Control of Programmed Cell Death by Rho-like GTPases-activated Signalling Cascades in the Developing Neuron

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A thesis submitted for the Degree of Doctor of Philosophy

Eisai London Research Laboratories,
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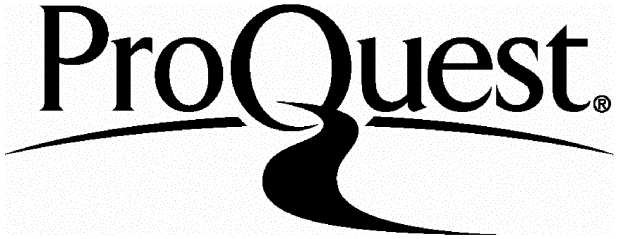
2001



ABSTRACT

Neuronal apoptosis is an essential process occurring not only during normal development but also in pathological situations including stroke, traumatic brain injury and neurodegenerative diseases. The mechanisms underlying neuronal cell death have begun to be unravelled. Rat superior cervical ganglion neurons (SCGs) have proven to be a good study model as they die by apoptosis when withdrawn from nerve growth factor (NGF). It has previously been shown that upregulation of the c-Jun transcription factor and of its phosphorylation are essential for NGF withdrawal-induced apoptosis in SCG neurons. This thesis tries to identify the signalling molecules that mediate apoptosis of SCG neurons via the c-Jun transcriptional pathway. The Rho-like GTPases Cdc42 and Rac1 have been shown to be involved in the activation of the c-Jun-NH_2-terminal kinase (JNK) pathway in other systems and therefore an investigation into the role of these GTPases was carried out. Here, it is demonstrated that Cdc42 and Rac1, but not RhoA or Ras, are required for cell death of SCG neurons and that they activate the JNK pathway, thereby inducing neuronal apoptosis. A further examination of the signalling molecules that would lie between Cdc42 and JNK on the death signalling pathway was then conducted. Here it is shown that two mitogen activated protein kinase kinase kinases (MAPKKKs), ASK1 and MLK3, are crucial elements of NGF withdrawal-induced activation of the Cdc42-c-Jun pathway and neuronal apoptosis.

ACKNOWLEDGMENTS

I would like to express my gratitude to my supervisor, Chantal Bazenet, for her expert guidance, help and encouragement throughout the course of this work.

My special thanks to Joanne Taylor for her advice and critical reading of this thesis.

I would also like to thank my office mates, Bina Shah and Jacqueline Catchick and everyone at Eisai for creating a friendly and enjoyable environment in which to work.

Finally, I would like to thank Marcus for his continuous support and encouragement.

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ABBREVIATIONS

AD	Alzheimer's disease
AP-1	activator protein-1
Apaf-1	apoptotic protease activating factor
ASK	apoptosis signal-regulating kinase
ATF	activating transcription factor
ATP	adenosine 5′-triphosphate
BDNF	brain derived neurotrophic factor
CAT	chloramphenicol acetyl transferase
CRIB	Cdc42/Rac interactive binding
DLK	dual leucine zipper bearing kinase
DNA	deoxyribonucleic acid
DRG	dorsal root ganglion
ERK	extracellular signal-regulated kinase
GAP	GTPase activating protein
GDI	guanine nucleotide dissociation inhibitor
GEF	guanine nucleotide exchange factor
GP-IgG	guinea pig IgG
GTP	guanosine 5′-triphosphate
IL	interleukin
JIP	JNK interacting protein
JNK	c-Jun NH_2-terminal kinase
MAPK	mitogen-activated protein kinase
MEKK1	MAPK/ERK kinase kinase 1
MLK	mixed lineage kinase
NF-κB	nuclear factor κB
NGF	nerve growth factor
p75NTR	p75 neurotrophin receptor
PAK	p21-activated kinase
PCD	programmed cell death
PI3K	phosphatidylinositol-3-kinase
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-PCR	reverse transcriptase-polymerase chain reaction
SAPK	stress-activated protein kinase
SCG	superior cervical ganglion
SEK1	SAPK/ERK kinase 1
SRE	serum response element
TNF-α	tumour necrosis factor α
Trk	tyrosine receptor kinase
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling
UV	ultra-violet
zVAD-fmk	N-benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone

1. Introduction

1.1 Apoptosis

1.1.1 History of cell death

Cell death has been observed and documented by scientists for decades. Indeed, in 1914 Ludwig Gräper published a paper entitled "A new point of view regarding the elimination of cells" (Gräper, 1914), which provided the first step towards understanding the mechanisms of cell death. However, the importance of this work was lost during the war years and it was not until 1951 that Glüksmann rediscovered the significance of cell death in development (Glücksmann, 1951). For instance, he recognised that cell death helps to shape the form of organs and to eliminate structures no longer useful. In the following years it became a well accepted fact that cells must be lost continuously from normal tissues to balance cell division and that this loss of cells accompanied atrophy and physiological involution of tissues and organs, but its morphological characteristics had not yet been clearly defined. At the time, the process of cell death best characterised was known as coagulative necrosis and it was the result of noxious stimuli or of irreversible disturbances of cellular homeostasis.

It was only in 1971 that John Kerr described other structural changes within dying cells that did not conform with the known concepts but that were consistent with an active, "inherently controlled phenomenon" (Kerr, 1971). Kerr used electron microscopy to look at lightly injured or mildly hypoxic livers. He found that, rather than swelling and rupturing, cells shrank, lost contact to neighbouring cells, condensed their nuclear chromatin and were phagocytosed and degraded. Kerr called this process "shrinkage necrosis" and later renamed it as "apoptosis" (*apoptosis* in Greek is used to describe the "dropping off" or the "falling off" of petals from flowers or leaves from trees).

However, it took almost 20 years before the idea that animal cells have a built-in cell death programme was broadly accepted. The term "programmed cell death" (PCD) was initially used to describe the cell death occurring at predictable

times and places in the developing embryo and in metamorphosing insects, to highlight the fact that it is an intrinsically programmed event in the developmental plan of an organism (Lockshin and Beaulaton, 1974). However, it was soon realised that these cell deaths could be modified by environmental factors and were not always unavoidable. Now PCD implies a pre-existing genetic control and the differential expression of genes that may regulate or be regulated by the activation and execution of cell death. This programme appears to be present in all the nucleated mammalian cells tested so far as staurosporine (a broad-spectrum kinase inhibitor) could induce apoptosis in the presence of cycloheximide (a protein synthesis inhibitor) (Weil *et al.*, 1996). This result was also observed in other organisms such as the fly *Drosophila melanogaster* and the nematode worm *Caenorhabditis elegans (C. elegans)*, suggesting that it might be present in every cell of all multicellular organisms as originally suggested by Umansky (Umansky, 1982; Steller, 1995; Shaham and Horvitz, 1996). These observations suggest that components of the cell death programme are in place and ready to go in the cytoplasm of all animal cells. The only exception found so far is the anucleated human red blood cell, which did not undergo apoptosis when treated with staurosporine in the presence or absence of cycloheximide (Weil *et al.*, 1996). This study demonstrates that anucleated erythrocytes do not seem to have the molecular machinery required for PCD and suggests that the nucleus might be responsible for their resistance. Indeed, in many cases of cell death, both *in vitro* and *in vivo*, a requirement for protein synthesis has been observed. For instance, the death of lymphocytes treated with glucocorticoids can be blocked by the inhibition of protein synthesis (Cohen and Duke, 1984), as is the developmental neuronal death in the chick embryo (Oppenheim *et al.*, 1990). Taken together, these results suggest that in most animal cells there is a pre-existing cytoplasmic death apparatus which may or may not require protein synthesis depending on the death stimulus and/or cell type.

1.1.2 Apoptosis and necrosis

There are certain morphological and biochemical events which occur in all cells undergoing apoptosis. These include a rapid condensation of cytoplasm and

nuclear chromatin, blebbing of the cell surfaces, a decrease in the rates of RNA (ribonucleic acid) and protein synthesis, and DNA (deoxyribonucleic acid) fragmentation [detected by DNA laddering and TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) staining]. The cells condense, lose cell-cell contact and fragment into membrane-bound apoptotic bodies that are rapidly phagocytosed. Because the contents of the apoptotic bodies are not released, there is no damage caused to the neighbouring tissues and an inflammatory response is not illicited. Within the phagocytic cells there is a further degradation of the fragments by lysosomes. The apoptotic process is highly dependent on energy, and affects characteristically single cells. A typical feature that is commonly described as a hallmark of apoptosis is the activation of nucleases that degrade chromosomal DNA. DNA damage represents an irreversible step in the death process. However DNA fragmentation is not observed in all cases of apoptosis (Cohen *et al.*, 1992; Oberhammer *et al.*, 1993; Tomei *et al.*, 1993). For instance, DNA fragmentation is not detected in cultured hepatocytes treated with tumour growth factor β 1 (TGF-β1) (Oberhammer *et al.*, 1993).

In contrast to this naturally occurring type of cell death which is observed during development and tissue homeostasis, necrosis, also termed oncosis (*oncos* meaning swelling in Greek), is known as a pathological form of cell death that results from severe cellular injuries such as toxins, ischaemia, complement attack or hypothermia which irreversibly disturb the cellular osmotic balance and that ultimately lead to lysis (Buckley, 1972; Hawkins *et al.*, 1972; Wyllie *et al.*, 1980; Laiho *et al.*, 1983; Trump *et al.*, 1984; Afanas'ev *et al.*, 1986; Borgers *et al.*, 1987). These insults destroy the plasma membrane integrity thereby disrupting the function of membrane pumps, allowing various ions, such as $Ca^{2+,}$ to move down their concentration gradients (Choi, 1992). In response to this passive ion influx, water comes in causing the cell to swell. In addition, an increase in intracellular Ca^{2+} concentration inhibits ATP (adenosine 5′-triphosphate) synthesis and stimulates phospholipases that further disrupt membranes by degrading phospholipids (Trump *et al.*, 1980). During the final stages of necrosis, lysosomes are ruptured and release hydrolases that accelerate cellular disintegration and

nucleases which cleave the DNA into fragments of various sizes (Hawkins *et al.*, 1972; Afanas'ev *et al.*, 1986). In vivo, in contrast to apoptosis, necrosis usually involves a large zone of cells such as in the liver following damage by hepatotoxins or in the brain, heart and kidney following ischaemia. As the cells die and burst, an acute inflammatory response is triggered at the periphery of the necrotic zone [for review see (Trump and Berezesky, 1994)].

