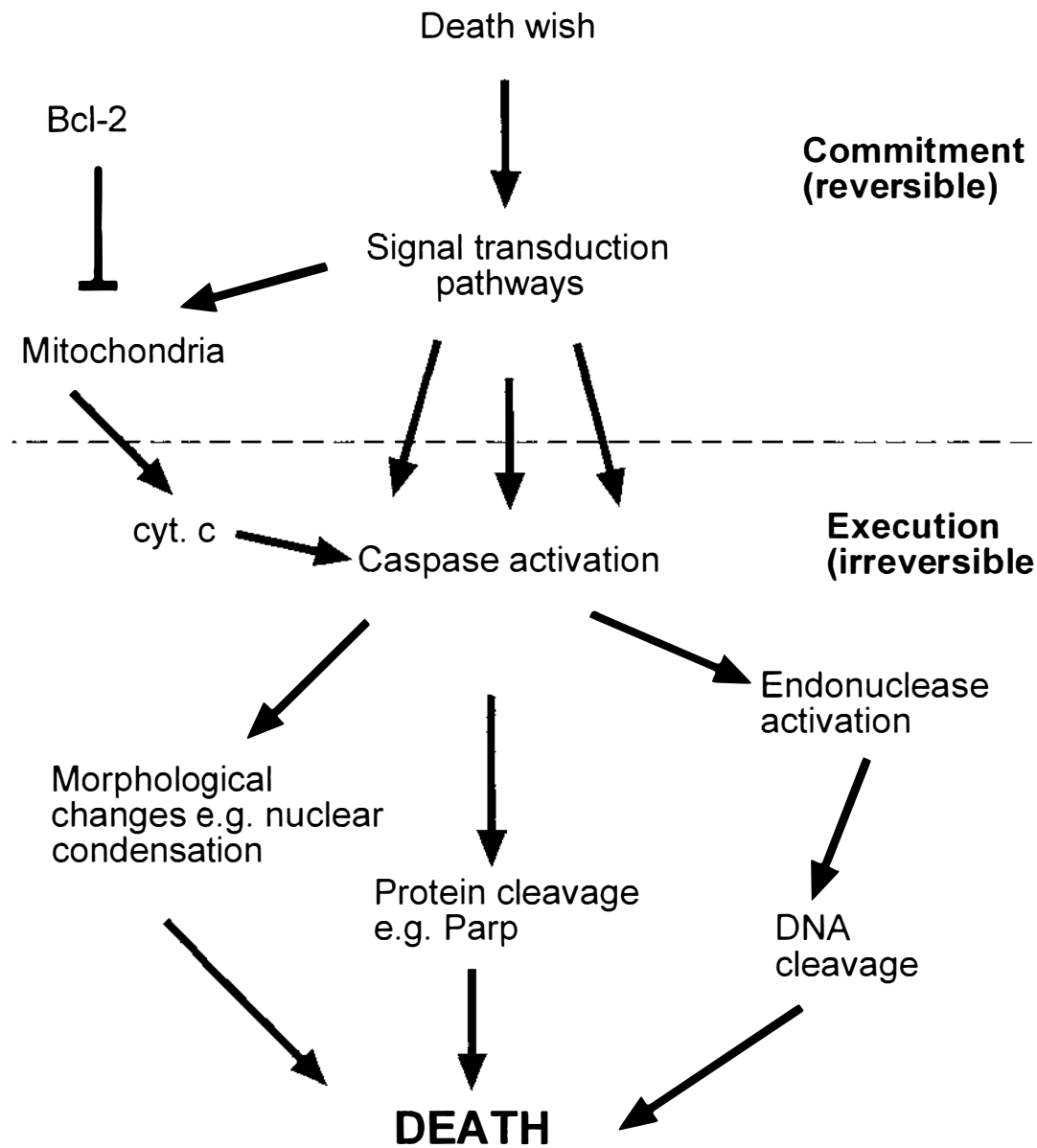

Figure 2: Outline of key steps in apoptotic regulation
Apoptosis can be divided into two stages. Following a death stimulus, the death signal is integrated and transmitted via signal transduction pathways, and the cell decides whether to commit to death. Once the caspases become activated, the execution phase has begun, which is irreversible.

this thesis seek to answer the question of what triggers and regulates apoptosis in neurons. An understanding of the mechanisms that regulate apoptosis in neurons will help in the development of therapies to combat neurodegenerative disease.

Chapter 2

Literature Review

“The strategy of maintaining metazoan life seems to involve investment in a superbly designed, highly efficient and tightly controlled removal kit that is competent not only to kill unwanted cells on cue, but to bury the evidence, and fast “ (Wyllie, 1998).

2.1 Molecular Machinery of Apoptosis

2.11 *C. elegans*

The molecular machinery for apoptosis appears to be conserved in all multicellular organisms. Activation of the apoptotic suicide mechanism is genetically encoded in the nematode, *C. elegans*. *C. elegans* is born with 1090 cells, 131 of which die by an intrinsic cell death programme during development. Two genes, *ced-3* and *ced-4* appear essential for the cells to undergo this programmed cell death because loss-of-function mutations in either of these genes prevents the death of all 131 cells (reviewed in Yuan, 1995; Hengartner, 1996). Another gene *ced-9* is necessary for the survival of most of the cells in the worm, including those not normally subject to programmed cell death. Mutation of *ced-9* is embryonic lethal and *ced-9* acts to suppress *ced-3/ced-4* -dependent cell death.

The nematode gene *ced-9* encodes a 280 amino acid protein with sequence and structural similarities to the mammalian proto-oncogene *bcl-2* (Hengartner & Horvitz, 1994a). Human *bcl-2* can functionally replace mutant *ced-9* in *C.elegans* which strongly suggests that the molecular mechanism of programmed cell death is evolutionarily conserved (Vaux, et al., 1992; Hengartner & Horvitz, 1994a). In mammals, Bcl-2 is a member of a family of related proteins, which includes both pro- and anti-apoptotic members. Further evidence that the molecular machinery is conserved comes from studies showing that over-expression of *ced-3* or *ced-4* in mammalian cells causes cell death (Miura,et al.,1993; Chinnaiyan, et al.,1997).

The *ced-3* gene was originally discovered to encode a protein with homology to the vertebrate protein interleukin-1β converting enzyme (Ice), a cysteine aspartic acid protease, which cleaves the inactive precursor of interleukin-1 to generate the active cytokine (Yuan, et al., 1993). Over 12 related cysteine aspartate proteases have since been discovered and renamed caspases. The caspases exist as inactive polypeptides (zymogens) which can be activated by

removal of the regulatory prodomain and assembled into active heteromeric proteases (reviewed in Vaux, et al., 1997). The Ced-3 caspase acts as an executioner of cell death by cleaving vital cellular proteins (reviewed in Thornberry & Lazebnik, 1998).

The mammalian homologue to *ced-4* was identified as *apaf-1,* for Apoptosis Promoting Factor (Zou, et al., 1997). The molecular mechanism by which Ced-4 functions also appear to be evolutionarily conserved in *Drosophila* because ectopic overexpression of Ced-4 in the *Drosophila* eye causes massive apoptotic death through caspase activation (Kanuka, et al.,1999).

Genetic studies in *C. elegans* suggest that *ced-4* acts upstream of or parallel to *ced-3* to induce cell death (Shaham & Horvitz, 1996a). In addition, every cell in the nematode contains the Ced-9, Ced-4 and Ced-3 proteins, which act in a cell-autonomous manner. So, there must be some sort of regulatory mechanism present to ensure the appropriate death of a subset of cells within the organism during development. Ced-4 is proposed to act as a positive regulator of caspases by enhancing their processing to mature forms. Ced-4 can directly recognize and bind to the caspase recruitment (CARD) prodomain of Ced-3. ATP-hydrolysis associated with Ced-4's ATP-binding site (P-loop) is required for Ced-3 processing mediated by Ced-4 (Chinnaiyan, et al., 1997). The Ced-3/Ced-4 complex is activated by oligomerization of Ced-4, which brings multiple proCed-3 molecules into close apposition (Yang, et al., 1998b). Ced-4 activity is normally antagonized by Ced-9 (Spector, et al., 1997; Chinnaiyan, et al., 1997), which sequesters it to intracellular membranes preventing its interaction with Ced-3 (Wu, et al., 1997). Another *C. elegans* protein Egl-1 is proposed to bind to the Ced-9/Ced-4 complex, releasing Ced-4 from its mitochondrial localisation, allowing it to activate Ced-3 (del Peso, et al., 1998) (Figure 3). The *egl-1* gene was originally identified by gain-of-function mutations that confer an egg-laying defect due to the ectopic deaths of HSN neurons in the hermaphrodite (Conradt & Horvitz, 1998). By contrast, loss-of-function *egl-1* mutations block most somatic programmed cell deaths.

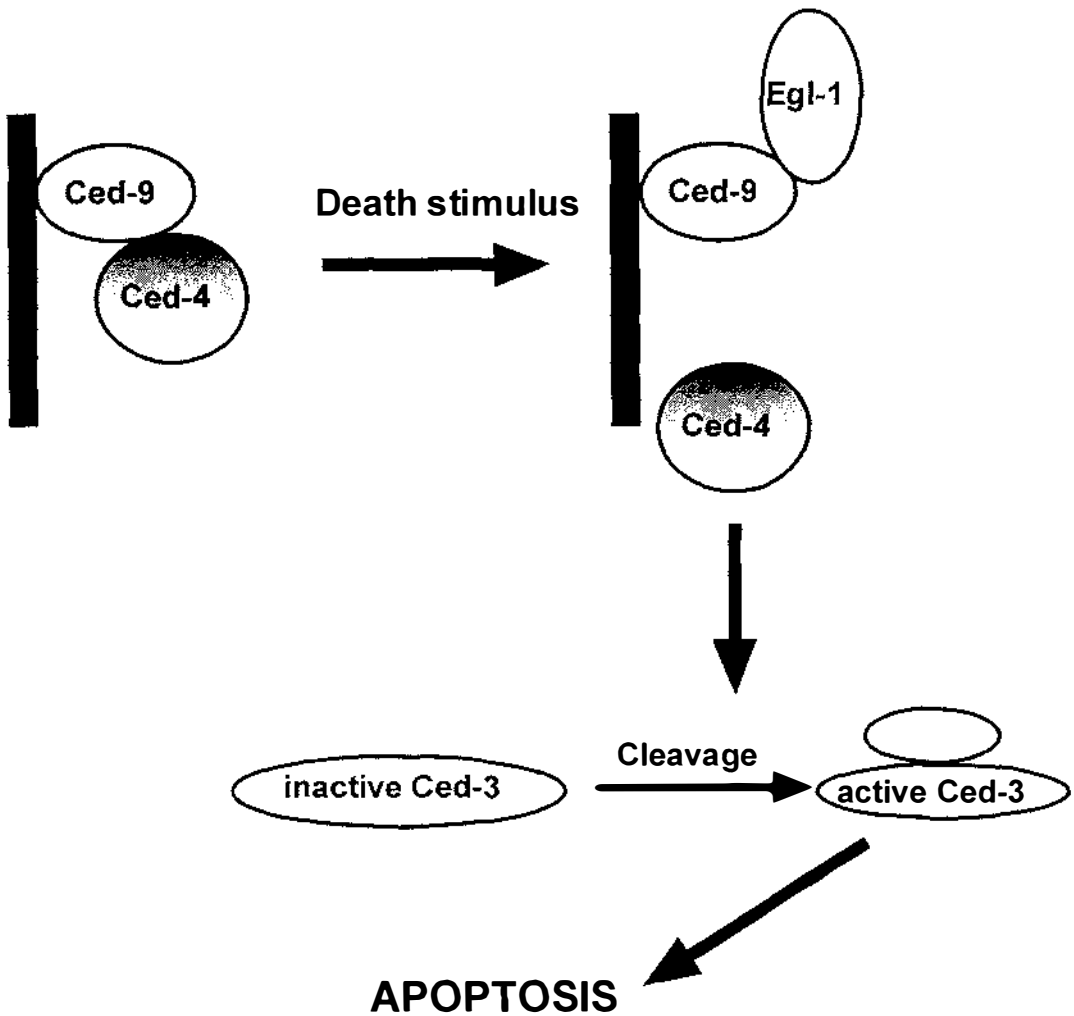

Figure 3: Model of activation of PCD in *C. elegans*
Normally, Ced-4 protein is sequestered away from Ced-3 by being bound by Ced-9 on intracellular membranes. In response to upstream signals, Egl-1 protein is produced, which can bind Ced-9 displacing Ced-4 from the complex. Cytosolic Ced-4 can then promote proteolytic processing of Ced-3 resulting in the activation of apoptosis within the cell.

This figure is adapted from Metzstein, et al., (1998) and del Peso, et al., (1998).

However this simple model does not explain all of the data. The *ced-9* gene may have both killing and protective activities. Loss-of-function alleles of *ced-9* can enhance survival of cells in weak *ced-3* mutants, while wild type *ced-9* can antagonize the gain-of-function *ced-9 n1950* mutant's ability to prevent cell death (Hengartner & Horvitz, 1994b). *Ced-9* homologs, *bcl-2* and *bcl-x*$_L$ also encode opposing cell death activities but these are generated by proteolytic processing, or by alternative splicing (Boise, et al., 1993; Cheng, et al., 1997).

Ced-4 isoforms, encoded by alternatively spliced transcripts, can function as a cell death activator or inhibitor (Shaham & Horvitz, 1996b). Ced-4L (L, long), contains 24 additional amino acids inserted between amino acids 212 and 213 of the 549-amino acid form, Ced-4S (S, short). Overexpression of the *ced-4S* transgene, causes cell death while *ced-4L* overexpression protects against programmed cell death. Ced-4L is believed to act as an interfering dominant negative form of the Ced-4 protein. Consistent with this hypothesis, Ced-4L has been shown biochemically to bind to, but not promote processing of the pro-enzyme form of Ced-3 (Chaudhary, et al.,1998).

Programmed cell death in *C. elegans* is proposed to be the consequence of a differentiated cell fate during development rather than reflecting an underlying cellular defect. A key question is how do developmental signals control the activities of the killing machinery comprising *egl-1, ced-9, ced-3* and *ced-4*? One possibility is that elevation of transcription of one of these genes could trigger cell death. In support of this hypothesis is the observation that overexpression of *egl-1* or *ced-3* is sufficient to cause cell death (reviewed in Metzstein, et al., 1998).

In addition, two cell death specification genes, *ces-1* and *ces-2*, have been identified that control a subset of programmed cell deaths in *C. elegans*. Mutations in these genes block the deaths of certain neural cells (Ellis & Horvitz, 1991). *Ces-2* encodes a member of the basic-leucine zipper (bZIP) family of transcription factors, which is consistent with the idea that PCD is controlled at the level of gene expression (Figure 4). Genetic studies indicate

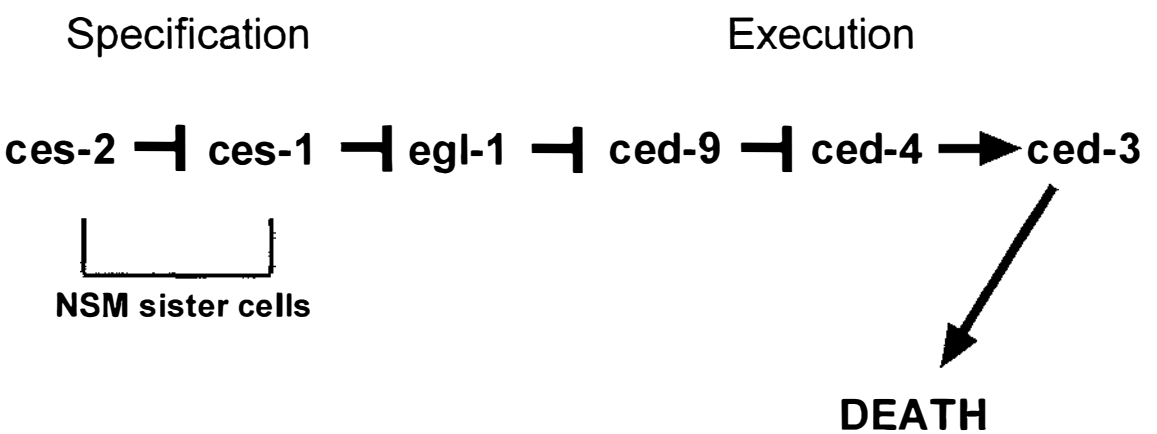

Figure 4: Genetic pathway for PCD in *C. elegans*
In neurosecretory motor neurons (NSM) sister cells, 2 genes, *ces-1* and *ces-2,* specify when a cell is to die. *Ces-1* negatively regulates *egl-1* which can induce cell death by preventing *ced-9* from inhibiting *ced-4's* activation of *ced-3.*

This figure is adapted from Conradt & Horvitz (1998).

that *ces-2* is a negative regulator of *ces-1*, possibly by repressing *ces-1* expression. *Ces-1* itself can negatively regulate cell death.

Six other *C.elegans* genes, *ced-1, ced-2, ced-5, ced-6, ced-7* and *ced-10,* have been characterized which are involved in phagocytosis of dying cells by their neighbours (Ellis, et al., 1991). *Ced-6* acts within engulfing cells and can promote engulfment of cells at both early and late stages of apoptosis (Liu & Hengartner, 1998). Based on its structure, Ced-6 protein is proposed to act as an adaptor protein in a signal transduction pathway mediating engulfment. It contains a phosphotyrosine-binding site and several potential SH3-binding sites. *Ced-7* has sequence similarity to the mammalian ABC transporter proteins and is localised to the plasma membrane in both dying and engulfing cells (Wu & Horvitz, 1998). Ced-7 protein is proposed to function by translocating molecules that mediate adhesion between the cell surfaces of the dying and engulfing cells. *Ced-2, Ced-5,* and *Ced-10* are required for cell corpse engulfment. *Ced-5* is similar to human *doc180* which is involved in cell shape changes (Wu & Horvitz, 1998), while *ced-2* encodes a CrkII-like SH2- and SH-3 containing adaptor protein, and *ced-10* encodes a Rac-like GTPase which is involved in cytoskeletal re-organisation. *Ced-2* and *ced-5* are believed to interact with each other and act upstream of *ced-10* in the phagocytosis process.

2.12 Drosophila

The Ced-3/caspase and Ced-4/Apaf-1 machinery is conserved in *Drosophila.* Five *Drosophila* members of the caspase family, Dcp-1, Drice, Decay, Dredd and Dronc, have been identified and implicated in the regulation of PCD (Song, et al., 1997; Fraser, et al., 1997; Chen, et al., 1998; Dorstyn, et al., 1999a; Dorstyn, et al., 1999b). Dcp-1, Decay and Drice have short pro-domains and resemble effector (downstream) caspases, while Dredd and Dronc have long pro-domains and resemble the initiator (upstream) caspases. *Dark*, a fly homologue of *ced-4* and *apaf-1* has also recently been identified (Rodrieguez, et al., 1999), which like its mammalian counterpart Apaf-1, is able to associate

with cytochrome c and an initiator caspase Dredd. Dark contains a caspase-recruitment domain (CARD), 2 ATP-binding domains and at least eight WD repeats. Loss-of-function mutations in *dark* prevent programmed cell deaths occurring during development, leading to enlarged nervous systems and defective wings in the mutant flies.

Drosophila, also possess additional apoptosis regulators upstream of the caspases. Deletion of the 75C region of the *Drosophila* genome will prevent all programmed cell deaths in the fly. This region contains 3 genes, *reaper, hid* and *grim,* which are potent death activators that function in parallel to engage a common set of effectors including the caspases (White, et al., 1994). *Reaper* encodes a small novel 65 amino acid protein that, when ectopically expressed induces programmed cell death. *Reaper* transcription is induced by a variety of conditions known to induce cell death, such as exposure to X-rays (reviewed in Hengartner, 1996). Mutations in the second gene, *hid (head involution defective),* result in an incompletely penetrant embryonic lethality, caused by failure of the developing head structures to internalize. Like *reaper,* overexpression of *hid*, causes PCD, which is particularly striking when expressed in the developing adult eye where there is dose-dependent ablation of the ommatidia (Grether, et al., 1995). Hid-induced apoptosis can be inhibited by activation of the Ras pathway. Ras can down-regulate *hid* expression, while Rolled, a Mapk p42/44 homologue, can directly phosphorylate Hid, inactivating the protein (Kurada & White, 1998; Bergmann, et al., 1998). *Reaper* and *hid* appear to act in parallel, because they show an additive, rather than synergistic effect on cell death and can induce cell death in the absence of each other.

The mechanism by which Grim, Reaper and Hid engage the core conserved apoptotic machinery is unknown because none of their homologues have been found in other organisms. Clues to the possible functions of Hid and Grim come from studies overexpressing these proteins in mammalian cells. Both Hid and Grim overexpression induces apoptosis in a caspase-dependent manner that can be antagonised by Bcl-2/Ced-9 in mammalian cells (Claveria, et al., 1998; Haining et al., 1999). Hid and Grim were able to localise to mitochondria in

those cells and may induce apoptosis through a mitochondrial-mediated pathway.

The N-terminal sequences of Reaper, Grim, and Hid, are highly conserved and are similar to N-terminal inactivation domains of voltage-gated potassium (K^+) channels. Synthetic Reaper and Grim peptides were found to induce fast inactivation of K^+ channels, which correlated with apoptotic activity in *Xenopus* oocytes (Avdonin, et al., 1998). Reaper and Grim may participate in initiating apoptosis by stably blocking K^+ channels. Sustained inactivation of K^+ channels will result in chronic membrane depolarization that may lead to the induction of apoptosis, possibly by increasing the level of cytosolic free Ca^{2+}.

The Inhibitor of Apoptosis Proteins (Iaps) may provide a link between Grim, Reaper and Hid, and the core apoptosis machinery. The function of the Iaps is not entirely understood, but they appear to bind and inhibit caspases (see section 2.23 below). Homozygous loss-of-function mutations in *Drosophila iap1 (diap1)* are lethal, and overexpression of Diap1 in the eye prevents apoptosis during eye development (Hay, et al., 1995). Diap1 can bind a processed form of the caspase Drice and inhibit the activity of a second caspase, Dcp-1 (reviewed in Deveraux & Reed, 1999). Hid has been shown to be able to bind Diap1 *in vitro* via its N-terminal domain and deletion of this domain results in a loss of Hid's ability to induce apoptosis (Vucic, et al., 1998;Wang, et al., 1999). It is proposed that Reaper, Hid, and Grim promote apoptosis by disrupting productive Iap-caspase interactions and that Diap1 is required to block apoptosis-inducing caspase activity (Figure 5) (Wang, et al., 1999). Consistent with this hypothesis, Diap1 mutants are embryonic lethal due to widespread cell death associated with a large increase in Diap1-inhibitable caspase activity. Also, Diap1 is still required for cell survival even when expression of *reaper, grim,* and *hid* is eliminated.

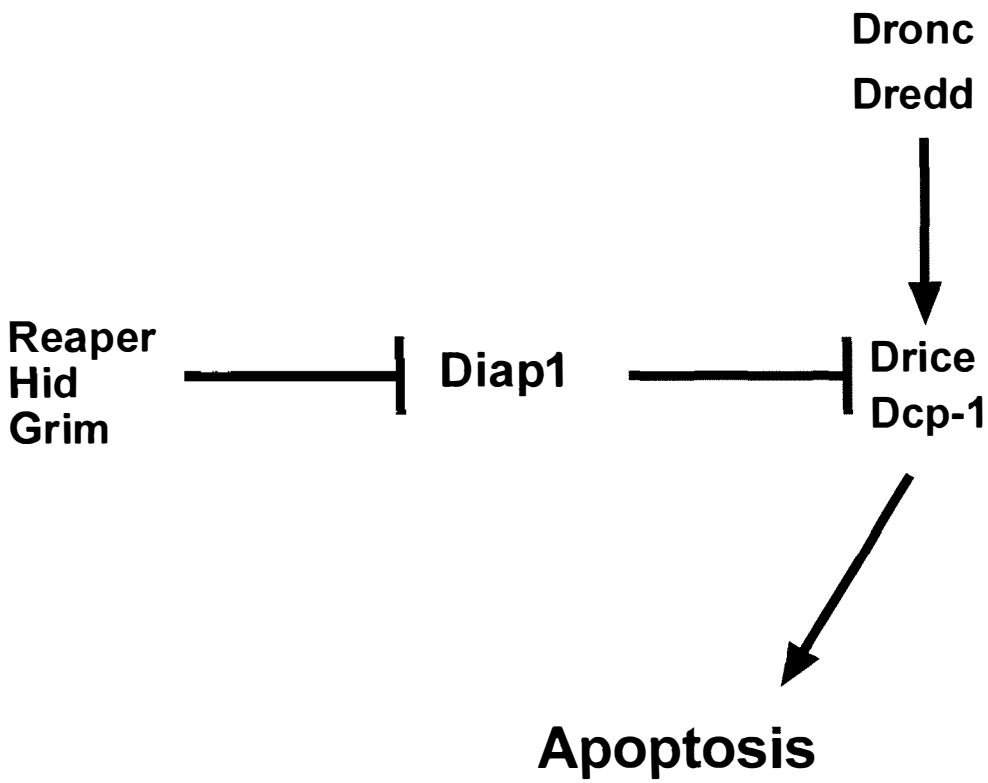

Figure 5: Model for PCD in *Drosophila*
Grim, Reaper, and Hid are believed to promote apoptosis by disrupting interactions between Iaps and caspases. Experimental evidence supports a model where Diap1 associates with and binds to the effector caspases Drice and Dcp-1, which lie downstream of the apical caspases Dredd and Dronc. It is proposed that Grim, Reaper and Hid bind Diap1, removing its inhibitory effect on the caspases.

2.13 Vertebrates

Caspases, Apaf-1 and Bcl-2 family proteins make up the core conserved apoptosis regulatory machinery in mammals.

Bcl-2 family of proteins

The proto-oncogene *bcl-2* appears to function in mammals as *ced-9* functions in *C. elegans* by offering protection against a variety of cell death stimuli. Bcl-2 can protect neurons from apoptosis caused by growth factor withdrawal (Garcia, et al., 1992; Batistatou, et al., 1993), PC12 cells from calcium-induced apoptosis (Mah, et al., 1993), or thymocytes from apoptosis induced by glucocorticoids or γ-irradiation (Sentman, et al., 1991; Strasser, et al., 1991). In contrast to Ced-9 knockouts, which are embryonic lethal, Bcl-2 knockout mice develop normally and only later exhibit marked lymphoid apoptosis, neuronal and intestinal lesions, and kidney disease (Veis, et al., 1993).

Bcl-2 is an integral membrane protein located mainly on the outer mitochondrial membrane but is also localised to nuclear and endoplasmic reticulum (ER) membranes (Hockenberry, et al., 1990; Krajewski, et al., 1993). Its related proteins, the Bcl-2 family, regulate apoptosis by interacting with one another (reviewed in Williams & Smith, 1993). Members of the Bcl-2 family are functionally classified into two groups (Figure 6). The first group, including Bcl-2, Bcl-X_L and Mcl-1, suppresses apoptosis. These proteins contain all 4 Bcl-2 homology (BH) domains, BH1, BH2, BH3 and BH4. The second group, including Bax, Bak, Bad, Bik, Bim, and Bcl-X_S, promotes apoptosis. Members of this group lack the BH4 domain and other domains and may only contain the BH3 domain. The BH3 domain is essential for the function of the “killer” proteins, including their *C. elegans* homolog Egl-1 (reviewed in Adams & Cory, 1998).

All pro-survival Bcl-2 family members are potentially oncogenic while proapoptotic family members may act as tumour suppressors. The *bcl-2* gene

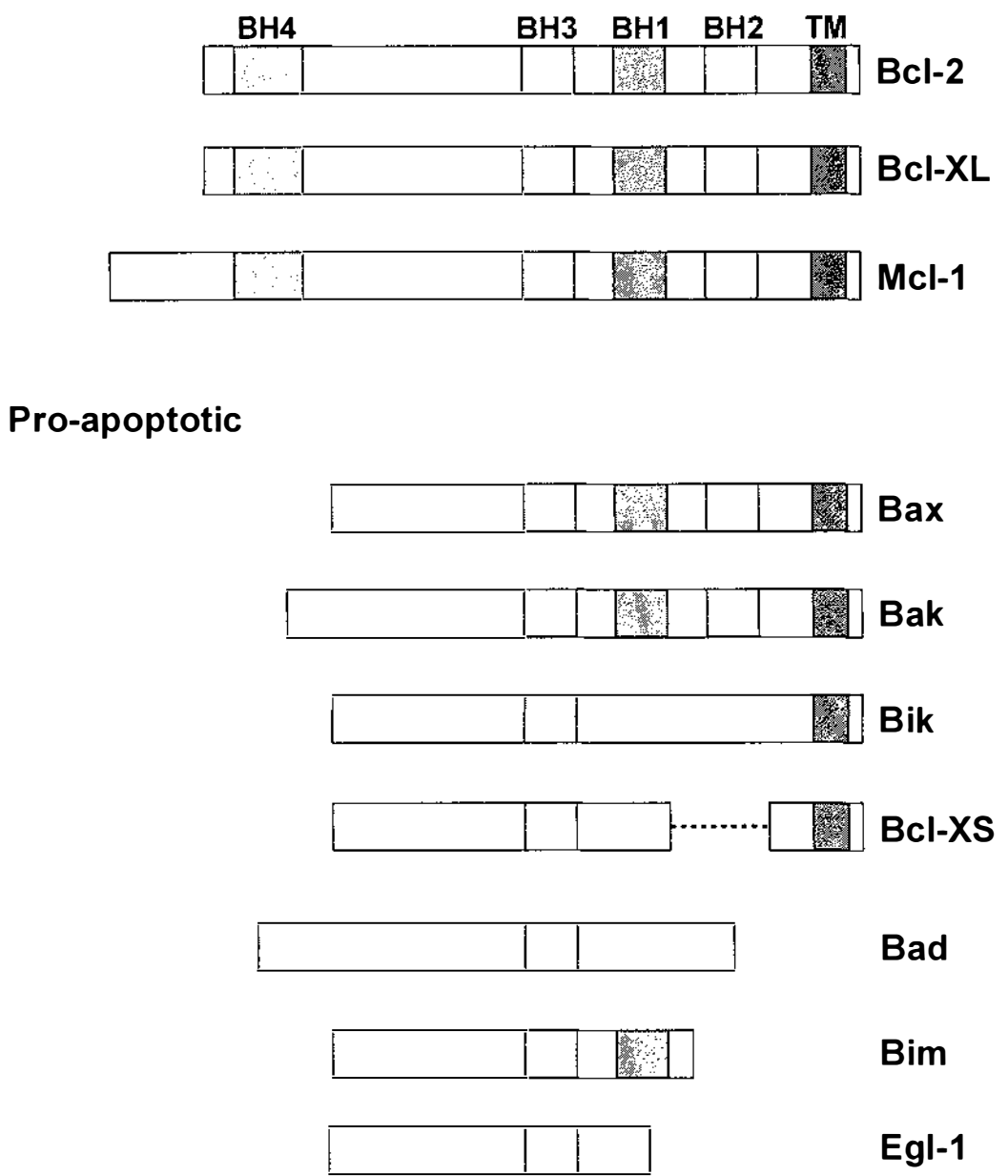

Figure 6: Bcl-2 related apoptotic regulators
Schematic representation of Bcl-2 family members and Egl-1 from *C. elegans* with their Bcl-2 homology (BH) domains and hydrophobic membrane targeting domain (TM) indicated.
The deletion in Bcl-XS resulting from differential splicing is indicated by dashed lines. The proteins are not drawn to scale.

This figure is adapted from Rao & White (1997) and Adams & Cory (1998).

was originally identified due to its translocation and amplification in follicular lymphoma and it demonstrates a profound ability to block apoptosis (reviewed in Fisher, 1994). Levels of Bcl-2 protein correlate with relative resistance to a wide spectrum of chemotherapeutic drugs and γ-irradiation (reviewed Reed, et al., 1996). In contrast, loss-of-function mutations in pro-apoptotic Bcl-2 family member, *bax*, occur in human gastrointestinal cancer and some leukemias (reviewed in Reed, et al., 1996; Adams & Cory, 1998). *Bax* expression is induced by p53 during apoptosis, and tumour growth is accelerated and apoptosis decreased by 50% in *bax*-deficient mice when compared to wild type mice (Yin, et al., 1997).

Bcl-2 family members can form homodimers and heterodimers with each other *in vitro* but the physiological significance of this is controversial. Bcl-2 and Bcl-X_L were originally thought to confer their cell death protection activities by heterodimerising with death-promoting members, thus preventing the formation of "killer" homodimers (Oltvai, et al., 1993). However, mutagenesis studies suggest that heterodimerization is not required for the pro-survival function of Bcl-2 and Bcl-X_L (Otter, et al., 1998; Minn, et al., 1999). In addition, Bax homodimer formation may not occur physiologically as it normally exists as a soluble monomer with homodimer formation being attributed as a detergent-induced phenomenon (Hsu & Youle, 1998). Instead, Bax is proposed to move from the cytosol to the mitochondria during apoptosis to cause release of cytochrome c leading to a cascade of caspase activation (see section below) (Hsu, et al., 1997; Wolter, et al., 1997; Jurgensmeier, et al., 1998; Rossé, et al., 1998).

Cellular localisation also appears to be important in regulating the activity of Bcl-2 family member Bim. Proapoptotic protein Bim is normally sequestered to the microtubular dynein motor complex by interaction with dynein light chain LC8 in live cells. Apoptotic stimuli provoke release of Bim and LC8, allowing Bim to associate with Bcl-2-like proteins (Puthalakath, et al., 1999). Bim knock-out mice have accumulations of lymphoid and myeloid cells, perturbed T cell development and autoimmune kidney disease (Bouillet, et al., 1999), so Bim

intracellular movement appears to be a key trigger of apoptosis in those cell types.

Caspase regulation

Caspases are produced as inactive zymogens and activated by two proteolytic cleavage events. The first, divides the proenzyme chain into large and small caspase subunits, while the second, removes the N-terminal prodomain. The subunits assemble into a tetramer with two active sites. The caspases can be divided into two groups; those with long prodomains (e.g. caspase -1,-2,-8,-9,-10,-11,-12) are referred to as initiator or apical caspases, while those with short prodomains (e.g. caspase-3,-6,-7) are effector or downstream caspases. The caspases are proposed to activate each other through a proteolysis cascade. In addition, the procaspases possess a small amount of catalytic activity and procaspase aggregation is believed to be an important step leading to caspase activation (Yang, et al., 1998a). Procaspase aggregation is mediated by binding of adaptor proteins and recruitment to an apoptosome complex. The apoptosome complex brings adaptor and caspase molecules into close proximity allowing intermolecular catalysis to occur.

Apaf-1 is one of these adaptor proteins, that binds caspases. It contains a caspase-recruitment domain (CARD) and bears homology to Ced-4 (Zou, et al., 1997). Despite their overall conservation of sequence and physical properties, Apaf-1 and Ced-4 differ in two important ways. The Apaf-1 protein contains an additional domain consisting of 12 WD-repeat sequences and requires cytochrome c as a co-factor for its activity (Li, et al., 1997). Apaf-1 forms a complex with caspase-9 in the presence of cytochrome c and dATP (Li, et al., 1997). Deletion of the WD-repeat region renders Apaf-1 constitutively active independent of ATP/dATP and cytochrome c (Hu, et al., 1998; Srinivasula, et al., 1998). Using purified components to reconstitute caspase-9 activation *in vitro*, Apaf-1 was found to bind and hydrolyse ATP or dATP to ADP or dADP, respectively (Zou, et al., 1999). The hydrolysis of ATP/dATP and binding of cytochrome c promoted Apaf-1 oligomerization, forming a large multimeric Apaf-

1:cytochrome c complex (Figure 7). This complex could be isolated by gel filtration chromatography and was sufficient to recruit and activate pro-caspase-9. Therefore, formation of a large multi-subunit Apaf-1:cytochrome c complex is proposed to be the functional apoptosome, that activates procaspase-9 by intermolecular catalysis (Zou, et al., 1999). Once activated, caspase-9 can dissociate from the complex and becomes available to cleave and activate downstream caspases such as caspase-3.

Like the Ced-3, Ced-4 and Ced-9 complex in *C. elegans*, Apaf-1, caspase-9 and Bcl-X_L are proposed to form an equivalent ternary complex in mammals. Caspase-9 and Bcl-X_L bound distinct regions in Apaf-1 and co-precipitated with Apaf-1 (Pan, et al., 1998). Recombinant Bcl-X_L could prevent Apaf-1 -dependent processing of caspase-9 but not processing mediated by a constitutively active Apaf-1 mutant (Hu, et al., 1998). This suggests that Bcl-X_L regulates caspase-9 through Apaf-1. However, recent *in vivo* association studies challenge this assumption that Apaf-1 binds Bcl-X_L. Morisiishi, et al., (1999), demonstrated that endogenous Apaf-1 does not interact detectably with either overexpressed or endogenous Bcl-2 or Bcl-X_L. Instead of sequestering Apaf-1, they propose an indirect role for Bcl-2 homologs in inhibiting Apaf-1 activity.

The function of Apaf-1 as an important apoptosis activator has been confirmed by knockout experiments in mice. Apaf-1 knockouts are embryonic lethal, have severe craniofacial malformations, brain overgrowth, interdigital webbing, and abnormal eye development (Cecconi, et al., 1998; Yoshida, et al., 1998). Apaf-1 deficient cells were resistant to a variety of apoptotic stimuli, and the processing of caspases -2, -3, and -8 were impaired. Mutant mice deficient in caspase-9 exhibit abnormalities similar, but not identical, to those observed in mice lacking Apaf-1 (Kuida, et al., 1998; Hakem, et al., 1998) which suggests that caspase-9 may not be the only binding partner for Apaf-1.

Significantly, Apaf-1 knockout mice lack apparent abnormalities in tissues, which depend on cellular apoptosis for development, such as thymus, suggesting the existence of additional Apaf-1 like caspase activators. Recently,

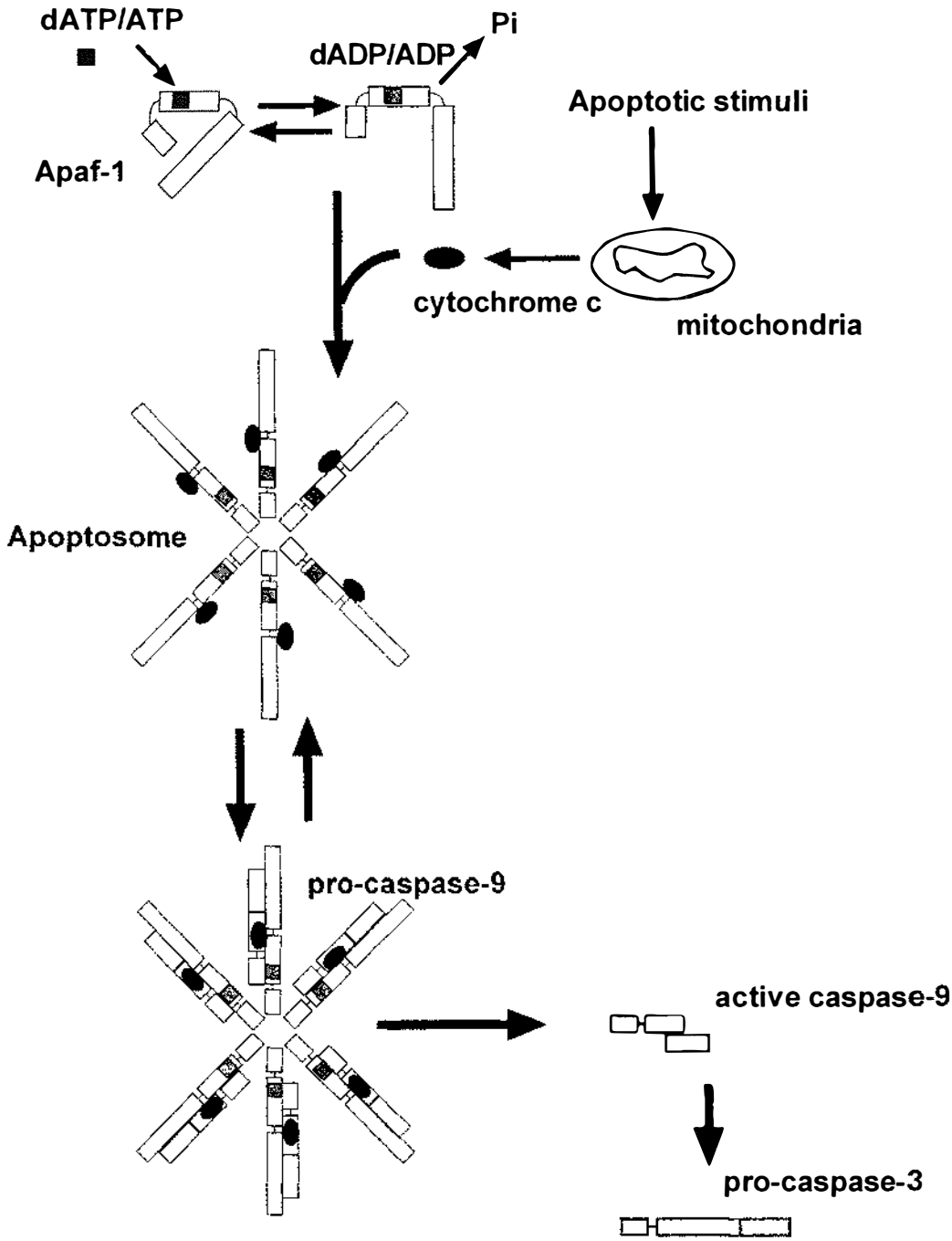

Figure 7: Model of caspase-9 activation
The hydrolysis of ATP/dATP promotes a conformational change in Apaf-1. Cytochrome c can bind this form of Apaf-1 and promote oligomerization into a complex which can recruit and activate caspase-9. Active caspase-9 then promotes the processing of downstream caspases. This figure is adapted from Zou, et al., (1999).

an Apaf-1-like protein, Nod1 has been identified which can bind to multiple caspases with long prodomains but specifically regulates caspase-9 (Inohara, et al., 1999). Unlike Apaf-1, Nod1 contains leucine-rich repeats and induces NF-κB activation. Another mammalian CARD-containing protein, Me10, has also been identified which can oligomerize and mediate activation of caspase-9 to cause cell death (Yan, et al., 1999).