In the last years variations in the characteristics of apoptosis have been observed. For instance, another type of PCD, in addition to apoptosis, is the so-called lysosomal cell death or autophagy. It is characterised by the primary expansion of lysosomes and other vacuoles which remove specific cell organelles and condense the cytoplasm and a late nuclear condensation and fragmentation (also detected by TUNEL staining) (Dunn, 1990a; Dunn, 1990b). As in apoptosis there is no membrane disruption and no induction of an inflammatory response. This type of PCD has been observed in longer living cells with massive cytoplasm such as rat mammary gland cells and *Manduca* labial gland cells (Lockshin and Zakeri, 1994; Zakeri *et al.*, 1996). More recently, autophagy has been observed as an integral part of the inductive phase of apoptosis in sympathetic neurons deprived of NGF (nerve growth factor), demonstrating that apoptosis and autophagy do not necessarily occur exclusive of one another and may overlap (Xue *et al.*, 1999). However it is not yet clear what the biochemical relationship between autophagy and apoptosis is.

In addition to these variations within PCD, it has been observed that many cells may exhibit characteristics of both apoptotic and necrotic death, and therefore the distinction between these two types of cell death has blurred (Clarke, 1998). For instance, in a model of excitotoxically lesioned newborn rat, kainic acid induced DNA laddering and death of neurons exhibiting a variety of morphologies, ranging from necrosis to apoptosis. Nowadays, it is conventionally believed that both types of cell death are two extremes in a continuum (Portera-Cailliau *et al.*, 1997). In addition, depending on the applied dose, the same toxin can induce either apoptosis or necrosis (Kroemer *et al.*, 1997). Therefore, apoptosis and necrosis are the terms normally used to discriminate between the activation of a intrinsic cellular suicide programme (PCD, apoptosis), and the death of cells by

external stimuli that does not involve the activation of a programmed mechanism (necrosis).

1.2 The role of apoptosis

1.2.1 Physiological apoptosis

Cell death by apoptosis is of central importance for both development and homeostasis of multicellular organisms. Recently, Jacobson *et al.* (Jacobson *et al.*, 1997) defined 5 functions of apoptosis during animal development:

(1) Sculpting of the body. For instance, interdigital cell death is necessary to create separate articulated digits (Saunders, 1966). Apoptosis has also been shown to be involved in the development of sexual dimorphism. In mammals, the female Mullerian and male Wolffian ducts are formed in both sexes during development, but are later deleted during sexual differentiation (Glücksmann, 1951; Saunders, 1966).

(2) Removal of unwanted structures. For example, during metamorphosis of amphibians, regression of the tadpole tail occurs by apoptosis (Yoshizato, 1989). Similarly, in some moths, the deletion of larval intersegmental muscles and abdominal prolegs are also a result of apoptosis (Schwartz *et al.*, 1990; Weeks *et al.*, 1992). Likewise, structures from ancestral species, such as pronephric tubules (which form kidneys in fish and amphibian larvae), that are no longer required in mammals, are deleted by apoptosis (Glücksmann, 1951; Saunders, 1966).

(3) Control of cell numbers. This is of crucial importance for the development of the nervous system where both neurons and oligodendrocytes are produced in excess and then eliminated by apoptosis (see section 1.3).

(4) Removal of abnormal, misplaced or harmful cells. For instance, immature B and T lymphocytes die by apoptosis because they express inappropriate antigen receptors, are self reactive or fail to detect a foreign antigen (Golstein *et al.*, 1991; Motyka and Reynolds, 1991; Cohen *et al.*, 1992; Rothenberg, 1992).

(5) Differentiation of cells without organelles. Skin keratinocytes and erythrocytes loose their nuclei in their highly specialised process of differentiation which have been suggested to be adapted forms of apoptosis (Nataraj *et al.*, 1994; Weil *et al.*, 1996; Weil *et al.*, 1999).

In developed organisms, apoptosis is also observed in the removal of harmful cells. For instance, in the immune system, self-reactive cells continue to be deleted, as are cells that are virally infected, as a protection mechanism to stop the infection spreading to neighbouring cells (Levine *et al.*, 1993). Also, as soon as neutrophils are no longer required they are eliminated by apoptosis to avoid inflammation (Savill *et al.*, 1993). Apoptosis is also observed in cells which have DNA damage and in tissues undergoing reversible expansion, like the hormone-dependent cells of the lactating breast (Bardon *et al.*, 1987). Moreover, apoptosis is an important mechanism in the elimination of cells that are carrying oncogenic mutations and that could potentially lead to cancer (Korsmeyer, 1992). All of these functions suggest that apoptosis is of prime importance in the homeostasis of organisms.

1.2.2 Pathological apoptosis

Because the control of cell number is the result of a balance between cell proliferation and cell death, deregulation of the death mechanisms can cause either uncontrolled growth or excessive death. Indeed, deregulation of apoptotic functions has been linked to the aetiology of many diseases.

Diseases characterised by an excessive number of cells include cancer, autoimmune diseases and viral illnesses. For instance, cells from many human cancers have decreased susceptibility to apoptosis, as they carry mutations that activate or inactivate key cell death regulatory genes (Hoffman and Liebermann, 1994). For instance, Bcl-2 was identified as a proto-oncogene because of its high frequency of constitutive activation following translocation at the chromosomal breakpoint of t(14;18)-bearing human B-cell lymphomas (Bakhshi *et al.*, 1985; Cleary and Sklar, 1985; Tsujimoto *et al.*, 1985; Reed *et al.*, 1998). Also, inactivation of the tumour suppressor gene p53 is a very common event in many human cancers (Symonds *et al.*, 1994; Bardeesy *et al.*, 1995). Autoimmune disease might be caused because of failure to delete autoreactive cells (Thompson, 1995). Viral illnesses can be developed because the viruses have evolved mechanisms of preventing cellular suicide. For instance, one of the genes expressed early after

infection with adenovirus is *e1b* (Rao *et al.*, 1992), which codes for a protein that is structurally homologous to the anti-apoptotic Bcl-2.

Alternatively, deregulation of apoptosis can cause excessive cell death and lead to a variety of diseases. For instance, in humans, reduction in lymphocyte numbers can be caused by the human immunodeficiency virus, leading to the acquired immunodeficiency syndrome (AIDS) (Meyaard *et al.*, 1992). A loss or decrease of blood flow (ischaemia) can lead to myocardial infarction or stroke and there is evidence that the damage occurring peripherally to the central ischaemic zone is due to delayed apoptosis (Thompson, 1995). Apoptosis also plays a role in the loss of specific neuronal populations which is the hallmark of many neurodegenerative diseases (section 1.3).

1.3 Apoptosis in the nervous system

1.3.1 Developmental apoptosis

Apoptosis plays a crucial role during normal development of the nervous system as, depending on the neuronal type, 20-80% of the neurons produced during neurogenesis die during synaptogenesis (Oppenheim, 1991). An important role of neuronal apoptosis is in the development of sexual dimorphism of both invertebrates and vertebrates. For instance, in the hermaphrodite *C. elegans*, the serotonergic motor neurons, which control egg laying and are formed in both sexes, are later eliminated in the male (Saunders, 1966). Neuronal apoptosis plays a role in the development of the neural song circuit in birds, whereby the male is able to learn intricate vocalisations and the female is not (Bottjer and Arnold, 1997). This is also a good illustration of synaptic plasticity, where there is a timely proliferation and cell death of the projecting neurons involved in the learning of bird song. Other functions of neuronal apoptosis includes the elimination of inappropriate projections, deletion of cells that have a transient function (e.g. Rohon-Beard cells in frogs), pattern formation and morphogenesis (e.g. the death of neural crest cells during neural tube closure) and assurance that the number of neurons does not exceed the number of target cells (Oppenheim, 1991; Burek and Oppenheim, 1996).

The most general function of vertebrate neuronal apoptosis is illustrated by the neurotrophic theory. This theory grew from pioneering studies by Rita Levi-Montalcini, Viktor Hamburger and Stanley Cohen on developing NGF-dependent sympathetic and sensory neurons (Cohen *et al.*, 1954; Hamburger *et al.*, 1981; Levi-Montalcini, 1987). The neurotrophic theory is based on the assumptions that the survival of developing vertebrate neurons depends on particular target-derived trophic factors (or neurotrophins) and that the neurons are produced in excess, so that only a proportion gets enough trophic support from their target cells for survival. The advantages of this neurotrophic system is that (1) inappropriate neuronal projections are eliminated as these neurons do not receive adequate neurotrophic factor; (2) the chance that all target cells become innervated increases; and (3) the number of target cells matches the number of neurons (Martin *et al.*, 1988). Therefore, this neurotrophic mechanism ensures that target cells become innervated in a precise and sufficient manner [reviewed by (Pettmann and Henderson, 1998)]. However, the regulation of neuronal cell death is much more complex than envisaged by the neurotrophic theory as developing neurons do not depend exclusively on the neurotrophins produced by their target cells (Korsching, 1993). Many require pre-synaptic input for survival, such as dorsal root ganglion (DRG) neurons [reviewed in (Oppenheim, 1991; Clarke, 1992)] and some require hormones, like hippocampal neurons (Sloviter *et al.*, 1989; McEwen and Gould, 1990).

1.3.2 Pathological apoptosis

A variety of neurological diseases are characterised by the loss of specific neuronal populations and there is increasing evidence that this cell death occurs by apoptosis. Indeed, neuronal apoptosis has been observed in post-mortem brains of patients with Alzheimer's disease (Su *et al.*, 1994), Parkinson's disease (Anglade *et al.*, 1997) and Huntington's disease (Portera-Cailliau *et al.*, 1995) as well as in brain tissue samples removed during surgery from traumatic brain injury patients (Clark *et al.*, 1999). Furthermore, there is increasing evidence that some of the cell death occurring following cerebral ischaemia is apoptotic (Liu *et al.*, 1996a). Secondary loss of neurons following a trauma may result from loss of

trophic factor, as illustrated by the fact that following global transient ischaemia, delayed death of the neurons in the CA1 region of the hippocampus can be prevented by injection of NGF into the ventricle (Shigeno *et al.*, 1991). However it is not always clear what the exact contribution of neuronal apoptosis towards disease progression is. Most cases of neurodegenerative diseases are sporadic and therefore can arise from a variety of different factors. Hence, studies of hereditary forms of neurodegenerative diseases have provided a lot of insight into the mechanisms of neuronal cell death. For instance, mutations in the β-amyloid and presenilin-1 and -2 genes have been linked to Alzheimer's disease (Yamatsuji *et al.*, 1996; Guo *et al.*, 1998; Mattson *et al.*, 1998), mutations in the Cu/Zn superoxide dismutase (SOD-1) gene are the cause of certain familial amyotrophic lateral sclerosis (Kunst *et al.*, 1997) and polyglutamine expansion of certain genes such as *huntingtin* cause Huntington's disease (Martindale *et al.*, 1998). Furthermore, these proteins such as β-amyloid, presenilin-2 and huntingtin, as well as downregulation of SOD-1 activity, have been shown to induce neuronal apoptosis *in vitro* (Forloni *et al.*, 1993; Rothstein *et al.*, 1994; Cotman and Anderson, 1995; Mark *et al.*, 1995; Wolozin *et al.*, 1996) and *in vivo* (huntingtin) (Zeitlin *et al.*, 1995).