2.2 Control of Apoptosis

In response to a death signal, depending on cell type, mammalian cells can utilise either a mitochondrial, or a death receptor -mediated pathway or both, to die (reviewed in Green & Reed, 1998).

2.21 Role of mitochondria

Mitochondria play an important role in inducing apoptosis by releasing cytochrome c (Liu, et al., 1996; Yang, et al., 1997; Kluck, et al., 1997; Narula, et al., 1999). During apoptosis, cytochrome c can relocalise from the mitochondria to the cytosol where it can interact with Apaf-1 and cause caspase activation. The addition of exogenous cytochrome c to cytosolic preparations from healthy cells activates caspases and causes DNA fragmentation in isolated nuclei (Liu, et al., 1996). Microinjection of cytochrome c into non-neuronal cells causes apoptosis which is caspase-dependent (Zhivotovsky, et al., 1998). In addition, active caspase-3 accelerates release of cytochrome c from mitochondria allowing positive feedback for amplification of the signal (Cosulich, et al., 1999). Overexpression of Bcl-2 and Bcl-X_L prevents release of cytochrome c following stimulation, suggesting that they act upstream of cytochrome c in the death pathway (Yang, et al., 1997).

Two competing models have been proposed as mechanisms for cytochrome c release from mitochondria (reviewed in Green & Reed, 1998). The first model involves a mitochondrial permeability transition (PT) resulting in swelling of the mitochondrial matrix and outer membrane rupture. In support of this model, a

wide variety of apoptotic stimuli induce progressive mitochondrial swelling and outer membrane rupture leading to cytochrome c release and inner mitochondrial membrane depolarization (Vander Heiden, et al., 1997). In a cell-free system, mitochondria induced to undergo PT by drug treatment caused apoptosis in isolated nuclei while PT inhibitor bongkrekic acid reduced apoptosis (Zamzami, et al., 1996). This model cannot account for all types of cell death because in many types of apoptosis the mitochondria remain morphologically normal.

The second model proposes the opening of a specific pore allowing release of factors without gross disruption of the outer mitochondrial membrane (reviewed in Green & Reed, 1998). Recent studies suggest that Bcl-2 family proteins bind the voltage-dependent anion channel (Vdac) and regulate mitochondrial membrane potential and cytochrome c release (Shimizu, et al., 1999). Bax and Bak can regulate release of cytochrome c by promoting opening of Vdac while Bcl-X_L closes Vdac. Other researchers have shown that the adenine nucleotide translocator (Ant) is also required for Bax-induced release of cytochrome c (Marzo, et al., 1998). Perhaps under some circumstances, Ant through binding Bax may open Vdac leading to the formation of a PT pore with mitochondrial swelling, membrane rupture and cytochrome c release as described in the first model.

Unfortunately, these two models do not account for all the data. High levels of Bcl-2 can also protect cells downstream of cytochrome c release, possibly by preventing caspase-mediated mitochondrial amplification of the death signal (Zhivotovsky, et al., 1998; Rossé, et al., 1998; Cosulich, et al., 1999). It is also important to note that cytochrome c relocalisation to the cytosol is not associated with all types of apoptotic stimuli. Apoptosis induced by dexamethasone or anti-Fas antibody in multiple myeloma cells is cytochrome c-independent (Chauhan, et al., 1997).

In addition to cytochrome c, mitochondria may also release other apoptosis-promoting factors. Apoptosis inducing factor (Aif) is released from mitochondria

during apoptosis and translocates to the nucleus where it causes chromatin condensation and DNA fragmentation (Susin, et al., 1999). There is evidence that mitochondria also contain pools of pro-caspase-9, pro-caspase-2, and pro-caspase-3 in the intermembrane space which are released during apoptosis (Krajewski, et al., 1999; Mancini, et al., 1998).

Mitochondria can also trigger apoptosis through the generation of reactive oxygen species (ROS). Bax expression in the yeasts, *S. cerevisiae* and *S. pombe,* causes cell death through generation of ROS (Zha, et al., 1996; Jurgensmeier, et al., 1997). Trace expression of human Bax can kill *E. coli* cells by increasing oxygen consumption, thus, triggering generation of superoxide (Asoh, et al., 1998). Antioxidants can also block or delay apoptosis and it has been suggested that Bcl-2 may function in an antioxidant pathway to prevent apoptosis. Bcl-2 was able to suppress lipid peroxidation and inhibit ROS-induced death in mammalian cells (Hockenberry, et al., 1993).

2.22 Death Receptors

Another pathway for signalling apoptosis is through the activation of death receptors (DR). Death receptors are cell surface receptors that transmit apoptotic signals initiated by specific death ligands. These receptors can activate caspases within seconds of ligand binding, causing demise of the cell within hours (reviewed in Nagata, 1997; Ashkenazi & Dixit, 1998). Death receptors belong to the TNF receptor superfamily and contain a homologous cytoplasmic death domain. Members of the family include TNFR1, Fas, DR3/Apo3, DR4, DR5, DR6 and $p75^{NTR}$. Another class of TNFR homologues are decoy receptors (DcR), DcR1, DcR2 and DcR3, which function as inhibitors of death signalling. These typically lack a functional cytoplasmic domain and prevent induction of apoptosis through death receptors by competing for ligand binding (reviewed in Ashkenazi & Dixit, 1999).

One of the best characterised death receptors is Fas/CD95/Apo1 which is capable of inducing apoptosis in a variety of cell types that express it. Mouse

knockouts in the Fas receptor (*lpr*) and Fas ligand (*gld*) demonstrate the importance of Fas-induced apoptosis in modulating T cells in the immune system. *lpr* and *gld* mutant mice develop an autoimmune disorder similar to the human condition lupus erythmatosus due to lack of removal of autoreactive T cells (Watanabe-Fukunaga, et al., 1992; Takahashi, et al., 1994).

Upon ligand binding, Fas receptor molecules aggregate into a membrane-bound complex. This signalling complex recruits the adaptor protein Fas-associated death domain (Fadd/Mort1) via death domain interactions (Figure 8). Fadd deficient mice die in utero which suggests that Fadd is essential for proper development (reviewed in Ashkenazi & Dixit, 1999). This then allows recruitment of a high local concentration of procaspase-8/Flice/Mach molecules to the complex via interactions through the CARD domain (Figure 8) (Muzio, et al., 1996; Boldin, et al., 1996). This aggregation of procaspase-8 is sufficient to drive intermolecular catalysis of the procaspase-8 molecules. In a similar manner to that in which aggregation of procaspase-9 is mediated by Apaf-1, oligomerization drives caspase-9 activation. Caspase-8 can process other caspases, including caspase-3 and cleave substrates within the cell (Muzio, et al., 1997; Stennicke, et al., 1998). Caspase-8 knockout studies indicate it is essential for apoptosis induction by Fas, TNFR1 and DR3 (Varfolomeev, et al., 1998). Caspase-8 knockouts are embryonic lethal due to impaired heart muscle development and accumulation of erythrocytes. Bcl-X_L can inhibit Fas- and TNF-induced apoptosis downstream of caspase-8 activation (Srinivasan, et al., 1998).

Defects in Fas-induced apoptosis are involved in Canale and Smith Syndrome/ Autoimmune lymphoproliferative syndrome (ALPS). ALPS type I arises from mutations in Fas receptor or Fas ligand, while ALPS type II arises from mutations in caspase-10 (Wang, et al., 1999). Caspase-10 can be recruited along with caspase-8 into apoptosis signaling complexes such as Fas, TNFRI, DR3, DR4 and DR5. Patients accumulate T cells with no CD4 or CD8, and circulating antibodies against their own red blood cells and platelets.

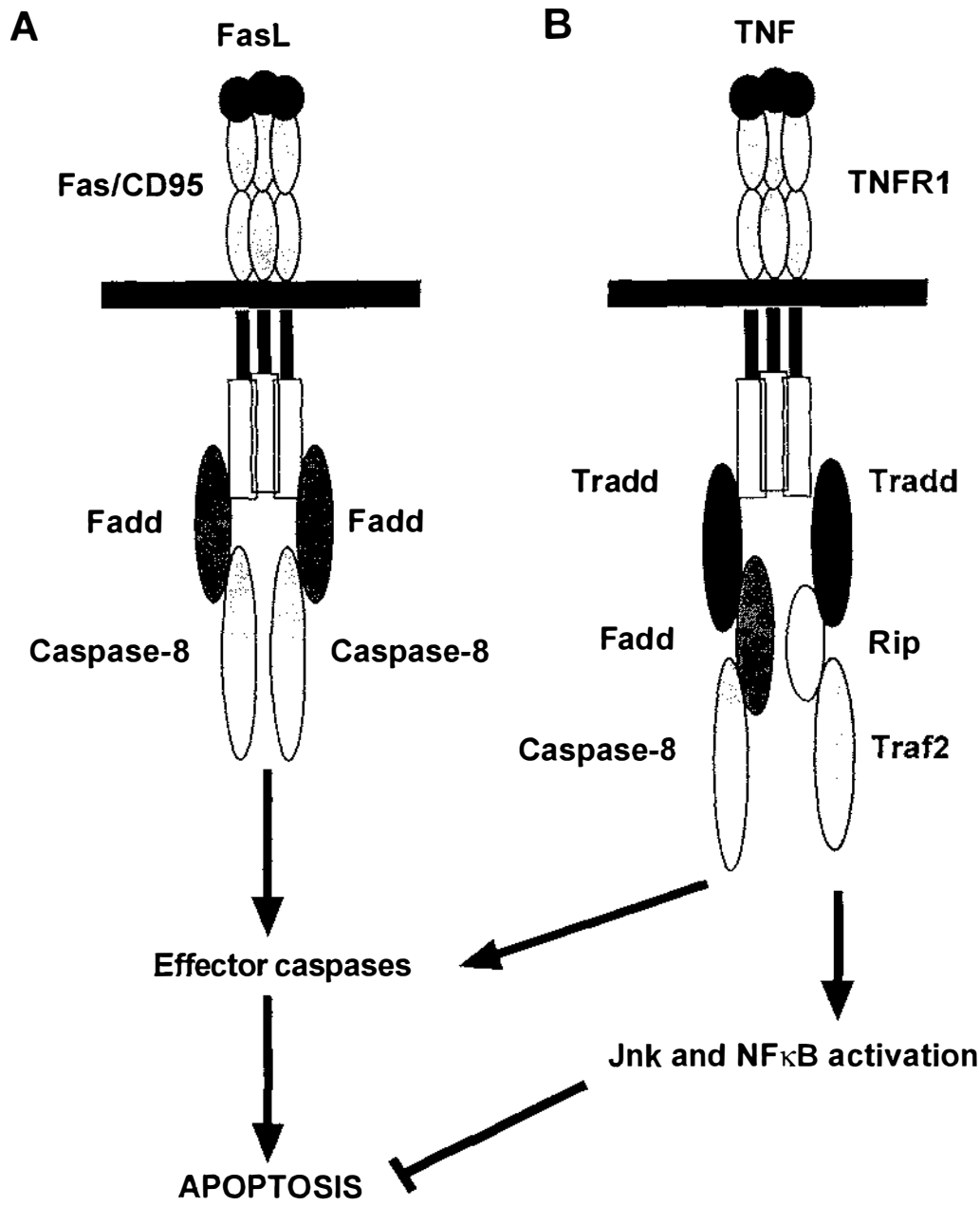

Figure 8:Signalling by Fas/CD95 and TNFR1
A. Fas receptor molecules aggregate following ligand binding which allows recruitment of Fadd and then caspase-8. Caspase-8 is activated in the complex and triggers an apoptotic cascade of events.
B. TNF can trimerise TNFR1 allowing recruitment of Tradd. Tradd can recruit either Fadd to trigger apoptosis or Rip to activate the Jnk and NF-κB pathways.
This figure is adapted from Ashkenazi & Dixit (1998).

Cancerous tumours have a mechanism to avoid a death signal. Tumours can produce a soluble decoy receptor 3 (DcR3) which binds to Fas ligand to block Fas-induced apoptosis (Pitt, et al., 1998). DcR3 is genetically amplified in a number of lung and colon carcinomas, suggesting that Fas/FasL interactions limit cancer growth, and that cells expressing higher levels of DcR3 may be more likely to become cancerous. In addition, Fas is downregulated or mutated in some cancers.

TNF can activate transcription factors via TNFR1 leading to induction of proinflammatory genes in response to infection (reviewed in Ashkenazi & Dixit, 1998). TNF can also induce apoptosis through TNFR1 but in distinct situations, for example, when protein synthesis is blocked. TNF trimerises TNFR1 upon binding allowing association of the adaptor protein TNFR-associated death domain (Tradd) with the receptor (Figure 8). Tradd can recruit several signalling molecules to the receptor such as TNFR-associated factor-2 (Traf2) and receptor-interacting protein (Rip) and Fadd. Rip and Traf2 stimulate pathways leading to activation of NF-κB and Jnk which is antiapoptotic, while Fadd mediates activation of apoptosis through caspase-8. In addition, a mechanism exists to protect against ligand-independent signalling by TNFR1 and DR3: Silencer of death domain (SODD) proteins associate with the death domain of TNFR1 preventing recruitment of Tradd (Jiang, et al., 1999).

There is cross-talk between the receptor-mediated caspase-8 pathway and the mitochondrial regulated caspase-9 pathway for apoptosis. Bid, a proapoptotic member of the Bcl-2 family, is cleaved by caspase-8 and the C-terminal cleavage fragment, truncated Bid (tBid), induces cytochrome c release from mitochondria (Li, et al., 1998; Luo, et al., 1998). The Apaf-1/caspase-9 pathway can then amplify the caspase-8 signal. Depletion of Bid from cytosolic extracts disrupted the ability of caspase-8 to cause cytochrome c release *in vitro.* Bid is proposed to bind Bax, leading to a change in Bax conformation allowing cytochrome c release from mitochondria (Desagher, et al., 1999). Bid-deficient mice have no apparent developmental abnormalities but are resistant to Fas-induced liver apoptosis (Yin, et al., 1999). Bid-deficient cultured cells exhibited

no cytochrome c release and reduced effector caspase activity in response to anti-Fas or TNF treatment. Interestingly, thymocyte sensitivity to Fas-induced apoptosis was unaffected. This indicates that Bid is a critical target *in vivo* for mitochondrial amplification of death receptor signals and is specific to liver cells.

2.23 Inhibitors of apoptosis

Because caspases have the potential to initiate cascades of proteolysis, it is important that their activity be tightly regulated. The only cellular caspase inhibitors that have been identified so far share homology with a family of proteins identified in baculoviruses called Inhibitors of Apoptosis Proteins (Iap) (reviewed in Uren, et al., 1998; Deveraux and Reed, 1999). It is believed that Iaps function in baculoviruses to prevent a host defensive apoptotic response, which would limit virus replication. Many Iaps have been reported to bind and inhibit caspases. These proteins contain one or more N-terminal repeats of a baculovirus Iap repeat motif (BIR) and a C-terminal RING finger motif.

The mammalian Iaps c-Iap1, c-Iap-2, and Xiap have been shown to bind directly to and inhibit caspases -3, -7 and -9 but not caspases -1,-6,-8,-10 or Ced-3. Survivin, another Iap, can also be coimmunoprecipitated with caspases -3, -7 and -9 and can suppress apoptosis caused by caspase overexpression (reviewed in Uren, et al., 1998; Deveraux & Reed, 1999). These mammalian Iaps are believed to block caspase activation and apoptosis downstream of Bax and cytochrome *c.*

2.3 Execution events

The final stage of apoptosis, called execution, occurs through the activation and function of caspases. Caspases act in an executive role in nuclear apoptosis by activating downstream factors that disassemble nuclei (Samejima, et al., 1998) and directly cleaving important structural and nonstructural proteins. It must be noted that while this is generally true there are exceptions. For example, cells can activate caspases without undergoing apoptosis (Boise & Thompson, 1997)

and cell death can also occur in the presence of caspase inhibitors (McCarthy, et al., 1997; Samejima, et al., 1998). Cells dying in the presence of caspase inhibitors display membrane blebbing and cell surface alterations but no changes in nuclear morphology (McCarthy, et al., 1997).

Caspases inactivate key enzymes and structural proteins during apoptosis. Cleavage of DNA dependent protein kinase and poly-(ADP)-ribose polymerase (Parp) unhooks DNA repair from DNA damage, lamin cleavage unpins the nuclear envelope while cleavage of keratin, actin and spectrin effects cell shape changes and movement during apoptosis.

Caspase-mediated proteolysis can activate cell death effectors. During apoptosis, caspase-3 activates gelsolin, which severs actin filaments and helps effect morphologic change during apoptosis such as cell rounding and blebbing (Kothakota, et al., 1997). Caspases can also cleave p21-activated kinase 2 (Pak2) activating it. Paks regulate membrane and morphological changes during apoptosis (Rudel & Bokoch, 1997). In addition, caspase cleavage activates the chromatin condensation factor Acinus (Sahara, et al., 1999).

Caspase-activated DNase, Cad (also called Dff40), is responsible for the DNA fragmentation that occurs during apoptosis. Chromosomal DNA is cleaved between nucleosomes to generate an internucleosomal ladder visible following gel electrophoresis. Cad is complexed with an inhibitor Icad (also called DFF45) in non-apoptotic growing cells, which also serves as a molecular chaperone to mediate correct Cad folding during translation (Enari, et al., 1998). Caspase-3 and caspase-7 activate Cad by cleaving its regulatory inhibitor Icad, unmasking a nuclear localisation signal, thus allowing it to translocate to the nucleus and cause DNA fragmentation (Liu, et al., 1997; Enari, et al., 1998; Sakahira, et al., 1998). Cad cleaves DNA by generating double strand breaks. Because double strand DNA breaks are themselves apoptotic signals, the cleavage of genomic DNA by Cad may trigger an amplification cycle that marks an irreversible step for apoptosis.

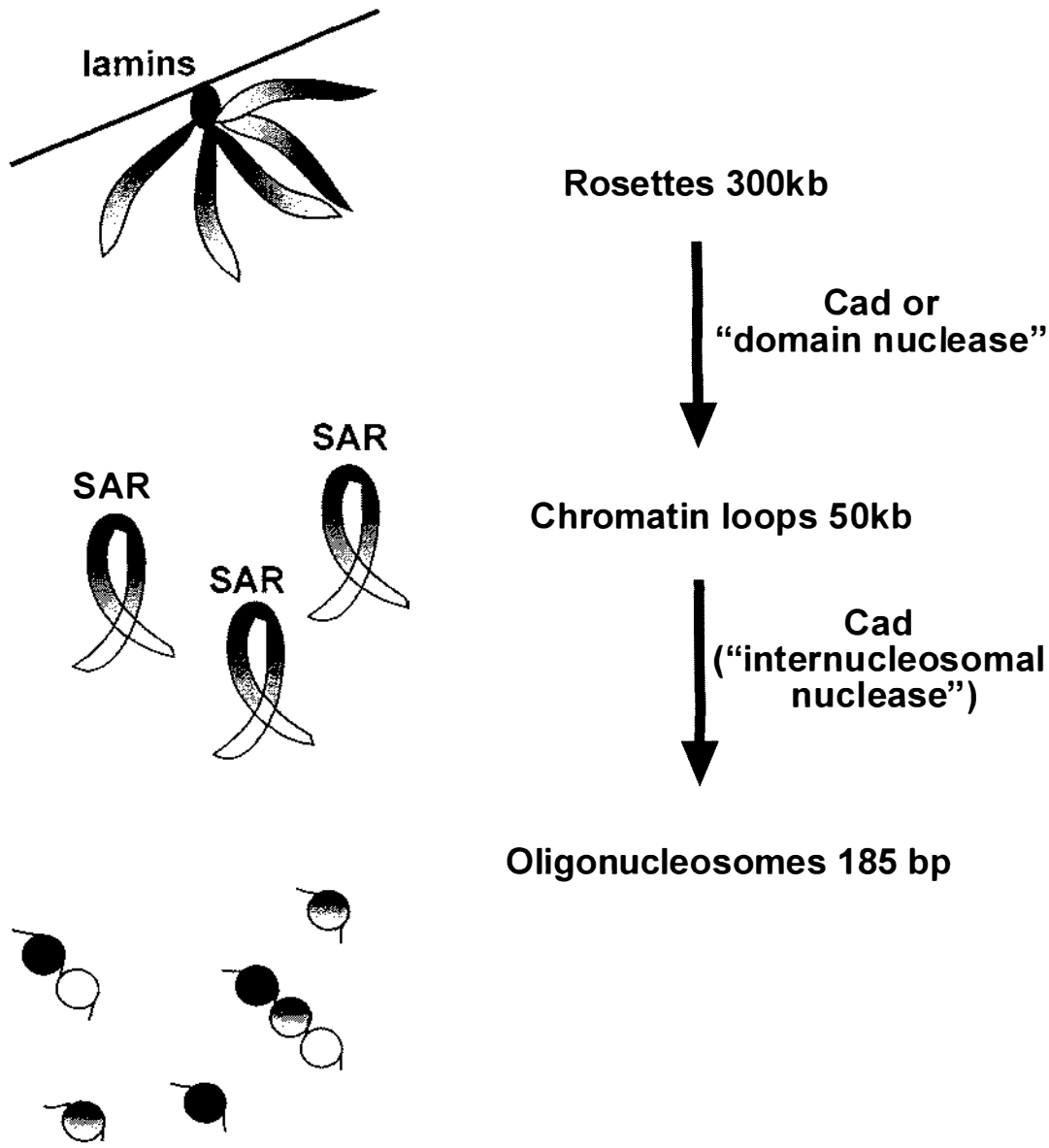

Figure 9: Multi-step chromatin degradation in apoptosis
Chromatin is cleaved first at scaffold attachment regions (SARS) to yield 50 and 300 kb DNA fragments and then between nucleosomes to generate the characteristic oligonucleosomal fragments.

Active Cad also cleaves chromatin to generate transient large fragments of uniform size before the appearance of the internucleosomal ladder (Enari, et al., 1998), suggesting that Cad may be responsible for this cleavage event also. Alternatively, a separate “domain” nuclease has been proposed (Figure 9) (Brown, et al., 1993; Oberhammer, et al., 1993). Cleavage of DNA to 300kb or 50 kb fragments occurs in many models of apoptosis, and is thought to indicate release of chromatin loops from attachment at nuclear scaffold attachment sites (Filipski, et al., 1990; Brown, et al., 1993; Oberhammer, et al., 1993; Lagarkova, et al., 1995).

DNA degradation and nuclear signaling are not required for induction of cell death. Blocking Cad activity by overexpression of Icad does not prevent apoptosis (Sakahira, et al., 1998). Enucleated cells and cytoplasts are still able to undergo apoptosis, which can be prevented by Bcl-2 (Schulze-Osthoff, 1994; Jacobson, et al., 1994; Nakajima, et al., 1995). Icad knockout mice have no Cad activity as Icad is needed for correct folding of Cad (Zhang, et al., 1998). These mice exhibit no obvious abnormalities so DNA degradation appears to be dispensible during apoptosis in mammalian cells.

Why does the cell destroy DNA during apoptosis? Perhaps because it is a much tidier way to die. Each cell contains about 1.5 metres of coiled DNA, which would definitely cause a problem for surrounding cells if structural integrity was lost. Also, free DNA is potentially dangerous because it could colonise or scramble the genomes of neighbouring cells.

2.4 Signal transduction in apoptosis

Signal transduction networks provide the nucleus with information concerning the cell’s environment and coordinate the cell’s response to environmental changes. Appropriate signal transduction pathways are turned on or off depending on a survival or death stimulus. Many of the pathways signalling for survival are potentially oncogenic, while molecules that down-regulate these

pathways are proapoptotic. Several of these signal transduction pathways are discussed in detail below.

2.41 PI3-K and Akt signalling in survival

Activation of class I phosphatidylinositol 3-kinases (PI3-K) are required for growth factor-induced survival in many cell types (Yao & Cooper, 1995; Yao & Cooper, 1996; Dudek, et al., 1997; D'Mello, et al., 1997). PI3-K inhibitors block NGF-promoted survival in PC12 cells and sympathetic neurons, while dominant negative PI3-K induces apoptosis in NGF-maintained sympathetic neurons (Yao & Cooper, 1995; Crowder & Freeman, 1998).

Growth factor activation of receptor tyrosine kinases causes association of SH2 domains on the 85 kDa regulatory subunit of PI3-K with specific phosphotyrosine residues on activated receptors (Figure 10). Binding of the 110 kDa catalytic domain of PI3-K brings it to the plasma membrane where it can phosphorylate the D-3 position of phosphatidylinositol (PtdIns), phosphatidylinositol-4- phosphate (PtdIns-4-P), and phosphatidylinositol-4,5-bisphosphate (PtdIns-4,5-P_2) to produce phosphatidylinositol-3-phosphate (PtdIns-3-P), phosphatidylinositol-3,4- bisphosphate (PtdIns-3,4-P_2), and phosphatidylinositol-3,4,5-triphosphate (PtdIns-3,4,5-P_3), respectively (reviewed in Franke, et al., 1997a; Coffer, et al., 1998). In the absence of growth factor stimulation PtdIns-3-P is constitutively made and is involved in vesicle trafficking. The lipids PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 are synthesized in response to extracellular stimuli as second messages. The signal generated by these lipids is attenuated by removal of the phosphate groups. Pten, a lipid phosphatase, antagonises PI3-K signalling by removing the 3'-phosphate from 3-phosphoinositides, thus inhibiting activation of the downstream kinase Akt and survival signalling (reviewed in Leevers, et al., 1999). Thus, Pten is a major tumour suppressor in human cancer.

The protein kinase Akt (also known as PKB-α or Rac-α) is the key effector of PI3-K in signaling cell survival. PI3-K activity is believed to be necessary and

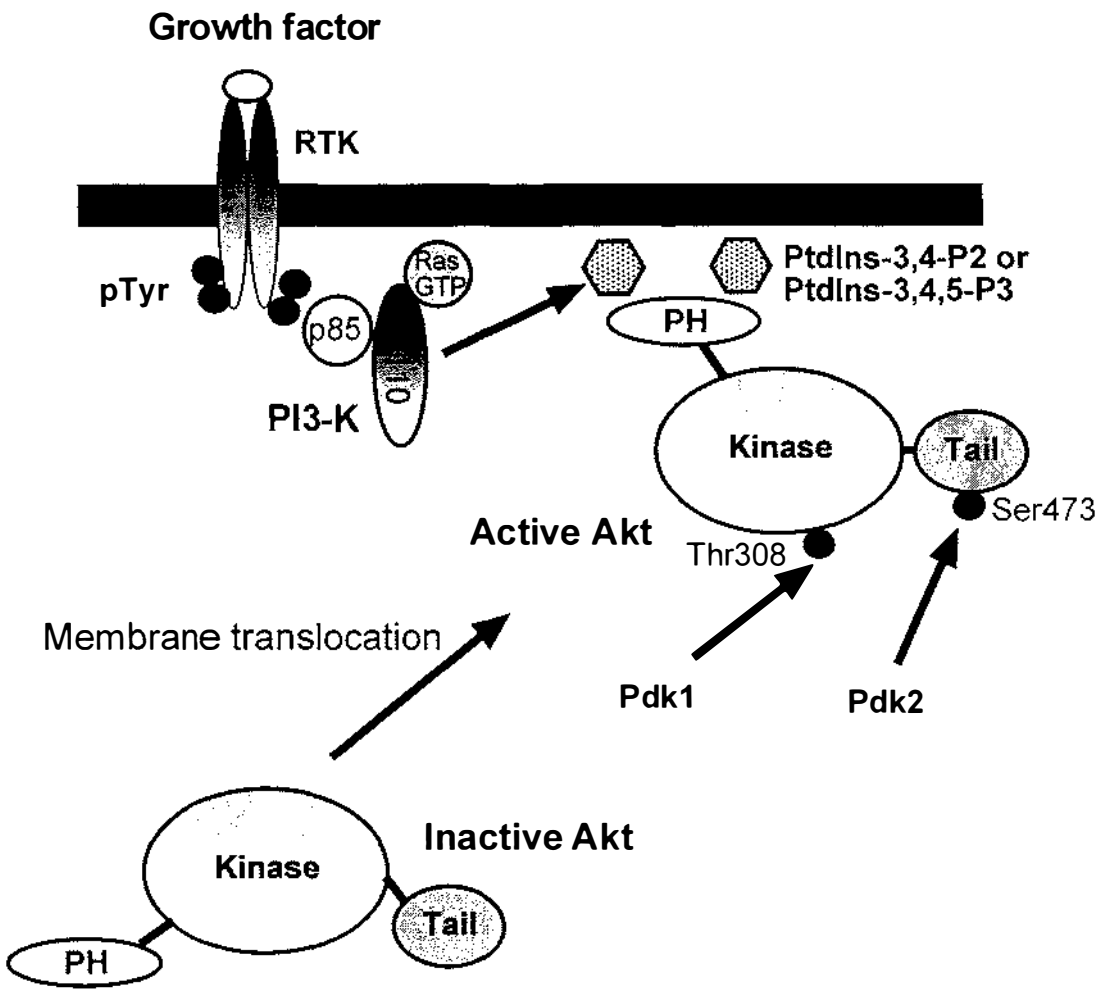

Figure 10: Growth factor-promoted activation of Akt by PI3-kinase

Following growth factor receptor stimulation, the p85 regulatory subunit of PI3-kinase can associate with the receptor through phosphotyrosine. p85 or activated GTP-bound Ras can activate the p110 catalytic subunit of PI3-K. PI3-K activation allows the production of PtdIns-3,4,5-P3 and PtdIns 3,4-P2. Akt is recruited to the membrane via an interaction between its pleckstrin homology domain (PH) and these lipids. Akt is then fully activated by phosphorylation at Thr308 and Ser473 by Pdk1 and Pdk2, respectively.

This figure is adapted from Franke, et al. (1997a), Marte and Downward (1998) and Coffer et al. (1998).

sufficient for activation of Akt. Dominant negative inhibitory alleles of PI3-K block activation of Akt, while the PI3-K inhibitor wortmannin blocks activation of Akt by growth factors (Burgering & Coffer, 1995). In addition, constitutively active PI3-K can activate Akt in the absence of growth factors (Franke, et al., 1997b). p38 Mapk/Hog and Ras have also been suggested to play a role in Akt activation possibly independently of PI3-K (Franke, et al., 1995).

Akt is a widely expressed cytoplasmic serine-threonine kinase and four isoforms are known to exist (Figure 11). Akt1/PKB-α was the first characterised member of the family, while PKB-β_1 and PKB-β_2 are alternatively spliced forms derived from the same gene, Akt2, and PKB-γ was isolated from rat. Akt contains a serine/threonine kinase domain, a carboxy-terminal tail region (amino acids 412-480) and its NH_2-terminus (amino acids 1-106) contains a pleckstrin homology (PH) domain. This PH domain can regulate Akt activity by binding D3-phosphorylated phosphoinositides, which are produced by PI3-K (Franke, et al., 1997b).

A model for Akt activation (Figure 10) involves PI3-K producing PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 at the membrane in response to growth factor stimulation. Akt binds to these lipids, dimerizes and is stabilised in this partially active state. Akt translocation to the membrane and/or dimerization then enhances the chances of Akt being phosphorylated at Thr308 and Ser473 by the phosphoinositide-dependent kinases (Pdks) to become fully activated (reviewed in Franke, et al., 1997a; Hemmings, 1997; Downward, 1998). Phosphoinositide-dependent kinase 1 (Pdk1) can phosphorylate the Thr 308 site and is constitutively active in the cell. However, Pdk1 will only phosphorylate PtdIns-3,4,5-P_3-bound Akt (Alessi, et al., 1997). The kinase responsible for phosphorylating Ser 473 on Akt *in vivo*, designated Pdk2, has yet to be identified but appears to be under the control of PI3-K (Alessi, et al., 1996).

Akt has been previously implicated in the control of glucose transport, glycolysis, nitric oxide synthesis and translation initiation (reviewed in Marte and Downward, 1997; Coffer, et al., 1998). More importantly, Akt also plays a key

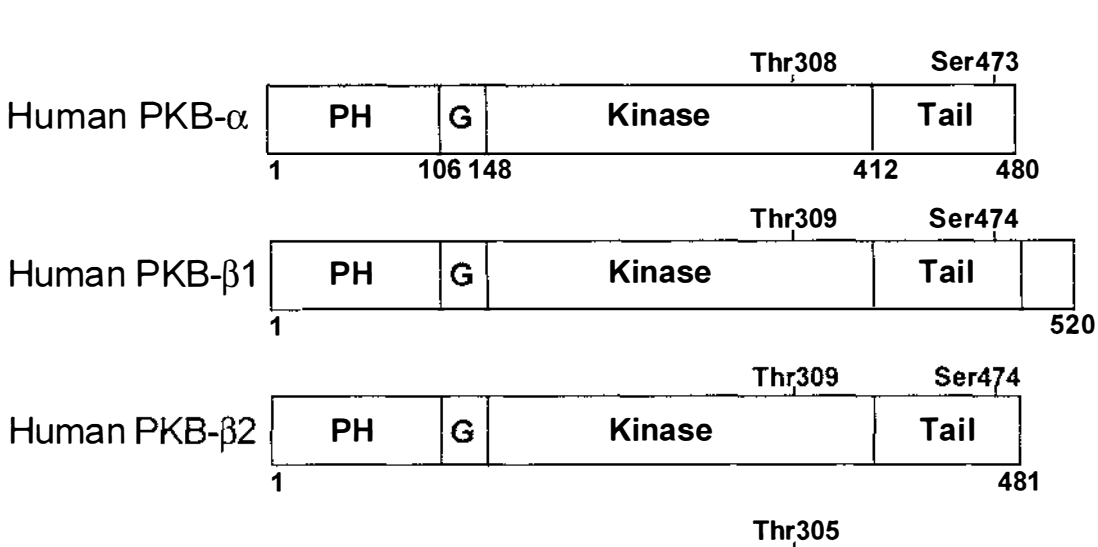

Figure 11: Schematic representation of the 4 isoforms of Akt
The domain structure, phosphorylation sites and sizes of the 4 isoforms of PKB/Akt are indicated. It contains an N-terminal pleckstrin homology domain (PH), followed by a glycine-rich region (G), catalytic domain and a C-terminal regulatory tail.

This figure is adapted from Coffer et al. (1998) and Downward (1998).

role in mediating cell survival. Loss of Akt function in *Drosophila* results in embryonic lethality due to widespread apoptosis (Stavely, et al., 1998). Expression of a dominant negative form of PI3-K in flies had a similar phenotype to the Akt loss-of-function mutation, which suggests that PI3-K signalling acts via Akt to repress apoptosis during development in flies (reviewed in Coffer, et al., 1998).

Akt also prevents apoptosis in higher organisms. Overexpression of constitutively activated Akt blocks UV-induced apoptosis in Rat-1 and COS-7 cells (Kulik, et al., 1997;Kennedy, et al., 1999), while activated Akt prevented apoptosis induced by detachment of MDCK cells from their extracellular matrix (Khwaja, et al., 1997).

Akt substrates for survival signalling

Akt may promote survival by a number of different mechanisms (Figure 12). Akt can directly inhibit apoptosis activators, regulate transcription factor activity and other signal transduction pathways or upregulate survival factors.

Akt can phosphorylate Bad (proapoptotic member of Bcl-2 family), *in vitro* and *in vivo* at Ser136 (del Peso, et al., 1997), blocking Bad-induced death of primary neurons (Datta, et al., 1997). Bad function is modulated by phosphorylation at two sites, serine 112 and serine 136 (Zha, et al., 1996) and phosphorylation is correlated with binding of Bad to 14-3-3 proteins. 14-3-3 protein can sequester Bad away from Bcl-X_L thus promoting cell survival. Bad is proposed to cause cell death by binding to Bcl-2 or Bcl-X_L and blocking their function.

Protein kinase A (PKA) and the pp90 ribosomal S6 kinases (Rsks) have been found to phosphorylate the Ser 112 site of Bad (Harada, et al., 1999; Bonni, et al., 1999). PKA must be anchored to the mitochondria by mitochondrion-specific A-kinase anchoring proteins (Akap) in order to phosphorylate and inactivate Bad in response to a survival factor. The presence of 2 phosphorylation sites on Bad suggests that simultaneous activation of different survival pathways can

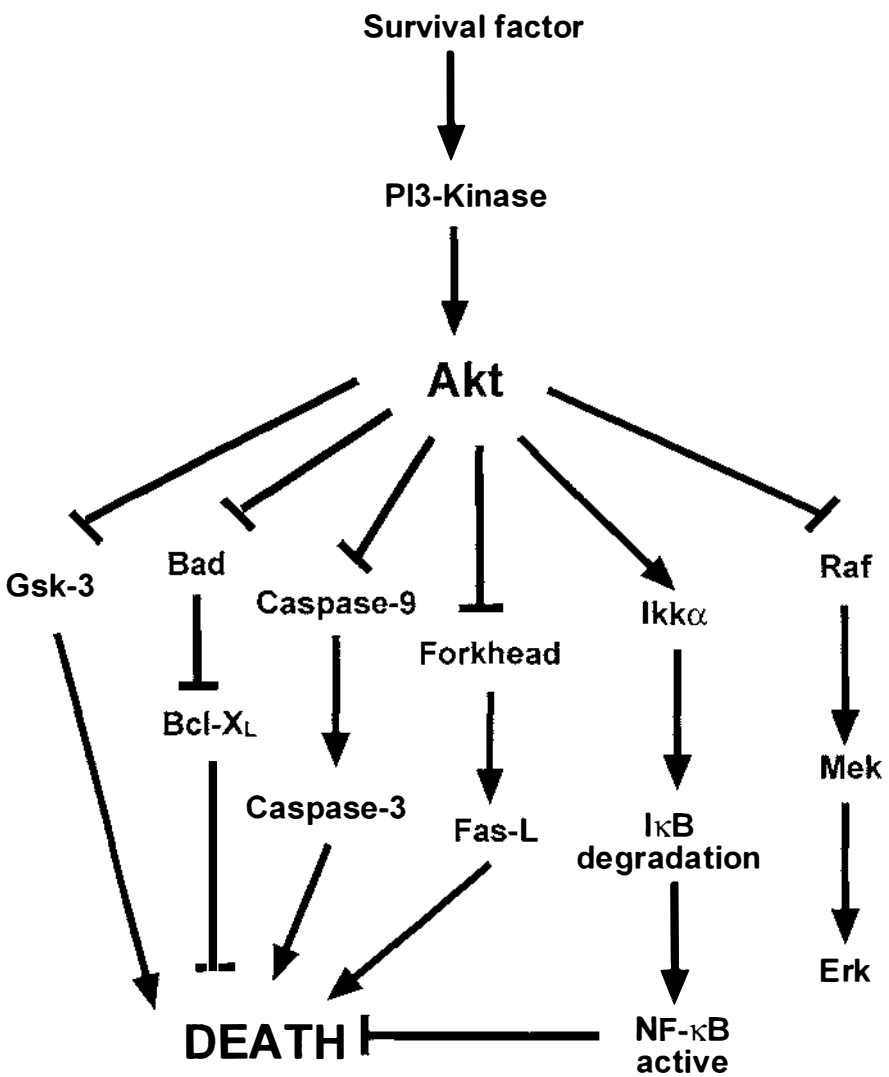

Figure 12: Possible downstream targets of Akt action

result in phosphorylation of Ser-112 and Ser-136 by different kinase pathways thus allowing cell survival in the presence of a combination of survival factors.

However, Bad is only expressed in a limited range of tissues and cell lines and Bad knockout mice are viable indicating that Bad may not be implicated in the death response in many cell types. Kennedy, et al. (1999), have also demonstrated that Akt can protect against the effects of unphosphorylated Bad and the overexpression of a non-phosphorylatable Bad mutant. These observations together with the fact that most experiments have relied on overexpressed Bad protein suggests that other substrates must be involved in the ability of Akt to protect cells from apoptosis independently of Bad expression (reviewed in Downward 1998).

Akt is also proposed to inhibit apoptosis by directly inhibiting caspases. Akt has been proposed to phosphorylate and inhibit caspase-9 activity (Cardone, et al., 1998). Overexpression of Akt or p21-Ras, an Akt activator, induced phosphorylation of pro-caspase-9 at Ser196 in cells and inhibited its activity *in vitro*. Mutant pro-caspase-9 (Ser196Ala) was resistant to Akt-mediated phosphorylation and inhibition *in vitro* and in cells, resulting in Akt-resistant induction of apoptosis. This suggests a role for Akt in preventing activation of apoptosis through the cytochrome c-mediated caspase-9 cascade. These findings contrast to others who propose that Akt inhibits caspase activity upstream of cytochrome c release because it couldn't protect against death caused by cytochrome c microinjection (Kennedy, et al., 1999).

Another apoptosis activator inhibited by Akt is Glycogen synthase kinase-3 (Gsk-3). In addition, to its function in regulating glycogen synthesis, Gsk-3 may induce apoptosis. Overexpression of catalytically active Gsk-3 induced apoptosis in Rat-1 and PC12 cells, while dominant-negative Gsk-3 prevented apoptosis in these cells (Pap and Cooper, 1998). Gsk-3 is involved in several intracellular signalling pathways including control of transcription factors AP1 and Creb, the tumour supressor APC and the *wingless* signalling pathway in flies (reviewed in Welsh, et al., 1996).

Akt also plays a role in regulating transcription and translocates into the nucleus upon exposure of cells to survival factors (Andjelkovic, et al., 1997). The human transcription factor Forkhead (Fkhrl1) is phosphorylated and inhibited by Akt *in vitro* and *in vivo* (Brunet, et al., 1999). Phosphorylation of Fkhrl1 results in its association with 14-3-3 proteins and its retention in the cytoplasm, thus preventing Fkhrl1-dependent transcription. During survival factor withdrawal, Fkhrl1 is dephosphorylated and is able to translocate to the nucleus where it can induce expression of target genes such as Fas ligand and triggers apoptosis. In fact, a mutant non-phosphorylatable form of Fkhrl1 induced apoptosis in transfected cerebellar neurons, fibroblasts and Jurkat cells in a manner dependent on Fas signalling. Therefore, Akt phosphorylation of Fkhrl 1 is proposed to suppress transcription of Fkhrl1-regulated death genes and promote cell survival.

Akt can up-regulate anti-apoptotic genes via the nuclear factor-κB (NF-κB) transcription factor pathway to increase cell survival (reviewed in Khwaja, 1999). Normally, NF-κB exists as an inactive complex with its inhibitor IκB in the cytoplasm. In response to cytokine or growth factor stimulation, NF-κB-inducing kinase (Nik) can phosphorylate the IκB-kinase (Ikk) complex which then phosphorylates IκB and targets it for degradation. This allows NF-κB to translocate to the nucleus, where it induces expression of genes involved in inflammation, host defence and survival. Kinase-dead Akt was found to inhibit TNF-mediated NF-κB activation while constitutively active Akt induced NF-κB activity (Ozes, et al., 1999). Akt can associate with, and phosphorylate the Ikkα subunit of the Ikk complex, activating it, thus, allowing NF-κB activation. NF-κB activation by Akt is also involved in survival signalling mediated by platelet-derived growth factor (PDGF) in fibroblasts. Upon PDGF stimulation, Akt transiently associates *in vivo* with Ikk and induces Ikk activation through the Ikkβ subunit (Romashkova & Makarov, 1999).

The anti-apoptotic gene *bcl-2* can be up-regulated by expression of catalytically active Akt and inhibit cytokine withdrawal-induced apoptosis in lymphoid cells (Ahmed, et al., 1997).

Finally, Akt can regulate other signal transduction pathways. Akt can phosphorylate Raf and inhibit the activation of the Raf-Mek-Erk signaling pathway in a human breast cell cancer line (Zimmerman & Moelling, 1999). This switches the biological response from growth arrest to proliferation in these cells. From growth arrest, a cell can choose to either proliferate or die by apoptosis. Phosphorylation of Raf by Akt allows cross-talk between the PI3-K–Akt and Raf-Mek-Erk pathways.

The research on Akt demonstrates how a single regulatory protein can exert its anti-apoptotic effects in a variety of ways (Figure 12). Depending on the particular cell type and survival signal different targets could be used to maintain cell survival.

2.42 Raf/Mek/Erk pathway

Ras is a key regulator of cell growth in all eukaryotic cells. *Ras* genes are mutated and activated in 20-30% of human cancers (reviewed in Heimbrook & Oliff, 1998). Growth factor receptors and tyrosine kinases activate Ras. Ras can activate three distinct Mapk cascades, Erk, Jnk and p38 (Figure 13). The biological activity of Ras is regulated by GDP and GTP. Guanine nucleotide exchange factors (GEFs) promote the formation of the active GTP-bound form of Ras (reviewed in Vojtek & Der, 1998). GTPase activating proteins (GAPs) promote hydrolysis of GTP to yield the inactive GDP-bound form of Ras. Ras can promote both cell death and cell survival through interactions with distinct effector proteins. Activation of Raf by Ras promotes apoptosis in fibroblasts overexpressing c-Myc, while activation of PI3-K by Ras promotes cell survival (Kauffmann-Zeh, et al., 1997).

Ras mediates its effects on cellular proliferation in part by activating the Raf/Mek/Erk kinase pathway (reviewed in Robinson & Cobb, 1997; Vojtek & Der, 1998). The binding of Ras to Raf promotes membrane translocation and activation of Raf. In mammals, three members of the Raf family of protein serine/threonine kinases have been identified, Raf-1, A-Raf, and B-Raf.

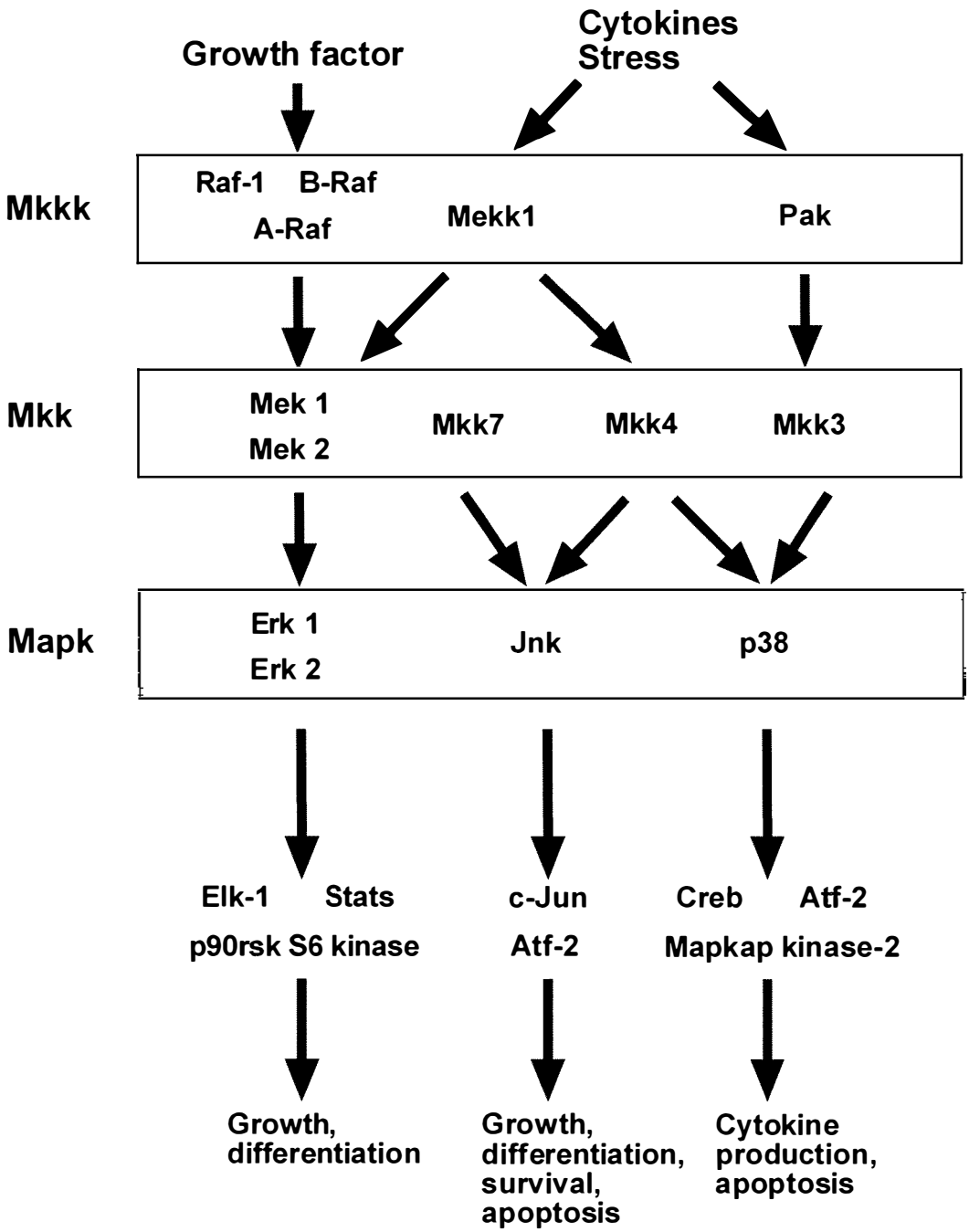

Figure 13: Organisation of Mitogen-activated protein kinase modules (Mapk)

This figure is adapted from Garington & Johnson (1999).

Activation of Raf then leads to activation of a kinase cascade consisting of Mapk/Erk kinase (Mek) and Extracellular-signal-regulated kinase 1/2 (Erk1/2) (Figure 14). Upon activation, Erks can phosphorylate cytoplasmic targets, Rsk and Mnk, and translocate to the nucleus where they can activate transcription factors, Elk1 and Stat3 (reviewed in Vojtek & Der, 1998).

Rafs may have specific functions in cell death regulation. B-Raf mouse knockouts, but not mice lacking Raf-1 or A-Raf, exhibit disturbances in cell survival (Wojnowski, et al., 1997). In addition, overexpression of B-Raf prevents apoptosis caused by growth factor withdrawal or PI3 kinase inhibition in Rat-1 fibroblasts (Erhadt, et al., 1999). This occurs through constitutive activation of the Mek/Erk pathway and occurs downstream of cytochrome c release. This raises the possibility that B-Raf/Mek signalling may affect the activity of caspase inhibitors such as Iaps. For example, the *Drosophila* death promoter Hid interacts with Iaps and is a direct target of Ras-dependent survival signalling by Erk (Bergmann, et al., 1998).

Biochemical studies suggest that Raf-1 also has an antiapoptotic function. Bcl-2 targets Raf-1 to mitochondria where it promotes cell survival without Erk activation (Wang, et al., 1996). Overexpression of constitutively active Raf-1 in serum deprived fibroblasts also blocks death but through activation of Mek (Erhadt, et al., 1999). So, the Raf/Mek/Erk pathway may regulate cell survival by both promoting cell proliferation and directly inhibiting cell death.

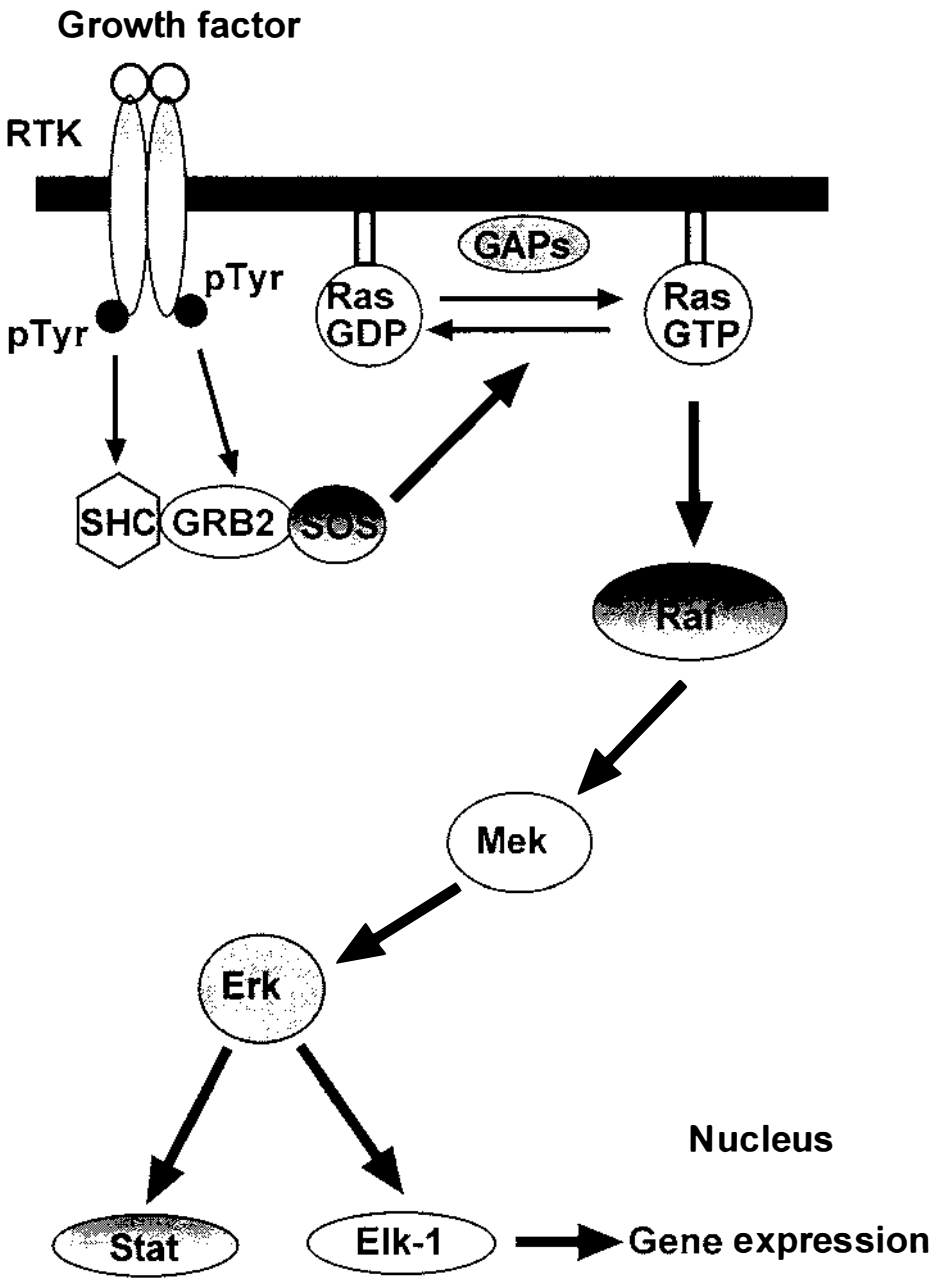

Figure 14: Raf/Mek/Erk pathway
Growth factors and receptor tyrosine kinases (RTK) activate Ras via exchange of GTP for GDP. Active GTP-bound Ras then activates Raf, which activates Mek and then Erk. Erk can translocate to the nucleus where it activates transcription factors.

This figure is adapted from Vojtek & Der (1998).

2.43 Sapk/Jnk pathway

c-Jun amino terminal kinase/stress-activated protein kinase (Jnk/Sapk) is activated by many pro-apoptotic stimuli. Stresses such as heat shock, osmotic shock, cytokines, protein synthesis inhibitors, antioxidants, UV, and DNA damaging agents will activate Jnk/Sapk through a kinase cascade in which Mapk/Erk kinase kinase 1 (MEKK1) phosphorylates and activates Sapk/Erk kinase 1/Mapk kinase 4 (Sek1/Mkk4) which then phosphorylates Jnk (Figure 15) (reviewed in Robinson & Cobb,1997; Ip & Davis, 1998). Jnk/Sapks are activated by dual phosphorylation at specific Thr and Tyr residues by Sek1/Mkk4 and Mkk7. In addition, Mkk4 may also activate the p38 Mapk pathway. The Mkk4 gene may suppress tumours because loss-of-function mutations were detected in a survey of human tumour cell lines (Teng, et al., 1997).

Jnks phosphorylate and activate transcription factors such as Atf-2, Atf-a, c-Jun, JunD, Elk-1 and Sap-1 (reviewed in Ip & Davis, 1998). These transcription factors have been associated with growth, differentiation, survival and apoptosis responses. Jnk phosphorylates c-Jun at Ser63 and Ser73 increasing its transcriptional activating potential. Once activated, c-Jun stimulates its own expression by interacting with two AP-1 sequence elements within its promoter. c-Jun can function as a homodimer or heterodimer with partner proteins such as Atf-2.

In many cell types Jnk/Sapk promotes apoptosis. Jnk is required for excitotoxic stress-induced neuronal apoptosis (Yang, et al., 1997). Jnks are activated in and promote anoikis (Frisch, et al., 1996a). Anoikis refers to the process where a cell loses integrin-mediated contacts with the extracellular matrix. Interestingly, caspases must be active for Jnks to respond to loss of cell-matrix contact (Frisch, et al., 1996b). In addition, induction of activated Mekk1 sensitises Swiss 3T3 cells to UV-induced apoptosis (Johnson, et al., 1996). However, dominant negative Jnk constructs were unable to attenuate Mekk1

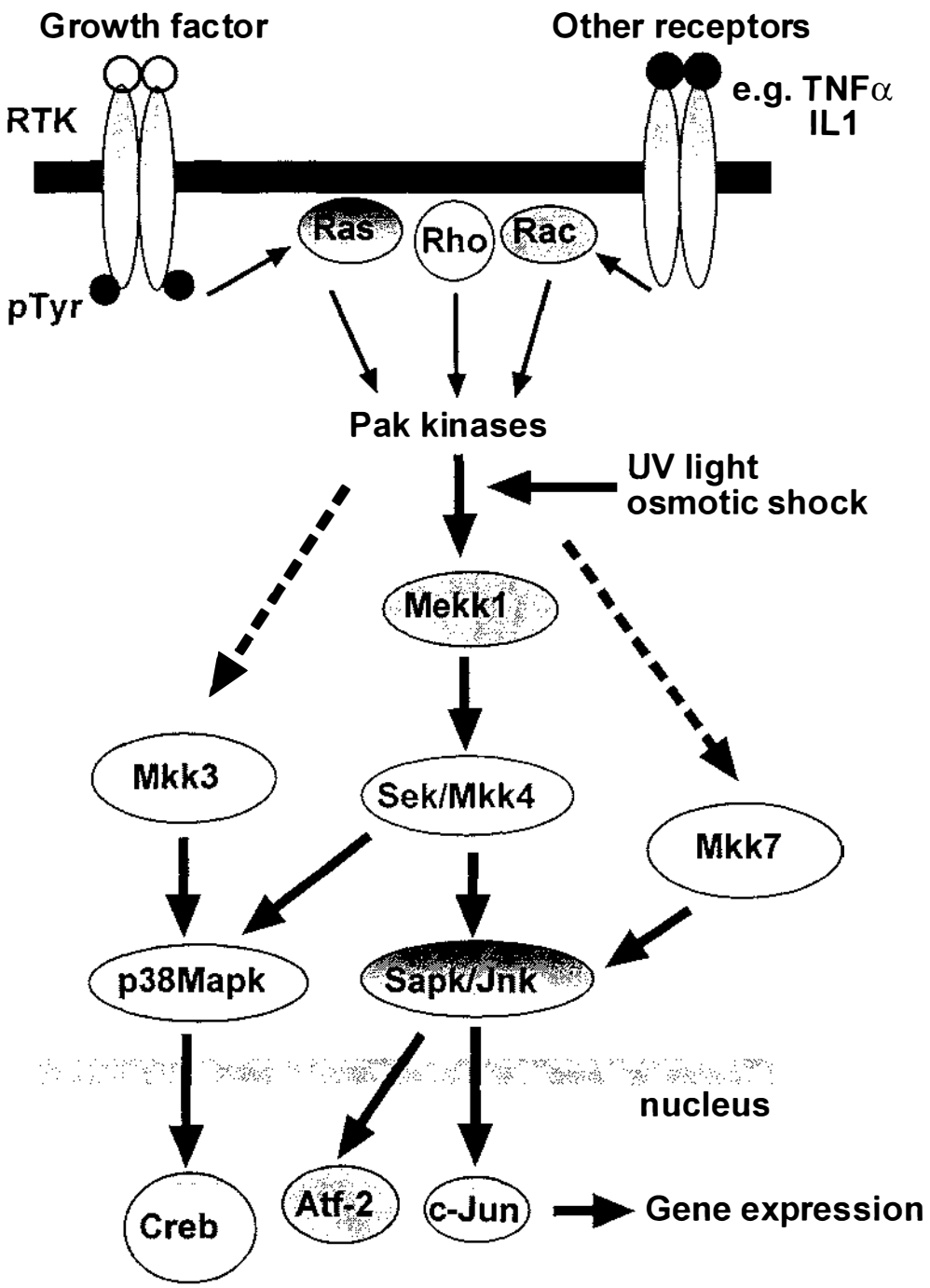

Figure 15: Schematic diagram of Sapk/Jnk and p38 Mapk pathways

Growth factors, cytokines, UV and osmotic shock result in the activation of the Jnk and p38 Mapk pathways. Mekk1 phosphorylates and activates Sek/Mkk4 which activates Sapk/Jnk. Mkk7 can also activate Jnk. Jnk can translocate to the nucleus and activate the transcription factors c-Jun and Atf-2. Sek acts a branchpoint for both pathways because it can activate p38Mapk as well as Jnk. Mkk3 also can activate p38Mapk. p38Mapk then activates transcription factors such as Creb.

-induced cell death suggesting that Mekk-induced death may occur independent of Jnk activation.

How might Jnks promote apoptosis? Bcl-2 has been proposed to act as a substrate for Jnk while the expression of proapoptotic proteins such as Bax, Fas, FasL, and caspase-3 may be upregulated by Jnk but the mechanism is unclear (reviewed in Ip & Davis, 1998). Jnk was activated and could phosphorylate Bcl-2 within its loop domain inhibiting its anti-apoptotic activity in response to microtubule-damaging drugs (Srivastava, et al., 1999).

Signalling by Jnk/Sapks is not associated with cell death in all circumstances. In human B cells, activation of Jnk by CD40 selectively rescues cells from apoptosis while activation of Erks in these cells by IgM crosslinking triggers apoptosis (Sakata, et al., 1995). In addition, the inhibitor of apoptosis protein hILP selectively activates Jnk1 and requires Jnk1 for protection against apoptosis (Sanna, et al., 1998).

2.44 p38 Mapk pathway and Creb

The p38 Map kinase (HOG kinase in yeast) pathway comprises a third Map kinase pathway. Like Sapk/Jnk, p38 is activated by several forms of environmental stress known to cause apoptosis. Inflammatory cytokines can signal to p38 through Mekk and Sek. As Sek can phosphorylate and activate both Jnk and p38, it serves as a branch point to activate both pathways. Mkk3 and Mkk6 also activate p38 Mapk. Activated p38 phosphorylates and activates Mapkap kinase-2, which activates the transcription factors Atf-2, Max, and Creb (Figure 15). In addition to p38, Protein kinase A (PKA), Akt, Ca^{2+}/calmodulin-dependent protein kinase II (CamkII) and CamkIV can stimulate Creb phosphorylation at Ser133. The transcription factor Creb binds the cAMP response element and activates transcription in response to extracellular signals including hormones, membrane depolarisation and neurotrophic factors. Creb can induce Bcl-2 expression in response to IGF-I in PC12 cells and Creb

overexpression can block apoptosis in neuronal cells (Pugazhenth, et al., 1999; Walton, et al., 1999).

p38 Mapk is important during cell death since its specific inhibitor SB203580 can prevent apoptosis (Kummer, et al., 1997). p38 activity increases following NGF withdrawal of differentiated PC12 cells and precedes apoptosis (Xia, et al., 1995). In addition, expression of a constitutively active Mkk3 induced apoptosis, while a dominant negative Mkk3 construct blocked apoptosis in these cells. p38 Mapk can also be activated by growth factors leading to promotion of differentiation and growth (Engelman, et al., 1998). Conflict in these functions can be explained by the existence of different p38 isozymes with distinct functions. The p38Mapkβ form has been associated with protection from apoptosis while the p38Mapkα form is proapoptotic (Pugazhenth, et al., 1999).

Mapks act in concert and with other cell signalling systems. Therefore, cross-talk between pathways is crucial to the coordinated responses of cells. Erk, Jnk and p38Mapk may act antagonistically in cells undergoing apoptosis, or may cooperate with, or antagonise, each other in supporting cell proliferation.

2.45 Caspase-mediated regulation of signal transduction pathways

Caspase cleavage of signal transduction molecules can regulate their function. Caspase-mediated cleavage of the protein kinases PKCδ (Emoto, et al., 1995), Pkn (Takahashi, et al., 1998), and Mekk1 (Cardone, et al., 1997) activates their kinase activities and is proapoptotic.

Mekk1 functions in the Jnk and Erk pathways and has been identified as a caspase target that is cleaved during anoikis, Fas ligation, and genotoxic stress (Cardone, et al., 1997; Deak, et al., 1998; Widmann, et al., 1998). Mekk1 is normally tethered to 14-3-3 proteins within the cell. During apoptosis, caspase-3 cleaves Mekk1, to remove its inhibitory N-terminal domain, releasing the kinase

domain, which is proapoptotic. In addition, a positive feedback loop is proposed to exist where activated Mekk1 can in turn activate caspases.

Caspases can also activate phosphatases. In Fas-induced apoptosis in Jurkat cells, caspase-3 cleaves the regulatory Aα subunit of protein phosphatase 2A (PP2A), increasing its activity (Santoro, et al., 1998). Erk is a substrate for PP2A. A decrease in Erk activity accompanied the increase in PP2A activity, suggesting a mechanism by which caspases might downregulate signaling through the Mapk/Erk pathway.

Caspase-mediated cleavage of survival molecules inactivates them and can help ensure the irreversibility of the process. Cleavage of RasGAP and Raf-1 are believed to contribute to the shut-off of the Erk pathway in apoptotic Jurkat cells (Widmann, et al., 1998). Bcl-2 can also be cleaved by caspases during Fas-, viral-, and IL-3 -withdrawal- induced apoptosis (Cheng, et al., 1997; Grandgirard, et al., 1998). The Bcl-2 cleavage product accelerated cell death while caspase-resistant Bcl-2 conferred protection against IL-3 -withdrawal - induced apoptosis (Cheng, et al., 1997).

In addition, cleavage of IκB-α by caspases can help facilitate apoptosis by inhibiting NF-κB activation. Caspase-3 can cleave IκB-α during apoptosis to create a constitutive inhibitor for NF-κB which blocks the ability of IκB-α to undergo signal-induced degradation (Barkett, et al., 1997).

2.5 Apoptosis in neurodegenerative disease

Apoptosis is a cellular defense against deregulated growth in multicellular organisms. A growing amount of work supports a role for abberant apoptosis in the pathogenesis of many diseases.

Evidence exists for inappropriate apoptotic neuronal death in stroke, Parkinson's disease, Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), and Huntington's disease (HD) (reviewed in Margolis, et al., 1993;

Dragunow, et al., 1998). Caspases are activated and cleave several gene products associated with polyglutamine disorders such as huntingtin, atrophin-1, ataxin-3 and the androgen receptor (reviewed in Kim & Tanzi, 1998). Caspase-3 mediated cleavage of huntingtin is associated with accelerated neuronal apoptosis in Huntington's Disease (Goldberg, et al., 1996). Mutant huntingtin can activate and recruit caspase-8 to the nuclei of neurons in HD brains and induce apoptosis (Sanchez, et al., 1999). In addition, caspase-1 is activated in HD and inhibition of caspase-1 slows disease progression in a mouse model of HD (Ona, et al., 1999).

Many of the mutations associated with neurodegenerative disorders have been shown to be proapoptotic within cell culture models. *Sod1* mutations, which encode a copper/zinc superoxide dismutase (CuZnSOD), are associated with familial ALS and induce apoptosis in cultured neural cells (Rabizadeh, et al., 1995). Mutant Sod1 appears to promote apoptosis by activating caspase-1 (Pasnelli, et al., 1998). In addition, downregulation of wild type Sod1 by antisense oligonucleotides causes apoptosis in PC12 cells (Troy & Shelanski, 1994).

Alzheimer's disease associated mutations in amyloid precursor protein (App) have also been shown to be proapoptotic in neuronal cells (Li, et al., 1996; Yamatsuji, et al., 1996; Zhao, et al., 1997). Caspase-3 mediated cleavage of App may facilitate formation of amyloid β–peptide, which can promote further caspase activation and apoptosis in neurons (Gervais, et al., 1999). Amyloid β–peptide induces apoptosis in PC12 cells (Ivins, et al., 1999) and is associated with senile plaques in AD brains. In addition, presenilin mutations can promote Aβ formation and apoptosis (reviewed in Hetts, 1998). Recently, caspase-12 has been demonstrated to be involved in mediating ER-regulated apoptosis induced by amyloid β–peptide (Nakagawa, et al., 2000).

Because apoptosis plays a role in so many neurological disorders it has been hoped that by blocking apoptosis in the affected neurons the progress of disease may be halted. Blocking apoptosis with p35 expression prevented

blindness in *Drosophila* retinal degeneration mutants (Davidson & Steller, 1998). This finding is significant because it showed that function can be retained in cells rescued from apoptosis and suggests possible therapeutic benefits for higher organisms.

2.6 Regulation of apoptosis in neurons

The core apoptotic Apaf-1/caspase-9 machinery plays a key role in mediating apoptosis in the nervous system. This is demonstrated by the grotesque increase in brain size observed in caspase-3, caspase-9, and Apaf-1 knockout mice (Kuida, et al., 1996; Hakem, et al., 1998; Yoshida, et al., 1998; Cecconi, et al., 1998; Kuida, et al., 1998). The mechanism for triggering activation of this core machinery is less well defined.

Raff's (1992) hypothesis states that somatic cell survival is an active process dependent upon extracellular signals. Cells compete for survival factors and lack of enough growth factor triggers suicide and removal of the affected cell. This theory is true of the nervous system where more than 50% of neurons created die before embryonic development is complete (Oppenheim, 1991). Developing sympathetic neurons compete with one another for limiting amounts of neurotrophins released by the target cells they innervate (Raff, et al., 1993).

The neurotrophin family consists of NGF, BDNF, NT-3 and NT4/5. Each of the neurotrophins acts by binding and activating specific receptors. NGF binds TrkA(p140^{trk}), BDNF and NT-4/5 binds TrkB, while NT-3 binds TrkC. The receptor p75^{NTR} can interact with all mammalian members of the neurotrophin family.

2.61 NGF signal transduction

NGF regulates the survival and differentiation of neurons in the peripheral and central nervous systems. All sympathetic neurons are lost in NGF-/- mice, as they are in the NGF receptor TrkA-/- mice (Crowley, et al., 1994; Smeyne, et al., 1994). NGF-withdrawal from sympathetic neurons causes apoptosis which is dependent on protein and RNA synthesis (Martin, et al., 1988). Studies using the rat pheochromocytoma PC12 cell line have elucidated the signal transduction pathways involved in promoting differentiation and survival of neural cells. NGF promotes the differentiation of PC12 cells into a phenotype resembling that of sympathetic neurons (Greene & Tischler, 1976).

NGF binding to TrkA stimulates Trk homodimer formation and activates it by autophosphorylation of the tyrosine kinase cytoplasmic domain. TrkA activation is necessary for neuronal survival (Belliveau, et al., 1997). The adaptor proteins SHC, phospholipase C-γ (PLC-γ), phosphotyrosine phosphatase SHP, directly associate with NGF-activated Trk (reviewed in Kaplan & Miller, 1997). These proteins couple Trk to several intracellular signalling pathways. SHC phosphorylation by Trk results in the rapid activation of PI3-kinase, Ras, B-Raf and Erk (reviewed in Kaplan & Miller, 1997). Mutagenesis studies with the TrkA receptor indicate that the SHC pathway acts in concert with PLC-γ to regulate elongation and maintenance of neurons and cell survival (Stephens, et al., 1994).

Because trophic factors bind to receptors at the neurite tip, a mechanism must exist to convey the signal to the cell body. One possibility is that NGF and activated TrkA are retrogradely transported in signalling vesicles (Grimes, et al., 1997; Bhattacharyya, et al., 1997; Riccio, et al., 1997). However, studies using rat sympathetic neurons in compartmented cultures suggest that at least some components of the retrograde signal are carried by a propagation mechanism (Senger & Campenot, 1997).

Ras, Mek, and Erk have been suggested to be necessary for NGF-induced neuronal differentiation and survival using dominant-inhibitory approaches and selective inhibitors. Erk activity is down-regulated during NGF-withdrawal induced apoptosis in PC12 cells and expression of constitutively active Mek blocks apoptosis caused by growth factor withdrawal (Xia, et al., 1995; Erhadt, et al., 1999). But, Ras activity was neither necessary nor sufficient for the NGF-mediated survival or neuritogenesis of chick sympathetic neurons (Borasio, et al, 1993). Mek inhibitors do not suppress NGF-induced survival of sympathetic neurons and Erks are not required for neuronal outgrowth or survival after growth factor withdrawal (Virdee & Tolkovsky, 1995; Virdee & Tolkovsky, 1996; Creedon, et al., 1996). Instead, Erks may be involved in regulating synaptic plasticity in postmitotic neurons (English & Sweatt, 1996). These results point to the existence of another Trk-regulated pathway mediating cell survival and differentiation.

The PI3-kinase pathway may contribute to signalling for cell survival through Trk. PI3-kinase activity is required for the prevention of apoptosis by NGF and IGF-1 (Yao & Cooper, 1995; D'Mello, et al., 1997). PI3-Kinase effector Akt plays a critical role in growth factor-induced neuronal survival. Dominant negative forms of Akt were able to cause apoptosis in cerebellar and sympathetic neurons while overexpression of wild type Akt could reduce apoptosis in the growth factor deprived neurons (Dudek, et al., 1997; Crowder & Freeman, 1998). Nerve growth factor (NGF) promotes Akt activation in PC12 cells and sympathetic neurons (Crowder & Freeman, 1998). In addition, mutant forms of presenilin-1(PS-1) implicated in familial Alzheimer's Disease downregulate Akt activity during apoptosis of hippocampal neurons and PC12 cells (Weihl, et al., 1999). Expression of a constitutively active Akt/PKB rescues hippocampal neurons from mutant PS-1-induced death, suggesting a potential therapeutic target for Alzheimer's Disease.

One transcription factor that is a key target of the NGF-stimulated pathway is Creb (Riccio, et al., 1997). In fact, PI3-K effector Akt may mediate Creb phosphorylation (Du & Montminy, 1998). Creb is phosphorylated in NGF-treated

PC12 cells and promotes NGF activation of the gene *c-fos* (Ginty, et al., 1994; Bonni, et al., 1995). Because many NGF-regulated genes contain Creb binding sites within their upstream regulatory regions, Creb is likely to be a mediator of the general transcriptional response to NGF. Creb regulates many aspects of neuronal function including excitation of nerve cells, circadian rhythms, CNS development and long-term memory formation (reviewed in Walton & Dragunow, 2000).

Creb also plays a key role in neuronal survival. Creb-mediated gene expression is both necessary and sufficient for NGF-dependent survival of sympathetic neurons (Riccio, et al., 1999). Creb can activate expression of Bcl-2 while inhibition of Creb causes cell death. Overexpression of Creb inhibited OKA-induced apoptosis in neural cells (Walton, et al., 1999). In addition, Creb immunoreactivity is lost in apoptotic neurons during hypoxic-ischemic brain damage while levels of phospho-Creb are increased in neurons resistant to damage (Walton, et al., 1996).

As well as mediating survival, TrkA can act as an apoptosis-inducing receptor in neural cells under certain conditions. NGF induces apoptosis through TrkA in medulloblastoma cells (Muragaki, et al., 1997) while high expression of TrkA correlates with favorable prognosis in neuroblastoma (reviewed in Kaplan & Miller, 1997).

In addition, to the TrkA receptor, the p75 neurotrophin receptor ($p75^{NTR}$) can also bind NGF. $p75^{NTR}$ is a member of the Fas/TNFRI (tumour necrosis receptor I) family. It may affect TrkA responsiveness and selectivity for NGF, or may signal independently of TrkA (reviewed in Kaplan & Miller, 1997). $p75^{NTR}$ can signal through ceramide, Jnk/Sapk, and NFκB like other members of the TNF receptor family. Cultured oligodendrocytes that expressed $p75^{NTR}$ but not TrkA and $p75^{NTR}$-transfected neuroblastoma cells underwent apoptosis in response to NGF accompanied by increases in ceramide levels, Jnk activity and caspase activity (Casaccia-Bonnefil, et al., 1996; Lievremont, et al., 1999). $p75^{NTR}$ activation and NGF-withdrawal cause increased expression of p53 and its target

Bax in sympathetic neurons (Aloyz, et al., 1998). p53 is essential for this neuronal death to occur and is positively regulated by the Jnk pathway. NGF-withdrawn or staurosporine treated PC12 cells show stimulation of Jnk and p38 Mapk activity which precedes the onset of apoptosis (Xia, et al., 1995). Treatment with agents that prevented apoptosis in these cells inhibited the activation of Jnk and p38. In addition, constitutively active Mekk1 stimulates Jnk/Sapk and induces apoptosis in PC12 cells and fibroblasts (Xia, et al., 1995; Johnson, et al., 1996).

$p75^{NTR}$ has been shown to play a role in developmental cell death of rat retinal neurons (Frade, et al., 1996) and mouse neonatal neurons (Bamji, et al., 1998). Endogenous NGF induced cell death in rat retinal neurons that express $p75^{NTR}$ but not TrkA (Frade, et al., 1996). BDNF-mediated activation of $p75^{NTR}$ in the absence of Trk activation induced apoptosis in sympathetic neurons (Bamji, et al., 1998). Sympathetic neuron death is developmentally delayed in $p75^{NTR}$ knockout mice and cultured $p75^{NTR}$-/- sympathetic neurons die more slowly after NGF withdrawal (Bamji, et al., 1998). In addition, expression of a constitutive truncated $p75^{NTR}$ domain in neurons of transgenic mice causes death of developing and sympathetic neurons (reviewed in Kaplan & Miller, 1997). These studies combined with work on the $p75^{NTR}$ mouse suggest that although sympathetic neuron death can occur without $p75^{NTR}$, this receptor is required for developmental apoptosis to occur rapidly and appropriately.