In addition, proteins that play an important role in the regulation of apoptosis in many different *in vivo* and *in vitro* systems, have also been shown to play a role in neurodegeneration. For instance, the level of mRNA transcripts of Caspase-3, which plays an essential role in the execution of the death programme in many systems [for review see (Robertson *et al.*, 2000)], was increased in the cerebral cortex of patients with Alzheimer's disease (Desjardins and Ledoux, 1998). Similarly, overexpression of Bcl-2, which prevents apoptosis in almost every experimental paradigm where it has been tested [for review see (Newton and Strasser, 1998)], in mice, conferred protection in models of Parkinson's disease (Offen *et al.*, 1998) and ischaemia (Martinou *et al.*, 1994) but only moderate protection in models of motor neuron disease (Sagot *et al.*, 1995; Kostic *et al.*, 1997). However, increased expression of the *bcl-2* gene was observed in post-mortem brains of patients with Alzheimer's (Satou *et al.*, 1995) or Parkinson's diseases (Mogi *et al.*, 1996), suggesting that Bcl-2 may be insufficient to

prevent these diseases. Taken together, these observations suggest that apoptosis plays a part in neurodegeneration although its requirement may vary from disease to disease.

1.4 Regulation of cell death

1.4.1 Balance between life and death

Tissue modelling during development and tissue homeostasis during adult life is regulated by a dynamic equilibrium between survival/growth and apoptosis. Survival and apoptosis are closely associated and a disturbance of the balance between these two processes often leads to pathological situations as described above. Some of the molecular mechanisms controlling cell survival and apoptosis are discussed below.

1.4.1.1 Survival pathways

Raff and co-workers (Raff, 1992) suggested that cellular suicide is regulated by a default death pathway present in every single cell and only those cells that manage to inactivate this pathway have the privilege to survive. It is a well-known fact that extracellular survival factors play a role in maintaining cell viability (Eastman, 1995). Therefore, if the cell death machinery is constitutively expressed, then one of the functions of survival factors must be to suppress those death pathways. The ability of these factors to promote survival have been attributed, at least in part, to the phosphatidylinositol-3-kinase (PI3K)/Akt kinase cascade.

Upon growth factor binding to a membrane receptor (e.g. insulin-like growth factor I receptor, integrin receptor, lysophosphatidic acid receptor) (Clark and Brugge, 1995; Segal and Greenberg, 1996; Weiner and Chun, 1999), PI3K is recruited to the membrane where it phosphorylates phosphoinositides [for review see (Rameh and Cantley, 1999)]. These lipids are known to function as secondary messengers that are able to activate several cellular intermediates including tyrosine kinases, GTPase activating proteins and serine/threonine kinases such as protein kinase C, the $pp70^{S6K}$ and Akt [for review see (Rameh and Cantley, 1999)]. Of all these, the activity of Akt, in particular, has been shown to be required for

growth factor-mediated survival and to be sufficient to prevent apoptosis induced by different death stimuli such as growth factor withdrawal, ultra-violet (UV) irradiation, DNA damage or treatment with anti-Fas [for review see (Kandel and Hay, 1999)]. Several targets of the PI3K/Akt cascade have been identified that may explain the survival function of this pathway. Some of the targets include components of the death pathway such as the pro-apoptotic, Bad, caspase-9 and ASK1 (see sections 1.4.2.2 and 1.5.3.2), transcription factors of the forkhead family and a kinase IKK that regulates the transcription factor NF-κB (nuclear factor κB) [for review see (Datta *et al.*, 1999)]. Certain members of the forkhead family of transcription factors have been shown to be phosphorylated by Akt (Brunet *et al.*, 1999; Kops *et al.*, 1999). In the phosphorylated form, these transcription factors remain in the cytoplasm and can no longer induce the expression of genes. The evidence that this family of transcription factors is involved in the regulation of cell death comes from experiments in which non-phosphorylatable mutants (which are potent transcriptional activators) induced apoptosis in a variety of cell types (Brunet *et al.*, 1999; Tang *et al.*, 1999). There is also evidence suggesting that some members of this family of transcription factors can trigger the expression of the *FasL* gene (Brunet *et al.*, 1999). Altogether, these facts demonstrate that Akt can mediate survival by blocking the expression of important cell death proteins. In addition to promoting survival by inducing post-translational modifications, the PI3K/Akt pathway can also prevent cell death by upregulating genes that are capable of promoting survival by indirectly mediating the release of NF-κB from its cytosolic sequester IκB. Some of the genes induced by NF-κB include the anti-apoptotic Bcl-2 member *Bfl-1* and the caspase inhibitors c-IAP1 and c-IAP2 [(see sections 1.4.2.2, 1.4.2.3 and (Chu *et al.*, 1997; You *et al.*, 1997; Zong *et al.*, 1999)], thereby illustrating the relationship between the PI3K/Akt pathway and survival promoting proteins. Figure 1.1 illustrates some of the ways by which the PI3K/Akt pathway mediates cell survival.

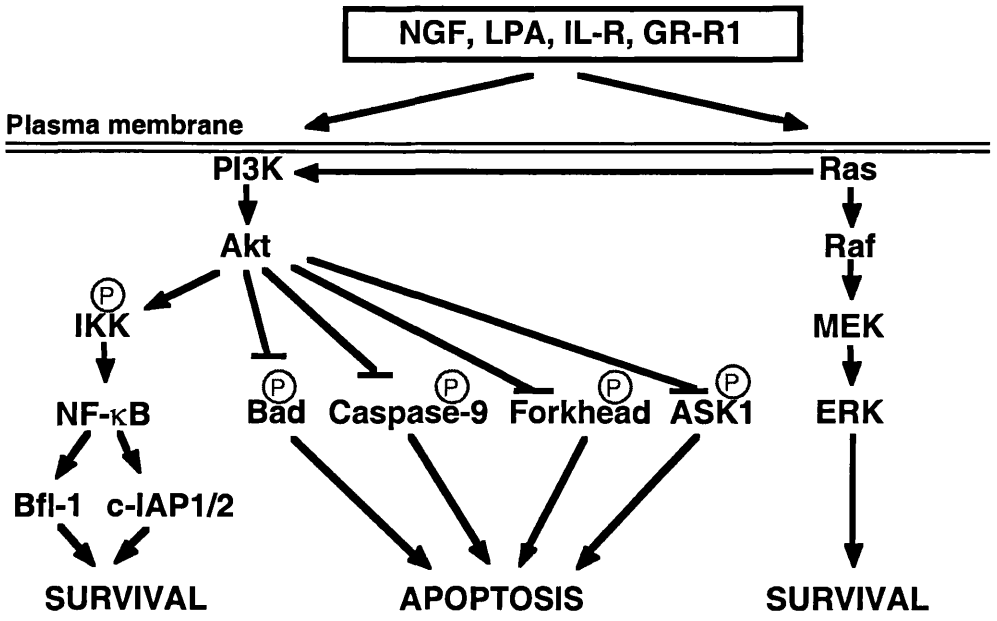


Figure 1.1 Survival pathways.

Diagram illustrating putative mechanisms by which extracellular factors are able to promote cell survival.

Although an increase in PI3K/Akt activity may be sufficient and necessary to promote survival, the existence of parallel survival pathways has questioned the absolute need for PI3K in cell survival. One of the pathways that can also promote survival upon growth factor binding is the Ras→Raf→MEK→ERK cascade. Indeed, some elements of this pathway have been shown to be critical in mediating growth factor-induced survival in a number of different cell types including neurons (Borasio *et al.*, 1989; Nobes and Tolkovsky, 1995; Markus *et al.*, 1997; Parrizas *et al.*, 1997). However, ERK (extracellular signal-regulated kinase) activity is not always required for the survival of hippocampal neurons (Marsh and Palfrey, 1996) nor for NGF-mediated survival of sympathetic neurons (Virdee and Tolkovsky, 1995; Creedon *et al.*, 1996; Virdee and Tolkovsky, 1996). In addition, Ras-mediated cell survival can also be mediated via the activation of PI3K (Kauffmann-Zeh *et al.*, 1997; Rodriguez-Viciana *et al.*, 1997; Xue *et al.*, 2000). Interestingly, recent studies have demonstrated that in superior cervical ganglion (SCG) neurons the Ras→PI3K pathway functions to inhibit apoptosis by NGF withdrawal, whilst the Ras→ERK pathway can only protect neurons from cytosine

arabinoside-induced death (Anderson and Tolkovsky, 1999; Xue *et al.*, 2000). The requirement for ERK activity in cell survival appears therefore to vary depending on the cell type as well as on the type of apoptotic insult. The possibility that there are other pathways to be discovered that could be more or less important depending on the cell type still remains.

1.4.1.2 Apoptotic pathways

Organisms have developed several mechanisms to rapidly and selectively eliminate cells by apoptosis. One of these mechanisms is regulated by the interaction of surface death receptors with their respective ligands. This is of particular importance in the immune system. These death receptors belong to the tumour necrosis factor (TNF) receptor superfamily, which are characterised by cysteine-rich extracellular domains and cytoplasmic "death domains" (DD) (Smith *et al.*, 1994). The DD links the receptor to the cellular apoptotic machinery. The best defined death receptors are Fas and tumour necrosis factor receptor 1 (TNF-R1). Their ligands, Fas ligand and TNF respectively, bind as homotrimers thereby inducing receptor trimer complex formation [for review see (Schulze-Osthoff *et al.*, 1998)]. In addition, the DD of the receptors can interact with each other and also recruit other DD containing molecules such as FADD (Fas associated death domain) or TRADD (TNF receptor associated death domain). FADD can recruit pro-caspase-8 via its death effector domain (DED), which results in the oligomerisation and consequent autocleavage and activation of caspase-8 (Muzio *et al.*, 1998). Caspase-8 can then activate downstream caspases such as caspase-3. The Fas receptor plays an important role in physiological apoptosis of immune cells (Nagata, 1997). The biological function of TNF-R1 is much more complex. For instance, by engaging TNF, TNF-R1 can induce the expression of pro-inflammatory and immunomodulatory genes (Tartaglia and Goeddel, 1992). In addition, in some cell types, TNF also induces apoptosis through TNF-R1 [for review see (Ashkenazi and Dixit, 1999)]. The TNF-R1 shares a signal cascade with Fas in one apoptotic pathway, but it also activates additional signalling pathways including one that activates a survival signal. Indeed, in addition to activating caspase-8 following ligand binding, TNF-R1-TRADD complex can also bind to RIP

(receptor-interacting protein) and TRAF2 (TNFR-associated factor 2), which induce pathways that lead to the activation of NF-κB and JNK/AP-1 (c-Jun NH_2-terminal kinase/activator protein-1) (Chinnaiyan *et al.*, 1996; Hsu *et al.*, 1996). The induction of JNK activity has been linked with TNF's ability to induce apoptosis (Ichijo *et al.*, 1997; Nishitoh *et al.*, 1998; Hoeflich *et al.*, 1999). As mentioned above, NF-κB can induce the expression of anti-apoptotic Bfl-1 and the caspase inhibitors c-IAP1 and c-IAP2, thereby suggesting a control system by which apoptosis induced by TNF is usually inhibited (Wang *et al.*, 1998a). Figure 1.2 illustrates some of the mechanisms by which death factors may mediate apoptosis.

Another member of the TNF receptor superfamily is the p75 neurotrophin receptor (p75NTR), which plays a role in the induction of apoptosis in the nervous system (see section 1.5.1).

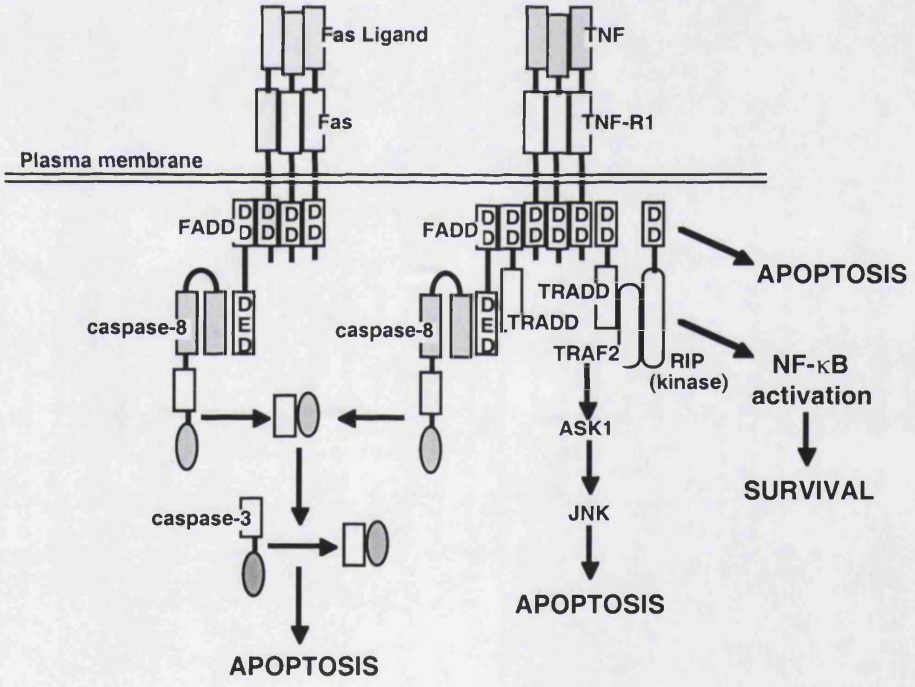


Figure 1.2 Apoptotic pathways by death factors.

Binding of ligand induces receptor trimerisation which recruits caspase-8 via the adaptors FADD and/or TRADD. Oligomerisation of caspase-8 may result in self activation of proteolytic activity and trigger the activation of downstream caspases. In another pathway, mediated only by TNF binding, RIP binds to TRADD and transduces an apoptotic signal through the death domain. In addition, TRAF2 may also recruit ASK1 which activates the JNK pathway. Furthermore, RIP together with TRAF2 can activate NF-κB, which induced the expression of survival genes.

1.4.2 Signal transduction players of apoptosis

1.4.2.1 Programmed cell death in the developing nematode worm

Genetic analysis of invertebrate models of PCD have been essential in providing important insights into the molecular mechanisms of apoptosis. Almost 25 years ago, Robert Horvitz began genetic studies on the developmental cell death in the nematode *Caenorhabditis elegans* (*C. elegans*) which were crucial for the understanding of PCD (Sulston and Horvitz, 1977). During development, 131 out of a total of 1090 cells undergo PCD and the position and timing of each cell is known (Ellis and Horvitz, 1991). Apoptosis is not restricted to particular cell types but most apoptotic events occur in neuronal cells (Sulston and Horvitz, 1977; Kimble and Hirsh, 1979; Sulston, 1983). Mutant *C. elegans* strains showing faulty developmental death were found to contain mutations in a variety of genes. Initially, *ced-3* and *ced-4* (*ce*ll *d*eath abnormal) were found to be necessary for cell death as loss-of-function (lf) alleles of both *ced-3* and *ced-4* resulted in the survival of all the 131 cells that normally die (Ellis and Horvitz, 1986; Shaham and Horvitz, 1996). Other nematode strains showing developmental defects were shown to contain mutations within the *ced-9* gene which was shown to prevent cell death (Hengartner *et al.*, 1992). Genetically *ced-9* acts upstream of *ced-3* and *ced-4*, as lf alleles of these genes are able to block the death induced by the lf *ced-9* allele (Hengartner *et al.*, 1992), thus suggesting that *ced-9* acts as a negative regulator of *ced-3* and *ced-4*. The *egl-1* (*egg l*aying abnormal) gene was discovered because gain-of-function mutations caused the death of the two hermaphrodite specific neurons that are required for egg laying (Conradt and Horvitz, 1998). Loss of this gene, however, allowed the survival of all the 131 cells that normally die. The proteins encoded by these genes are now known to function in a pathway (Figure 1.3). Other *ced* genes have been shown to play a role in the recognition and engulfment of dying cells (Hedgecock *et al.*, 1983; Ellis *et al.*, 1991).

Figure 1.3 *C. elegans* cell death pathway.

Egl-1 inhibits Ced-9, which itself inhibits Ced-4, which activates Ced-3.

Cloning of the above-mentioned genes led to the identification of mammalian homologues which performed similar functions in the regulation of apoptosis. For instance, *ced-3* possesses 29% homology with the human interleukin 1β-converting enzyme (ICE) (Yuan *et al.*, 1993) which is a member of the caspase family of proteases (Alnemri *et al.*, 1996). CED-4 shares homology with the recently identified Apaf-1 (Apoptotic protease activating factor-1) (Zou *et al.*, 1997). The sequence of *ced-9* revealed 24% homology with the mammalian *bcl-2* oncogene, which also negatively regulates cell death (Hengartner and Horvitz, 1994); whilst *egl-1* has homology to pro-apoptotic members of the Bcl-2 family (Conradt and Horvitz, 1998). The role of these in the regulation of apoptosis will be explained in the sections below.

1.4.2.2 The Bcl-2 family

Anti-apoptotic Bcl-2 is the founding member of an expanding gene family that includes the anti-apoptotic Bcl-X_L, Bcl-w, Bfl-1 and Mcl-1, as well as pro-apoptotic members such as Bax (Bcl-2 associated protein X), Bak, Bcl-X_S, Bad, Bik, Bid, Bim, Bok, Mtd, Krk and Hrk [(Hsu *et al.*, 1997a; Inohara *et al.*, 1998; O'Connor *et al.*, 1998) also reviewed in (Reed, 1997)].

Bcl-2 has been shown to delay multiple cell death pathways in many cell types, including those mediated by growth factor deprivation (neuronal, haemopoietic, lymphoid and fibroblastic cells), tumour necrosis factor α (TNF-α), neurotrophin withdrawal, UV and γ-radiation, heat shock, reactive oxygen species (ROS), calcium ionophores, glutamate excititoxicity, p53 and caspase activation [reviewed in (Reed, 1994; White, 1996)]. By contrast, pro-apoptotic members of the Bcl-2 family appear to promote apoptosis (Sakakura *et al.*, 1996; McCurrach *et al.*, 1997).

The overall sequence homology between different Bcl-2 family members is low. It is concentrated around four specific regions called the Bcl-2 homology domains (BH 1-4), which correspond to α-helical segments which mediate protein interactions [for review see (Adams and Cory, 1998; Antonsson and Martinou, 2000)]. Bcl-2 family members can interact in different ways to form homo- or heterodimers and their relative abundance might play a major role in determining the response to a death signal (Oltvai and Korsmeyer, 1994; Sedlak *et al.*, 1995). For instance, the BH1 and BH2 domains have been shown to be required for Bcl-2 and Bcl-X_L to bind Bax and block apoptosis (Borner *et al.*, 1994). Many of the anti-apoptotic members show homology on all four domains (e.g. Bcl-2, Bcl-X_L, Bcl-w). In contrast, all pro-apoptotic members, except for Bcl-X_S, lack the BH4 domain which is crucial for the anti-apoptotic function. This is illustrated by the fact that mutants of Bcl-2 that lacked the BH4 domain behaved like killer proteins (Cheng *et al.*, 1997). The BH3 domain, on the other hand, is a crucial death domain in all pro-apoptotic members (Zha *et al.*, 1997) and several BH3-only proteins exist (e.g. Bid, Bad, Bim, Hrk).

The biochemical mechanisms by which Bcl-2 family members regulate cell death are complex and not yet completely clear. The majority of anti-apoptotic members are integral membrane proteins which are localised in the mitochondria, endoplasmic reticulum (ER) or nuclear membranes (Hockenbery *et al.*, 1990; Krajewski *et al.*, 1993; de Jong *et al.*, 1994; Zhu *et al.*, 1996), whereas most pro-apoptotic members are found in the cytosol or cytoskeleton before a death signal (Hsu *et al.*, 1997b; Gross *et al.*, 1998; Puthalakath *et al.*, 1999). Following a death stimulus, cytosolic monomeric Bax undergoes a conformational change that allows it to homodimerise and to translocate to the mitochondrial outer membrane (Wolter *et al.*, 1997; Gross *et al.*, 1998). The presence of anti-apoptotic proteins such as Bcl-2 or Bcl-X_L can prevent Bax activation in response to a death signal (Gross *et al.*, 1998). Besides oligomerisation and translocation, the activation state of Bcl-2 family members can also be regulated by post-translational modifications such as phosphorylation or proteolytic cleavage. Bad, for instance, is regulated by phosphorylation. In the presence of a survival factor, Akt or PKA (a cAMP-dependent protein kinase) phosphorylates Bad on two serines residues

(Datta *et al.*, 1997; del Peso *et al.*, 1997; Harada *et al.*, 1999), resulting in its sequestration to the cytosol by 14-3-3 proteins (Zha *et al.*, 1996). Recently, Bad has also been shown to be phosphorylated by Akt at serine 155 and its mutation enhanced the pro-apoptotic activity of Bad (Virdee *et al.*, 2000). Serine 155 locates within the BH3 domain and it has been suggested that its phosphorylation may induce a conformational change which favours the dissociation of Bad from anti-apoptotic Bcl-2 family members (Virdee *et al.*, 2000), thereby neutralising Bad's pro-apoptotic activity. Phosphorylation of Bcl-2 has also been shown to affect its anti-apoptotic activity (Haldar *et al.*, 1995; Ito *et al.*, 1997; Poommipanit *et al.*, 1999). A good example of activation by cleavage is the proteolysis of cytosolic Bid by caspase-8 upon Fas or TNF-α treatment (Li *et al.*, 1998; Gross *et al.*, 1999; Han *et al.*, 1999). The truncated carboxy terminus of Bid translocates to the mitochondria, where it seems to be required for the release of cytochrome c (Gross *et al.*, 1999), which then complexes with cytosolic caspase-9 and Apaf-1 (the mammalian CED-4 homologue) (Liu *et al.*, 1996b; Zou *et al.*, 1997; Zou *et al.*, 1999) to cleave and activate the effector caspase-3 (Li *et al.*, 1997) which ultimately lead to cell death (Figure 1.4 and see section 1.4.2.3). There is also evidence that the expression of some Bcl-2 family members is transcriptionally regulated. For instance, the pro-apoptotic Hrk and Bim are upregulated in response to a death signal (Inohara *et al.*, 1997; Whitfield *et al.*, in press).