The mechanism by which $p75^{NTR}$ causes apoptosis may be mediated by c-Jun as hyperphosphorylation of c-Jun occurred following $p75^{NTR}$ stimulation (Bamji, et al., 1998). Studies using neutralising antibodies or dominant negative interfering mutants of c-Jun suggest a critical role for this transcription factor in neuronal cell death (Estus, et al., 1994; Ham, et al., 1995; Xia, et al.,1995). Induction of c-Jun expression is observed after ischemia and in chronic neurodegenerative disease as well as prior to NGF-deprivation induced death in sympathetic neurons (reviewed in Ip & Davis, 1998).

$p75^{NTR}$ signalling can be antagonised by TrkA. PC12 cells coexpress TrkA and $p75^{NTR}$ receptors. Only ligands unable to bind TrkA were able to activate $p75^{NTR}$ signalling in PC12 cells and sympathetic neurons (Dobrowsky, et al., 1995). NGF decreased ceramide elevation following serum-withdrawal in PC12 cells (Edsall, et al., 1997). Therefore, the amount of $p75^{NTR}$ activity relative to the amount of TrkA activity may determine a cell's response to NGF. If there is high $p75^{NTR}$ activity in the presence of low or no TrkA activity, survival is suppressed and apoptosis is induced. In the presence of high TrkA activity and high $p75^{NTR}$ activity, apoptosis is suppressed and survival responses predominate (reviewed in Kaplan & Miller, 1997).

2.62 Competence to die

Various findings link inadequate neurotrophic support and mitochondrial dysfunction with neuronal death. NGF- and serum- withdrawn PC12 cells undergo a decrease in mitochondrial membrane potential which can be blocked by NGF readdition (Wadia, et al., 1998). Reactive oxygen species (ROS) are produced during apoptosis and can act at a late stage of the apoptotic pathway in certain neuronal cells (Schulz, et al., 1997). NGF-deprivation induced apoptosis is delayed by microinjecting SOD into sympathetic neurons (Greenlund, et al., 1995).

Bcl-2 family member, Bax, a key regulator of mitochondrial apoptosis, is required for the death of many but not all neurons. Bax knockout mice have increased numbers of neurons in the superior cervical ganglia and facial motoneurons (Knudson, et al., 1995; Deckwerth, et al., 1996). Bax is required for apoptosis of cerebellar and sympathetic neurons induced by NGF-withdrawal (Miller, et al., 1997; Deckwerth, et al., 1996). In addition, dorsal root ganglion neurons that normally die following NGF-deprivation, survive in mice double null for Bax and NGF or TrkA (Patel, et al., 2000). These studies demonstrate that apoptosis induced by neurotrophin deprivation *in vivo* requires Bax.

Cytochrome c redistributed to the cytosol of neurons following NGF withdrawal and apoptosis was prevented in these neurons by microinjection of an antibody to cytochrome c (Neame, et al., 1998). However, unlike in other cell types, cytochrome c microinjected into neurons did not induce death in NGF-maintained neurons suggesting that redistribution of cytochrome c is not the factor required for neuronal cell death. Neurons had to be deprived of NGF for 15-20 hours before they acquired competence to die by cytochrome c microinjection. It has been proposed that another factor referred to as "competence to die" is acquired during NGF withdrawal which in addition to cytochrome c allows neuronal death to occur (Deshmukh & Johnson, 1998; Neame, et al., 1998). Possible factors include post translational modification of the apoptotic machinery, accumulation of cofactor, or loss of an inhibitor such as an Iap.

2.7 Research Aims & Strategy

Although some form of the suicide machinery appears to be present in every cell, different signal transduction pathways and caspases are used depending on the cell type and cell death stimulus. The aim of these studies was to identify signal transduction pathways and caspase substrates specific to neural apoptosis. Neuronal apoptosis has been notoriously difficult to study at the molecular level due to small numbers of cells available for biochemical studies and the asynchronous nature of the death process. Initial work involved devising two strategies to overcome asynchrony during cell death. The first strategy exploited differences in cell density to separate PC12 cells into 3 populations at different stages of apoptotic commitment. These populations were used to identify biochemical and cellular events correlating with the stage of commitment to cell death. The second strategy involved creating a cell-free system to reconstitute the biochemical events of apoptosis. This system was particularly useful for characterising the conditions required for caspase activation and identifying potential caspase substrates such as molecules involved in survival signalling. Finally, an understanding of these modes of cell death was used to return to whole cell models of apoptosis to assess that the

events identified *in vitro* were specific to a particular death stimulus or cell type. Particular attention was focused on the survival signalling pathways mediated by Akt, Raf, and Creb, and the death-promoting Jnk pathway.

Chapter 3

Materials & Methods

3.1 Materials

All reagents were purchased from Sigma (St. Louis, MO) unless otherwise noted. New England Biolabs primary antibodies were a kind gift from Michael Comb (New England Biolabs, Inc., Beverly, MA). Solutions were made using ddH_2O unless otherwise stated.

3.2 Cell Culture

Rat pheochromocytoma (PC12) cells obtained from Lloyd Greene (Columbia University, NY), were cultured on collagen-coated dishes in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum and 5% foetal calf serum (Life Technologies, Inc., Gaithersburg, MD) as described (Greene & Tischler, 1976). Human neuroblastoma SY5Y cells obtained from Mark Israel (UCSF, CA) were grown in RPMI 1640 medium supplemented with 10% foetal calf serum.

Cells were frozen in liquid nitrogen for long term storage in medium containing 12% DMSO (Greene, et al., 1987). Frozen stocks were reconstituted following thawing at 37°C and washed in serum-containing RPMI 1640 medium prior to plating.

3.3 DNA extraction

DNA was extracted for analysis of internucleosomal cleavage by a procedure modified from Gross-Bellard, et al. (1973). Cell suspensions (about 10^6 cells per sample) were centrifuged at 200 x g for 15 minutes at 4°C to separate cells from the incubating medium. Cells were resuspended in homogenisation solution (0.1M NaCl, 0.01M EDTA pH 8.0, 0.3M Tris pH 8.0, and 0.2M sucrose) and incubated for 3 hours at 50°C with 1% SDS, and proteinase K (Life Technologies, Inc.) was added to give a final concentration of 200 μg/ml. 0.8M potassium acetate was added (4°C, 60 minutes) and samples were centrifuged

5,000 x g for 10 minutes at 4°C to pellet cellular debris. The supernatant was extracted with phenol/chloroform and chloroform. DNA was ethanol precipitated with 0.3M sodium acetate and 2.5 volumes of ethanol followed by centrifugation at 13,000 rpm, 30 minutes, 4°C. The pellet was resuspended in TE buffer and incubated (37°C, 60 minutes) with DNase-free RNAse (0.5μg per sample) (Roche Molecular Biochemicals, Auckland, NZ), then phenol/chloroform extracted and ethanol precipitated as above.

In experiments where DNA released by cultured cells was analysed, the media was removed from cells following centrifugation at 200 x g for 3 minutes. DNA was recovered by ethanol precipitation and then incubated in PK buffer (10mM Tris, 10mM EDTA, 150mM NaCl, 0.5% SDS, 200 μg/ml proteinase K) at 37°C for 1 hour (Joseph, et al., 1993). Samples were extracted sequentially with phenol/chloroform and chloroform, and ethanol precipitated as above.

3.4 Internucleosomal DNA Fragmentation Analysis

DNA extracted as above, from PC12 cells or nuclei, was end-labelled with [α-^{32}P]ddATP (Amersham, Buckinghamshire, UK) using terminal deoxynucleotidyl transferase enzyme (Life Technologies, Inc.) according to the method of Tilly and Hseuh (1993). DNA samples and the 1 kb ladder marker (100 ng) were incubated in TdT reaction buffer [0.5M potassium cacodylate (pH 7.2), 10mM cobalt chloride, 1 mM DTT] containing [α-^{32}P] ddATP and terminal deoxynucleotidyl transferase enzyme (14.9 units) for 1 hour at 37°C. Reactions were stopped by addition of EDTA to a concentration of 0.25mM and tRNA to 5μg/μl. DNA was ethanol precipitated twice and pellets were washed in 80% ethanol. DNA was resuspended in 1 x TE buffer and electrophoresed on a 2% agarose gel at 150 V for 90 minutes. The gel was washed overnight in an aqueous solution containing 10% isopropanol, 10% acetic acid to remove unincorporated label, then dried under a gel dryer without heat for 2 hours and exposed to Fuji Medical X-ray film under intensifying screens.

3.5 Pulse field gel electrophoresis

Agarose plugs were prepared according to Ramachandra & Studzinski (1995). Cells were resuspended in nuclear buffer (0.15M NaCl, 2mM KH_2PO_4 pH 6.4, 1mM EGTA, 5mM $MgCl_2$) centrifuged at 2,000 x g, 2 minutes, 4°C, then at 12,000 x g, 10 seconds. Cells were resuspended in nuclear buffer containing 0.4 mg/ml Proteinase K and mixed with an equal volume of 1.5% low melting point agarose at 50°C before pouring into plug moulds. The agarose plugs were allowed to solidify at 4°C and incubated in lysis buffer (10mM NaCl, 10mM Tris-HCl pH 9.5, 25mM EDTA, 10% N-lauroyl sarcosine) supplemented with 100 µg Proteinase K, with agitation at 37°C overnight. Plugs were rinsed in TE buffer and stored in 0.5M EDTA pH 8.0 at 4°C. Samples, *S. cerevisiae* 225kb-2200 kb and lambda 0.1 –200 kb markers were electrophoresed using a CHEF apparatus (BioRad, Hercules, CA) with an initial ramping rate of 2-8 seconds for 4 hours, followed by a ramping rate of 8-15 seconds for 14 hours at 200V. The gels were stained with SYBRI Green Nucleic Acid Stain (Molecular Probes, Inc., Eugene, OR).

3.6 Visualisation of Chromatin Condensation

Cells or nuclei were fixed with 4% paraformaldehyde and stained with 10 µg/ml Hoechst 33342 (Molecular Probes, Inc.) for 30 minutes at room temperature in the dark. Cells were then washed in PBS, resuspended in 10% glycerol and examined using a Zeiss Axioskop fluorescence microscope. Nuclei were scored for the presence of apoptotic morphology (brightly stained clumped chromatin) (see Figure 19) and photographed with 35 mm film.

3.7 Cytochrome c immunofluorescence

Cells were fixed in suspension with 4% paraformaldehyde, washed with PBS, and permeabilised in NBS/PBS (1% normal goat serum, 0.5% BSA, 0.1% saponin in PBS). Cells were incubated with primary mouse monoclonal anti-

cytochrome c antibody (Pharmingen, San Diego, CA) at 1:200 dilution overnight at 4°C. Cells were washed 3 times in NBS/PBS before incubation with Rhodamine-conjugated anti-mouse secondary antibody (Molecular Probes, Inc.) at 1:200 dilution for 1 hour at room temperature. Cells were washed twice in NBS/PBS and stained with 10 μg/ml Hoechst 33342 for 15 minutes. After a final wash in PBS, cells were resuspended in SlowFade reagent (Molecular Probes, Inc.) and placed on microscope slides. Cells were scored for the presence of apoptotic morphology (brightly stained clumped chromatin) and mitochondrial localisation of cytochrome c using a Zeiss Axioskop fluorescence microscope and photographed with 35 mm film.

3.8 Preparation of Cytosolic Extracts from SY5Y Cells

Cytosol was prepared from healthy growing cells, or those induced to undergo apoptosis by incubation in serum-free RPMI 1640 media with 0.5μM staurosporine for 24 hours at 37°C. 2.5 x 10^8 cells were harvested by centrifugation, washed in PBS and resuspended in Cytosol Buffer (38mM each of the potassium salts of aspartic, gluconic and glutamic acids, 20mM MOPS pH 7.1, 10mM potassium carbonate, 5mM reduced glutathione, 0.5mM magnesium carbonate, 1mM EGTA, 1mM EDTA, and 1mM PMSF). Cells were mechanically permeabilised by passing through a ball homogeniser (8.020mm cylinder with an 8.0186mm ball) and centrifuged at 1,000 x g for 10 min at 4°C. The supernatant was centrifuged at 100,000 x g for 1 hour in a Beckman TiSW55 rotor. The resulting supernatant was stored at -70°C and used for the *in vitro* reconstitution of apoptosis.

3.9 Purification of Nuclei from PC12 cells

Nuclei were isolated by a procedure modified from Lazebnik, et al.(1993). The cells (1.5 x 10^8) were harvested by centrifugation, washed in PBS and resuspended in Nuclear Buffer (10 mM PIPES, pH 7.4, 10mM KCl, 2mM $MgCl_2$, 1mM DTT, 10μM cytochalasin B, 1mM PMSF). Cells were passed 8 times

through a ball homogeniser (8.020 mm cylinder with an 8.008 mm ball) and nuclei were pelleted by centrifugation at 800 x g for 10 minutes at 4°C. Nuclei (8 x 10^8 nuclei/ml) were resuspended in Nuclear Storage Buffer (10mM PIPES, pH 7.4, 80mM KCl, 20mM NaCl, 250mM sucrose, 5mM EGTA, 1mM DTT, 0.5mM spermidine, 0.2mM spermine, 1 mM PMSF, plus 50% glycerol) and stored at -70°C.

3.10 Preparation of total protein lysates from intact cells

Protein lysates were prepared from cells according to the method of Wang, K., et al. (1996). Cells were washed twice in 5 ml TBS-EDTA (20mM Tris-HCl, pH 7.4, 155mM NaCl, 1mM EDTA) at 200 x g for 3 minutes at room temperature. 500 µl of lysis buffer (2% w/v SDS, 5mM EGTA, 5mM EDTA, 0.5mM PMSF, 10µg/ml pepstatin, 10µg/ml leupeptin, 20mM Tris-HCl, pH 7.4) was added and the cell pellets were incubated for 15 minutes at room temperature to lyse the cells. 100 µl of trichloroacetic acid (TCA) (10% final concentration) was added to the lysates, to precipitate DNA and protein. Aggregated DNA was removed with a pipette tip and the suspension was centrifuged at 3,600 x g for 5 minutes at room temperature. The protein pellet was washed twice with 1 ml 2.5% TCA at 3,600 x g for 5 minutes and dissolved in 25 µl 3M Tris base for 30-60 minutes followed by 25 µl of water. Samples were stored at -20°C until use.

3.11 Lowry Assay for Protein Concentration

Protein concentrations were measured by the method of Lowry, et al.(1951). Unknowns and standard protein samples were made up to a final volume of 1 ml. Reaction mix was made by adding Solution C (2% w/v NaK tartrate in H_2O) to Solution A (2% w/v Na_2CO_3 in 0.1M NaOH) and then adding Solution B (1% w/v $CuSO_2.5H_2O$ in ddH_2O) in the ratio 1 Solution C: 100 Solution A: 1 Solution B. 1ml of reaction mix was added to the samples and incubated at room temperature for 10 minutes. 100 µl of Folin-Ciocalteau reagent (diluted 1:3 in ddH_2O) was added and the samples were incubated at room temperature for 30

minutes. Absorbance was read at 550 nm and a standard curve for protein was used to determine the protein concentration of samples.

3.12 Immunoblotting

Equivalent amounts of protein were loaded onto SDS gels except where noted, along with colour stained (Life Technologies Inc.) and biotinylated markers (New England Biolabs). Proteins were resolved on 8-12% SDS-polyacrylamide gels according to Laemelli (1970) and transferred to nylon-reinforced nitrocellulose (OptiTran, Schleicher & Schull, Dassel, Germany) using method of Towbin, et al. (1979). Membranes were blocked for 1 hour in 1XTBS + 0.1% Tween-20 + 5% dry milk or 1XTBS + 0.3% Tween-20 + 5% dry milk (Parp, Raf-1 and caspase-9 antibodies) at room temperature. Membranes were probed overnight at 4°C with rabbit polyclonal antibodies against Parp (1:2000; Roche Molecular Biochemicals), phospho-specific Akt (Ser473) (1:1000; New England Biolabs), Akt (1:1000; New England Biolabs), phospho-specific IκB (Ser32) (1:1000; New England Biolabs), total IκB (1:1000; New England Biolabs), phospho-specific Mek1 (1:1000; New England Biolabs), total Mek1 (1:1000; New England Biolabs), phospho-specific Bad (Ser136) (1:1000; New England Biolabs), Bad (1:1000; New England Biolabs), phospho-specific Gsk-3α(Ser21) (1:1000; New England Biolabs), phospho-specific Creb (Ser133) (1:1000; New England Biolabs), total Creb (1:1000; New England Biolabs), caspase-9 (1:2000; Pharmingen) and Erk1/2 (1:2000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), goat polyclonal antibody to Raf-1 (1:500; Santa Cruz Biotechnology Inc.), and a mouse monoclonal antibody to caspase-3 (1:1000; Transduction Laboratories, Lexington, KY). Blots were washed three times for 15 minutes each wash in 1XTBS + 0.1%Tween-20 (0.3% Tween-20 for Parp, caspase-9 and Raf-1). Proteins were detected using HRP-conjugated secondary antibodies: anti-rabbit (1:2000 or 1:5000 dilution), anti-mouse (1:5000), anti-goat (1:5000), and anti-biotin (1:2000). All immunoblots were visualized using enhanced chemiluminescence Western blotting detection reagents (Amersham).

3.13 Stripping and reprobing of membranes

To strip antibodies from blots for subsequent reprobing, nitrocellulose membranes were soaked in 1XTBS pH 2.0 (10 mM Tris, 150 mM NaCl) for 10 minutes exactly. Membranes were washed 3 times, 5 minutes each wash with distilled water. They were then soaked in 1XTBS pH 8.0 for 10 minutes before blocking in 1XTBS + 5% dry milk + 0.1% Tween-20 for 1 hour at room temperature. Appropriate primary antibody was then added as above (Section 3.12).

3.14 Image analysis

The figures of x-ray films in this thesis have been digitally scanned and processed to remove background. The image of a blank film was subtracted from data using the digital analysis programme NIH image 1.62 (download at http://rsb.info.nih.gov/nih-image/download.html). This removed lighting variations from the lightbox and camera that would otherwise interfere with the densitometry measurements. The signal intensity was measured in the pixels of a defined area (a box encompassing the desired band), creating a graph of the result. The area under the curve was computed and used as a measurement of density. Density values for Akt and Raf-1 were expressed as a ratio relative to Erk1, which provided a control for protein loading.

3.15 Calculations

Statistical p values were calculated for comparing two independent sample means using the student's t-test.

Chapter 4

Separation of PC12 cells at different stages of apoptotic commitment

4.1 Introduction

4.11 Serum-withdrawal model of apoptosis in PC12 cells

Rat pheochromocytoma (PC12) cells are a useful model for studying the mechanism of apoptosis and its reversal. Normally, these cells proliferate in serum-containing medium. In response to NGF, they stop proliferating, differentiate, and acquire a sympathetic neuron-like phenotype (Greene & Tischler, 1976). Proliferating PC12 cells (naive) depend upon serum, while differentiated postmitotic (neuronal) PC12 cells depend upon serum and NGF for their survival. When serum is withdrawn from naive cells and both serum and NGF from neuronal-differentiated PC12 cells, they die by apoptosis (Batistatou & Greene, 1991; Rukenstein, et al., 1991; Batistatou & Greene, 1993; Pittman, et al., 1993). NGF-withdrawal -induced death in neuronal PC12 cells resembles that of sympathetic neurons (Mesner, et al., 1992). In this system, a number of factors can prevent cell death, including NGF, FGF, insulin, cAMP analogues, and aurintricarboxylic acid. Jnk is activated upstream of caspase-3 in serum- and NGF- withdrawn PC12 cells (Park, et al., 1996; Stefanis, et al., 1996; Haviv, et al., 1997). Exogenous Bcl-2 expression inhibits apoptosis in PC12 cells and prevents Jnk activation (Mah, et al., 1993; Park, et al., 1996). Apoptosis induced by serum withdrawal is not preceded by growth arrest and can occur at each phase of the cell cycle (Lindenboim, et al., 1995). In addition, death of serum-withdrawn PC12 cells does not require RNA or protein synthesis (Rukenstein, et al., 1991; Pittman, et al., 1993).

4.12 Asynchrony in apoptosis

Asynchronous death is a feature of apoptosis occurring in serum- and NGF-deprived rat pheochromocytoma (PC12) cells (Mills, et al., 1997; Messam & Pittman, 1998). Following a death signal, the cell enters a condemned (committed) phase that is terminated in a cell-autonomous fashion by transition to a final execution phase (Figure 16) (reviewed in Earnshaw, 1995a).

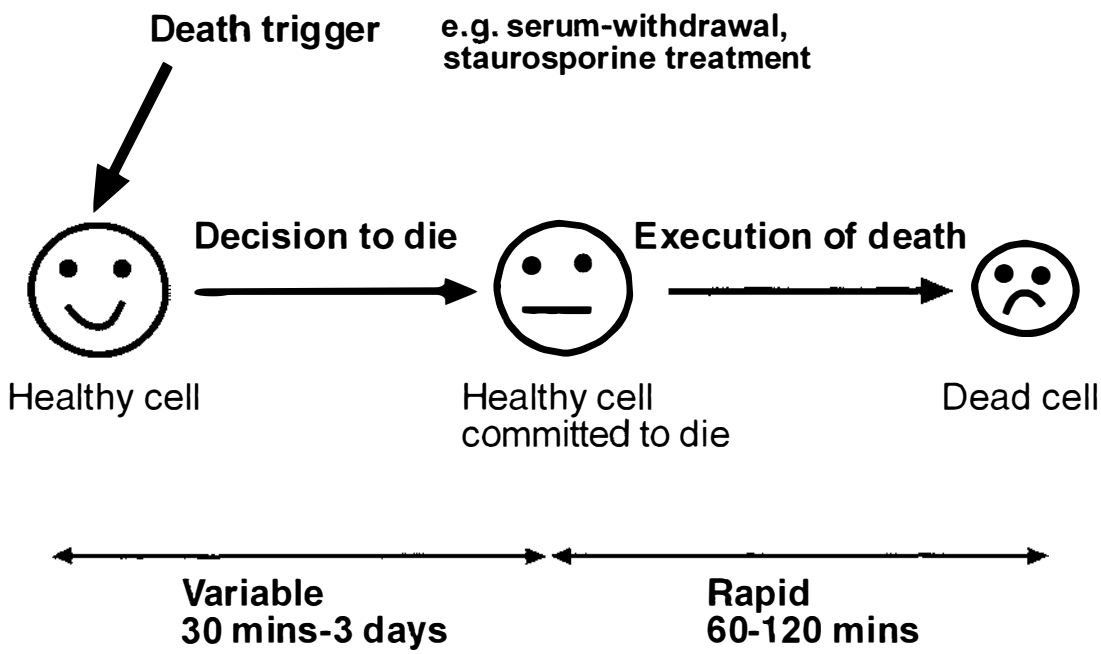

Figure 16: Asynchrony during apoptosis
Asynchrony arises during cell death due to the variable length of the commitment phase and its stochastic transition to the execution phase. This means that only a small number of cells within a population will be exhibiting the morphological and biochemical features of apoptosis at any one time.

Execution is brief and decisive, and results in the disappearance of the cell within 15 minutes to 1 hour. The stochastic nature of the transition from the condemned to execution phase results in a condemned phase that varies in length; only about 5-10% of the cells are in the execution phase at any one time. Thus, key molecular events that initiate execution may be occluded when using biochemical analyses on a whole population of cells.

Several approaches have been used to overcome limitations created by asynchrony, and to define cellular and biochemical changes occurring during apoptosis. These include reconstituting apoptosis in nuclear cell-free systems (Lazebnik, et al., 1993), performing analyses on individual cells (McCarthy, et al., 1997; Messam & Pittman, 1998), and isolating an enriched population of apoptotic cells (Wyllie & Morris, 1982; Cohen, et al., 1993; Desjardins & MacManus, et al., 1995; Mills, et al., 1998).

A key to understanding apoptosis will be characterising the point at which cells irreversibly commit to die. Events occurring upstream of this point represent potential regulatory pathways that can be manipulated to modulate apoptosis, and events downstream represent cellular degradation events.

The aim of these studies was to characterise the events occurring during the transition from the condemned phase to the execution phase in serum-deprived PC12 cells. This involved studying the timing of morphological changes and DNA fragmentation during apoptosis and confirming the asynchronous nature of the process. We employed density gradient sedimentation to separate populations of cells at three different stages of commitment to, and execution of, cell death. Serum-deprived PC12 cells were centrifuged through a discontinuous gradient of iodixanol (Optiprep™). This experimental system separates different populations of apoptotic cells by their density and thus permits a more detailed study of cells at different stages of cell death. Based on their chromatin morphology and extent of DNA cleavage, the three populations were defined and named live, dead, and deciding. Cytochrome c localisation, caspase activity, and c-Jun, Creb and Akt expression were characterised in

each population. To assess their commitment to cell death, the ability of NGF to rescue cells from death was also studied in each population.

4.2 Experimental Procedures

4.21 Serum-Deprivation of PC12 cells

Cells were serum-deprived by washing three times in serum-free RPMI 1640 medium and incubated in the same medium at 37°C for 1 to 24 hours. Samples were taken at time points 1, 2, 3, 4, 6, 8, 12 and 24 hours after serum withdrawal.

PC12 cells were harvested and recultured in RPMI 1640 medium containing 10% heat-inactivated horse serum and 5% foetal calf serum for 4 hours at 37°C as a negative control.

4.22 Differential centrifugation

Serum-withdrawn cells were harvested by pipetting and centrifuged at 200 x g, 15 minutes, 4°C to separate cell pellet (P1) from media. The supernatant was then subjected to differential centrifugation at 1,000 x g, 10 minutes (P2), 10,000 x g, 20 minutes (P3), and 100,000 x g, for 60 minutes, resulting in a final pellet (P4) and supernatant (S4).

4.23 Discontinuous Density Gradient Centrifugation and Recovery of Cell Populations

PC12 cells deprived of serum for 4 hours were washed in PGB (0.1% Glucose, 0.1% BSA in PBS) and resuspended in 1.10g/ml Optiprep™ (18.3% Optiprep™ in PGB) (Life Technologies Inc.). Cells were loaded into the middle of a discontinuous density gradient of Optiprep consisting of the concentrations, 1.06, 1.08, 1.10, 1.12, 1.14, 1.17, and 1.20 g/ml (10.6%, 14.5%, 18.3%, 22.2%, 26.0%, 31.8%, and 37.5%, in PGB respectively). Gradients were centrifuged at 1,000 x g, 30 minutes at 4°C. 5 drop fractions were collected, refractive indexes measured, and densities calculated. Fractions were pooled as follows:

population (1) 1.12-1.16 g/ml; population (2) 1.08-1.11 g/ml; population (3) 1.04-1.08 g/ml. Populations were recultured in serum-free RPMI 1640 medium in the absence or presence of 1 nM NGF for 3 hours at 37°C.

4.24 c-Jun Immunocytochemistry

Immunostaining of cells from the PC12 populations for c-Jun and Creb were performed by Mike Dragunow's lab at Department of Pharmacology, Medicine and Health Sciences Campus, University of Auckland, Auckland.

4.3 Results

4.31 Timetable of DNA fragmentation events in serum-withdrawn PC12 cells

DNA degradation, which ensures irreversible commitment to cell death, is a characteristic event in apoptotic execution. Serum-withdrawal of PC12 cells induces apoptotic DNA fragmentation to both high molecular weight and low molecular weight DNA fragments. During the course of my Honour's project I demonstrated that a continuous smear of 50-700kb fragments is visible from 1 hour after serum-withdrawal following CHEF pulse-field gel electrophoresis (Figure 17A) (François, 1996). The pulse field data was analysed by densitometry, and arbitrary units of DNA were plotted against time following serum-withdrawal for different DNA fragment sizes (Figure 17B). The largest fragments appeared first, and as time after serum withdrawal increased, fragments 50kb in size accumulated, peaking at 3-4 hours after serum-withdrawal. After 3 hours, the quantity of very large fragments decreased, and low molecular weight DNA below 50kb appeared, indicating further fragmentation had occurred.

Oligonucleosomal DNA fragments have been difficult to detect in some PC12 cell lines (Mesner, et al., 1992), so internucleosomal DNA fragmentation was analysed by radioactively end-labelling DNA fragments prior to electrophoresis. Cleavage of DNA into the 185 bp 'internucleosomal ladder' was detected from 1 hour after serum-withdrawal and increased over time (Figure 18A, left panel).

The fate of disassembled chromatin and the release of DNA from cells have not been greatly studied. We found that internucleosomal DNA fragments in the cell incubation media could be detected at earlier times but were greatly increased 4 hours after serum-withdrawal (Figure 18A, right panel). DNA in the media could represent free DNA or DNA contained within fragments of cells (apoptotic bodies). Differential centrifugation experiments were performed to distinguish

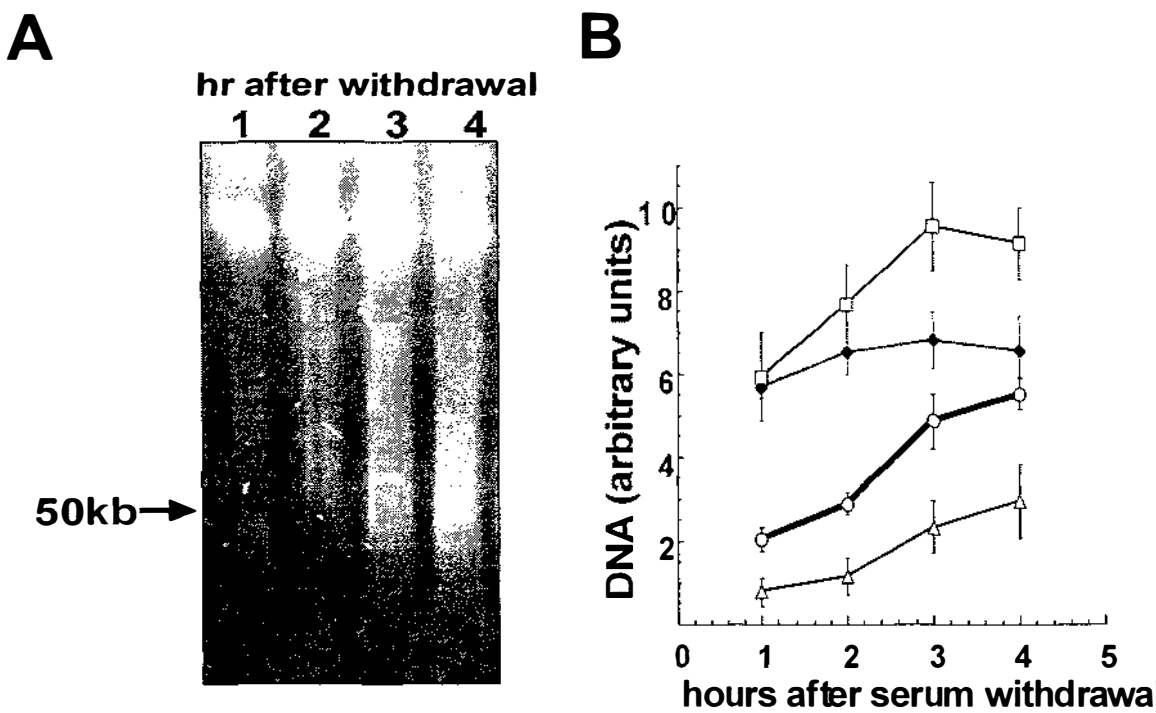

Figure 17: High molecular weight DNA fragmentation in serum-deprived PC12 cells.
PC12 cells were deprived of serum as described in Experimental Procedures. A. High molecular weight DNA from serum-deprived cells was analysed by CHEF pulse-field gel electrophoresis and stained with SYBRI Green nucleic acid stain. The arrow indicates the accumulation of fragments of 50kb in size.
B. Quantitation of data in (A). DNA in segments of the gel corresponding to 700-2000kb (◆), 100-700kb (❑), 40-100kb (○), and <40kb (Δ) were quantified by densitometry and plotted in arbitrary units versus time after serum withdrawal. Error bars represent SEM from the plotted average of 4 experiments.

This figure is adapted from François (1996).

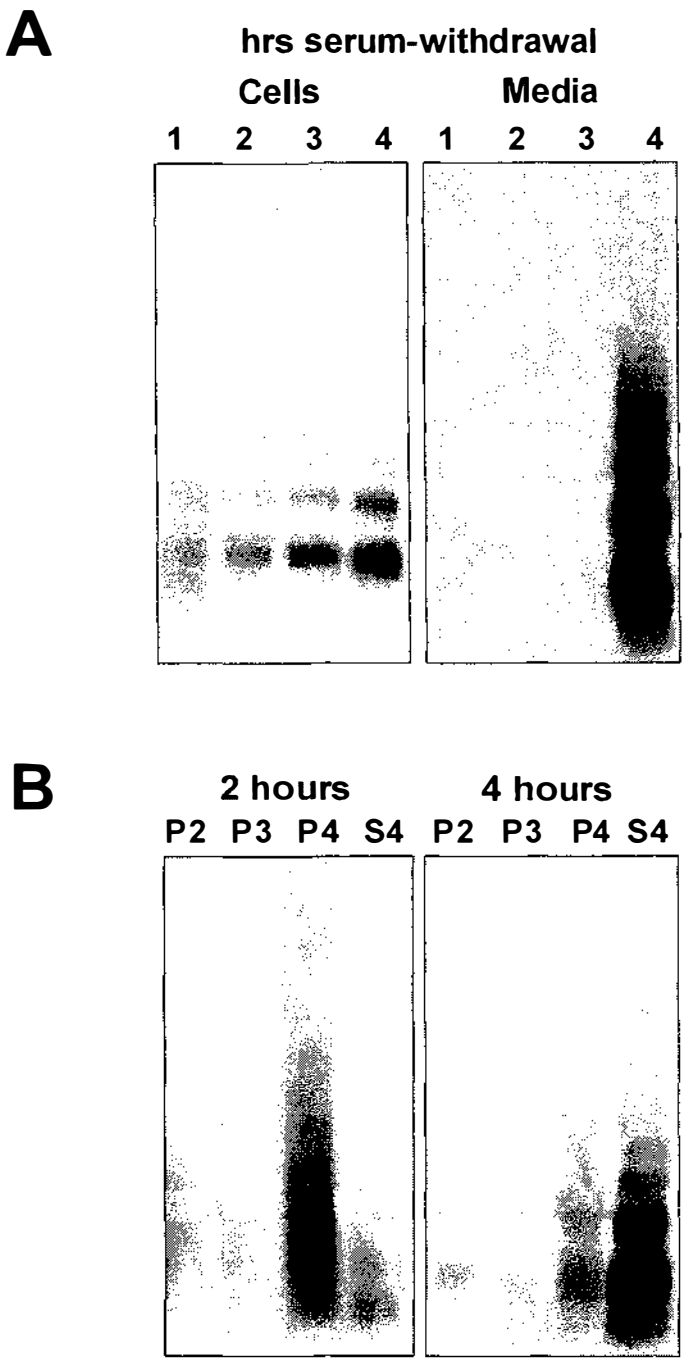

Figure 18: Multi-step degradation of DNA in serum-withdrawn PC12 cells.

A. DNA was extracted from serum withdrawn PC12 cells and the incubation media and radioactively end-labelled with [α-^{32}P]-ddATP. DNA was resolved by 2% agarose gel electrophoresis and visualised by autoradiography. Results are representative of 5 independent experiments.

B. Differential centrifugation of DNA released into the media. DNA was recovered from the incubation media of cells deprived of serum for 2 and 4 hours in centrifugation pellets P2, P3 and P4, and supernatant S4. DNA was radioactively end-labelled and analysed as above . Data are representative of 6 independent experiments.

between these alternatives. We used successive centrifugation steps of increasing g-force to collect different sized cell fragments or particles containing DNA. Serum-deprived cell suspensions were spun first at 200 x g for 15 minutes to remove the cell pellet (P1) and supernatants were sequentially spun at 1,000 x g for 10 minutes (P2), 10,000 x g for 20 minutes (P3), and 100,000 x g for 60 minutes (P4). Free DNA would be expected to be in the supernatant (S4) of the high-speed centrifugation. 2 hours after serum withdrawal, DNA was predominantly found in the 100,000 x g pellet (P4) (Figure 18B). 4 hours after serum-withdrawal, DNA extracted from the media was recovered predominantly in the soluble fraction (S4). Some DNA was also detected in larger cell fragments (P2, P3) at both times (Figure 18B). That most of the apoptotic bodies were recovered in the 100,000 x g pellet (P4) suggests that most of them are relatively small, the size of small organelles. The mechanism by which DNA is released into apoptotic bodies, and then subsequently into the soluble pool, remains unclear.

These data suggest that DNA is broken down into progressively smaller fragments while the cell is intact and that the destruction of DNA continues within apoptotic bodies.

4.32 Serum-withdrawn PC12 cells die asynchronously

End-labeling with radioactive nucleotide is a sensitive method for detecting DNA fragments, but does not indicate what fraction of cells are undergoing apoptosis. Oligonucleosomal DNA fragmentation can be discerned when as little as 2% apoptosis is observed morphologically (Collins, et al., 1992). Morphological markers in addition to DNA fragmentation analysis should be used because internucleosomal DNA cleavage can also occur during necrosis (Collins, et al., 1992). Cells were stained with the DNA-specific dye Hoechst 33342 and examined by fluorescence microscopy to determine the extent of chromatin condensation. Cells were scored as apoptotic if they exhibited chromatin condensation and/or nuclear fragmentation (Figure 19 inset). In the control sample (C), cells recultured in serum for 4 hours, very few cells showed

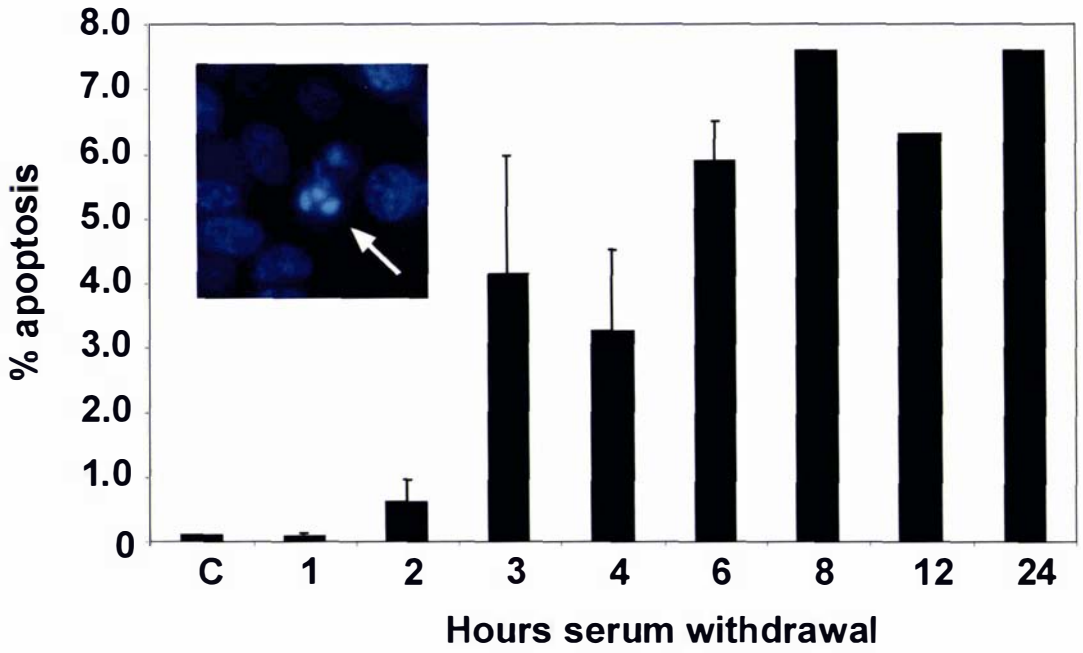

Figure 19:Quantification of apoptotic morphology in serum-withdrawn PC12 cells.

PC12 cells were deprived of serum for various times or recultured in serum for 4 hours (C) as a control. Cells were stained with Hoechst 33342 and scored for apoptotic nuclear morphology as demonstrated by arrow in inset photo. Standard errors are indicated by bars. Results are averages from 3 or more independent experiments (time points containing error bars). Samples have only been taken once at 8, 12 and 24 hours following serum-withdrawal so there are no error bars calculated for those time points.

apoptotic nuclear morphology (<0.1%) (Figure 19). The proportion of apoptotic cells began to rise 3 hours following serum withdrawal, reaching a maximum of about 8% at 8 hours, thereafter remaining relatively constant up to 24 hours without serum (Figure 19). These results are consistent with Messam & Pittman (1998) who demonstrated that serum-deprived PC12 cells die asynchronously, with only 5-10% of the cells in the execution phase at any one time. This is problematic for the goal of biochemically analysing the molecular mechanism of apoptosis triggering in these cells, as there will always be a high background of non-apoptotic cells that will occlude analyses. Therefore we sought a method to separate cells that are at different stages of triggering and execution.

4.33 Separation of PC12 cells at different stages of cell death

In previous studies, the higher density of apoptotic, compared with normal, thymocytes have been used as the basis for their separation and purification by isopycnic centrifugation (Arends, et al., 1990; Cohen, et al., 1992). Apoptotic cells increase in buoyant density when compared to healthy viable cells (Wyllie & Morris, 1982), so density gradient centrifugation was used to separate apoptotic cells from non-apoptotic cells (Figure 20). PC12 cells deprived of serum for 4 hours were separated on discontinuous iodixanol (Optiprep™) gradients and fractions were analysed for the appearance of apoptotic morphology (Figures 21A & 22A). Most of the cells floated to the least dense fractions at the top of the gradient (fractions 15-18). Very few or none of these cells floating exhibited apoptotic chromatin condensation (Figures 21A, 22A & 22B). Cells in fractions with higher densities (fractions 4-11) showed apoptotic morphology with increasing frequency that correlated with density (Figures 21A, 22A & 22B).

Gradient fractions were also analysed for DNA fragmentation (Figure 21B) and the results were consistent with morphology data. The least dense fractions (fractions 16-18) exhibited no internucleosomal DNA laddering, while cells in the densest fractions (fractions 3-9) showed pronounced DNA laddering. Interestingly, fractions of intermediate density (fractions 10-15), which exhibited

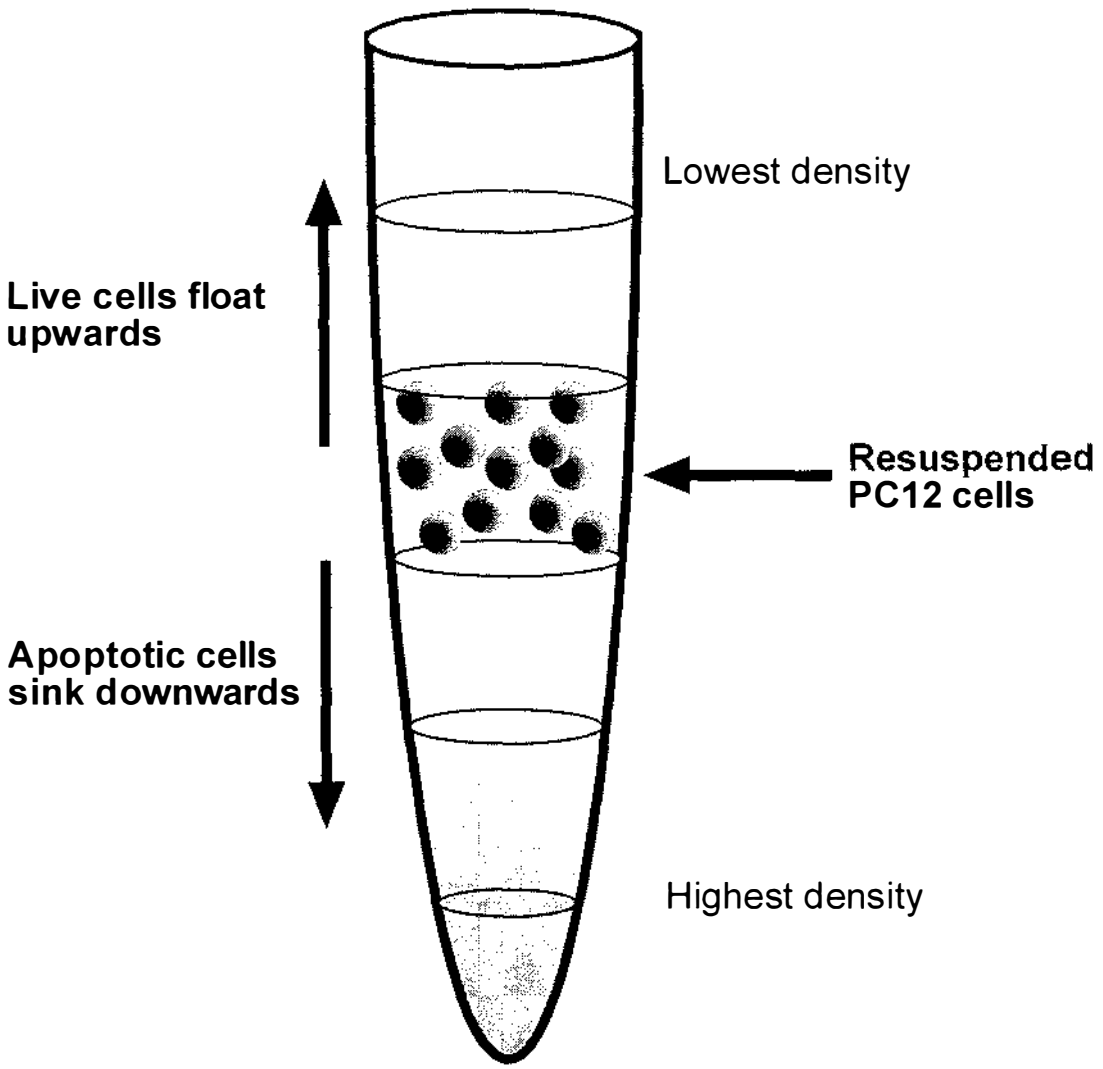

Figure 20: Discontinuous density gradient fractionation of apoptotic PC12 cells
Cells were resuspended within a discontinuous density gradient of Optiprep. Following centrifugation, apoptotic cells should sink downwards, as they have a higher density than live cells, which float upwards through the gradient.

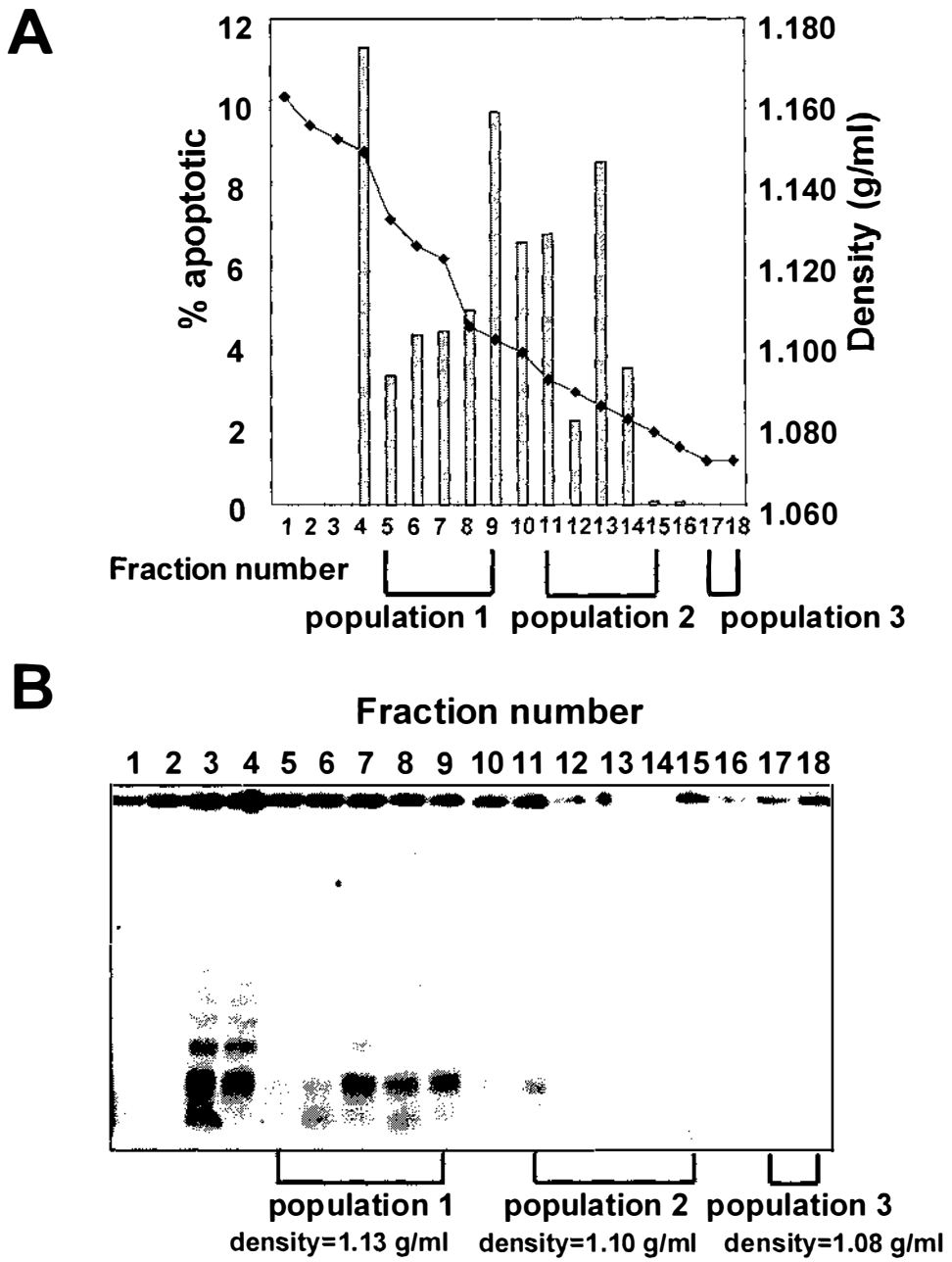

Figure 21: Density gradient separation of serum-deprived PC12 cells.

PC12 cells were deprived of serum for 4 hours before separation on Optiprep density gradients as described in Materials and Methods.

A. Fractions collected from the gradients were scored for apoptotic chromatin condensation (bar graph) as in Figure 19. Gradient fraction density was measured by refractometry (line graph). Fractions pooled to give populations 1, 2 and 3 are indicated. Data are representative of 4 independent experiments.

B. DNA was extracted from fractions collected in (A) and radioactively end- labelled with [α-^{32}P]-ddATP as in Figure 18. Equivalent amounts of DNA (50 ng) were used in the end-labelling reactions. DNA was resolved by gel electrophoresis followed by autoradiography. Fraction pools along with their average densities are indicated. Results shown are representative of 4 independent experiments.

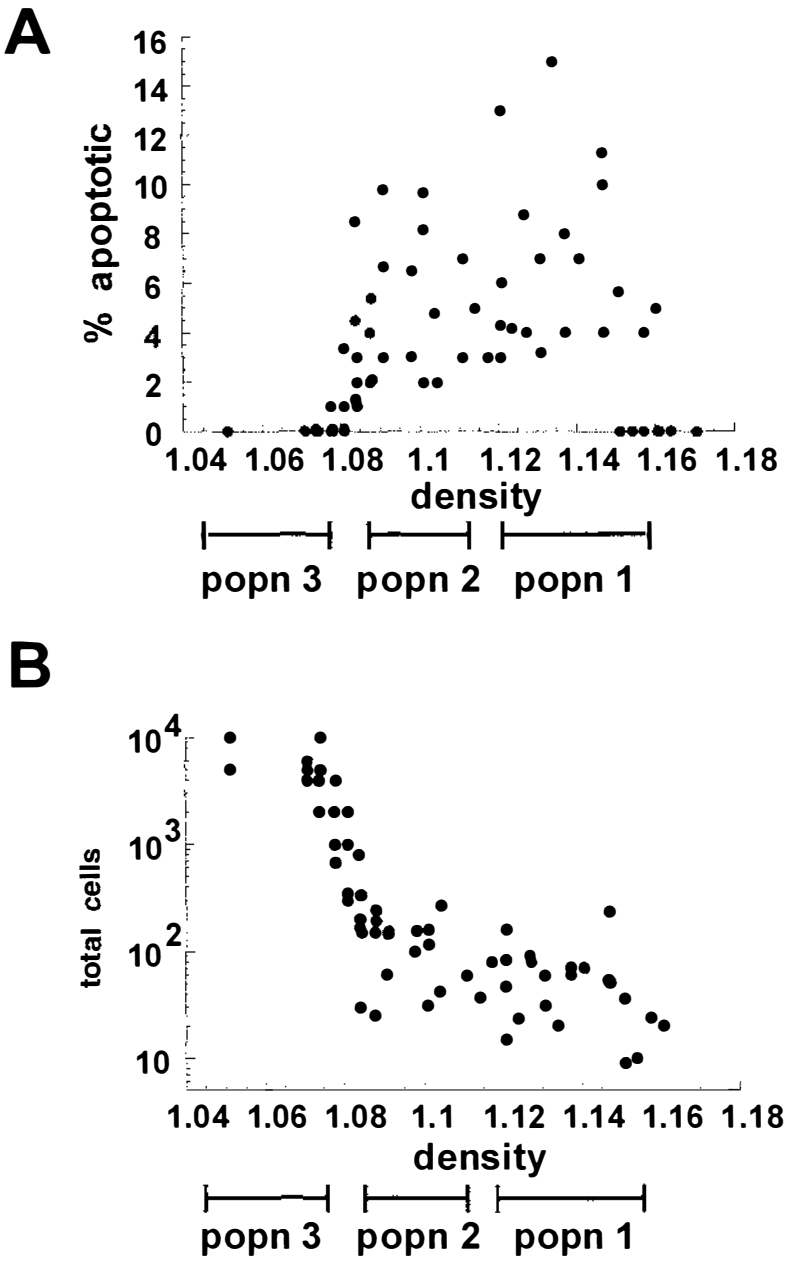

Figure 22: Pooled data for gradient separation of apoptotic PC12 cells

Data from 4 independent experiments, such as shown in Figure 21, were pooled to yield summary scatter plots. Populations 1, 2 and 3 are indicated below the graphs. There were no cells present in the densest fractions collected (fractions 1-2). Note that density on the x-axis is presented in the opposite orientation to that of fraction number in Figure 21.

A. The percentage of apoptotic cells at different densities.

B. The total number of cells at each density. Note the logarithmic scale.

apoptotic chromatin condensation (Figure 21A) showed only a very small amount of DNA fragmentation (Figure 22B).

On the basis of these data, the fractions were pooled to give three distinct cell populations. Population 1 in the densest fractions (1.12-1.16 g/ml) displayed the highest proportion of apoptotic cells but contained fewer cells than populations 2 and 3. Population 3 in the lightest fractions (1.04-1.08 g/ml) was made up of non-apoptotic, live cells that floated to the top of the gradient. Population 2 was contained in fractions of intermediate density (1.08-1.11 g/ml). Since these cells displayed some chromatin condensation but little DNA fragmentation, we hypothesised that population 2 contains cells that are at an intermediate stage in the cell death process.

4.34 Population 2 but not population 1 was rescued from cell death by NGF

To determine if differences existed between the three populations in their stage of commitment to cell death, the cells from pooled gradient fractions were recultured 3 hours in the absence or presence of NGF. Population 1, which contained the highest proportion of apoptotic cells, was not rescued from cell death by NGF treatment (Figure 23A). In fact, less DNA was recovered after reculturing from this fraction, suggesting that DNA may have been more extensively degraded (Figure 23B). In contrast, cells in population 2 could be rescued from cell death by NGF treatment (Figure 23). Without NGF 6% of cells had condensed chromatin, where as with NGF, only 1% of cells had apoptotic morphology. DNA fragmentation was pronounced when cells in population 2 were recultured without NGF, but in the presence of NGF was completely absent. Population 3, when recultured without serum and NGF began to display chromatin condensation, which was prevented by NGF. The large number of cells in Population 3 increased background detection of DNA fragmentation in the presence of NGF, but fragmentation was more pronounced in the absence of NGF. These results suggest that populations 2 and 3 were not yet fully committed to cell death because NGF could rescue them. Reculturing in the absence of serum and NGF drives more of the cells towards execution.

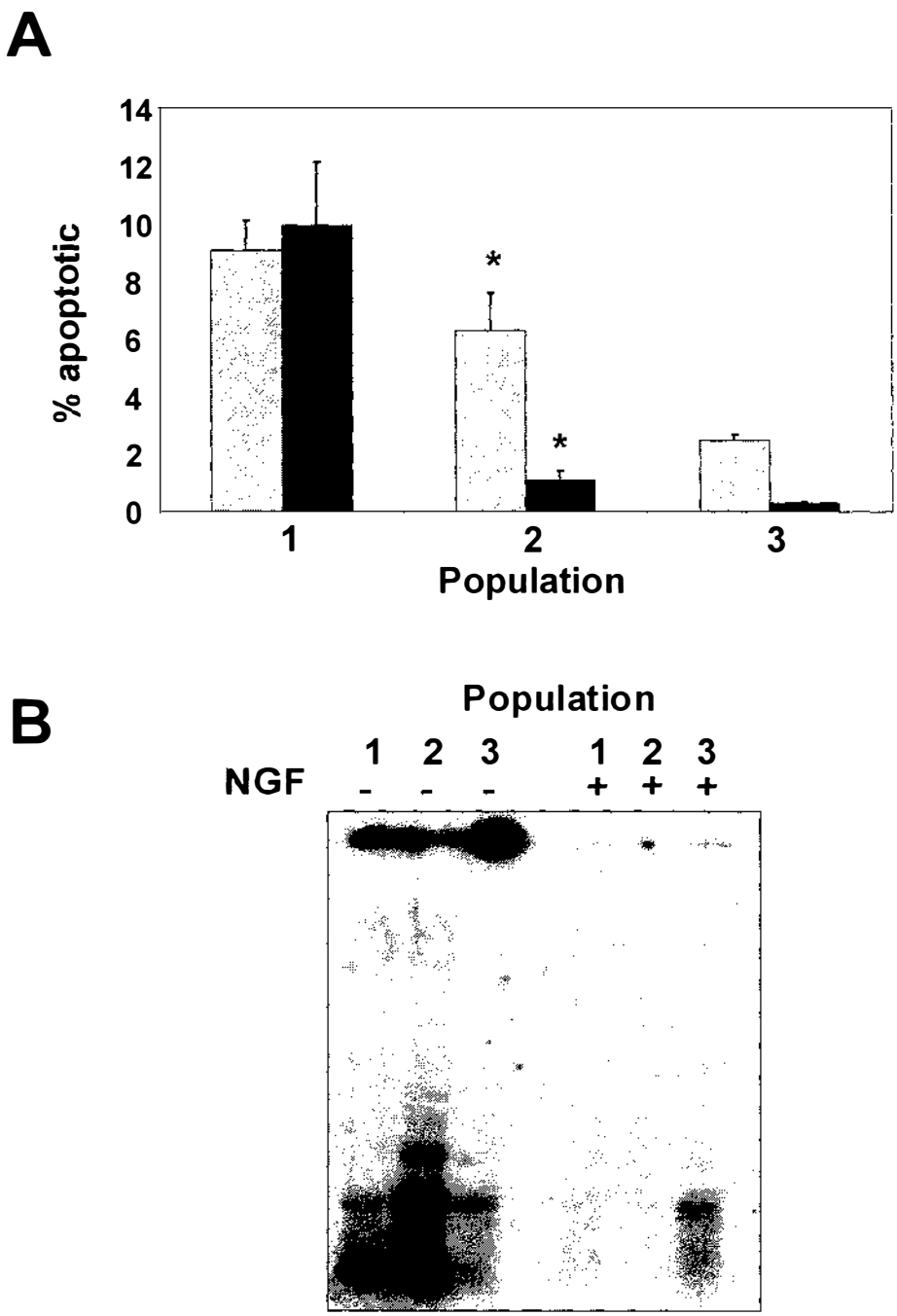

Figure 23:Survival of NGF-recultured PC12 cell populations
Serum-withdrawn PC12 populations recovered from Optiprep gradients were pooled into 3 populations as indicated in Figure 21 and recultured for 3 hours in the absence (grey bars) or presence of 1 nM NGF (black bars).
A. Populations were scored for apoptotic chromatin morphology. Data are averages from 3 independent experiments. Standard errors are indicated.
* $p<0.05$ for population 2 +/- NGF.
B. DNA fragmentation was analysed in the populations by end-labelling as in Figure 21. Equivalent amounts of DNA (50 ng) were used in the end-labelling reactions. Data are representative of 3 independent experiments.

Population 2 may represent cells at an intermediate stage where they are in the process of triggering apoptosis.

4.35 The separated populations differ in their cytochrome c immunoreactivity

Cytochrome c translocation from mitochondria to cytosol is believed to be important in regulating the onset of cell death. In order to explore further the stage of triggering and commitment between the three populations, cells were stained with antibodies to cytochrome c to assess its release from mitochondria. Release is typically followed by degradation (Neame, et al., 1998), so in this assay cytochrome c-negative cells were assumed to have released it from mitochondria. Populations 1, 2 and 3 all differed in their intensity of cytochrome c staining. Population 3, the live cells, had a bright punctate cytoplasmic cytochrome c distribution, clearly excluded from the nuclear space and consistent with mitochondrial localisation in over 98% cells (Figure 24A right panels & 24B). In contrast, population 1 showed a diffuse and reduced cytochrome c staining in two thirds of the cells (Figure 24A, left panels & 24B), which is consistent with loss of mitochondrial localisation and its later degradation within the cytosol. Population 2 exhibited mostly strong punctate staining consistent with a mainly mitochondrial location, but there was a loss of localisation in 10-12% of the cells (Figure 24A, middle panels & 24B). Reculturing with NGF did not restore cytochrome c immunoreactivity in population 1 or 2.

4.36 c-Jun protein levels were upregulated in Population 2

Changes in transcription factor levels and activation states are proposed to be involved in commitment to cell death. mRNA levels of c-jun, c-myb, c-fos, fosB, cyclin D1 are increased in dying neurons (Estus, et al., 1994; Freeman, et al., 1994). There is considerable evidence that c-Jun expression plays a role in cell death (Colotta, et al., 1992; Dragunow, et al., 1994; Estus, et al., 1994). Populations 2 and 3, recultured in the absence or presence of NGF, were fixed

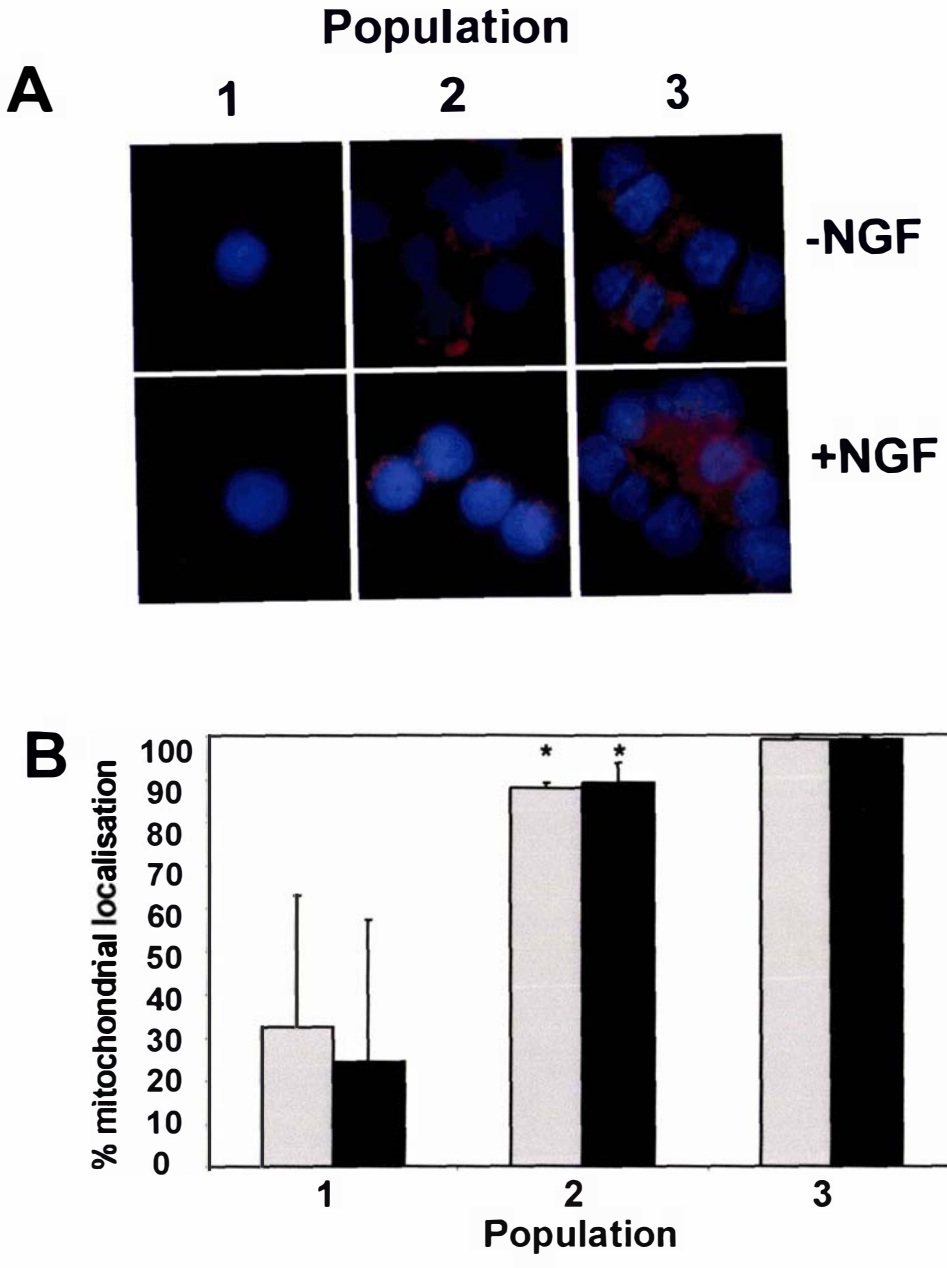

Figure 24:Cytochrome c staining of recultured PC12 populations
A. Serum-withdrawn PC12 cell populations recultured in the presence or absence of NGF were fixed with paraformaldehyde and stained with antibodies to cytochrome c. Secondary antibody is anti-mouse IgG conjugated to rhodamine (red). Nuclei were counter-stained with Hoechst 33342 (blue). Cells were photographed using a 35mm camera attached to a Zeiss Axioskop fluorescence microscope. Double photographic exposures are shown and magnification used was 1000 X. Photos are representative of 3 independent experiments.
B. Quantitation of data in (A). Populations were scored for mitochondrial localisation of cytochrome c in the absence (grey bars) or presence of 1 nM NGF (black bars). Results are averages from 3 independent experiments for populations 2 and 3 and standard errors are indicated by bars. Results are averages from 2 experiments for population 1 and range is indicated by bars.
*$p<0.02$ for population 2 and 3 without NGF.
*$p<0.2$ for population 2 and 3 recultured with NGF.

and then stained with antibodies to c-Jun. Population 2 showed an increase in c-Jun staining when compared to population 3 (Figure 25). In addition, population 2 recultured in the absence of NGF had slightly more c-Jun positive cells than the same population recultured with NGF.

Levels of the transcription factor Creb, and its activated form phosphorylated-Creb, were also examined in the populations. As mentioned previously (Chapter 2), Creb plays an important role in neuronal survival and is downregulated during cell death (Walton, et al., 1996). No changes were detected in Creb or phospho-Creb levels between the populations (data not shown).

4.37 Caspase activity and Akt protein downregulation

Caspase activation is believed to occur downstream of commitment as part of a set of terminal execution events. The DNA repair enzyme Poly-ADP-ribose polymerase (Parp) is a substrate for a number of caspases, including the effector caspase, caspase-3. We examined whole cell lysates from populations 2 and 3 for the appearance of caspase cleavage products of Parp. There were too few cells in population 1 to extract protein for analysis. Only the full-length form of Parp (113 kD) was detected in populations 2 and 3 (Figure 26A, top panel). Although, there was less 113kD Parp present in population 2, this may be due to unequal protein loading. The caspases that cleave Parp do not appear to be active in populations 2 and 3.

Akt kinase is important in regulating cell survival. Dominant negative Akt causes apoptosis in insulin-treated cerebellar neurons while overexpression of wild type Akt prevents death of growth factor deprived cerebellar neurons (Dudek, et al., 1997). In addition, we have demonstrated cleavage of Akt by caspases during *in vitro* apoptosis (François & Grimes, 1999; see Chapter 5). We examined Akt protein levels in populations 2 and 3 (Figure 26A & B). Akt protein levels, relative to the Erk1 control, were decreased 4-fold in population 2 when compared to population 3 (Figure 26B). Thus, Akt protein appears to be specifically downregulated in population 2.

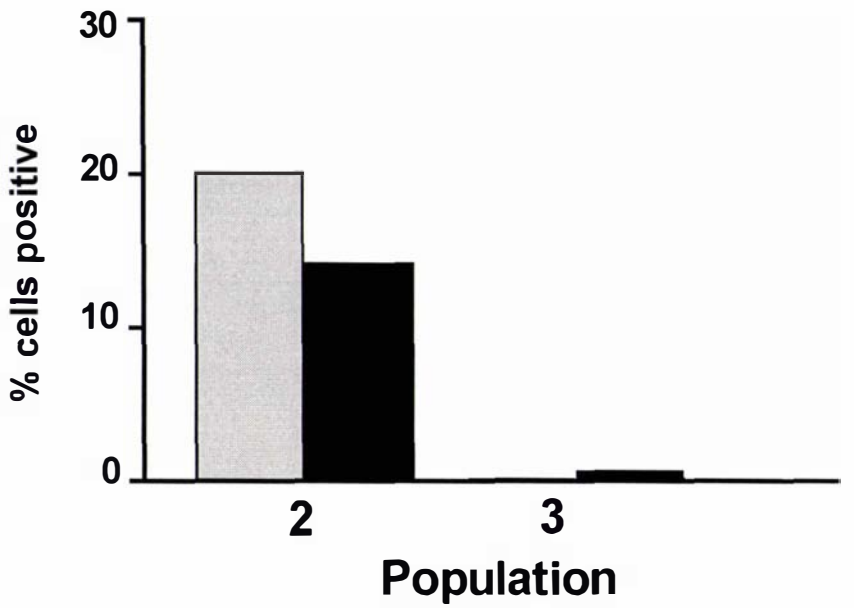

Figure 25: Populations 2 and 3 differ in reactivity to c-Jun antibodies

Serum-withdrawn PC12 cell populations recultured in the presence (black) or absence (grey) of NGF as described above were fixed with paraformaldehyde and stained with antibodies to c-Jun. The percentage of cells stained positive with c-Jun antibody was scored. Results are averages of five slides from one experiment. c-Jun immunocytochemistry was performed by Mike Dragunow, University of Auckland.

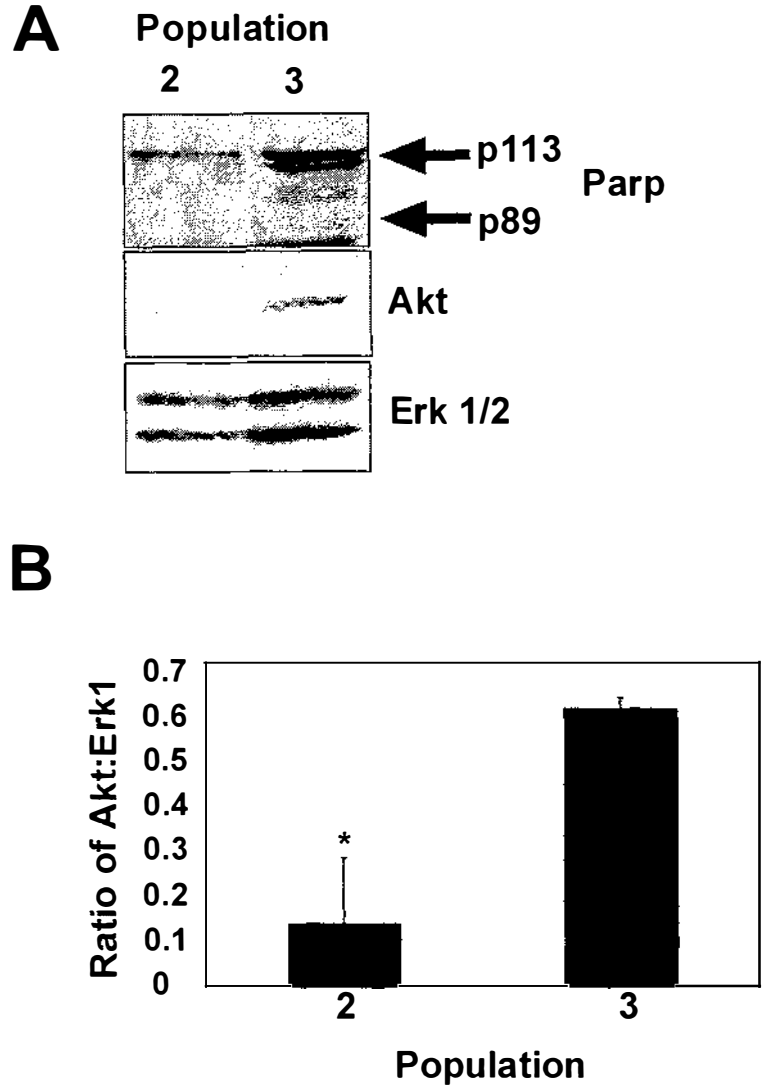

Figure 26: Immunoblotting of populations for Akt, Parp and Erk

Total cell lysates from PC12 cell populations 2 and 3 without subsequent reculturing were separated by SDS-PAGE and blotted onto nitrocellulose membrane.

A. Cell lysates were probed with antibodies to total Akt protein, Parp, and Erk. The position of the p89 Parp cleavage fragment, which was not detected in population 2 or 3, is shown.

B. Because there is unequal protein loading of the samples, levels of Akt protein were determined by densitometry and expressed as ratios relative to the control protein Erk. Data are averages from two independent experiments. Ranges are indicated by bars.

*$p<0.05$ comparing populations 2 and 3 calculated using t-test.

4.4 Discussion and Future Work

The present study was designed to address several features of apoptosis in neural cells that have not been directly addressed previously. These include: (1) defining the sequence of DNA fragmentation events accompanying apoptosis, (2) defining cell populations at different stages of commitment to apoptosis, and (3) determining if commitment to die can be defined relative to characteristic morphological and biochemical changes.

4.41 Multi-step degradation of DNA during apoptosis

Consistent with other researchers (Pandey, et al., 1994), we have detected the activity of an endonuclease in serum-deprived PC12 cells which cleaves DNA to approximately 50kb fragments. These large fragments accumulated as time increased after serum withdrawal. Consistent with the appearance of low molecular weight DNA fragments below 50kb, internucleosomal DNA laddering was detected in the samples at the same times following serum-withdrawal. The pattern of DNA fragmentation observed is believed to reflect chromatin structure and nuclease accessibility to DNA. The 50kb fragments may reflect excision of DNA loops at scaffold attachment regions of the nuclear matrix, and the 185 bp oligomers reflect DNA cleaved between nucleosomes (Filipski, et al., 1990). Our results are consistent with either a single endonuclease such as Cad mediating all stages of DNA degradation, or with a "domain nuclease" mediating 50 kb DNA cleavage, followed by internucleosomal cleavage by Cad. Our results do not distinguish between these two possibilities. The discovery of Aif supports the existence of a separate domain nuclease (Susin, et al., 1999). Recombinant Aif added to isolated nuclei caused high molecular weight DNA fragmentation to 50kb fragments but not oligonucleosomal cleavage characteristic of Cad activity.

Experiments where apoptosis has been induced in both sympathetic neurons and PC12 cells support our finding that DNA fragments are released into the

media by apoptotic cells (Edwards & Tolkovsky, 1994; Joseph, et al., 1993). However, none of these researchers have performed experiments to assess the origin of this extracellular DNA. Our differential centrifugation studies suggest that the extracellular DNA is associated with apoptotic bodies of different sizes, which have been released from dying cells. Our data suggets that DNA fragmentation continues within these bodies, because the amount of extracellular DNA in the soluble fraction (S4) increased with time following serum-withdrawal. The mechanism by which DNA is released into apoptotic bodies and then into the soluble pool remains unclear. Very late during apoptosis and in the absence of phagocytosis, membrane integrity is lost and apoptotic bodies may degrade by a process termed “secondary necrosis” (Wyllie, et al., 1980; Collins, et al., 1992). This process could occur in our system, which could explain the release of DNA into the soluble pool. Analysing the samples by electron microscopy would help characterise these apoptotic bodies.

We propose a multi-step chromatin degradation model where DNA is first cleaved to 50kb loops and then further degraded to 185 bp oligonucleosomal fragments, which are released from the cell enclosed in apoptotic bodies.

4.42 PC12 cells were separated at different stages of cell death

We have identified three populations of PC12 cells following serum-withdrawal and density gradient fractionation. Most cells floated to a light density and were live (population 3). These cells displayed no DNA laddering or gross morphological changes, while the densest cells (population 1) exhibited extensive internucleosomal DNA laddering and chromatin condensation. In addition, we identified an intermediate population (population 2) containing cells in the process of deciding to die. Significantly, this population could be rescued from cell death by NGF.

Other researchers have separated populations of cells at different stages of apoptosis. Thymocytes have been separated by density centrifugation on

Percoll gradients (Cohen, et al., 1993; Cohen et al., 1994), while HT29 tumour cells have been separated into 4 different stages on the basis of differences in cell adherence (Desjardins & MacManus, et al., 1995). However, only the degree of DNA fragmentation was studied in these separated cells and they were not characterised further biochemically. Biochemical characterisation of our isolated cell populations has allowed us to order events occurring during the onset of apoptosis.

4.43 Ordering events occurring during commitment to cell death

Populations 2 and 3 could be rescued from death by NGF, so we believe that we have isolated 2 separate populations of cells upstream of commitment to cell death (Figure 27). These populations were characterised further biochemically and used to order events occurring during commitment to cell death. Mitochondrial cytochrome c localisation appeared to correlate with the ability of NGF to rescue cell populations from death. Most cells in populations 2 and 3 maintained a mitochondrial localisation of cytochrome c, while population 1 lost cytochrome c into the cytoplasm. That 10-12% of the cells in population 2 had lost cytochrome c from the mitochondria suggests that these cells are further along towards commitment than population 3. Loss of mitochondrial cytochrome c was not reversed by reculturing with NGF. Consistent with the lack of commitment to death in populations 2 and 3, Parp-cleaving caspases did not appear to be active to the extent that the caspase-cleaved fragment was detected.

Jnk is activated upstream of the caspases in trophic factor-deprived PC12 cells (Park, et al., 1996; Stefanis, et al., 1996). The Jnk effector, c-Jun, has been shown to be necessary for neuronal cell death (Estus, et al., 1994). We found that population 2, but not population 3, exhibited upregulation in c-Jun protein levels. There was a decrease in c-Jun expression upon reculturing with NGF. Further work will be required to determine if this is significant.