The mechanism by which this family of proteins functions at the mitochondrion has recently been under intense investigation. The release of cytochrome c has been observed in many cell types, including neurons, undergoing apoptosis (Liu *et al.*, 1996b; Bossy-Wetzel *et al.*, 1998; Neame *et al.*, 1998; Rosse *et al.*, 1998) and it has been considered a crucial event in the decision of a cell fate (Green and Reed, 1998). The release of cytochrome c results in the uncoupling of oxidative phosphorylation and consequent loss of mitochondrial membrane potential ($\Delta\Psi m$) and swelling of the mitochondria (Green and Reed, 1998). Pro-apoptotic Bcl-2 family members seem to regulate the release of cytochrome c from the mitochondria either by forming pores in the outer mitochondrial membrane or by regulating the activity of existing channels. For instance, Bad is known to influence mitochondrial membrane integrity and the

release of cytochrome c from the mitochondria by interacting with anti-apoptotic Bcl-2 or Bcl-X_L (Yang *et al.*, 1995). Furthermore, Bid seems to mediate the release of cytochrome c by inducing a conformational change in Bax. Following a conformational change, translocation to the mitochondria and oligomerisation, Bax is thought to insert into the mitochondrial outer membrane and trigger the release of cytochrome c (Eskes *et al.*, 1998; Jurgensmeier *et al.*, 1998), probably through the opening of a specific channel such as the PTP (permeability transition pore) (Marzo *et al.*, 1998), VDAC (voltage-dependent anion channel) (Shimizu *et al.*, 1999), or even the Bax pore itself. The release could be blocked by overexpression of Bcl-2 (Kluck *et al.*, 1997; Yang *et al.*, 1997b), suggesting that anti-apoptotic Bcl-2 family members may exert their functions by blocking the release of cytochrome c.

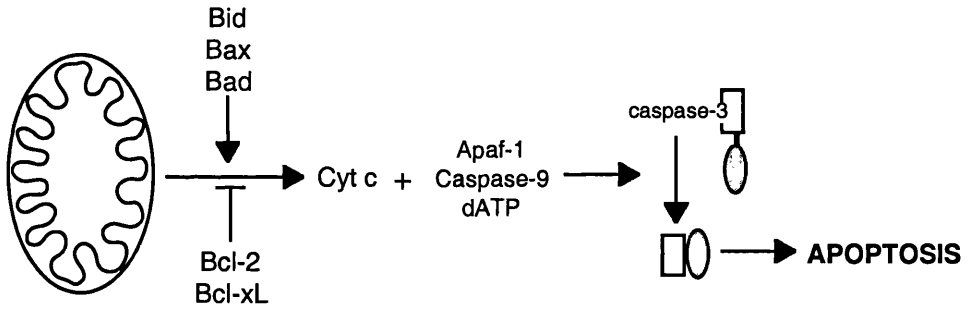


Figure 1.4 The role of cytochrome c release in apoptosis.

Various cell death stimuli cause cytochrome c to be released from the mitochondria into the cytosol. Cytosolic cytochrome c forms a complex with pro-caspase-9, Apaf-1 and in the presence of dATP, pro-caspase-9 is activated and leads to the activation of caspase-3 and apoptosis.

1.4.2.3 Caspases

As discussed in section 1.4.2.1 cloning of the *C. elegans* gene *ced-3* led to the identification of the mammalian homologue, ICE, a cysteine protease responsible for the processing of interleukin (IL) precursor to its mature secreted form (Yuan *et al.*, 1993). Cleavage of precursor-IL-1β (pro-IL-1β) by ICE to yield the active cytokine occurs after Asp116. This cleavage after an aspartic residue is a feature

common to all of these proteases, with the four amino acids immediately N-terminal to the cleavage site defining the specificity of the substrate [reviewed in (Nicholson and Thornberry, 1997)]. The CED-3 homologues are now known as caspases (cysteine proteases that cleave their substrates after an aspartate residue) and to date, 14 members have been identified (caspase-1 to 14) [for review see (Shearwin-Whyatt and Kumar, 1999)].

Of the caspases known to date, not all seem to play a role in apoptosis. Caspase-1, -4, -5 and -11 appear to be primarily involved in cytokine processing, whereas caspase-2, -3, -7, -8, -9 and -10 have been shown to play a direct role in apoptosis. A lot of the evidence supporting this has come from studies on knockout mice. For instance, caspase-1 and -11 knockout mice have defects in IL-1β and IL-1α production (Kuida *et al.*, 1995; Li *et al.*, 1995; Wang *et al.*, 1998b). Caspase-3 and -9 knockout mice die soon after birth and show profound developmental defects such as a higher brain mass due to lack of neuronal cell death, thereby demonstrating the importance of these caspases in neuron cell death (Kuida *et al.*, 1996; Hakem *et al.*, 1998; Kuida *et al.*, 1998; Woo *et al.*, 1998).

The current evidence points to two mechanisms of caspase activation: autocleavage and cleavage by other caspases. Caspase-3 and -7 have been shown to be activated by caspase-10 (Fernandes-Alnemri *et al.*, 1996), suggesting that caspases are able to interact and activate each other in a caspase cascade. Because there are multiple caspases within one cell, the ability of one caspase to activate several others creates an amplification of the cascade. How is this cascade of proteases initiated? Structurally caspases can be divided into two groups: (1) caspases that contain a long amino-terminal pro-domain similar to those found in adaptor molecules such as FADD (caspase-1, -2, -8, -9 and -10); and (2) caspases containing small pro-domains (caspase-3, -6 and -7). The present model is that following an apoptotic stimulus, long pro-domain containing caspases, are recruited to death complexes where they dimerise through their pro-domain region and are consequently self-activated. Because of their direct interaction with the upstream apoptotic machinery, these caspases are often called the upstream, initiator or class I caspases. Caspases containing small pro-domains are unlikely to interact with the upstream death complexes and because they have been shown to

be activated by class I caspases, they are termed downstream, effector or class II caspases. These effector caspases have been shown to be responsible for the cleavage of many proteins involved in the process of cell death [see below and for review see (Nicholson and Thornberry, 1997; Cryns and Yuan, 1998)].

In addition to being activated by death pathways, caspases can also be a target of regulation by components of survival pathways. For instance, caspase-9 phosphorylation by Akt renders it inactive probably by blocking the intrinsic catalytic activity of caspase-9 (Cardone *et al.*, 1998).

To date about 40 proteins have been shown to be cleaved by downstream caspases during apoptosis. Cleavage can result in either the activation or the inactivation of that protein and some of the morphological and biochemical changes observed in apoptotic cells may result from the cleavage of specific proteins that are important in maintaining cellular homeostasis. For instance, some of the enzymes cleaved are involved in DNA repair and function, like the poly ADP-ribose polymerase (PARP) and the 70 KDa subunit of the U1 small nuclear ribonucleoprotein (U1-70 snRNP) (Casciola-Rosen *et al.*, 1994; Fernandes-Alnemri *et al.*, 1995a; Fernandes-Alnemri *et al.*, 1995b). Interestingly, a protein responsible for the DNA fragmentation observed during apoptosis, caspase activated DNase (CAD), is normally bound to a cytoplasmic inhibitor (ICAD) which is cleaved and degraded by caspase-3 (Enari *et al.*, 1998). Several structural proteins are also cleaved by caspases during apoptosis. For instance PAK2 (p21-activated kinase 2) which is involved in the regulation of the actin cytoskeleton is activated by caspase cleavage [see section 1.5.3.1 (Lee *et al.*, ; Rudel and Bokoch, 1997)]. Other structural proteins that are cleaved include the lamins (Lazebnik *et al.*, 1995; Neamati *et al.*, 1995), actin (Mashima *et al.*, 1995), fodrin (Martin *et al.*, 1995b) and Gas2 (Brancolini *et al.*, 1995). Some of the substrates include cell cycle proteins, protein kinase C δ and proteins involved in plasma membrane integrity. A comprehensive list of known caspase targets can be found in recent reviews (Nicholson and Thornberry, 1997; Cryns and Yuan, 1998) and it continues to grow.

1.4.2.4 Involvement of the JNK pathway in transcription-dependent death

The most compelling evidence for the role of transcription in apoptosis came from studies performed on neurons. In different types of primary neurons cultured *in vitro*, inhibitors of transcription or translation block cell death induced by survival factor withdrawal (Martin *et al.*, 1988; Scott and Davies, 1990; D'Mello *et al.*, 1993; Milligan *et al.*, 1994). These observations suggested that the removal of survival signals may activate regulatory pathways which lead to the activation of specific genes whose products promote cell death (Johnson and Deckwerth, 1993). Some of the signalling pathways that lead to apoptosis have begun to be defined and many molecules that either induce or block apoptosis have been identified. The c-Jun NH_2-terminal kinase (JNK; also referred to as stress-activated protein kinase or SAPK) pathway has been shown to play an important role in both the induction of apoptosis and cell survival *in vivo* and in a variety of cell systems [for review see (Leppa and Bohmann, 1999)].

The most convincing data for c-Jun acting as an inducer of apoptosis has been obtained from studies in developing neurons. In cultured rat sympathetic neurons, NGF-deprivation induced a specific and significant increase in the level of the c-Jun transcription factor (Ham *et al.*, 1995), suggesting that AP-1 activity participates in the transcriptional programme of neuronal cell death. Furthermore, the importance of c-Jun in neuronal apoptosis has been demonstrated in studies where NGF-withdrawal induced death of PC12 cells and sympathetic neurons could be blocked by microinjection of anti-c-Jun antibodies or by overexpression of a dominant negative mutant of c-Jun (Estus *et al.*, 1994; Ham *et al.*, 1995). In addition to the above *in vitro* studies, in the rat nervous system, an increase in c-Jun expression was shown to correlate with ischaemia (Dragunow *et al.*, 1993) and ionising radiation (Ferrer *et al.*, 1995). Furthermore, in humans, increased expression of c-Jun has been observed in post-mortem specimens from patients with amyotrophic lateral sclerosis (ALS) (Virgo and de Belleroche, 1995), multiple sclerosis (Martin *et al.*, 1996) and Alzheimer's disease (Anderson *et al.*, 1994) suggesting that c-Jun may play a role in the pathology of different neurodegenerative diseases.