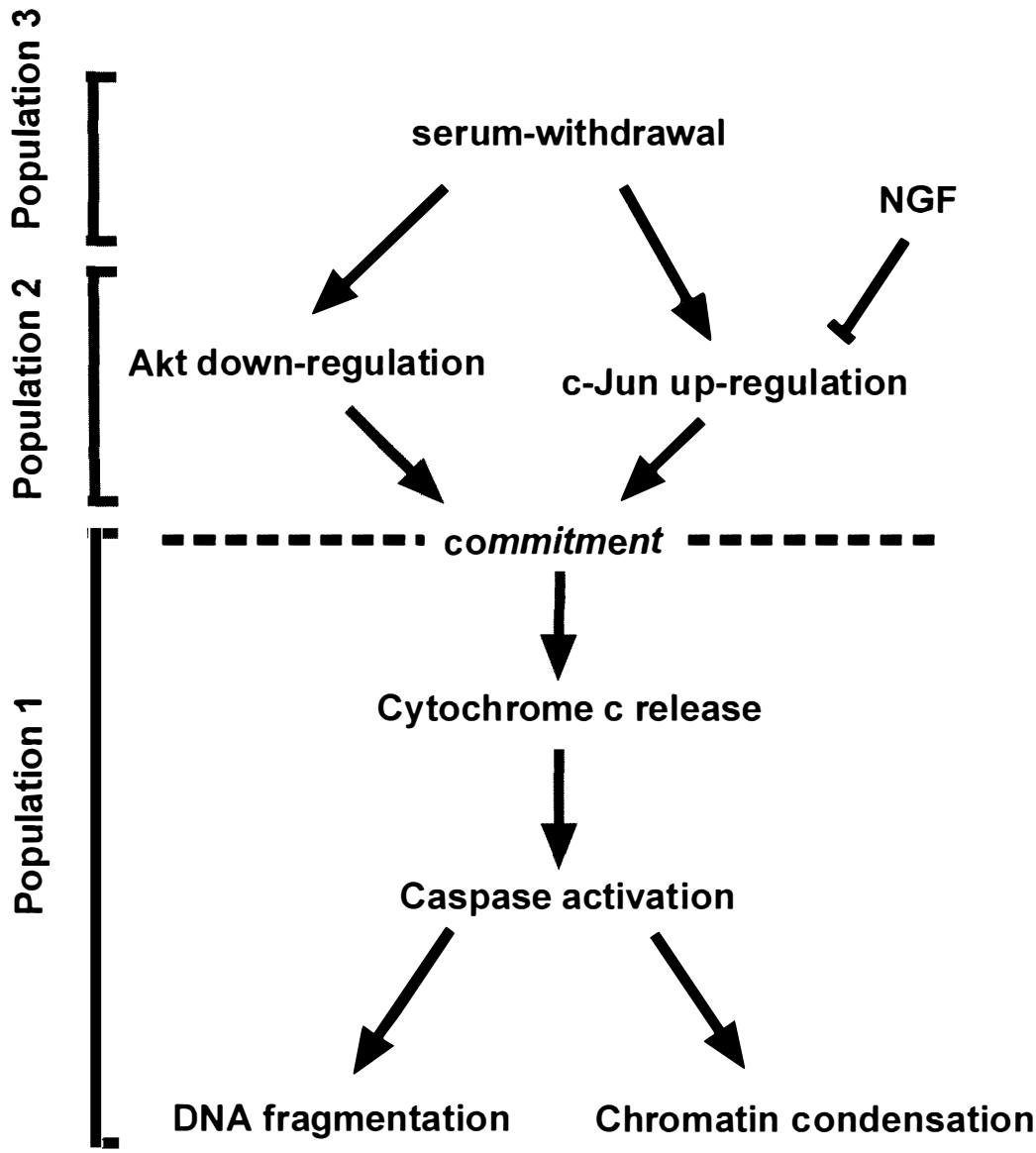

Figure 27: Model of events occurring in each of the cell populations

Following serum-withdrawal PC12 cells were separated into 3 different populations based on their density. Each population is characterised by distinct biochemical events. Population 3 shows no obvious morphological or biochemical changes and resembles live cells. Population 2 shows up-regulated c-Jun protein levels and decreased Akt protein levels. Cells up to this point can be rescued by reculturing with NGF. Population 1 represents the committed cells, which have released cytochrome c from mitochondria, and exhibit extensive DNA fragmentation and chromatin condensation.

Levels of the pro-survival signalling molecule, Akt, were downregulated in population 2. Further experiments are required to test whether Akt downregulation in population 2 may be rescued by reculturing with NGF.

We propose an order of events occurring following serum-withdrawal (Figure 27). In this model cells initially show no obvious morphological or biochemical changes after serum-withdrawal (population 3). Cells then begin the process of deciding to die by upregulating levels of the pro-death effector, c-Jun, and downregulating pro-survival kinase, Akt (population 2). Cells at this stage can be rescued from death by NGF, because they are upstream of cell death commitment. Once the cell becomes committed to die (population 1), cytochrome c is released from mitochondria, caspases are activated, DNA destroyed, and the cell exhibits the gross morphological events of apoptosis. The precise nature of the transition to irreversible execution is unknown.

What is the molecular basis of NGF's ability to rescue population 2 from death? NGF re-addition to NGF-deprived sympathetic neurons can lead to the restoration of cytochrome c content by the mitochondria if caspase activation was prevented by inhibitors (Martinou, et al., 1999). In our studies, population 2 displayed only a small amount of cytochrome c release from mitochondria, which was not affected by reculturing with NGF. NGF does more than simply inhibit caspases. Caspase inhibition prolongs the life of NGF-deprived neurons, but only for a limited time, cell bodies continue to shrink and mitochondria deteriorate. NGF reverses all of these conditions (Deshmukh, et al., 1996; Martinou, et al., 1999). Recent work has demonstrated that NGF can increase the expression of a caspase inhibitor protein, Ita, which could block death mediated by the caspases (Wiese, et al.,1999). However, Parp-cleaving caspases were not active in population 2 and our data is consistent with NGF-mediated rescue of serum-withdrawn cells occurring upstream of caspase activation.

Further experiments are required to test the validity of our model. The mechanism of c-Jun upregulation and Akt downregulation requires further work

to assess whether it occurs at the level of transcription or occurs post-translationally. Because of NGF's overall effect on cell body size and the general robustness of the neuron, we hypothesise that NGF can inhibit downregulation of Akt protein levels. Although no cleavage of Parp was detected after 4 hours serum withdrawal, other caspases may be active on other substrates. The use of more sensitive assays for caspase activation, such as cleavage of fluorometric caspase substrates that can be monitored spectroscopically would help determine whether there is any caspase activity in population 2.

Chapter 5

In vitro reconstitution of apoptosis

5.1 Introduction

5.11 In vitro models of apoptosis

Since neuronal apoptosis is stochastic and asynchronous (Mills, et al., 1997; Messam & Pittman, 1998), biochemical features of execution are difficult to study in whole cells. If cell death is asynchronous, apoptotic cells are a small population compared to a large number of viable cells, as discussed in Chapter 4. Cell-free models that reconstitute apoptotic events have proven to be very useful in overcoming problems of asynchrony. *In vitro* incubations using cell-free systems complement studies in which proteins are overexpressed in cells or genetically ablated in whole animals. Advantages of cell-free systems are: experiments are rapid; it is possible to add and deplete proteins and other reagents that may not normally cross the cell membrane; and it is possible to study apoptotic events without worrying about the effects of various treatments on other aspects of cellular physiology (reviewed in Earnshaw, 1995b).

One of the first reported cell-free systems for apoptosis used highly concentrated extracts prepared from DU429 chicken hepatoma cells after perturbation of the cell cycle. These extracts could promote both chromatin condensation and DNA fragmentation in nuclei isolated from a variety of cell types and species (Lazebnik, et al., 1993). Another early system used apoptotic *Xenopus laevis* (African clawed frog) egg extracts, which are a model for the process of oocyte atresia (Newmeyer, et al., 1994). Extracts are made from oocytes harvested from frogs treated with hormones to trigger the atretic program. Following incubation at room temperature for several hours, the extracts cause nuclear and DNA fragmentation characteristic of apoptosis. These events can be inhibited by Bcl-2.

Initial studies with cell-free extracts have yielded several notable findings. First, essential factors triggering the onset of apoptotic execution are present in the mitochondria (Newmeyer, et al., 1994). Cell-free studies employing

deoxyadenosine-5-triphosphate (dATP) to activate apoptosis in cytosol prepared from healthy growing HeLa cells identified cytochrome c as the factor released from mitochondria involved in caspase activation (Liu, et al., 1996). Using the same system, Apaf-1, the human homologue of Ced-4, and caspase-9 were identified as being part of a complex activated by cytochrome c that is responsible for triggering a cascade of caspase activation in the cytosol (Li, et al., 1997; Zou, et al., 1997). Addition of cytochrome c and dATP to cytosol derived from healthy cells will induce caspase-3 activation and apoptosis (Li, et al., 1997).

Protein modifications such as phosphorylation/dephosphorylation appear to play an important role in the mechanism of apoptosis in human cells. Okadaic acid prevents radiation-induced apoptosis in human lymphoid tumour lines (Baxter & Lavin, 1992), but at the same concentration, induces apoptosis by itself in various other human or rodent cell lines (Boe, et al.,1991; Davis et al., 1994). Staurosporine, a relatively nonspecific protein kinase C inhibitor, induces apoptosis very rapidly in numerous cell lines (Bertrand, et al., 1994). Expression of the Ca^{2+}/camodulin-dependent protein phosphatase, calcineurin, predisposes neuronal cells to apoptosis and is associated with areas of the brain susceptible to stroke, epilepsy and neurodegenerative diseases (Asai, et al., 1999). Taken together, these data suggest that activation of a phosphatase(s) or loss of activity of a kinase(s) could be of central importance in triggering the apoptotic process.

The aim of experiments reported in this chapter was to create a cell-free model of neuronal apoptosis to study interactions among key regulators of apoptosis. The long term goal of these studies is to understand the upstream regulation of apoptotic triggering. First, however, the system required characterisation, so execution was artificially initiated by the addition of cytochrome c, and the effects of caspase activation on several key signalling molecules was investigated. In addition, the effects of kinase and phosphatase inhibitors on the execution machinery were investigated.

5.2 Experimental Procedures

5.21 *In Vitro* Reconstitution of Apoptosis

Aliquots of 40 µl of SY5Y cytosol (500 µg protein) were incubated with 5 x 10^6 PC12 nuclei in the absence or presence of 2µM cytochrome c, 2µM DEVD-CHO (Biomol, Plymouth Meeting, PA), 100µM z-VAD-fmk (Enzyme Systems Products, Livermore, CA), 1mM sodium orthovanadate, 5µM okadaic acid (Life Technologies Inc.), 5 units recombinant activated Gsk-3β enzyme (New England Biolabs Inc.), 0.1µg recombinant activated Erk1 enzyme (Upstate Biotechnology, Lake Placid, NY), an ATP-regenerating system (8mM creatine phosphate, 1mM ATP, and 5µg/ml creatine kinase) or ATP-depleting system [5mM glucose and 14.2 units/ml hexokinase (Serva, Heidelberg, Germany)] and made up to a final volume of 50 µl with Cytosol Buffer (described in Chapter 3). Reactions were incubated at 37°C for 4 hours. Preliminary experiments established that an incubation time of 4 hours was optimal for observing both the morphological and biochemical events of apoptosis.

5.22 DNA Fragmentation Analysis and Visualisation of Chromatin Condensation

DNA was extracted from PC12 nuclei by 2 hour incubation at 50°C in lysis buffer (10mM Tris-HCl, pH 8.0, 100mM EDTA, 0.5% SDS, 200 µg/ml proteinase K), followed by phenol/chloroform extraction and ethanol precipitation. DNA samples were end-labelled with [α -^{32}P] ddATP (Amersham, Buckinghamshire, UK) using terminal deoxynucleotidyl transferase enzyme (Life Technologies, Inc.) as described in Chapter 3. DNA samples were electrophoresed on a 2% agarose gel, in 1X TAE buffer, dried and exposed to Fuji Medical X-ray film.

Nuclei were fixed with 4% paraformaldehyde in PBS, washed with PBS, and stained with 10 µg/ml Hoechst 33342 (Molecular Probes, Inc.).

5.23 Immunoprecipitation of Bad protein

450 µl of a 1:500 dilution of anti-Bad primary antibody (New England Biolabs Inc.) diluted in cell lysis buffer (1% Triton X-100, 50mM Tris pH 7.5, 10mM EDTA, 0.02% sodium azide, 1mM PMSF, 1µM mixed protease inhibitors (pepstatin, chymostatin, leupeptin, aprotonin), 1mM okadaic acid, 2mM sodium orthovanadate) were added to the 50 µl *in vitro* reactions and incubated overnight at 4°C. 50 µl of 20% protein A sepharose beads (Pierce, Rockford, IL) was added to the samples and they were rotated at 4°C for 2 hours. Samples were then centrifuged at 1,000 x g for 5 minutes at 4°C. The pellets were washed twice in Buffer A (10mM Tris-HCl, pH 7.5, 100mM NaCl, 2mM EDTA, 0.2% Igepal, 0.5mM PMSF), and once in Buffer B (10mM Tris-HCl, pH 7.5, 500mM NaCl, 2mM EDTA, 0.2% Igepal, 0.5mM PMSF) and Buffer C (10mM Tris-HCl, pH 7.5, 0.5mM PMSF), then respun at 1,000 x g, 5 minutes. Beads were then boiled in 2X SDS sample buffer for 5 minutes and centrifuged at 5,000 x g for 3 minutes. Samples were stored at –20°C until use.

5.24 Immunoblotting

Immunoblotting was performed as described in Chapter 3. Due to differences in signal intensity and background levels the order of primary antibodies used to probe the same membrane was very important. Phospho-specific antibodies were always used before their non-phospho-specific counterparts. In addition, caspase-9 was always used after caspase-3, and Erk was always used last to probe membranes.

5.25 Sequence Analysis

Protein amino acid sequences were obtained using Entrez at http://www.ncbi.nlm.nih.gov/Entrez/. Potential caspase cleavage sites were identified using the caspase substrate consensus sequence specificities (Nicholson & Thornberry, 1997):

Caspase-1,-4,-5	W/L	EHD
Caspase-3,-7	D	EXD
Caspase-6,-8,-9	I/L/V	EXD

Theoretical molecular weights for cleavage fragments were calculated using the compute pI/MW tool at http://www.expasy.ch/tools/pi_tool.html

5.3 Results

5.31 Creation of a cell-free model for apoptosis in neural cells

Cytosol harvested from cells primed to undergo apoptosis induces apoptotic changes in isolated nuclei. For example, cytosol harvested from staurosporine- or camptothecin-treated human promyelocytic HL-60 cells, and anti-Fas or UV-treated Jurkat and U937cells induces apoptotic changes in added nuclei (Solary, et al., 1993; Bertrand, et al., 1994; Martin, et al., 1995; Chow, et al., 1995; Shimizu & Pommier, 1996).

Cytosol was harvested from healthy cells or cells induced to undergo apoptosis and incubated with isolated PC12 nuclei in the presence of an ATP-regenerating system for 2-4 hours at 37 °C (Figure 28). Nuclei were then assayed for DNA fragmentation and chromatin condensation (Figures 29 & 30). The timing of key execution events has been previously characterised in serum-withdrawn PC12 cells (Chapter 4), so cytosol was harvested from cells that had been primed to undergo apoptosis by serum-withdrawal for 3-6 hours. Cytosols harvested from serum-withdrawn (data not shown) and control PC12 cells (Figure 29, lane 1) were unable to induce DNA fragmentation in PC12 nuclei. Data from a number of pilot experiments similar to that shown in Figure 29 are summarised in Table 2.

Cytoplasmic extracts prepared from PC12 cells were not competent to induce apoptosis *in vitro* under any conditions tested (Table 2). Since apoptotic events have been characterised in human neuroblastoma SY5Y cells induced to die by staurosporine treatment (Postmantur, et al., 1997; also see Chapter 6), cytosol was prepared from healthy SY5Y cells and SY5Y cells treated with 0.5μM staurosporine for 12-24 hours.

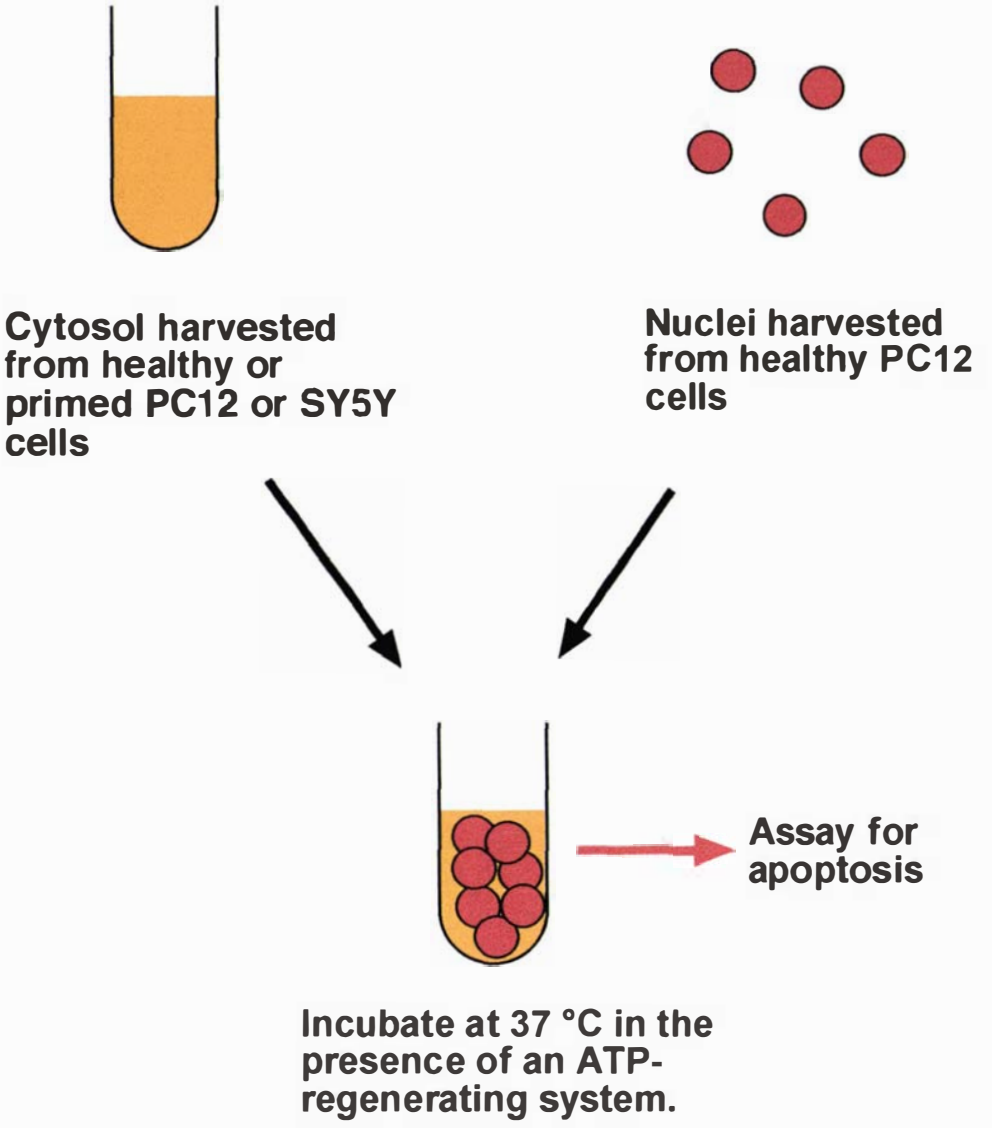

Figure 28: Cell-free reconstitution of apoptosis

Table 2: Summary of preliminary attempts to reconstitute apoptosis *in vitro*

PC12 nuclei were incubated with cytosol harvested from growing PC12 or SY5Y cells (Control), serum-withdrawn PC12 cells or SY5Y cells treated with 0.5 μM staurosporine for 12-24 hours (STS-SY5Y), in the presence or absence of 3 μM cytochrome c and 1 mM dATP. *In vitro* reactions were incubated for 2-4 hours at 37°C in the presence of an ATP-regenerating system. Apoptosis was scored by the absence or presence of an internucleosomal DNA ladder. N refers to the number of times each reaction has been performed.

Control PC12 cytosol	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Serum-wth PC12 cytosol	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-
Control SY5Y cytosol	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-
STS-SY5Y cytosol	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+
1mM dATP	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+
3 μM Cyt. c	-	-	+	+	-	-	+	+	-	-	+	+	-	-	+	+
Apoptosis (Y/N)	N	N	N	N	N	N	N	N	N	N	Y	Y	Y *	Y	Y	Y
N=	8	2	4	1	7	2	3	1	5	2	5	1	7	1	2	1

* DNA fragmentation was not present in every experiment performed using these conditions.

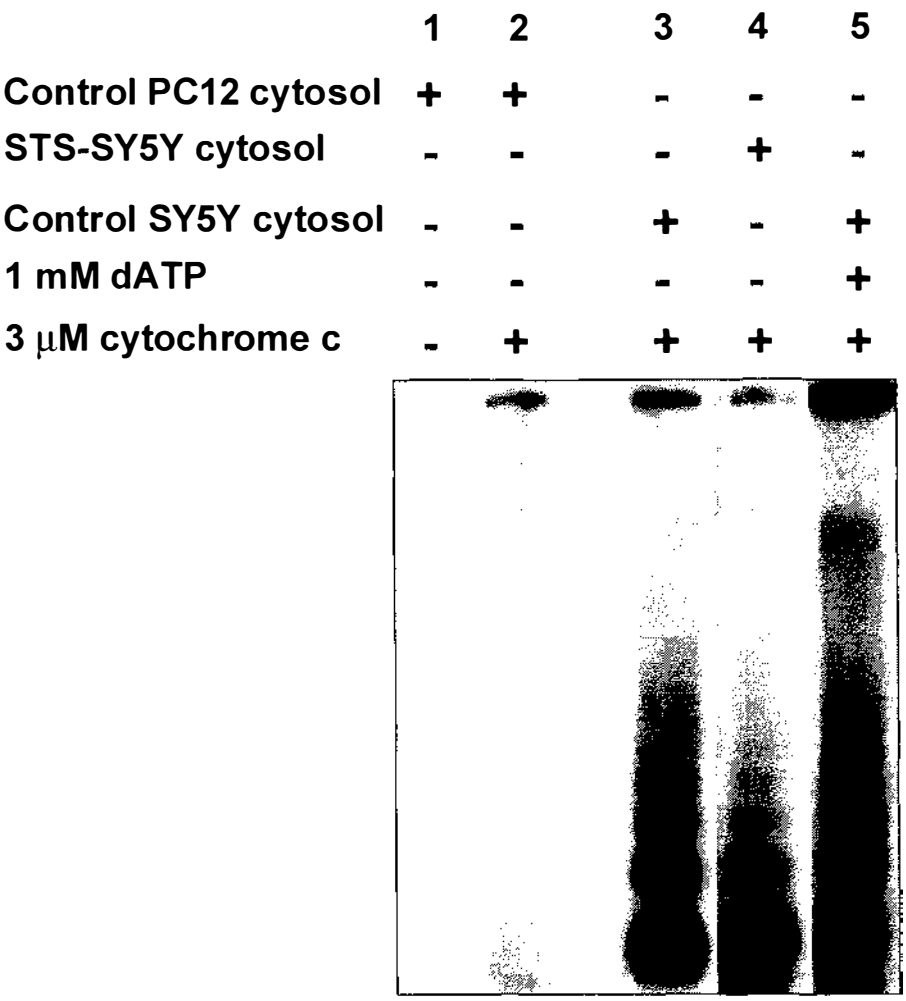

Figure 29: Apoptotic DNA fragmentation in *in vitro* reactions.
PC12 nuclei were incubated with cytosol harvested from growing PC12 cells (lanes 1 & 2), growing SY5Y cells (lanes 3 & 5), STS-treated SY5Y cells (lane 4), together with 3 μM cytochrome c (lanes 2,3,4 & 5), and 1 mM dATP (lane 5). *In vitro* reactions were incubated for 4 hours at 37 °C in the presence of an ATP-regenerating system. DNA was extracted, radioactively end-labelled and subjected to 2% agarose gel electrophoresis and autoradiography.
This figure is an example of data used to generate Table 2.

The SH-SY5Y cell line is the third successive subclone of the SK-N-SH line originally established from a bone marrow biopsy of a neuroblastoma patient. Neuroblastoma cells arise from neuroblasts of the neural crest, which have failed to develop normally to postmitotic, functional neurons. SY5Ycells, like PC12 cells, can be induced to differentiate and acquire a neuronal phenotype in response to NGF (Jensen, 1987). Differentiation requires selection for postmitotic cells with aphidicolin. Differentiated SY5Y cells can also be induced to die by NGF withdrawal (Jensen, et al., 1992).

Cytosol harvested from staurosporine-treated SY5Y cells, but not that from healthy SY5Y cells, was able to induce DNA fragmentation in PC12 nuclei (Table 2, Figure 30A lane 5). Despite the display of this characteristic feature of apoptosis, none of the nuclei incubated with cytosol from staurosporine-treated cells exhibited another characteristic of apoptosis, chromatin condensation (data not shown).

It has previously been shown that the addition of cytochrome c along with dATP induces caspase-3 activation and apoptosis in some cell-free extracts (Li, et al., 1997). In an attempt to find a system which recapitulated both morphological and DNA fragmentation events of apoptosis *in vitro*, the cofactors dATP and cytochrome c were added to activate the extracts. 1mM dATP by itself was unable to induce apoptosis in isolated nuclei in reactions containing cytosol prepared from both PC12 and SY5Y cells (Table 2). We found that the addition of cytochrome c alone activated the execution mechanism in extracts from SY5Y cells but not those from PC12 cells (Table 2, Figure 30A). dATP appears to be dispensable for apoptosis in this system because when it was added in the presence of cytochrome c and an ATP-regenerating system no greater induction of apoptosis was observed (Figure 29, lane 5). Therefore, dATP was not added in subsequent experiments. Due to the highly reproducible dramatic induction of apoptosis in this system, cytochrome c-activated SY5Y extracts were used for further studies.

5.32 Cytochrome c- activated neural cell extracts induced apoptosis in isolated PC12 nuclei

To ensure that our *in vitro* incubations with cytosol from SY5Y cells were faithfully reconstituting biochemical features of apoptosis, we further characterised the DNA fragmentation and also assayed chromatin condensation in PC12 nuclei (Figure 30). The addition of cytochrome c (3 μM) to reactions containing cytosol harvested from growing SY5Y cells and PC12 nuclei caused internucleosomal DNA fragmentation (Figure 30A, lane 4), nuclear shrinkage, and widespread chromatin condensation (Figure 30C). Cytosol or cytochrome c alone were not sufficient to induce DNA fragmentation (Figure 30A, lanes 1 & 2) or chromatin condensation (Figure 30B). In addition, the caspase inhibitor DEVD-CHO (2 μM) completely prevented the appearance of internucleosomal DNA fragmentation (Figure 30A, lane 3) and chromatin condensation (Figure 30D). These data are consistent with a model where cytochrome c initiates the activation of caspases whose activities are necessary for chromatin condensation and DNA fragmentation (Enari, et al., 1998; Liu, et al., 1996). The addition of cytochrome c to SY5Y cytosol was sufficient to reproduce caspase-dependent nuclear apoptosis in this *in vitro* system.

5.33 Caspase activation in cell-free extracts

To confirm that caspases were activated in the cell-free incubations caspase activation and cleavage of the caspase substrate Parp were examined by immunoblotting. Caspase-9 and caspase-3 were activated and Parp was cleaved in the cytochrome c-containing *in vitro* reactions (Figure 31, lane 2). This cleavage could be inhibited by DEVD-CHO (Figure 31 lane 3). In addition, we tested whether ATP was required for *in vitro* reconstitution of apoptosis.

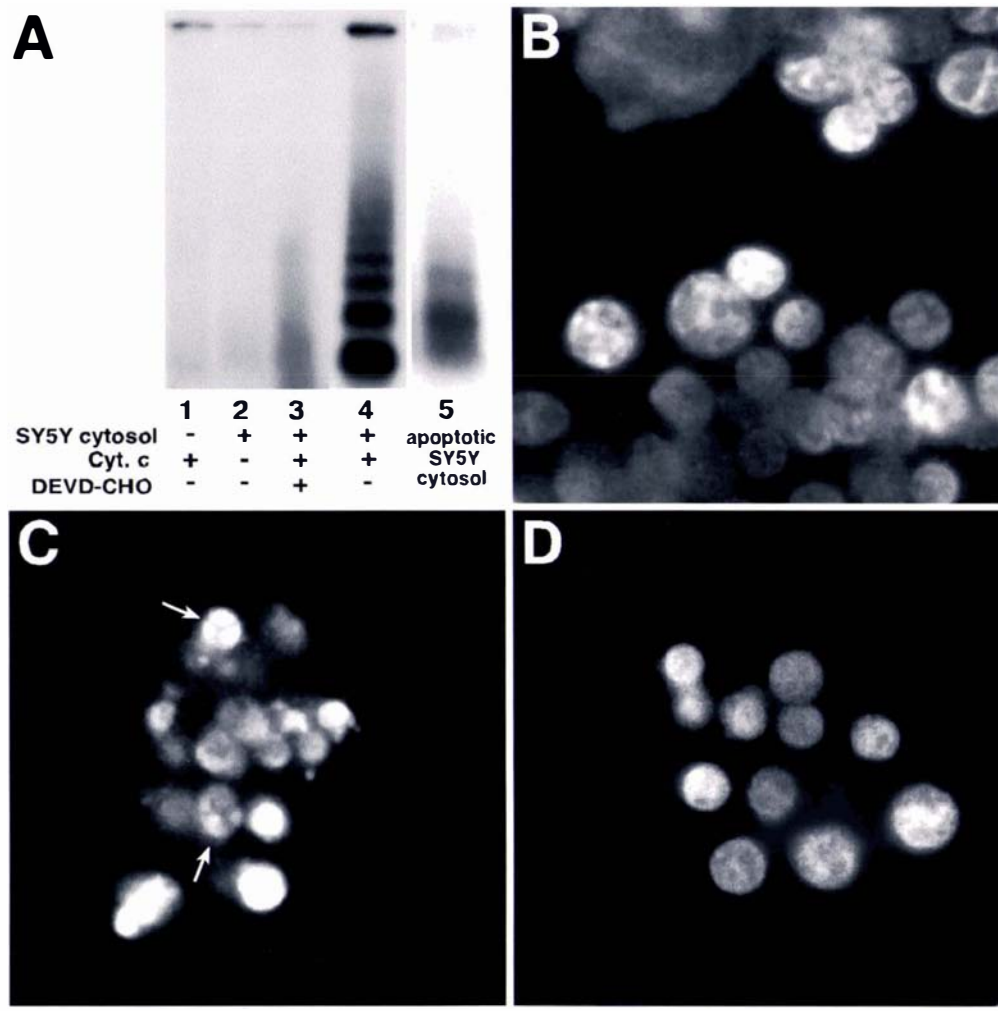

Figure 30: Cytochrome c-activated neural cell extracts induced apoptosis in isolated PC12 nuclei

A. PC12 nuclei were incubated alone (lane 1), or in the presence of SY5Y cytosol harvested from growing SY5Y cells (lanes 2, 3 and 4), together with 3 μM cytochrome c (lanes 1, 3, and 4), and 2 μM DEVD-CHO (lane 3). *In vitro* reactions were incubated for 4 hours at 37°C in the presence of an ATP-regenerating system (see Materials & Methods). DNA was extracted, radioactively end-labelled and subjected to 2% agarose gel electrophoresis and autoradiography. In lane 5, PC12 nuclei were incubated with cytosol harvested from SY5Y cells treated for 24 hours with 0.5 μM staurosporine. The reaction was incubated and DNA extracted as above. Data are representative of 3 independent experiments.

B,C & D. PC12 nuclei were incubated with SY5Y cytosol with an ATP-regenerating system as in (A), in the absence (B), or presence of 3 μM cytochrome c (C and D). 2 μM DEVD-CHO was added to the reaction in (D). Nuclei were fixed and stained with Hoechst 33342 and photographed with a fluorescence microscope. Magnification is 630X. Arrows in (C) point out examples of condensed chromatin. Data are representative of 4 independent experiments.

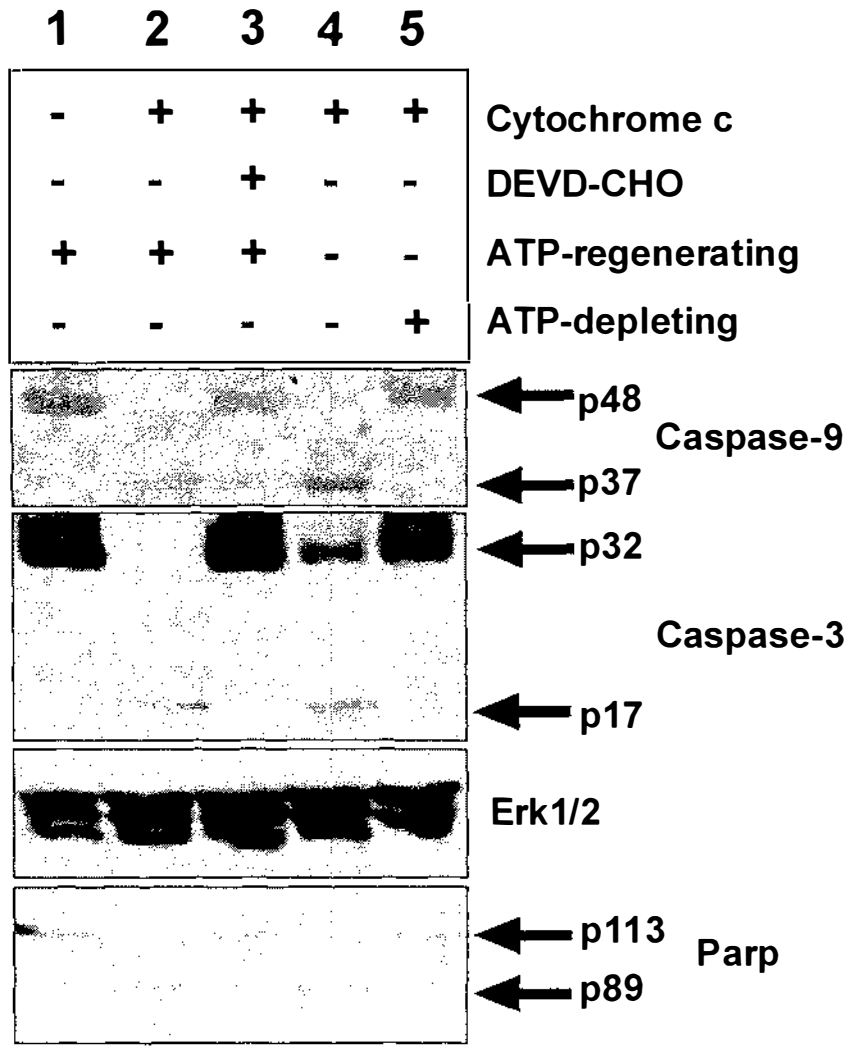

Figure 31: Caspase activation in cell-free extracts
PC12 nuclei were incubated in the presence of cytosol harvested from growing SY5Y cells, together with 3 μM cytochrome c (lanes 2,3,4 & 5), 2 μM DEVD-CHO (lane 3), in the absence (lanes 4 & 5) or presence of an ATP-regenerating system (lanes 1, 2 & 3), or presence of an ATP-depleting system (lane 5). *In vitro* reactions were incubated for 4 hours at 37 °C. After incubation, the samples were analysed by immunoblotting using an antibody specific for proteins labelled at right of panels. Cleavage fragment sizes are indicated for caspase-9, caspase-3, and Parp. The blot for Erk 1 & 2 shows that equal protein was loaded on the gel.
Data are representative of 4 or more independent experiments.

Caspase activation and Parp cleavage were completely inhibited by functional depletion of ATP (Figure 31 lane 5). If the ATP-regenerating system was ommitted from the reaction, but ATP was not functionally depleted, Parp was still processed, but activation of caspase-3 and caspase-9 was partially inhibited (Figure 31 lane 4). This suggests that endogenous ATP present in the SY5Y cytosol allowed partial caspase activation but that was limiting. Thus, ATP is required for caspase activation in our *in vitro* system.

5.34 Akt was cleaved during cell-free apoptosis; cleavage was prevented by phosphatase inhibitors

One key factor mediating neuronal survival appears to be Akt kinase, a member of the phosphatidylinositol 3-kinase (PI3-K) signalling pathway. Inhibition of Akt signalling causes apoptosis, while Akt overexpression blocks apoptosis in a variety of cell types (Yao & Cooper, 1995; Dudek, et al., 1997; Philpott, et al., 1997; Kulik, et al., 1997). The status of Akt was examined in cell-free incubations by Western blotting with antibodies to the active form of Akt (phosphorylated at serine 473) and total Akt protein. We found that Akt was depleted in the reactions where cytochrome c initiated the apoptotic mechanism (Figure 32, lane 3). Both total Akt and phospho-Akt were selectively depleted in these reactions relative to the control proteins Erk 1 and 2. We were unable to detect Akt cleavage fragments by immunoblotting. Caspase activity was necessary for removal of Akt, because its disappearance was prevented by the caspase inhibitor DEVD-CHO, which prevented activation of caspase-9 and caspase-3 (Figure 32, lane 5). Functionally depleting ATP from the *in vitro* reactions also completely prevented caspase-9 and -3 activation and Akt digestion (Figure 32, lane 7). This is consistent with a requirement for ATP in caspase-9 and caspase-3 activation (Li, et al., 1997; Liu, et al., 1996), and a

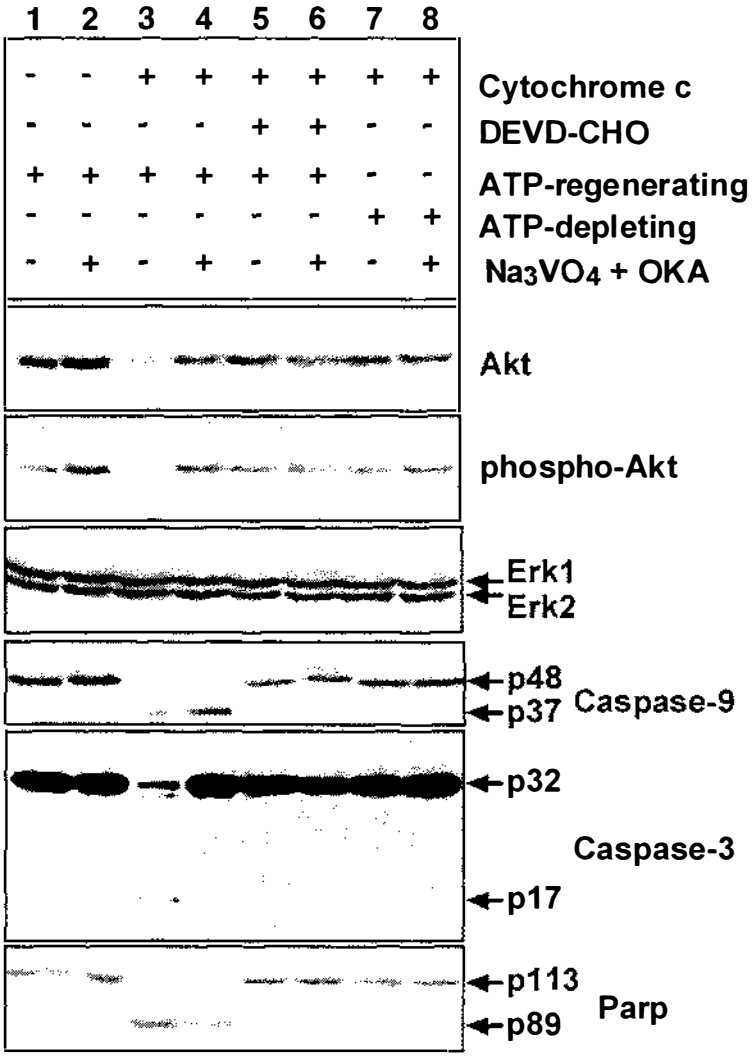

Figure 32: Akt kinase cleavage and caspase-9 and caspase-3 activation are inhibited by phosphatase inhibitors in cytochrome c-activated neural cell extracts

SY5Y cytosol and PC12 nuclei were incubated together as in Figure 31 in the absence (lanes 7 & 8) or presence of an ATP-regenerating system (lanes 1-6), with 2 μM cytochrome c (lanes 3-8), an ATP-depleting system (lanes 7 & 8), or 2 μM DEVD-CHO (lanes 5 & 6). The phosphatase inhibitors orthovanadate (Na_3VO_4) and okadaic acid (OKA) were included in some of the reactions (lanes 2, 4, 6 & 8). After incubation, the samples were analyzed by immunoblotting using an antibody specific for proteins labelled at the right of the panels. Cleavage fragment sizes are indicated for caspase-9, caspase-3, and Parp. The anti-Akt antibody did not detect any cleavage fragments in the apoptotic reactions. The blot for Erk 1 & 2 shows that equal protein was loaded on the gel and that Akt specifically disappeared in the apoptotic reactions. Data are representative of 4 or more independent experiments.

caspase-dependent mechanism for Akt cleavage. In this respect, Akt cleavage resembled that of poly ADP-ribose polymerase (Parp) in nuclei, which also required ATP (Figure 32, bottom panel).

5.35 Caspase-9 and caspase-3 activation and Akt cleavage were inhibited by phosphatase inhibitors and functional depletion of ATP

Since Akt cleavage was dependent on caspase activation, and Akt has been shown to regulate by phosphorylation the activity of caspase-9 (Cardone, et al., 1998), we asked if phosphatase inhibitors affected the apoptotic mechanism in our *in vitro* system. Caspase-9 cleavage was partially inhibited by orthovanadate and okadaic acid (Figure 32, lane 4). In addition, phosphatase inhibitors caused the appearance of a higher molecular weight form of caspase-9, consistent with a phosphorylated form (Figure 32, lanes 2 & 7). However, its downstream target caspase-3 appeared to be entirely unprocessed in the presence of phosphatase inhibitors. Parp cleavage was inhibited, but not blocked entirely by phosphatase inhibitors under these conditions, suggesting that this substrate is sensitive to proteolysis and cleaved early in apoptotic execution. These data suggest inhibiting phosphatases mitigates caspase-9's activation and ability to activate caspase-3, but this is not sufficient to completely block activation of caspase-9 or Parp cleavage.

We also asked whether protein phosphorylation plays a role in the degradation of Akt. The disappearance of phospho-Akt and total Akt protein was completely prevented by the addition of phosphatase inhibitors in the cytochrome c - activated cell-extracts (Figure 32, lane 4). Not surprisingly, the amount of phospho-Akt increased in the presence of phosphatase inhibitors (Figure 32,

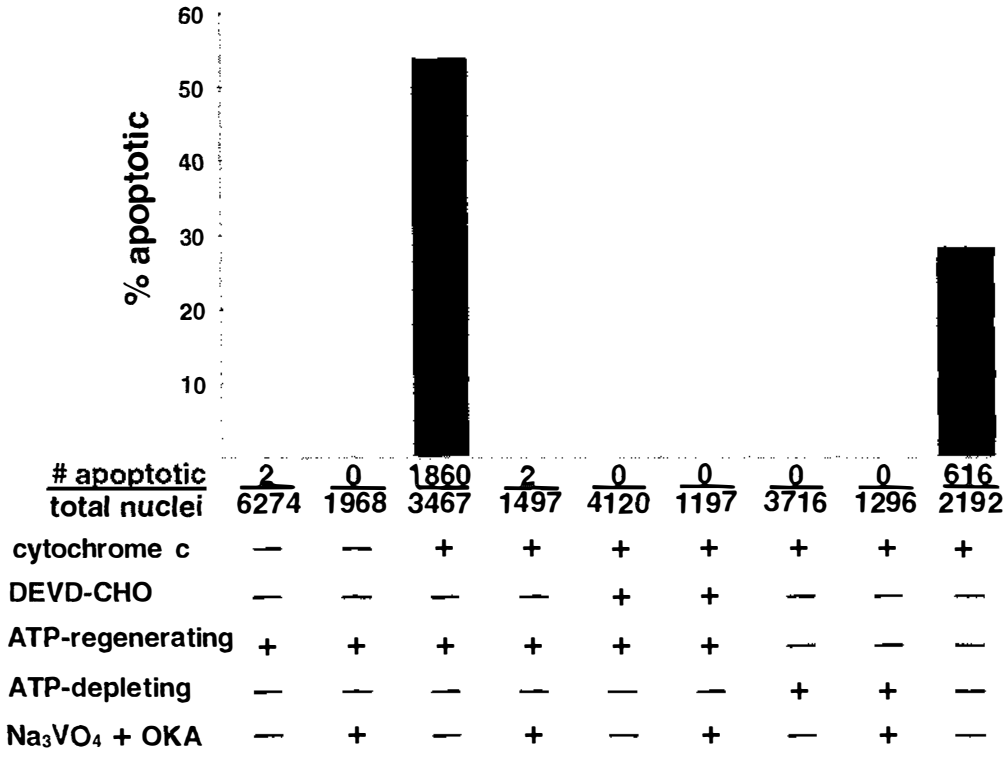

Figure 33: Phosphatase inhibitors prevented nuclear chromatin condensation

Nuclei from the same reactions as in Figure 32 were fixed and stained with Hoechst 33342 and scored for apoptotic morphology. In addition, both the ATP-regenerating and ATP-depleting system were omitted in one reaction (far right). The total number of nuclei counted in 3 independent experiments is shown under each bar.

compare lanes 1 and 2). The inhibitors had no effect in the non-apoptotic reactions lacking cytochrome c, ATP, or containing the caspase inhibitor DEVD-CHO (Figure 32, lanes 2,6, and 8).

Phosphatase inhibitors and ATP-depletion completely inhibited nuclear chromatin condensation in the *in vitro* reactions (Figure 33). This mechanism, like Akt cleavage but unlike cleavage of the nuclear protein Parp, appears to be very sensitive to phosphatase inhibition. In addition, where the ATP-regenerating system was omitted and in the absence of an ATP-depleting system, an intermediate amount of nuclei were morphologically apoptotic (Figure 33). Partial activation of caspase-9, caspase-3, and Parp cleavage (Figure 31 lane 4) was also observed under conditions where endogenous cytoplasmic ATP levels were not manipulated by regeneration or depletion, but only rarely was Akt cleavage observed (data not shown).

5.36 Other proteins downstream of Akt are unaffected in the cell-free system

Potential downstream targets of Akt kinase are presented in Figure 34 (reviewed in Khwaja, 1999). Because Akt and its putative substrate caspase-9 were affected during *in vitro* apoptosis, we examined the status of other putative Akt targets in our system. Akt-mediated phosphorylation targets IκB for degradation resulting in activation of NF-κB (Ozes, et al., 1999; Romashkova & Makarov, 1999). We hypothesised that, since Akt is deactivated and degraded in our system, levels of phosphorylated IκB should decrease in the apoptotic reactions. However, we detected phosphorylated IκBα only in the reactions containing the phosphatase inhibitor okadaic acid (Figure 35, middle panel, lanes 4 & 5) and there were no differences in total IκB levels between the

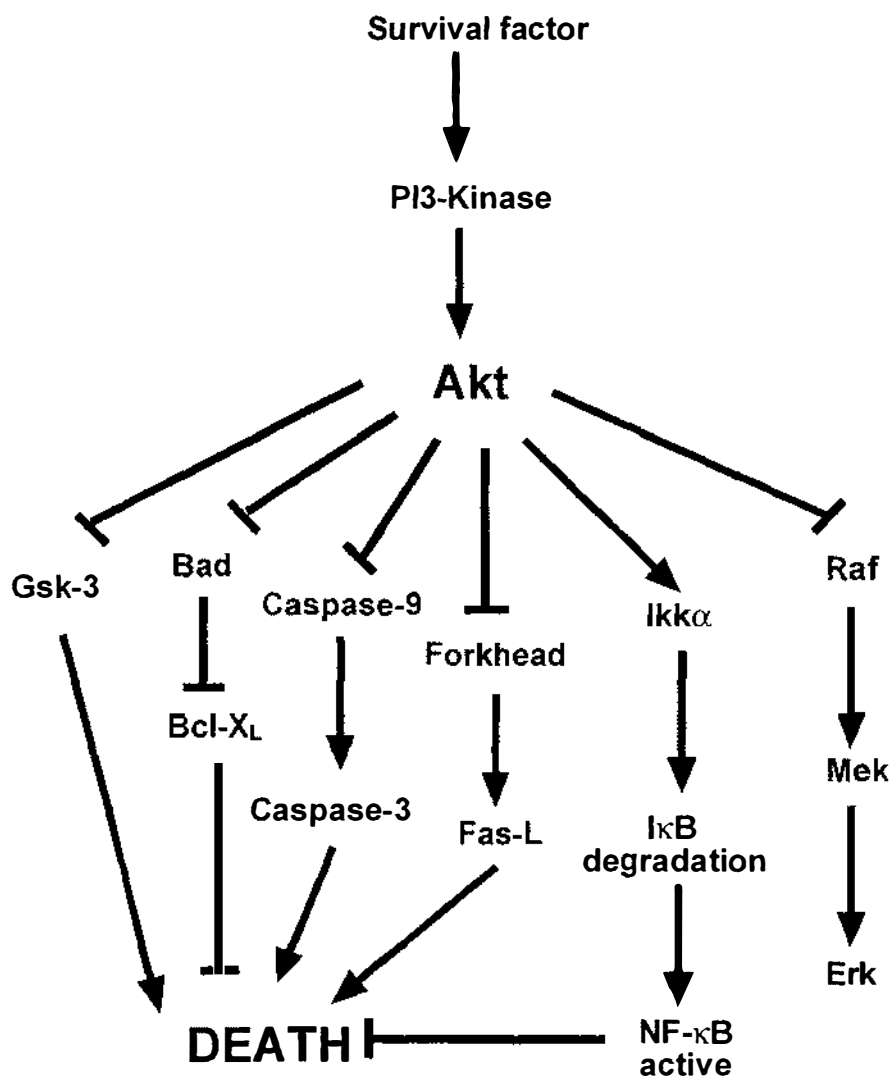

Figure 34: Possible downstream targets of Akt action

control and apoptotic reactions (Figure 35, bottom panel).

Akt is also proposed to phosphorylate Raf-1 (Zimmerman & Moelling, 1999), so we examined Raf-1 levels by immunoblotting in the reactions. We found that Raf-1 levels were unaffected during *in vitro* apoptosis (Figure 35, top panel). However, we have demonstrated down-regulation of Raf-1 protein levels in staurosporine-induced apoptosis in PC12 cells (see Chapter 6).

Next, we examined the status of Bad. Akt can phosphorylate Bad in some cell types allowing it to be sequestered by 14-3-3 proteins, thus preventing cell death. Using polyclonal antibodies against phosphorylated Bad at Serine 136 and total Bad protein, no endogenous Bad protein could be detected in the *in vitro* reactions. The manufacturer of the Bad antibodies (New England Biolabs Inc.) stated that Bad is not expressed, or only at very low endogenous levels, in some cell types. So we tried immunoprecipitating Bad protein from *in vitro* reactions prior to Western blotting, but still no Bad could be detected. To rule out removal of Bad protein during cytosol preparation from SY5Y cells, immunoprecipitations were also carried out on whole cell lystaes from SY5Y cells. Again, no Bad was detected following immunoprecipitation and Western blotting with Bad antibody. These data suggest that Bad is not expressed at detectable levels in SY5Y cells using these reagents.

Akt phosphorylates and inhibits Gsk-3 activity (Cross, et al., 1995). Since Gsk-3β is pro-apoptotic in some systems (Pap & Cooper, 1998), we tested whether the addition of activated Gsk-3β enzyme could induce apoptosis in cell-free extracts. Exogenous Gsk-3β was unable to induce caspase-9 or caspase-3 activation in our system (Figure 36 lane 4). In addition, we tested whether exogenous Erk1, a pro-survival signalling kinase, could block apoptosis *in vitro.* The addition of Erk1 was unable to block cytochrome c-mediated caspase-9 and caspase-3 processing in our system (Figure 36 lanes 5 & 6).

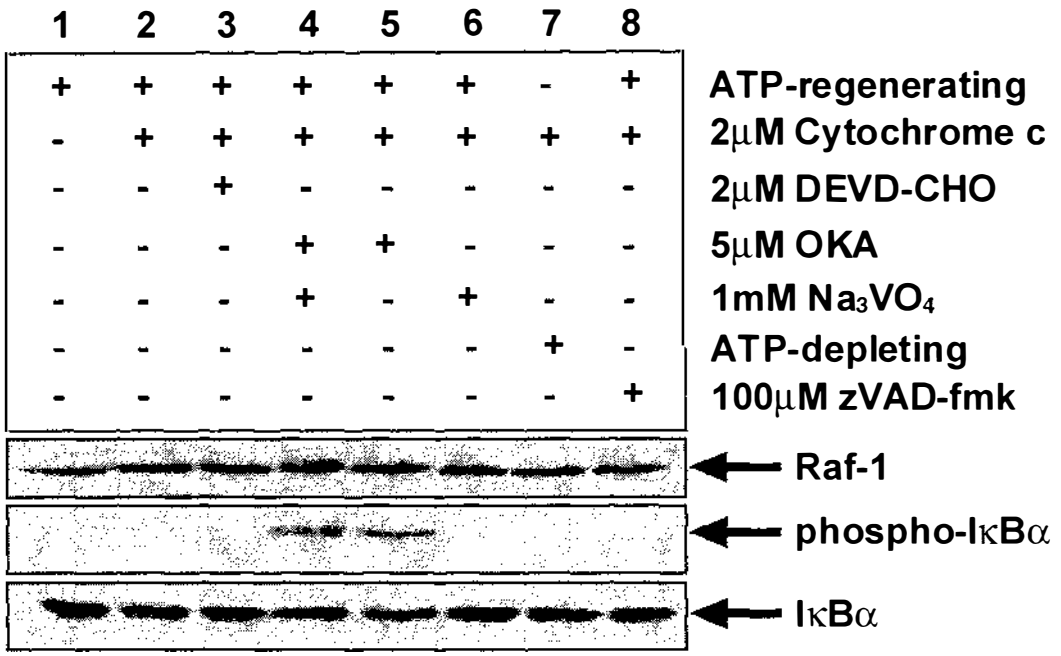

Figure 35: Raf-1 and IκBα are unaffected during *in vitro* apoptosis.
SY5Y cytosol and PC12 nuclei were incubated together as in Figure 31 in the absence (lane 7) or presence of an ATP-regenerating system (lanes 1-6, & 8), with cytochrome c (lanes 2-8), an ATP-depleting system (lane 7), DEVD-CHO (lane 3), orthovanadate (lanes 4 & 6), okadaic acid (lanes 4 & 5), z-VAD-fmk (lane 8). After incubation the samples were analysed by immunoblotting using an antibody specific for proteins labelled at the right of panels. Data are representative of 4 independent experiments.

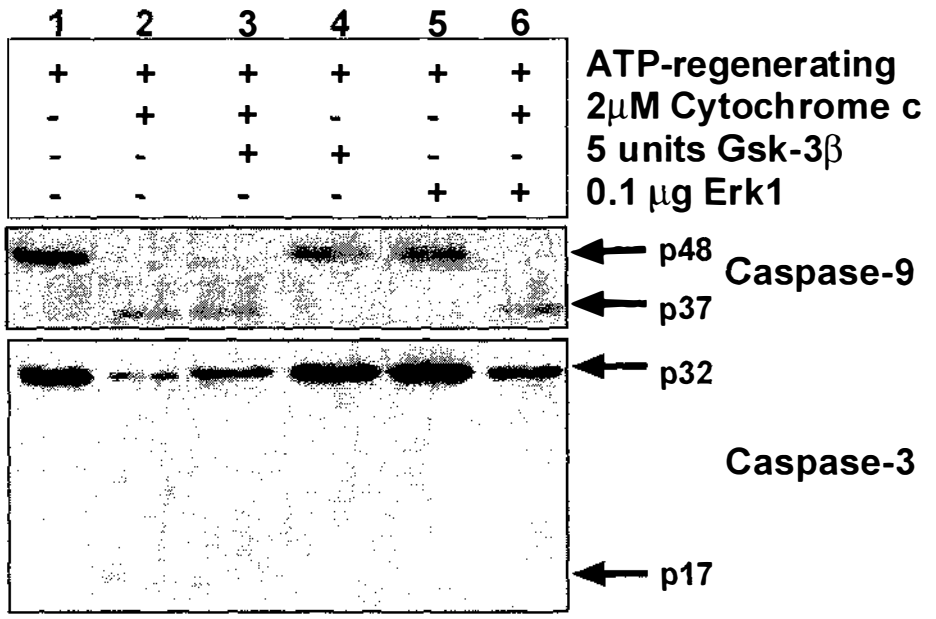

Figure 36: Gsk-3β does not activate apoptosis in *in vitro* reactions

SY5Y cytosol and PC12 nuclei were incubated as in Figure 31 with an ATP-regenerating system (lanes 1-6), cytochrome c (lanes 2,3, & 6), recombinant activated Gsk-3β (lanes 3 & 4), and recombinant activated Erk1 (lanes 5 & 6). After incubation the samples were analysed by immunoblotting using antibodies specific for caspase-3 and caspase-9. Cleavage fragment sizes are indicated for the caspases. The p17 caspase-3 fragment was weakly detected in lanes 2, 3 & 6. Results are representative of at least 2 independent experiments for all conditions.

5.37 Creb is a caspase substrate during apoptosis

The transcription factor Creb has recently been shown to be both necessary and sufficient to promote neuronal survival in NGF-dependent cultured sympathetic neurons (Riccio, et al., 1999). We examined whether Creb was affected during apoptosis in our neural cell-free extracts. Creb is activated by phosphorylation at serine 133 and the phosphorylated form translocates to the nucleus. Consistent with this, no phosphorylated Creb was detected in the SY5Y cytosol not subjected to the *in vitro* incubations with nuclei. Active Creb, phosphorylated at serine 133, was readily detectable in the reactions containing the phosphoserine phosphatase inhibitor, okadaic acid (Figure 37, lanes 4 & 5). Incubation with the phosphotyrosine phosphatase inhibitor, orthovanadate, by itself, did not cause the appearance of phosphorylated Creb (Figure 37, lane 6). Phospho-Creb was also detected in lower amounts in the reactions containing the caspase inhibitors DEVD-CHO and z-VAD-fmk (Figure 37, lanes 3 & 8).

Western blots with an antibody to total Creb protein revealed that Creb was cleaved in the apoptotic reactions yielding a cleavage fragment of approximately 30 kD (Figure 37, lanes 2 & 5). Interestingly, total Creb protein levels were reproducibly decreased in the ATP-depleted reaction even though caspase-3 and caspase-9 weren't activated and no Creb cleavage fragment was observed (Figure 37, lane 7). Specific cleavage, which produced the cleavage fragment of Creb, correlated with caspase-9 and caspase-3 activation. Creb cleavage was specific because there was no change in the amounts of the control protein Erk. Creb cleavage was inhibited in the presence of z-VAD-fmk and DEVD-CHO (Figure 37, lanes 3 & 8). These results suggest that Creb can be cleaved during apoptosis in a process mediated by caspases.

To characterise the nature of the phosphatase activity necessary for caspase activation (section 5.35), either okadaic acid or orthovanadate were used alone in the *in vitro* reactions. 1mM orthovanadate but not 5 μM okadaic acid could inhibit caspase-3 and caspase-9 processing (Figure 37, lanes 5 & 6), which suggests that the phosphatase involved in caspase activation has a phospho-

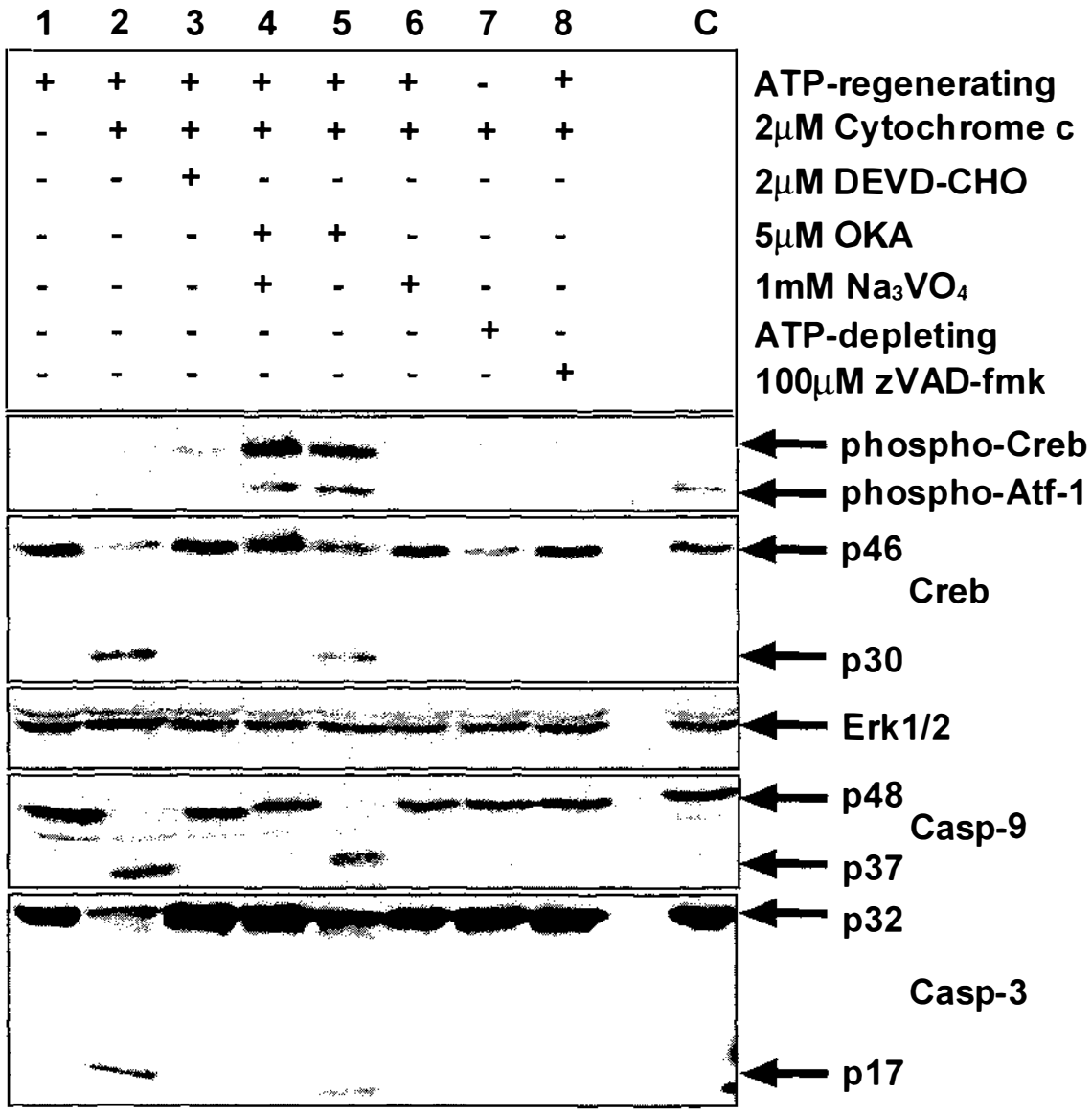

Figure 37: Creb is cleaved during apoptosis
SY5Y cytosol and PC12 nuclei were incubated together as in Figure 31 in the absence (lane 7) or presence of an ATP-regenerating system (lanes 1-6, & 8), with cytochrome c (lanes 2-8), an ATP-depleting system (lane 7), DEVD-CHO (lane 3), orthovanadate (lanes 4 & 6), okadaic acid (lanes 4 & 5), z-VAD-FMK (lane 8). Sample (C) is SY5Y cytosol not subjected to the *in vitro* reaction. After incubation the samples were analysed by immunoblotting using an antibody specific for proteins labelled at the right of panels. The phospho-Creb antibody cross-reacts with the transcription factor Atf-1. Cleavage fragment sizes are indicated for caspase-9, caspase-3, and Creb. The blot for Erk1/2 shows that equal protein was loaded on the gel and that Creb is specifically cleaved in the apoptotic reactions. Data are representative of 4 independent experiments.

tyrosine specificity. Orthovanadate, but not okadaic acid, prevented Creb cleavage (Figure 37, lanes 5 & 6).

5.4 Discussion and Future Work

We have created a cell-free model of neural cell apoptosis that recapitulates the apoptotic events of caspase activation, DNA fragmentation and chromatin condensation. Using this system we were able to demonstrate interactions between endogenous proteins that regulate apoptosis.

5.41 A neural cell-free model of apoptosis

We report here that the addition of cytochrome c to neural SY5Y cell extracts induces apoptosis. While these experiments were being performed, a cell-free system for neuronal apoptosis was established that also used cytochrome c to activate extracts prepared from cerebellar neurons and human teratocarcinoma (NT2) cells that were treated with retinoic acid, which causes neuronal differentiation (Ellerby, et al., 1997). Other studies also support our finding that exogenous dATP isn't required for cell-free apoptosis activated by exogenous cytochrome c (Slee, et al., 1999).

It is interesting that exogenous cytochrome c could not activate apoptosis in PC12 cell extracts. However, exogenous cytochrome c does not always induce apoptosis in extracts derived from other cell types. 2 μM cytochrome c and 1mM dATP did not activate caspase-3 in extracts derived from human promyelocyte leukemic HL60 or rat glioblastoma A15A5 cells (Juin, et al., 1998). In addition, PC12 cytosol could be made competent to induce apoptosis by adding 0.5 mM $CaCl_2$ (Juin, et al., 1998), which suggests that other triggers might be necessary to induce apoptosis in extracts from this particular cell type.

The inefficiency of the staurosporine-primed SY5Y cell extracts in inducing apoptotic changes in nuclei may be attributed to the low number of cells undergoing apoptosis following staurosporine treatment. Due to asynchrony during neural apoptosis, only 14-18% of SY5Y cells were apoptotic 12–24 hours after staurosporine treatment (Figure 36A, Chapter 6) so there would only be a low concentration of active apoptosis effectors in the cytosolic extracts.

5.42 Akt is cleaved by caspases during cell-free apoptosis

Our data showed that Akt protein is degraded during apoptosis in cytochrome c-activated extracts and correlated with caspase activity. DEVD-CHO, ATP-depletion and phosphatase inhibitors, all of which inhibited caspase-3 and -9 activation, prevented Akt cleavage. These results are consistent with results that demonstrated down-regulation of Akt protein levels *in vivo* in Jurkat and U937 cells stimulated to undergo apoptosis by Fas ligation, UV exposure, or treatment with etoposide, and during p53-mediated apoptosis in carcinoma cells (Widmann, et al., 1998; Bachelder, et al., 1999). These researchers have also been unable to detect Akt cleavage fragments *in vivo* by immunoblotting.

Analysis of the sequence of human Akt kinase showed the presence of one potential caspase cleavage site for caspase-3 or -7 and 7 potential sites for caspase-6,-8 or -9 (Figure 38). Recently, Bachelder, et al.(1999) demonstrated that recombinant caspase-3 can cleave baculovirus-expressed Akt *in vitro* to yield a fragment of about 49 kD by silver staining. Cleavage of Akt at the proposed caspase-3 cleavage site would yield 2 fragments of theoretical molecular weights 53 kD and 2.7 kD. The 2.7 kD fragment would contain the epitope to the Akt antibody, which could explain why an Akt cleavage fragment was not detected in our experiments. In addition, if additional cleavage events occurred at any of the other potential sites, Akt would be further degraded, complicating detection. The exact mechanism for Akt cleavage is yet to be determined.

We tested the effect of apoptosis in our system on a number of potential Akt substrates: caspase-9, Raf-1, IκB, Bad and Gsk-3. Caspase-9 was the only potential Akt target tested that was affected during *in vitro* apoptosis. Caspase-9 was activated during apoptosis in a manner dependent on phosphatase activity and ATP. Activation states and protein levels of other putative Akt targets, Raf-1 and IκB, were unaffected during *in vitro* apoptosis, and Bad protein could not be detected in our cell-free extracts. Further experiments are required to rule out whether Bad is expressed at low levels in SY5Y cells. Recombinant Gsk-3β was

ORIGIN

1 msdvaivkeg wlhkrgeyik twrpryfllk
ndgtfigyke rpqdvdqrea plnnfsvaqc
61 qlmkterprp ntfiirclqw ttviertfhv
etpeereewt taiqtvadgl kkqeeeemdf
121 rsgspsdnsg aeemevslak pkhrvtmnef
eylkllgkgt fgkvilvkek atgryyamki
181 lkkevivakd evahtltenr vlqnsrhpfl
talkysfqth drlcfvmeya nggelffhls
241 rervfsedra rfygaeivsa ldylhseknv
vyrdlklenl mldkdghiki tdfglckegi
301 kdgatmktfc gtpeylapev lendygrav
dwwglgvvmy emmcgrlpfy nqdheklfel
361 ilmeeirfpr tlgpeaksll sgllkkdpkq
rlgggsedak eimqhrffag ivwqhvyekk
421 lsppfkpqvt setdtryfde eftaqmitit
ppdqddsmec vdserrphfp qfsysasst a
481

Figure 38: Amino acid sequence of Human Akt1 Kinase
There is one potential caspase-3/-7 cleavage site (red) and 7 potential caspase-6,-8 or-9 cleavage sites (blue) within the Akt sequence. 2 caspase sites overlap between amino acids 280 and 285 (blue). The epitope to which the Akt antibody was raised is indicated (green).

This sequence was obtained from the SwissProt database (accession number P31749).

also unable to induce apoptosis in our system; it may require cofactors and/or compartmentalisation not available in this cell-free system for its apoptosis-inducing activity. As discussed in the literature review, these results are consistent with the view that Akt's physiological targets may differ according to cell type and context.

The data by Cardone, et al. (1998) together with our results, suggest a model in which caspases and Akt have a mutually antagonistic relationship (Figure 39). In this model, when survival signals dominate, Akt phosphorylates caspase-9, inhibiting its activity. If the cell is stimulated to undergo apoptosis, caspase-9 becomes dephosphorylated and activated, activates downstream caspases, which cleave and inactivate Akt.

Future work would involve mapping the exact caspase cleavage sites within Akt by mutagenesis studies combined with the use of different recombinant caspases to digest the translated protein *in vitro*. This information will allow the generation of caspase-resistant Akt constructs, which can be used to assess the *in vivo* physiological significance of caspase-mediated degradation of Akt in turning off cell survival and switching on death.

5.43 Role of phosphatases in triggering apoptosis

We found that phosphatase inhibitors could inhibit caspase-3 activation and chromatin condensation during apoptosis, while caspase-9 and Parp processing was only partially inhibited. That phosphatase inhibition incompletely inhibits caspase activation could be explained if phosphorylated caspase-9 can still be proteolytically processed, but has an altered substrate specificity. The phosphorylated form may be unable to associate with and activate caspase-3 but could still cleave Parp directly, or activate other caspases that cleave Parp. Coimmunoprecipitation experiments could be performed to test whether phosphorylated caspase-9 loses its ability to associate with caspase-3. In addition to caspase-3, cytochrome c can initiate the processing of caspase-2,

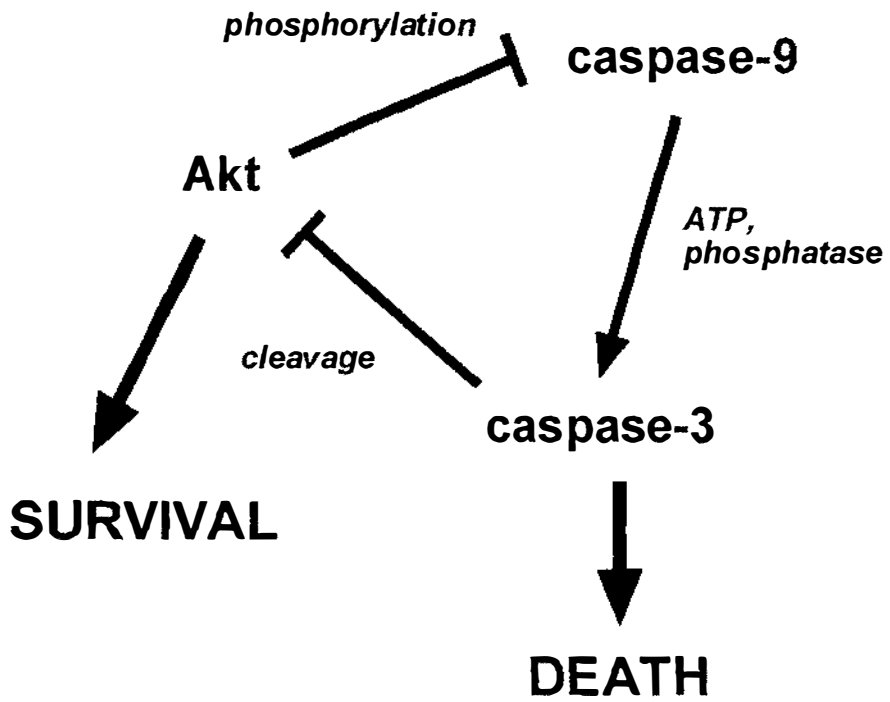

Figure 39: Mutually antagonistic model of caspase-9 and Akt action
When survival signals predominate, Akt phosphorylates caspase-9 inhibiting its activity. In response to an apoptotic signal, phosphatases are activated and caspase-9 is dephosphorylated. Caspase-9 can then activate other caspases which in turn cleave and inactivate Akt.

-6, -7, -8, and -10 in cell-free extracts, all through caspase-9 (Slee, et al.,1999). Any of these caspases can cleave Parp *in vitro*, but because activated caspase-9, but not the other caspases, can translocate to the nucleus, it is likely that caspase-9 may cleave Parp directly (Krajewski, et al., 1999). Caspase-3 activity is required for chromatin condensation (Sahara, et al., 1999), which may explain why phosphatase inhibition resulted in the complete inhibition of chromatin condensation.

Cardone et al. (1998), proposed that caspase-9 is phosphorylated by Akt at serine196 which suggests that okadaic acid, a serine phosphatase inhibitor, would prevent its dephosphosphorylation and activation. Instead, we found that orthovanadate, which has specificity for phospho-tyrosine, and not okadaic acid, was the most effective phosphatase inhibitor in preventing caspase activation (Figure 37). In addition, the serine196 phosphorylation site on caspase-9 is not conserved in other species, such as mice or dogs. Our findings suggest that a tyrosine phosphatase may lie upstream of, and inhibit, caspase activation during apoptosis. This is supported by studies in *Xenopus* cell-free extracts where 10-20 mM phosphotyrosine or 1-10 mM pervanadate, but not okadaic acid, could inhibit apoptosis (Newmeyer, et al., 1994; Farschon, et al., 1997). In addition, there is evidence for interactions between phosphotyrosine and SH2-containing proteins in regulating the onset of apoptosis upstream of caspase activation (Farschon, et al., 1997).

Further evidence of a role for phosphatase activity in triggering apoptosis in neuronal cells comes from studies overexpressing the phosphatase calcineurin. High level forced expression of calcineurin induced cytochrome c/caspase-3 dependent apoptosis in neurons (Asai, et al., 1999). However, calcineurin has a Ser/Thr phosphate specificity, so its relevance to our studies is unclear.
A tyrosine phosphatase, or another, may play a role in the regulation of caspase-9 activity by Akt and Akt activity by caspase-dependent proteolysis.

5.44 Role of ATP during apoptosis

We demonstrate for the first time that ATP is absolutely required for the biochemical and morphological events occurring during apoptosis. Functional depletion of ATP blocked caspase-3, and -9 activation, Parp and Akt cleavage, and chromatin condensation. A requirement for ATP for chromatin condensation and DNA fragmentation has been reported previously using a cell-free system of cytosol prepared from Fas-treated Jurkat cells (Kass, et al., 1996). However, the researchers did not functionally deplete ATP from their reactions, instead, they relied on whether an ATP-regenerating system was present or absent in interpreting their results. Because, they did not functionally deplete ATP from their reactions, their observation that DNA fragmentation occurs independent of ATP could be incorrect due to low endogenous levels of ATP present in the cell-free extracts.

What is the role of ATP during cell-free apoptosis? One possibility is that ATP may substitute for dATP as a cofactor in Apaf-1/caspase-9 activation (Li, et al., 1997), which is an event that lies upstream of caspase-3, Parp, and Akt cleavage, and chromatin condensation. It is also possible that ATP hydrolysis is required for one or more steps in the execution mechanism.