In addition to an increase in c-Jun expression, c-Jun phosphorylation by JNK has been shown to be required for the mediation of apoptosis in many neuronal cell types. An increase in JNK activity has been observed soon after NGF withdrawal from SCG neurons (Virdee *et al.*, 1997; Eilers *et al.*, 1998) and in differentiated PC12 cells, NGF withdrawal induced an activation of JNK and of p38/HOG1 mitogen activated protein kinase whereas the ERK pathway was inhibited (Xia *et al.*, 1995). Similarly, JNK activation has been observed in embryonic motoneurons after removal of their survival factor (Maroney *et al.*, 1998). In vivo evidence for the importance of JNK in neuronal apoptosis has been obtained from *jnk* knockout mice. Indeed, knockout of the brain-specific *jnk3* gene, but not *jnk1* or *jnk2*, protected hippocampal neurons from kainate-induced apoptosis (Yang *et al.*, 1997a; Dong *et al.*, 1998; Yang *et al.*, 1998). In addition, the hippocampi of mice that have a *c-jun* locus carrying mutations in the phosphorylation sites (Ser63→Ala and Ser73→Ala) are also protected from kainic acid-induced apoptosis (Behrens *et al.*, 1999). On the whole, these findings suggest that pathways regulating the level of c-Jun protein and its phosphorylation are important in neuronal cell death. Consequently, upstream regulators of these pathways might be involved in mediating neuronal cell death (section 1.5).

1.4.2.5 The JNK family of kinases

To date, three human JNK genes have been identified: *jnk1* (Derijard *et al.*, 1994), *jnk2* (Kallunki *et al.*, 1994; Sluss *et al.*, 1994) and *jnk3* (Mohit *et al.*, 1995; Gupta *et al.*, 1996). In addition the corresponding rat genes have also been isolated (Kyriakis *et al.*, 1994). The *jnk1* and *jnk2* genes are expressed ubiquitously, whilst the expression of the *jnk3* gene is restricted to the brain, heart and testis. The mRNAs of these genes are differentially spliced to yield 46 KDa and 55 kDa JNK isoforms, however the functional significance of these splice variants is unclear (Gupta *et al.*, 1996; Widmann *et al.*, 1999). A second site of alternative splicing has been identified within the kinase domain of *jnk1* and *jnk2* but not *jnk3* (Gupta *et al.*, 1996). It influences the substrate specificity by changing the ability of JNK to interact with docking sites on substrates (Gupta *et al.*, 1996). Initial studies suggested that the substrate specificities of JNK1 and JNK2 were different. For

instance, JNK1 was thought to preferentially bind and phosphorylate c-Jun whereas JNK2 would preferentially bind and phosphorylate ATF-2 (activating transcription factor 2) (Kallunki *et al.*, 1994; Sluss *et al.*, 1994). However, it is now clear that those differences reflected the particular splice variants examined. Indeed, distinct tissues express different amounts of the various spliced JNK isoforms and the specific splice variant that binds and phosphorylates a particular substrate can be expressed by either the *jnk1* or the *jnk2* genes (Gupta *et al.*, 1996). Studies on *jnk* knockout mice have provided some clues towards the understanding of the function of these kinases. Mice lacking JNK1 or JNK2 seem to be morphologically normal. However, they are immunodeficient because of defects in T cell function (Constant *et al.*, 2000). In addition, *jnk3*$^{-/-}$ mice are developmentally normal but are defective in the apoptotic response to excitotoxins (Yang *et al.*, 1997a). The *jnk1/jnk3* and *jnk2/jnk3* deficient mice have also no phenotypic abnormalities. However, *jnk1/jnk2 knockout mice* are embryonically lethal and have defects in neuronal apoptosis and exencephaly (Kuan *et al.*, 1999; Sabapathy *et al.*, 1999). Primary murine fibroblasts from *jnk1/jnk2* knockout embryos totally lack JNK illustrating the specific neuronal expression pattern of the *jnk3* gene (Tournier *et al.*, 2000). Altogether, the studies of *jnk* knockout mice do confirm that there is a high degree of complementation between the *jnk* genes and that the tissue-specific defects in signal transduction may reflect a differential expression profile of the various JNK isoforms. This may complicate the analysis of *jnk* knockout mice and demonstrate the need for studies of animals that are deficient in all JNK isoforms (Tournier *et al.*, 2000).

The JNK pathway is activated by treatment of cells with pro-inflammatory cytokines such as TNF and IL-1β, by exposure of cells to environmental stresses such as UV light, X-rays, hydrogen peroxide (H_2O_2), heat, osmotic shock and withdrawal of growth factors (Derijard *et al.*, 1994; Kallunki *et al.*, 1994; Kyriakis *et al.*, 1994; Minden *et al.*, 1994; Sluss *et al.*, 1994; Gupta *et al.*, 1996). These stimuli activate protein kinase cascades usually consisting of three hierarchical protein kinases including mitogen-activated protein kinase (MAPK), MAPK kinase (MAPKK) and MAPKK kinase (MAPKKK) (Errede and Levin, 1993; Davis, 1994; Waskiewicz and Cooper, 1995; Kyriakis and Avruch, 1996; Schaeffer and Weber,

1999; Widmann *et al.*, 1999). MAPKKK phosphorylates and hence activates MAPKK which in turn phosphorylates and activates MAPK. The JNKs (MAPK) are activated by dual phosphorylation within the protein kinase subdomain VIII, more specifically on Thr-183 and Tyr-185 in JNK1 and JNK2 (Derijard *et al.*, 1994; Whitmarsh and Davis, 1996) and Thr-221 and Tyr-223 in JNK3 (Lisnock *et al.*, 2000). Following phosphorylation, cytosolic JNKs translocate to the nucleus where they can phosphorylate transcription factors. This phosphorylation has been shown to be mediated by the MAPKKs MKK4 (Derijard *et al.*, 1994; Sanchez *et al.*, 1994; Lin *et al.*, 1995) and MKK7 (Tournier *et al.*, 1997). Distinct MAPKs are activated by different kinase modules which respond to specific stimuli (Davis, 1994; Derijard *et al.*, 1994; Whitmarsh and Davis, 1996). Interestingly, a new group of proteins termed JNK interacting proteins (JIPs) has been shown to interact with members of the MLK (mixed lineage kinase) family of MAPKKKs such as MLK3 (mixed lineage kinase 3) and DLK (dual leucine zipper bearing kinase), two kinases upstream of JNKK, with MKK7 and with JNK thereby linking these kinase-signalling components (Dickens *et al.*, 1997; Whitmarsh *et al.*, 1998; Yasuda *et al.*, 1999). The JIPs are therefore thought to act as molecular scaffolds that organise the JNK signal transduction pathway in response to specific stimuli (Whitmarsh *et al.*, 1998). The translocation of the activated JNKs into the nucleus seems to be controlled by JIPs (Dickens *et al.*, 1997) which maintain the JNKs in the cytoplasm by acting as an anchor.

1.4.2.6 Substrates of the JNKs

Targets of the JNKs include the transcription factors ATF-2, c-Jun (Whitmarsh and Davis, 1996) or Elk-1 (Cavigelli *et al.*, 1995). ATF-2 and c-Jun are members of the AP-1 family of transcription factors. The AP-1 family has three subfamilies of bZIP-domain (basic region leucine zipper) proteins: Jun, Fos and ATF-2 (Angel and Karin, 1991). The Jun subfamily includes c-Jun, JunB, and JunD; the Fos subfamily is composed of c-Fos, Fos, Fra-1 and Fra-2; and the ATF proteins include ATF-2 and ATF-α. Like all bZIP transcription factors, AP-1 proteins form homo- or heterodimer complexes and bind to AP-1 (identified by the sequences TGACTCA, TGACGTCA) and AP-1-like sites in the promoters of many genes

(Curran and Franza, 1988). JNK binds ATF-2 and c-Jun at an N-terminal region and phosphorylates Thr69 and Thr71 in ATF-2 and Ser63 and Ser73 in c-Jun, which lie within the activation domain of these transcription factors (Pulverer *et al.*, 1991; Derijard *et al.*, 1994; Gupta *et al.*, 1995; Karin and Hunter, 1995; Livingstone *et al.*, 1995; van Dam *et al.*, 1995). Phosphorylation of ATF-2 and c-Jun leads to increased transcriptional activity, including AP-1's own transcriptional activity through the induced expression of c-Fos and c-Jun (Whitmarsh and Davis, 1996). c-Fos expression is regulated by activation of the serum response element (SRE) in the *c-fos* promoter by Elk-1 (Cavigelli *et al.*, 1995; Gille *et al.*, 1995; Whitmarsh *et al.*, 1995). The increase in c-Jun expression is mediated by two mechanisms: an increase in AP-1 activity which then turns on the *c-jun* promoter by binding to its AP-1-like site (Whitmarsh and Davis, 1996); and phosphorylation of c-Jun which causes a decrease in ubiquitin-induced degradation of c-Jun, thereby increasing the half life of c-Jun (Fuchs *et al.*, 1996; Musti *et al.*, 1997). The above studies clearly show that the JNK pathway can regulate the AP-1 transcriptional activity in response to different stimuli.

In addition to transcription factors, JNKs phosphorylate a variety of cytoplasmic substrates which are important for physiological and apoptotic functions of JNK in the nervous system. For instance, the JNKs have been shown to phosphorylate cytoskeleton proteins such as the neurofilament heavy chain (Giasson and Mushynski, 1997; Brownlees *et al.*, 2000); and Tau (Reynolds *et al.*, 1997). It is interesting to note that phosphorylation of the neurofilament heavy chain by JNKs leads to an increased vulnerability to neuronal stress (Giasson and Mushynski, 1997) and also that Tau is hyperphosphorylated in neurofibrillary tangles of Alzheimer's disease patients (Ksiezak-Reding *et al.*, 1992; Kopke *et al.*, 1993). These observations suggest a link between JNK activation and the development of neurodegenerative diseases such as Alzheimer's disease. The JNKs have also been shown to antagonise the function of the anti-apoptotic Bcl-2 and Bcl-X_L proteins (Park *et al.*, 1997) probably by phosphorylation (Maundrell *et al.*, 1997; Kharbanda *et al.*, 2000). Finally, the JNK pathway activates the tumour suppressor p53 (Fuchs *et al.*, 1998), a pro-apoptotic transcription factor that suppresses *bcl-2* and induces *bax* expression (Miyashita *et al.*, 1994). These studies

demonstrate a way in which JNK may modulate the regulation of important cell death and survival proteins.

1.4.2.7 Targets of c-Jun

One of the transcriptional targets of c-Jun includes the *Fas ligand* gene which has consensus AP-1 binding sites in its promoter (Yang *et al.*, 1997a; Kasibhatla *et al.*, 1998). Recent findings have demonstrated that the upregulation of the expression of the *Fas ligand* gene is a common characteristic of trophic factor deprivation-induced neuronal cell death (Herdegen *et al.*, 1998; Le-Niculescu *et al.*, 1999; Martin-Villalba *et al.*, 1999; Raoul *et al.*, 1999). These studies suggest a model in which growth factor withdrawal induces the transcription of a potent death protein, such as Fas ligand, thereby amplifying the initial death stimulus. However, no induction in the expression of Fas ligand was observed in sympathetic neurons deprived of NGF (Cesare Spadoni, unpublished observations), suggesting that the mechanisms of cell death might differ depending on the neuronal type.

As discussed in section 1.4.2.2, there is evidence that the expression of some Bcl-2 family members, such as Hrk, is transcriptionally regulated (Inohara *et al.*, 1997). Recent work from colleagues has shown that FLAG-Δ169, a c-Jun deletion mutant that acts as an inhibitor of AP-1 activity (Hirai *et al.*, 1989; Castellazzi *et al.*, 1991), can block NGF-withdrawal-induced release of cytochrome c in sympathetic neurons (Whitfield *et al.*, in press). This observation suggests that AP-1 transcription is required for the release of cytochrome c, which is regulated by Bcl-2 family members. In addition, Whitfield *et al.*. (Whitfield *et al.*, in press) demonstrate that following NGF deprivation, pro-apoptotic Bim is upregulated and this increase in expression is significantly reduced by FLAG-Δ169. Furthermore, microinjection of anti-sense Bim oligonucleotides can block NGF withdrawal-induced release of cytochrome c and death. This study provides strong evidence that Bim upregulation by c-Jun is a critical step in NGF withdrawal-induced release of cytochrome c and death of sympathetic neurons. Figure 1.5 illustrates putative ways by which JNK mediates apoptosis.

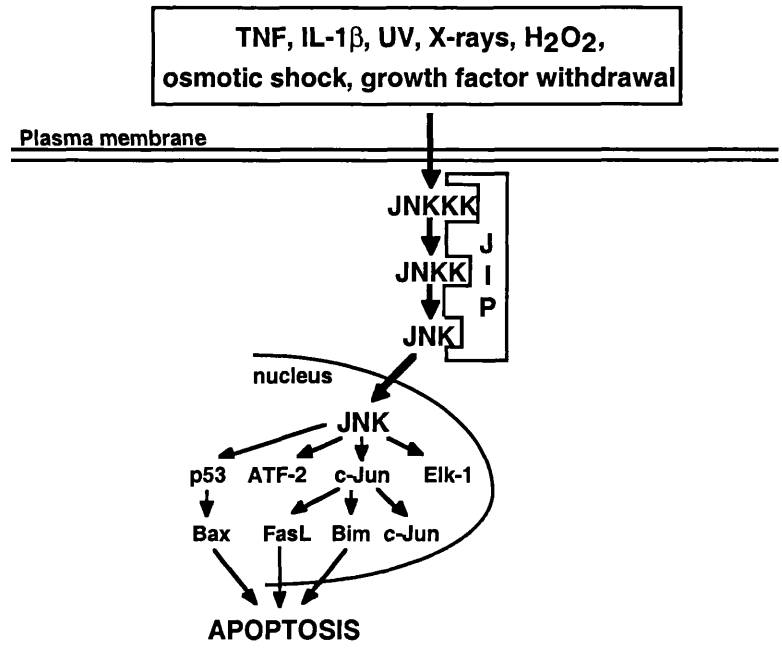


Figure 1.5 The JNK pathway and apoptosis.

The JNK cascade can be activated by cytokines, environmental stresses, osmotic shock and withdrawal of growth factors. Following phosphorylation, JNK translocates to the nucleus where it phosphorylates p53, ATF-2, c-Jun and Elk-1. p53 and c-Jun can activate target genes that promote apoptosis.

1.5 Upstream activators of the JNK pathway

As discussed in 1.4.2.5, the JNK pathway has been shown to be activated in response to a variety of extracellular stimuli, including growth factor withdrawal (Derijard *et al.*, 1994; Kallunki *et al.*, 1994; Kyriakis *et al.*, 1994; Minden *et al.*, 1994; Sluss *et al.*, 1994; Gupta *et al.*, 1996). Recently, several studies have shown that binding of neurotrophins to p75NTR can induce JNK activation (see section 1.5.1). However it is not yet clear what the mechanisms of JNK activation following NGF withdrawal are. There are several lines of evidence indicating that Rho family GTPases mediate the activation of JNK by some stimuli (see section 1.5.2). Some of the signalling transduction pathways that activate Rho GTPases include receptor tyrosine kinases (Schlessinger, 2000), suggesting that Rho proteins may mediate the activation of JNK caused by membrane receptor activation. In addition, some of the kinases activated by the Rho GTPases, such as the PAKs (p21-activated kinases) and the MLKs, have also been shown to play a role in the

activation of the JNK pathway in some cellular systems (see section 1.5.3). The upstream activators of the JNK pathway that were most relevant for the work presented in this thesis are discussed in more detail in the sections below.

1.5.1 The p75 neurotrophin receptor

Neuronal apoptosis is usually regulated by the limited availability of neurotrophins or neurotrophic factors, which are required for both the development and the maintenance of neuronal populations by binding to cell surface receptors. Soluble neurotrophins have been shown to form dimers which then bind to a high affinity receptor complex that generates a signalling phosphorylation cascade (McDonald and Hendrickson, 1993). NGF, for instance, binds to two types of receptors: the tyrosine receptor kinase A (TrkA) and the p75NTR (Meakin and Shooter, 1992; Huber and Chao, 1995; Ross *et al.*, 1996). TrkA and p75NTR can heterodimerise via their extracellular domains and form high-affinity receptors for NGF, whereas both TrkA and p75NTR homodimers bind NGF at a lower affinity (Hempstead *et al.*, 1991). Upon NGF binding, Trk can autophosphorylate thereby initiating a signalling cascade that mediates survival, growth and neuronal plasticity probably through an increase in NF-κB expression. Although the involvement of p75NTR in the formation of high-affinity neurotrophin receptor complex and in the mediation of neuronal survival (in the presence of Trk receptors) is well studied, there is increasing evidence that p75NTR is also involved in neuronal cell death [for review see (Kaplan and Miller, 2000)]. For instance, p75NTR has been shown to mediate apoptosis in neuronal cells and, in the absence of TrkA, in response to NGF (Rabizadeh *et al.*, 1993). In addition, decreased expression of p75NTR in the presence of antisense oligonucleotides increased the survival of DRG neurons (Barrett and Bartlett, 1994), whilst treatment with antibodies that block the binding of NGF to p75NTR inhibited the death of chick retinal ganglion cells which express p75NTR but not TrkA (Frade *et al.*, 1996). Furthermore, overexpression of the transmembrane and cytoplasmic domain of p75NTR in neurons in transgenic mice induced a marked increase in neuronal cell death during development in sensory and sympathetic ganglia (Majdan *et al.*, 1997). Together, these data indicate that p75NTR can signal

to mediate apoptosis and that this mechanism is essential for naturally occurring neuronal death. Moreover, p75NTR knockout mice have increased numbers of sympathetic and forebrain neurons (Van der Zee *et al.*, 1996; Bamji *et al.*, 1998) but also reduced numbers of DRG neurons (Lee *et al.*, 1992) therefore displaying a phenotype of both survival and neuronal cell death. This dual nature of the p75NTR can be explained in the following way: the decreased numbers of DRG neurons is a result of the inability to form high-affinity NGF receptors at the period of target innervation which naturally occurs before birth (Lee *et al.*, 1992; Murray *et al.*, 1999), whereas because the period of innervation for sympathetic neurons normally occurs after birth, these neurons undergo a delayed cell death (Van der Zee *et al.*, 1996; Bamji *et al.*, 1998). These studies suggest that the development and survival of the neurons is based upon the functional interplay of the signals generated by Trk and p75NTR. p75NTR expression is high during development and is downregulated during postnatal development (Yan and Johnson, 1987). However, p75NTR is rapidly induced following ischaemia or nerve lesion (Moix *et al.*, 1991; Lee *et al.*, 1995). Interestingly, in aged rat brain and Alzheimer's patients, high levels of p75NTR are observed in the basal forebrain and hippocampus, correlating with the regions where extensive cell death is observed (Kerwin *et al.*, 1993; Wiley *et al.*, 1995). Taken together, these results suggest that p75NTR might be involved not only in developmental neuronal cell death but also in the mechanisms of pathological neuronal loss.

The signalling molecules recruited by p75NTR, that are important in the mediation of cell death, have only recently started to be identified. These include Zinc finger proteins such as NRIF (neurotrophin receptor interacting factor) and SC-1 (Casademunt *et al.*, 1999; Chittka and Chao, 1999), FAP-1 (Fas-associated phosphatase-1) (Irie *et al.*, 1999), NADE (p75NTR-associated cell death executor) (Mukai *et al.*, 2000), NRAGE (neurotrophin receptor-interacting MAGE homolog) (Salehi *et al.*, 2000) and TRAF family proteins (Khursigara *et al.*, 1999; Ye *et al.*, 1999). Of particular interest are NRIF, NADE and NRAGE. NRIF is a Zinc finger protein containing a nuclear localisation signal and it associates with the cytoplasmic domain of p75NTR. Mice with the *nrif* gene deleted displayed reduced cell death in early retinal cells, indistinguishable from *p75NTR* -/- mice

(Casademunt *et al.*, 1999), demonstrating the importance of NRIF in developmental cell death. NADE is an adaptor molecule that specifically binds the DD of p75NTR (Mukai *et al.*, 2000). Co-expression of NADE with p75NTR induced cell death in 293 HEK cells, PC12 cells ($p75NTR^{+++}$, $TrkA^{+}$) PC12 nnr5 cells ($p75NTR^{++}$, $TrkA^{-}$) and oligodendrocytes ($p75NTR^{+++}$, $TrkA^{-}$) in response to NGF, suggesting that NADE plays an important role in NGF-induced death by transmitting the signal downstream of p75NTR. Overexpression of NRAGE allowed NGF-dependent apoptosis within sympathetic neuron precursor cells (Salehi *et al.*, 2000). This seems to occur because NRAGE competes with TrkA for the same p75NTR binding site. Finally, p75NTR has also been shown to bind and activate the small GTP-binding protein RhoA and neurotrophin binding abolished RhoA activation (Yamashita *et al.*, 1999). This study provided the first evidence that p75NTR can modulate the activity of cytoskeletal proteins in a ligand-dependent manner and it suggests a way in which the morphological changes that occur during apoptosis might be regulated.