5.45 Creb is a caspase substrate during cell-free apoptosis

The survival factor Creb was cleaved in apoptotic reactions to yield a 30 kD fragment detected by immunoblotting (Figure 37). Cleavage of Creb correlated with caspase-3 and caspase-9 activation and was inhibited by caspase- and phosphatase- inhibitors. Examination of the Creb amino acid sequence (Figure 40) reveals the presence of two potential caspase-6,-8 and -9 cleavage sites. Cleavage at either of these two sites would yield a fragment containing the antibody epitope with a theoretical molecular weight of about 15 kD which is half the size of the actual fragment observed by SDS-PAGE (Figure 37). However, the theoretical molecular weight calculated for the intact Creb protein is 36.7 kD,

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ORIGIN
1    mtmesgaenq qsgdaavtea enqqmtvqaq
     pqiatlaqvs mpaahatssa ptvtlvqlpn
61   gqtvqvhgvi qaaqpsviqs pqvqtvqssc
     kdlkrlfsgt qistiaesed sqesvdsvtd
121  sqkrreilsr rpsyrkilnd lssdapgvpr
     ieeekseeet sapaittvtv ptpiyqtssg
181  qyiaitqgga iqlanngtdg vqglqtltmt
     naaatqpgtt ilqyaqttdg qqilvpsnqv
241  vvqaasgdvq tyqirtapts tiapgvvmas
     spalptqpae eaarkrevrl mknreaarec
301  rrkkkeyvkc lenrvavlen qnktlieelk
     alkdlychks d
```

Figure 40: Amino acid sequence of Human Creb
There are 2 potential caspase-6,-8 or-9 cleavage sites (blue) between amino acids 137 and 144 within the Creb sequence. The epitope to which the Creb antibody was raised is indicated (green). The epitope and caspase cleavage site overlap at amino acid 137 (red).

This sequence was obtained from the SwissProt database (accession number P16220).

which is much smaller than the observed migration of the protein at 46 kD by SDS-PAGE. Structural changes and post-translational modifications may help explain the differences between observed and theoretical molecular weights for the Creb protein and cleavage fragment. Further work combining mutagenesis of potential caspase cleavage sites within Creb with caspase digestion of the *in vitro* translated protein would help clarify the mechanism of Creb cleavage.

It is interesting to ask why Creb is a caspase substrate. Why inactivate it by proteolysis, rather than by dephosphorylation? It has been reported that non-phosphorylated Creb has anti-apoptotic activity and can block c-Jun function (Masquilier & Sassone-Corsi, 1992). Non-phosphorylated Creb can suppress AP-1 transcriptional activity by competing with c-Jun protein for the AP-1 binding site on target genes. Thus non-phosphorylated Creb may have a function of its own.

The decrease in total Creb protein observed in the reaction functionally depleted of ATP was unexpected because no caspases were activated in this reaction (Figure 37). ATP-depletion is associated with necrotic death, while apoptosis is energy dependent (Ha & Snyder, 1999). Other proteases, such as calpains, are activated during necrosis and may be responsible for degrading Creb in this reaction. This would result in a different fragmentation pattern than that mediated by the caspases. Further work is required to test this hypothesis, for example by adding different calpain and other protease inhibitors to the *in vitro* reactions.

These experiments constitute the first demonstration that two signalling molecules that are implicated in neuronal survival, Akt and Creb, are cleaved by caspases during apoptosis in neural cells. Caspase-mediated cleavage of proteins required for survival makes their deactivation irreversible and eliminates the cell's ability to activate survival signals. That this occurs at a late stage enforces no possibility of escape from execution.

Chapter 6

Staurosporine- and camptothecin-induced apoptosis in SY5Y cells

6.1 Introduction

6.11 Apoptosis in SY5Y cells

Cell-free reconstitution studies of apoptosis (see Chapter 5) employed cytosol from SY5Y cells, so we investigated apoptosis in this cell type in order to test the biological relevance of events we observe during *in vitro* reconstitution of apoptosis. Two drugs that induce cell death in SY5Y cells were employed for these studies: the kinase inhibitor staurosporine and the topoisomerase inhibitor camptothecin. We asked whether Akt or other signalling molecules were downregulated during apoptosis.

Staurosporine (STS) induces apoptosis in a wide variety of cell types including cortical neurons and SY5Y cells (Koh, et al., 1995; Postmantur, et al., 1997). Staurosporine is a broad spectrum protein kinase inhibitor and its targets include Protein Kinase C, Ca^{2+}/calmodulin-dependent kinase II (Camk II) and tyrosine kinases.

DNA topoisomerase inhibitors are also commonly used to induce apoptosis in cell culture (Solary, et al., 1993; Shimizu & Pommier, 1996; Morris & Geller, 1996). The top1 inhibitor, camptothecin (CPT), an alkaloid from the Asian tree *Camptotheca acuminata,* damages DNA by trapping top1-cleavable complexes, and inducing DNA strand breaks and enzyme-DNA adducts during active DNA replication (Pommier, et al., 1994). DNA damage then triggers apopotosis. Although camptothecin is an S-phase-specific agent, it can also induce apoptosis in postmitotic rat cortical neurons independently of DNA synthesis (Morris & Geller, 1996).

6.2 Experimental Procedures

6.21 Staurosporine- and camptothecin-induced apoptosis in SY5Y cells

6-8 plates of confluent SY5Y cells (passage 3-25 from original UCSF stocks) were washed and harvested in serum-free RPMI 1640 media. Cells were recultured in serum-free RPMI 1640 media containing 0.5μM staurosporine (Sigma, St. Louis, MO) or 10 μM camptothecin (Sigma, St. Louis, MO) in the absence or presence of 20 or 100 μM z-VAD-fmk (Enzyme Systems Products, Livermore, CA). A sample was taken prior to reculturing in the durgs as a control. Cells were incubated at 37°C for various times before preparation of protein lysates.

6.3 Results

6.31 Staurosporine-induced apoptosis in SY5Y cells

SY5Y cells treated with 0.5 μM staurosporine (STS) underwent apoptosis as measured by the appearance of condensed chromatin (Figure 41A) and internucleosomal DNA fragmentation (Figure 41B). SY5Y cells showed DNA laddering and apoptotic morphology from 6 hours and increasing up to 24 hours after treatment with staurosporine.

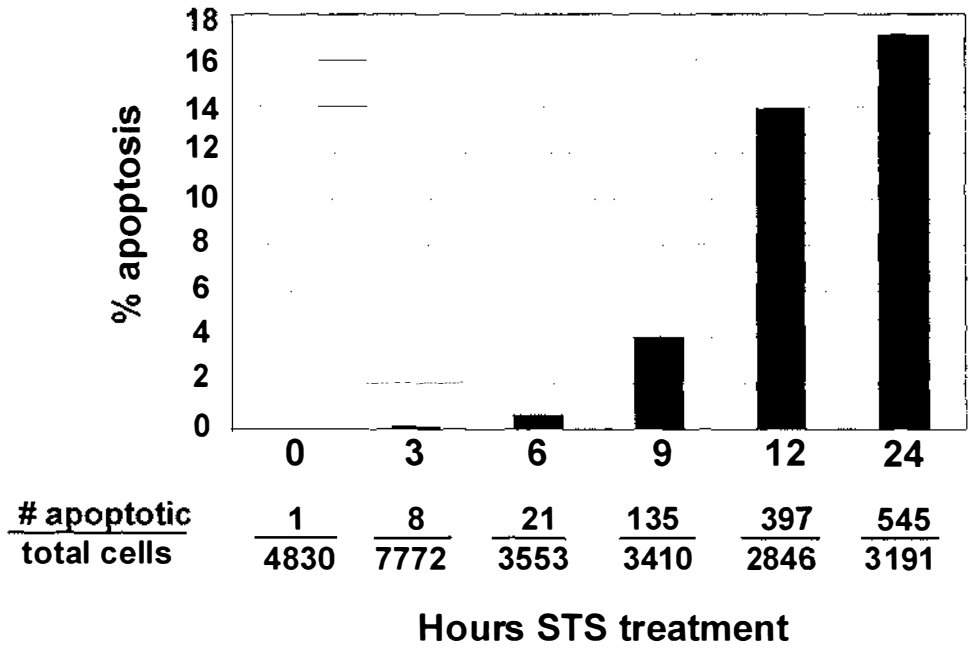
Caspase activation was then examined within the STS-treated cells by immunoblotting. Caspases were active from 24 hours after STS treament as shown by cleavage of Parp (Figure 42, top panel). However, caspase-9 and caspase-3 only showed weak activation 48 hours after STS treatment (Figure 42, bottom and middle panels).

6.32 Raf-1 but not Akt is downregulated during STS-induced apoptosis in SY5Y cells

We have shown that Akt is downregulated in uncommitted dying PC12 cells (population 2, see Chapter 4), and is degraded during *in vitro* apoptosis in SY5Y cell extracts (see Chapter 5, François & Grimes, 1999). To test whether Akt was downregulated during STS-induced apoptosis, lysates from STS treated cells were immunoblotted with antibodies to Akt. Akt protein did not decrease relative to the control protein Erk, even after 48 hours of staurosporine treatment (Figure 43A).

Raf-1 has been shown to be downregulated during etoposide, UV- and Fas-induced apoptosis (Widmann, et al., 1998). We observed that Raf-1 protein levels were decreased in cytosol harvested from staurosporine-treated SY5Y cells (data not shown), so we asked if Raf-1 was downregulated in whole cell lysates following treatment with STS. Raf-1 protein levels decreased 6 hours

A



B

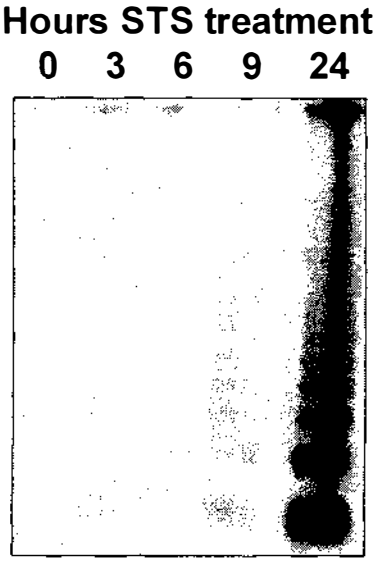


Figure 41:Staurosporine-induced apoptosis in SY5Y cells
SY5Y cells were treated with 0.5 μM staurosporine (STS) for times indicated.
A. Cells were stained with Hoechst 33342 and scored for apoptotic morphology as in Figure 19. The total number of cells counted in 2 or more independent experiments is shown under each bar.
B. DNA was extracted from the STS-treated cells, radioactively end-labelled, and subjected to 2% agarose gel electrophoresis and autoradiography.

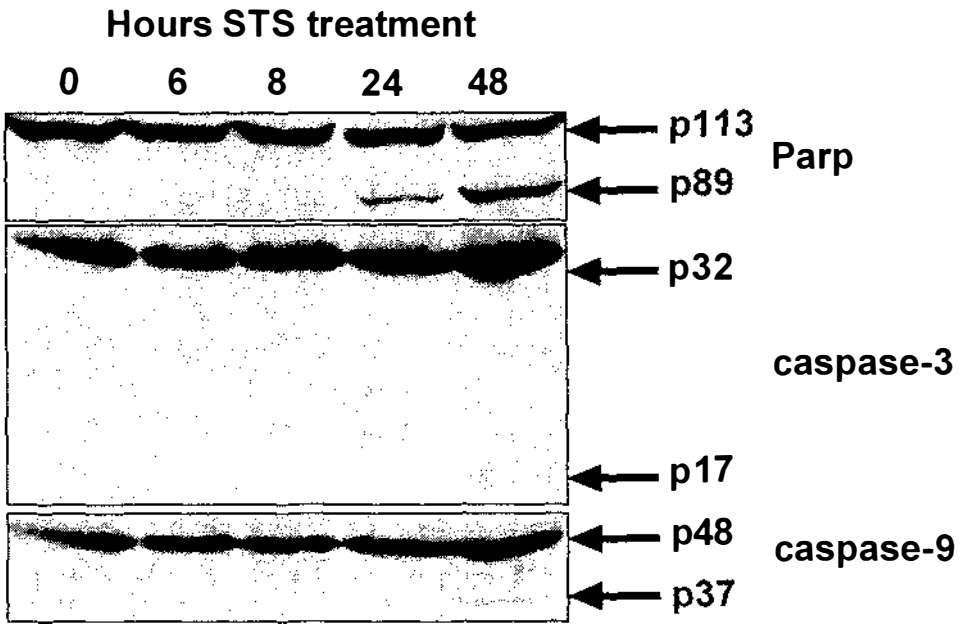


Figure 42: Caspase activation in staurosporine-treated SY5Y cells

SY5Y cells were treated with 0.5 μM STS for times indicated. Whole cell lysates were then analysed by immunoblotting using antibodies specific for the proteins labelled at the right of the panels. Cleavage fragments for Parp, caspase-3 and caspase-9 are indicated. Weak signals for p17 (caspase-3) and p37 (caspase-9) were seen only after 48 hours of STS treatment.
Data are representative of 3 independent experiments.

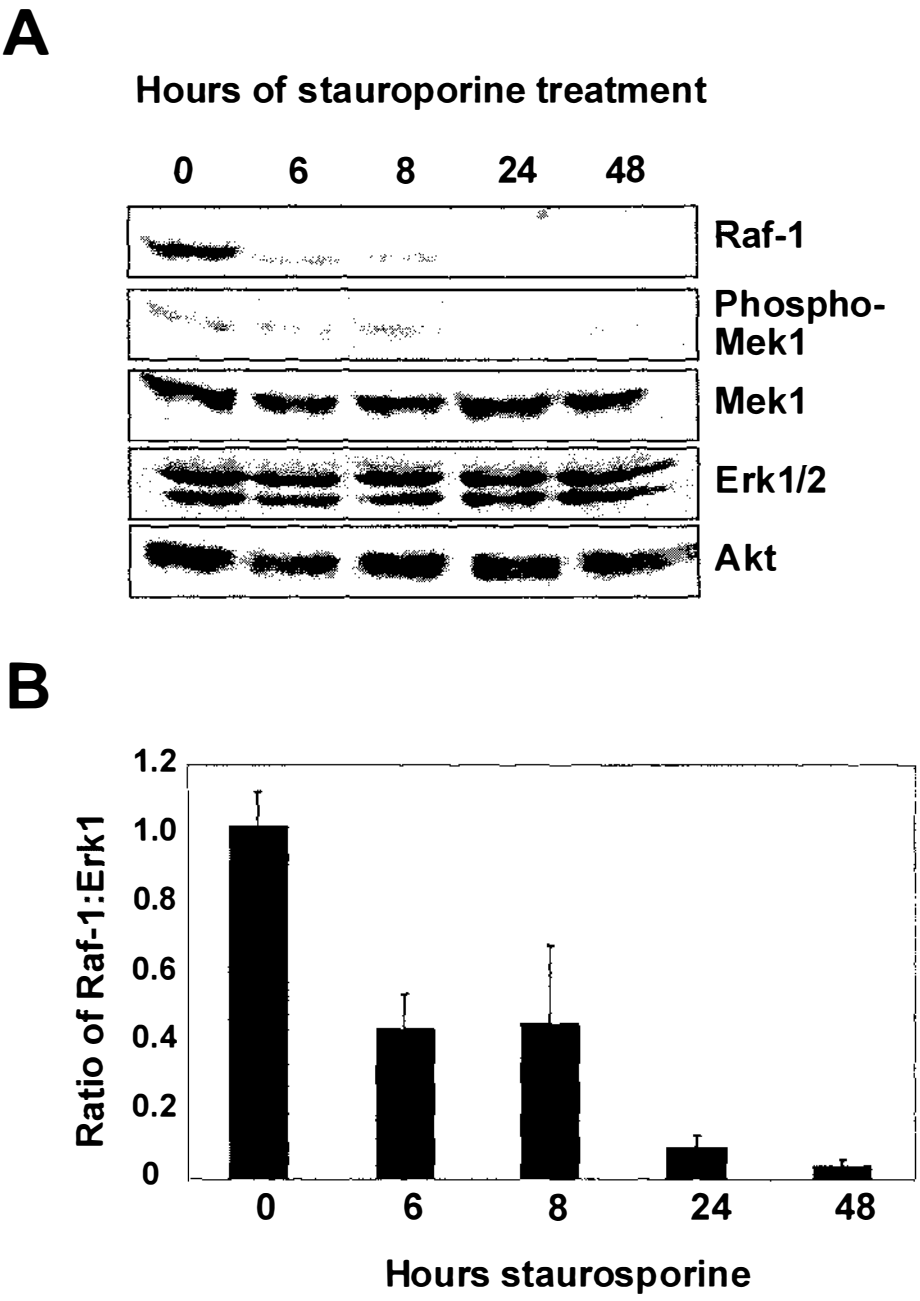


Figure 43: Raf-1 and phospho-Mek1 were downregulated during STS-induced apoptosis

A. SY5Y cells were treated with 0.5 μM STS for times indicated and lysates were analysed by immunoblotting using antibodies specific for proteins labelled to the right of panels. Results are representative of 3 independent experiments.

B. Densitometry was performed on the immunoblots and levels of Raf-1 protein are expressed as a ratio relative to the Erk1 control for protein loading. Data are averages of 2 experiments and standard errors are indicated.

after STS treatment and had almost disappeared after 24 hours of STS treatment (Figure 43A & B). Raf-1 cleavage fragments could not be detected with the antibody used. Analysis of immunoblots by densitometry showed that levels of Raf-1 protein decreased 10-fold relative to the Erk1 control between 0 and 24 hours of staurosporine treatment (Figure 43B).

Mek1 lies immediately downstream of, and is phosphorylated by Raf-1, so whole cell lysates were also examined for changes in the level of Mek1 and phoshorylated Mek1 following treatment with STS. Phospho-Mek 1 decreased with similar kinetics to Raf-1, while total Mek1 levels remained constant (Figure 43A). The downregulation of phospho-Mek1 could be explained by the action of the kinase inhibitor, staurosporine. That Parp cleavage and caspase activation was observed (Figure 42) suggests that the downregulation of Raf-1 may possibly be associated with caspase activity.

6.33 z-VAD-fmk did not prevent Raf-1 disappearance in STS-induced apoptosis

To test whether Raf-1 down-regulation was caused by caspase activity, SY5Y cells were treated with staurosporine in the absence or presence of benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk). z-VAD-fmk is a cell-permeable broad spectrum caspase inhibitor (Slee, et al., 1996), which can inhibit staurosporine-induced cell death in a wide variety of cell types (Jacobson, et al., 1996). Initial experiments using 20 μM z-VAD-fmk, did not inhibit caspase-3, caspase-9, and Parp processing in STS-treated SY5Y cells (data not shown), so the concentration of z-VAD-fmk was increased to 100 μM. This concentration suppressed caspase activation in our *in vitro* reconstitution studies (see Chapter 5) and suppresses STS-induced death in a wide variety of mammalian cell types (Jacobson, et al., 1996).

However, 100 μM z-VAD-fmk did not inhibit Raf-1 down-regulation in STS-treated SY5Y cells (Figure 44A). z-VAD-fmk at this concentration only partially inhibited caspase-9, caspase-3, and Parp cleavage in intact whole cells; Parp

Raf degradation is probably mediated by other caspases since degradation precedes activation of caspase-3 and caspase-9.

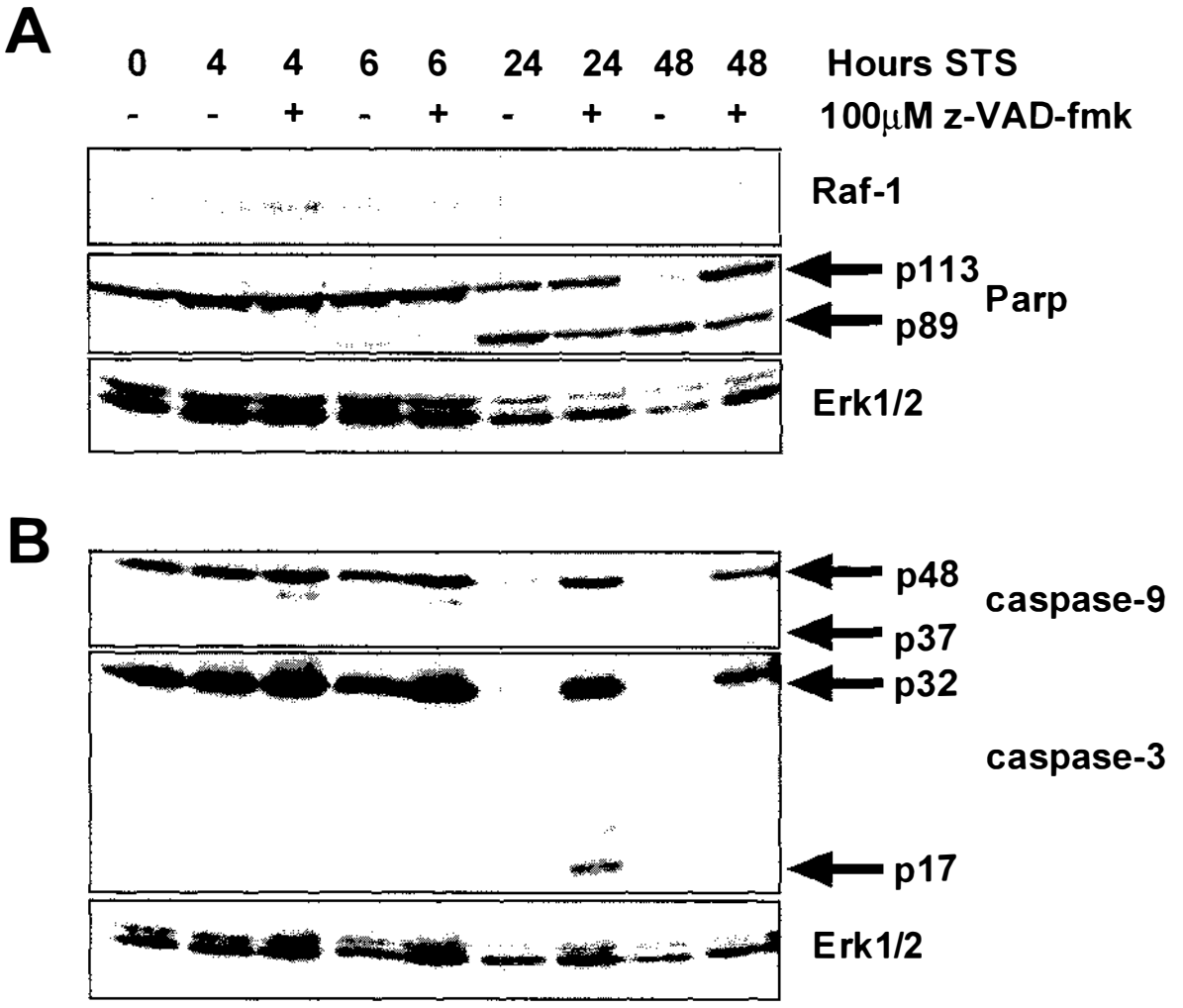


Figure 44: z-VAD-fmk doesn't inhibit Raf-1 downregulation or caspase activation during STS-induced apoptosis

SY5Y cells were treated with 0.5 μM STS in the absence or presence of 100 μM z-VAD-fmk for the times indicated. Whole cell lysates were then analysed by immunoblotting using antibodies specific for the proteins labelled at the right of the panels. Cleavage fragments for Parp, caspase-3, and caspase-9 are indicated. There is unequal protein loading in some of the lanes so Erk1/2 is shown as a control for protein loading. Note the decrease in p32 caspase-3, and therefore a decrease in the p17 form, at 48 hours STS treatment, due to unequal protein loading.
These data are from a single experiment.

cleavage was probably partially inhibited at all time points (Figure 44). Because caspase activation was not completely blocked in the presence of 100 μM z-VAD-fmk, this experiment was inconclusive. Further work will be necessary to ascertain whether Raf-1 down-regulation during STS-induced apoptosis is a caspase-dependent event.

6.34 Akt and Raf-1 are downregulated during camptothecin-induced apoptosis in SY5Y cells

Camptothecin (CPT) treatment was used as a second method to induce apoptosis in SY5Y cells. Parp was cleaved 6 hours after CPT treatment, which suggests that caspases are active at this time, but caspase-3 and caspase-9 cleavage fragments were detected only 24 hours after CPT treatment (Figure 45).

Samples from CPT-treated cells were immunoblotted with antibodies to Akt, Raf-1 and Erk. Both Akt and Raf-1 levels decreased 24 hours after CPT treatment (Figures 45 & 46). This decrease in total Akt and Raf-1 protein was specific when compared to the Erk control (Figures 45 & 46). Levels of Akt protein decreased over 3-fold, while levels of Raf-1 decreased 2-fold, relative to the Erk1 control 24 hours following camptothecin treatment (Figure 46A & B).

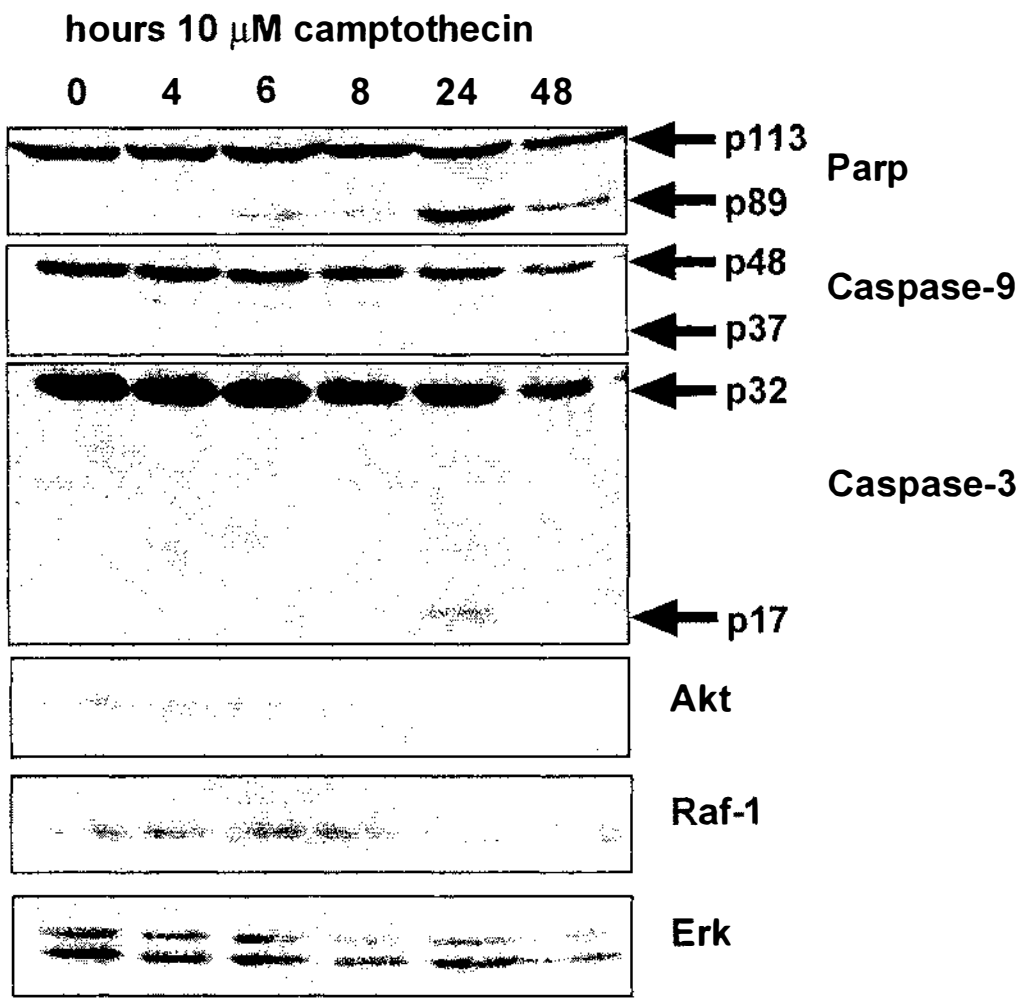


Figure 45: Akt and Raf-1 are downregulated during CPT-induced apoptosis

SY5Y cells were treated with 10 μM camptothecin for the times indicated and analysed by immunoblotting using antibodies specific for the proteins indicated to the right of panels. Note that the decrease in p32 caspase-3, and therefore a decrease in the p17 form at 48 hours CPT treatment, was due to unequal protein loading as shown by the blot for Erk. .
Data are representative of 4 independent experiments.

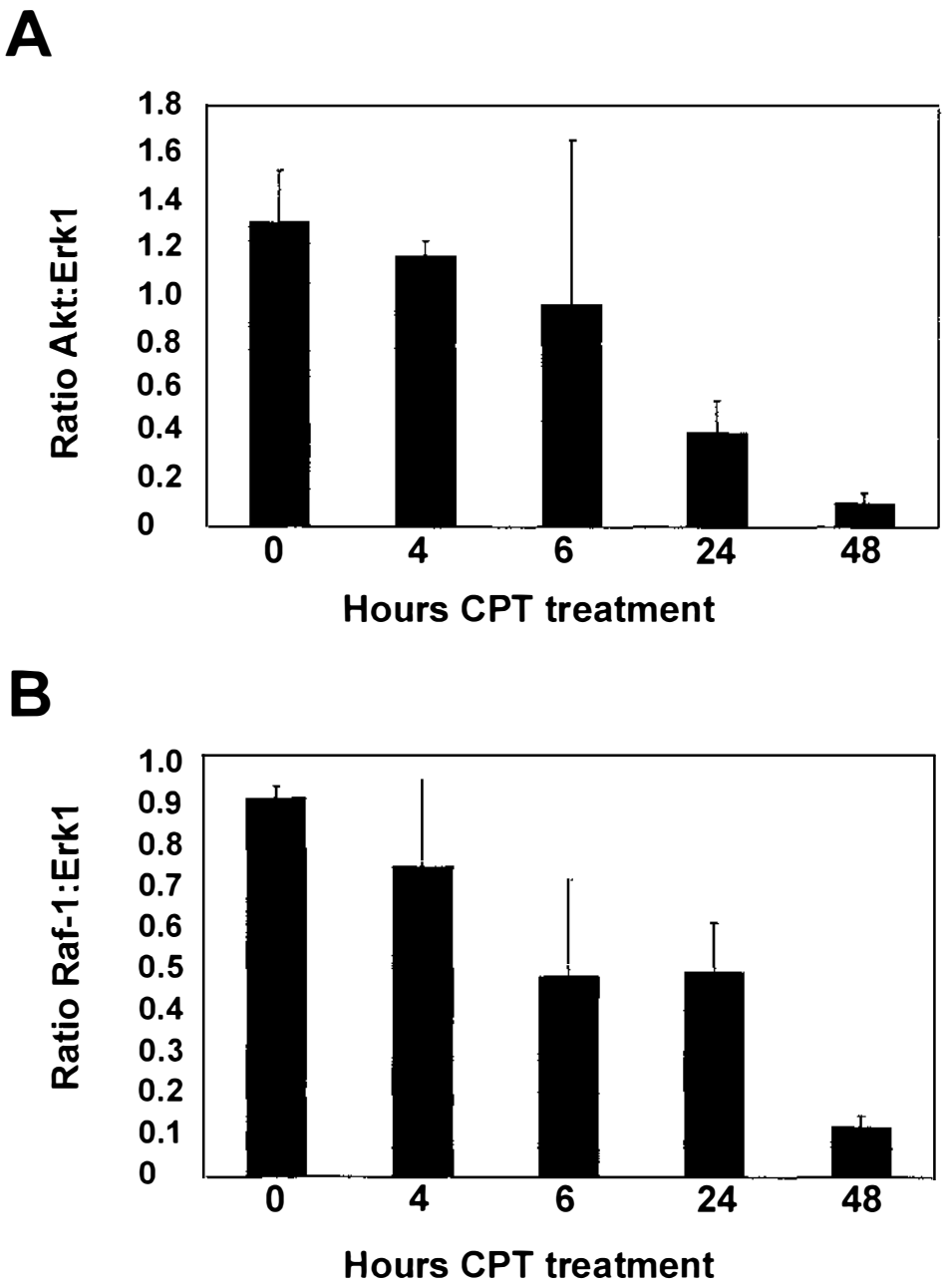


Figure 46: Analysis of Akt and Raf-1 protein levels by densitometry during CPT-induced apoptosis
Densitometry was performed on the immunoblots (shown in Figure 45) and levels of Akt (A) and Raf-1 (B) protein are expressed as ratios relative to the Erk1 control for protein loading. Data are averages of 2 experiments and ranges are indicated by bars.

6.4 Discussion and Future Work

6.41 Raf-1 is downregulated during STS- and CPT-induced apoptosis

Raf-1 protein levels specifically decreased in SY5Y cells, 24 hours following camptothecin or staurosporine treatment (Figures 43B & 46B). The downregulation of Raf-1 could be at the level of transcription initiation or could occur post-translationally, for example, by caspase-mediated degradation. Examination of the amino acid sequence for human Raf-1 revealed the presence of one potential consensus cleavage site for caspase-2,-3 or -7 and four potential consensus sites for caspase-6,-8 or -9 (Figure 47). Calculation of theoretical molecular weights for potential cleavage fragments yields a range of possible fragment sizes which could react with the Raf-1 antibody: 6.8kD (LVAD site), 20.3kD (IHRD site), 42.4kD (LPVD site), 58.6kD (LQVD site) and 58kD (DFLD site). However, Raf-1 cleavage fragments could not be detected by immunoblotting (Figures 43A & 45). It is unclear whether caspase-mediated degradation, or an alternative mechanism, is involved in the down-regulation of Raf-1. Examination of the literature revealed that although 100 μM z-VAD-fmk can block the nuclear morphological events of apoptosis, processing of Parp and caspase-3 are not completely inhibited by z-VAD-fmk in other cells (Jacobson, et al., 1996; McCarthy, et al., 1997). The caspase-inhibitor z-VAD-fmk did not block down-regulation of Raf-1 and only partially inhibited caspase activation in STS-treated SY5Y cells. It will be of interest to determine whether z-VAD-fmk has any effect when CPT is used to induce apoptosis.

z-VAD-fmk more effectively inhibited caspase activation during *in vitro* reconstitution of apoptosis. 100 μM z-VAD-fmk completely blocked caspase-3, -9 and Parp cleavage *in vitro* (see Chapter 5) but only partially inhibited these events in STS-treated SY5Y cells. Intact SY5Y cells may handle the drug differently, for example, they may exclude it, pump it out, or inactivate it in some way. These experiments illustrate the difficulty in trying to extrapolate results

ORIGIN
1 mehiqgawkt isngfgfkda vfdgsscisp
tivqqfgyqr rasddgkltd psktsntirv
61 flpnkqrtvv nvrngmslhd clmkalkvrg
lqpeccavfr llhehkgkka rldwntdaas
121 ligeelqvdf ldhvpltthn farktflkla
fcdicqkfll ngfrcqtcgy kfhehcstkv
181 ptmcvdwsni rqlllfpnst igdsgvpalp
sltmrrmres vsrmpvssqh rystphaftf
241 ntsspssegs lsqrqrstst pnvhmvsttl
pvdsrmieda irshsesasp salssspnnl
301 sptgwsqpkt pvpaqrerap vsgtqeknki
rprgqrdssy yweieasevm lstrigsgsf
361 gtvykgkwhg dvavkilkvv dptpeqfqaf
rnevavlrkt rhvnillfmg ymtkdnlaiv
421 tqwcegssly khlhvqetkf qmfqlidiar
qtaqgmdylh akniihrdmk snniflhegl
481 tvkigdfgla tvksrwsgsq qveqptgsvl
wmapevirmq dnnpfsfqsd vysygivlye
541 lmtgelpysh innrdqiifm vgrgyaspdl
sklykncpka mkrlvadcvk kvkeerplfp
601 qilssiellq hslpkinrsa sepslhraah
tedinactlt tsprlpvf

Figure 47: Amino acid sequence of human Raf-1
There is one potential caspase-2,-3 or -7 cleavage site (red) between amino acids 128/129 and 132 within the Raf-1 sequence. There are an additional 4 potential cleavage sites for caspase-6,-8 or -9 (blue) within the Raf-1 sequence. There is overlap between two caspase cleavage sites at amino acids 128 and 129 (green). The epitope to which the antibody was raised maps to the carboxy terminal sequence of the protein.

This sequence was obtained from the SwissProt database (accession number P04049).

obtained *in vitro* with those obtained in intact cells. Cellular organelles, membranes, and transport mechanisms, which confer additional complexity, are omitted from the *in vitro* system.

Quantification of Raf-1 mRNA using Northern blots or RT-PCR would show whether Raf-1 down-regulation during STS or CPT treatment occurred at the level of gene expression. Alternatively calpains, Ca^{2+}-activated cysteine proteases that cleave a number of different proteins, including known caspase substrates such as spectrin, keratin, tau, Parp, and Camk IV (reviewed in Wang, 2000), may be employed to cause Raf-1 downregulation. Calpains have been shown to be activated in staurosporine-treated SY5Y cells undergoing apoptosis and are not inhibited by caspase inhibitors (Nath, et al., 1996; Postmantur, et al., 1997). The use of calpain inhibitors could help determine whether Raf-1 disappearance during drug-induced apoptosis in SY5Y cells is due to calpain activity.

What is the physiological significance of Raf-1 down-regulation? As we observed in STS-treated SY5Y cells, when Raf-1 decreases, levels of its downstream target, phosphorylated Mek1, also decrease (Figure 43A). So, by decreasing levels of an upstream effector, downstream targets in the signal transduction pathway may be turned off. Down-regulation of Raf-1 protein levels and its kinase activity have also been observed during Fas-induced apoptosis in Jurkat cells (Widmann, et al., 1998). As mentioned in the Introduction (Chapter 2), signalling by the Raf/Mek/Erk pathway is important for cell proliferation, differentiation and survival responses. Overexpression of activated Raf-1 prevents apoptosis caused by growth factor deprivation through activation of Mek1 (Erhardt, et al., 1999). By downregulating Raf-1 during apoptosis, the cell can ensure that it does not propagate conflicting signals for cell survival.

6.42 Akt is downregulated during CPT-induced apoptosis in SY5Y cells.

Levels of Akt protein were specifically downregulated during camptothecin-induced apoptosis in SY5Y cells. Total Akt protein disappeared 48 hours after CPT treatment (Figure 45 & 46A). *In vitro* experiments suggest that Akt is cleaved by caspases during apoptosis (see Chapter 5), but experiments using caspase inhibitors need to be performed *in vivo* to ask whether Akt disappearance in this particular model is indeed a caspase-dependent event.

In contrast to Raf-1, Akt protein was not downregulated during staurosporine-induced apoptosis in SY5Y cells (Figure 43A). This demonstrates how in the same cell type, targets affected during the apoptotic response may differ according to the death stimulus received.

6.43 Caspases other than caspase-3 and –9 are activated early during STS- and CPT-induced apoptosis

In both models of drug-induced apoptosis, cleavage of Parp occurred 16-24 hours before caspase-3 and caspase-9 activation could be detected. Parp can be cleaved *in vivo* by other caspases such as caspase-7 (Salvesen & Dixit, 1997), which suggests that other caspases are active prior to caspase-3 in STS- and CPT-treated SY5Y cells. This suggests that an alternative mechanism may be operating prior or parallel to the cytochrome c/caspase-9/caspase-3 pathway in these cells during apoptosis. A study showing that staurosporine causes death in SY5Y cells by a p53-independent mechanism supports this theory (Ronca, et al., 1997). Apoptosis may be induced by staurosporine through an alternative pathway to the Apaf-1/caspase-9 pathway. Other studies disagree with this. It has been shown that Bax can translocate to the mitochondria and cause cytochrome c release and caspase activation after staurosporine treatment in SY5Y cells (McGinnis, et al.,1999). However, these researchers relied on monitoring caspase-3 activation by proteolysis of a

fluorogenic peptide. This type of assay is more sensitive than immunoblotting in detecting any caspase-like activity, but is relatively non-specific for determining activity of a particular caspase. For example, caspase-3, caspase-6, and caspase-7 can all cleave the fluorogenic substrate Ac-DEVD-MCA (Biomol Research Laboratories, Inc., Plymouth Meeting, PA). Therefore, this study does not rule out the possibility that caspases other than caspase-3 and –9 are activated early during apoptosis in STS-treated SY5Y cells. Further experiments, which look at cytochrome c localisation and other caspases by immunoblotting, will help clarify which mechanism of cell death is relevant in STS- and CPT-induced apoptosis in SY5Y cells.

In addition to the mitochondrial- and death receptor- mediated pathways, a third pathway exists for regulating apoptosis. Recently, an endoplasmic reticulum (ER)- mediated pathway has been implicated in the regulation of apoptosis (Nakagawa, et al., 2000). Stress to the ER triggers release of $Ca^{2}+$ from intracellular stores and activation of caspase-12, which resides in the ER. This ER-specific pathway may also play a role in amyloid-β toxicity. Which of these pathways is activated during apoptosis depends on both the specific cell type and death stimulus involved. For example, targeting of Bcl-2 to the ER and not the mitochondria will protect Rat-1 cells but not MDCK cells from serum-deprivation-induced apoptosis (Zhu, et al., 1996). Also, in Rat-1 cells, ER-targeted Bcl-2 will inhibit apoptosis induced by Myc but not by etoposide (Lee, et al., 1999). It will be interesting to test whether an ER-mediated pathway is operating in addition to, or instead of, the mitochondrial-mediated pathway during drug-induced apoptosis in SY5Y cells.

Chapter 7

General Discussion and Future Directions

General Discussion & Future Directions

The overall goal of this thesis was to gain an understanding of what triggers and regulates apoptosis in neurons. Several different techniques using neural cells were used to answer this question. PC12 cells were separated at different stages of cell death on density gradients (see Chapter 4). This allowed the ordering of events occurring during commitment to cell death following serum-deprivation. Akt protein levels were downregulated while c-Jun protein levels were upregulated prior to irreversible commitment to cell death. Cytochrome c translocated from the mitochondria to the cytosol and caspases were activated following commitment. The downregulation of Akt observed after serum-withdrawal suggests that this paradigm represents a mitochondrial-mediated pathway where the PI3-K signalling pathway is specifically downregulated during apoptosis. This study is the first where cells separated at discrete stages of apoptosis could be used to order events occurring during the onset of cell death.

Neurons differ from other cell types in that cytochrome c release alone is not sufficient to trigger apoptosis. Another factor, "competence-to-die" is acquired during trophic factor deprivation and is required in addition to cytochrome c to trigger apoptosis in sympathetic neurons (Deshmukh & Johnson, 1998). The nature of this "competence-to-die" factor is unknown. It may represent removal of an antiapoptotic factor (e.g. Iap) or accumulation of a cofactor required for caspase activation. It is interesting to speculate whether Akt downregulation or c-Jun upregulation which occur during commitment in the serum-withdrawal paradigm represent potential "competence-to-die" factors. Further work is needed to answer the question of what makes neurons competent to die.

We have created a cell-free system for apoptosis, which recapitulated the post-mitochondrial events of apoptosis following cytochrome c release (Chapter 5). This system showed strong activation of caspase-3 and caspase-9, which required ATP and phosphatase activity for full caspase activation. This system demonstrated downregulation of two signalling molecules implicated in neuronal

survival, Akt and Creb. Creb and Akt cleavage could be prevented by caspase inhibitors, which strongly suggests that their downregulation occurs by a caspase-mediated mechanism. This is the first time Creb and Akt have been shown to be caspase substrates during apoptosis. This cytochrome c- activated *in vitro* paradigm, like the serum-withdrawal model discussed above, represents a mitochondrial-regulated pathway characterised by specific downregulation of PI3-K signalling effectors during apoptosis.

In contrast, the two drug-induced models of apoptosis in SY5Y cells appear to occur by an alternative mechanism (Chapter 6). Parp-cleaving caspases other than caspase-3 and –9 were activated early during STS and CPT-induced apoptosis, while Raf-1 was specifically downregulated in apoptotic cells. Although unaffected during STS-induced apoptosis, Akt was specifically downregulated during CPT treatment in SY5Y cells. Therefore, even within the same cell type, different death stimuli may affect different signal transduction pathways. These results point to the existence of an alternative execution pathway to the cytochrome c/caspase-9 –mediated pathway during drug-induced apoptosis in SY5Y cells, which causes downregulation of survival signalling by the Raf/Mek/Erk pathway. Further work is required to determine the nature of this alternative execution pathway.

These studies illustrate the complexity of apoptosis signalling in neural cells and the differences in the mechanisms of cell death in neurons and other cell types. Why is the regulation of neurons so complex and why are they so difficult to kill? The decision to undergo apoptosis needs to be tightly regulated in neurons since these cells cannot be easily replaced. This is in contrast to the death of many other cell types, which can be replaced by normal cell proliferation. Cytochrome c release could occur during normal mitochondrial turnover and biogenesis in neurons, so neurons, being irreplaceable may have evolved additional regulatory mechanisms to prevent the accidental activation of caspases.

Chapter 8

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Appendix