Mechanisms of p75NTR induction of apoptosis - a putative role for JNK

As discussed in section 1.4.2.4, signalling through JNK is required for apoptosis of sympathetic neurons, motoneurons and PC12 cells. Recently, several lines of evidence have demonstrated that binding of neurotrophins to p75NTR can induce JNK activation. Firstly, in oligodendrocytes overexpressing p75NTR but not TrkA, JNK activation and apoptosis were induced by binding of NGF to p75NTR (Casaccia-Bonnefil *et al.*, 1996). In addition, NGF-induced apoptosis could be prevented by an alkaloid that blocks the JNK pathway (Yoon *et al.*, 1998). Furthermore, the co-expression of TrkA suppressed JNK activation and induced the activity of ERK1 and blocked NGF-induced apoptosis (Yoon *et al.*, 1998). These results indicate that the expression of TrkA changed the NGF-signalling cascade from JNK to ERK1 and the NGF effect from pro-apoptotic to anti-apoptotic. The outcome of NGF signalling seems therefore to depend on the ratio of particular Trk receptors to p75NTR, suggesting that Trk activation might inhibit p75NTR apoptotic signalling. In sympathetic neurons, which express both p75NTR and TrkA, NGF did not induce c-Jun phosphorylation whilst BDNF (brain derived neurotrophic factor) did (Bamji *et al.*, 1998). It is important to point out that

because p75NTR binds each of the neurotrophins with similar affinity, the specificity of the high-affinity neurotrophin receptor is dependent on Trk (Chao and Hempstead, 1995). Because sympathetic neurons do not express TrkB (the specific receptor for BDNF), BDNF's pro-apoptotic response seems to be mediated by p75NTR.

1.5.2 The Rho family of GTPases

The Rho-subfamily of GTPases form a subgroup of the larger Ras superfamily of small GTP-binding proteins. The Ras superfamily comprises over 50 members which have been classified into 5 sub-families: Ras, Rho, Rab, Arf and Ran (Hall, 1990; Bourne *et al.*, 1991; Wagner and Williams, 1994). These proteins share many common features including sequence homology, similar molecular weights (20-25 kDa) and the ability to bind guanine nucleotides and hydrolyse GTP. The Rho family of proteins is highly conserved in eukaryotes and in mammals and it includes Rho (A, B, C, D, E, F, G), Rac (1, 2, 3), Cdc42 (two splice variants), TC10 and TTF (Ridley, 1996). The best characterised members are RhoA, Rac1 and Cdc42.

1.5.2.1 Primary structure

The main conserved regions within the Rho proteins are: (1) a GTP binding and hydrolysis region (split into four or five separate domains that come together in the protein's tertiary structure), (2) an effector domain, (3) a Rho insert domain and (4) a C-terminus CAAL motif needed for proper membrane localisation (Figure 1.6).

(1) The GTP binding and hydrolysis domains have been deduced from structural similarities to other GTPases and from the analysis of activated and dominant negative mutations and as the name indicates are important for the binding and the hydrolysis of GTP [for review see (Halliday, 1983; Schweins and Wittinghofer, 1994)].

(2) The effector domain was originally defined by mutations in Ras and then by the crystal structures of Rho, Rac and Cdc42 (Hirshberg *et al.*, 1997; Wei *et al.*, 1997; Hoffman *et al.*, 2000). This domain forms an extended β-strand/loop structure which covers a large proportion of one side of the protein. It is thought that

different effectors or regulators of Cdc42, such as guanine nucleotide exchange factors (GEFs), may bind to different subdomains of the effector domain, possibly even simultaneously. In addition, the binding of one effector to this domain could interfere with the binding of other proteins to this domain, providing another way of regulating the Rho-GTPases (see below). The predominant binding partner for the effector domain in Cdc42 and Rac has been shown to be the CRIB (Cdc42/Rac interactive binding) domain which is found in many of their downstream effectors [see below and (Burbelo *et al.*, 1995)]. However, not all proteins that interact with Cdc42 and Rac have a CRIB domain, suggesting that there might be other mechanisms by which proteins interact with these GTPases.
(3) It is the Rho insert domain that makes Rho GTPases unique within the Ras superfamily. This domain contains 13 extra amino acids and has been shown to play a role in Cdc42 interactions with its effector IQGAP and BNIP-2 (McCallum *et al.*, 1996; Wu *et al.*, 1997; Low *et al.*, 2000) and its GDIs (guanine nucleotide dissociation inhibitor) (Wu *et al.*, 1997).

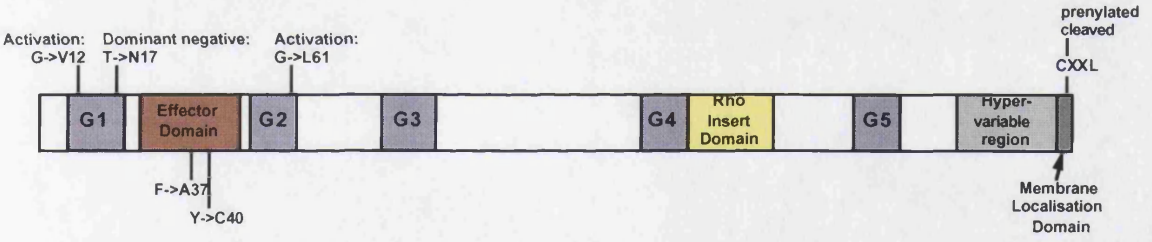


Figure 1.6 General structure of the Rho GTPases.

Regions G1 to G5 are highly conserved and essential for GTP binding and hydrolysis. The amino acids that are commonly mutated and that were the subject of this investigation are noted. All Rho GTPases are geranylgeranylated and methylated except RhoB, which is further modified by a palmitoyl group and RhoE which is farnesylated.

(4) As with the members of the Ras superfamily, all Rho GTPases are modified in the CALL motif by prenylation of a conserved cysteine, four amino acids from the C-terminus, followed by proteolytic cleavage of the last three amino acids and carboxymethylation of the now C-terminal Cys residue (Adamson *et al.*, 1992; Marshall, 1993; Foster *et al.*, 1996). Rho, Rac and Cdc42 are prenylated by a 20-carbon chain geranylgeranyl group (Adamson *et al.*, 1992). Although prenylation

of these proteins is not required for their GTPase activity, it is crucial for their plasma membrane localisation and their biological functions (Glomset and Farnsworth, 1994).

The greatest differences between the Rho-GTPases are found in the C-terminus hypervariable region.

1.5.2.2 Regulation and mediation of the activity of the Rho GTPases

Rho GTPases, like Ras proteins, function as molecular switches which regulate the transmission of an upstream signal to downstream effectors. These proteins are thought to interact with their downstream effectors only when bound to GTP and this interaction is terminated following a conformational change induced by the hydrolysis of GTP. The GTP hydrolysis *per se* is an irreversible process, however the reversibility of the conformational change can be achieved by the exchange of GDP for GTP. It is these two opposing reactions that make this cycling mechanism possible (Figure 1.7).

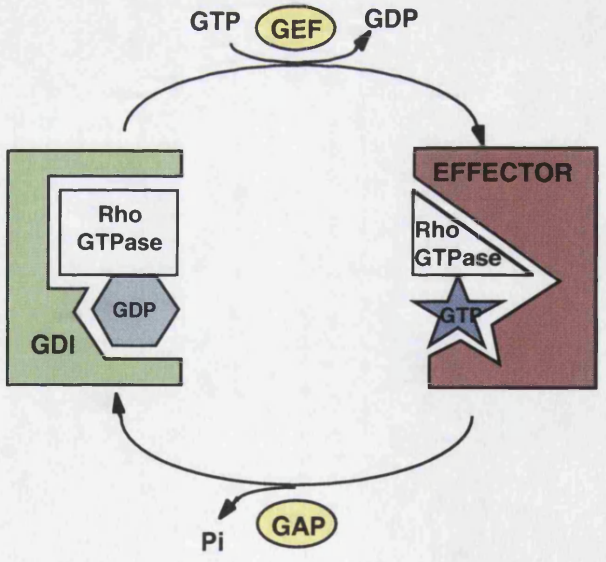


Figure 1.7 The GTPase molecular switch.

Rho GTPases are inactive when bound to GDP, however exchange of GDP for GTP by a GEF converts the GTPase into an active conformation which allows it to interact with a downstream effector. This interaction is terminated following a conformational change induced by the hydrolysis of GTP which is catalysed by a GAP. The intrinsic or catalysed GTP hydrolysis activity ensures that the GTPase only remains active for a limited amount of time.

1.5.2.2.1 Regulators

The interconversion between the GTP and the GDP-bound forms is mediated by three types of regulatory proteins: the GTPase activating proteins (GAPs), the guanine nucleotide exchange factors (GEFs) (Whitehead *et al.*, 1997) and the guanine dissociation inhibitors (GDIs) (Fukumoto *et al.*, 1990).

The GAPs catalyse the GTP hydrolysis (Boguski and McCormick, 1993). To date, more than 25 members of Rho-GAPs have been identified from yeast to human and they include p50 Rho-GAP, Bcr, Abr, N-chimaerin, β-chimaerin, p190GAP, RalBP1 and Graf, to mention only a few (Diekmann *et al.*, 1991; Settleman *et al.*, 1992; Barfod *et al.*, 1993; Leung *et al.*, 1993; Tan *et al.*, 1993; Lancaster *et al.*, 1994; Cantor *et al.*, 1995; Hildebrand *et al.*, 1996). The substrate specificity of the Rho GAPs towards members of the Rho subfamily varies with each GAP protein as well as their tissue distribution. For instance p50 Rho-GAP is expressed ubiquitously and Cdc42 is its preferred GTPase, whereas N-chimaerin is only expressed in the brain and is specific for Rac (Diekmann *et al.*, 1991; Barfod *et al.*, 1993; Lancaster *et al.*, 1994).

The GEFs disrupt the Mg^{++} and nucleotide binding sites thereby releasing the bound GDP which is rapidly exchanged for GTP due to the high intracellular ratio of free GTP/GDP in vivo. The GEFs stabilise the Rho-GTPases in a nucleotide free state required for further formation of an active GTP-bound state (Whitehead *et al.*, 1997). Like the GAPs, GEFs also display specificity towards different GTPases and tissue distribution. For instance, Lbc is specific for Rho and expressed in heart, lung and skeletal muscle, whereas FGD1 is specific for Cdc42 and expressed in brain, heart, lung and kidney (Glaven *et al.*, 1996; Zheng *et al.*, 1996). Recent studies have shown that the Rho GEF proteins have functions similar to their respective GTPases when overexpressed in cells. For instance, microinjection of Lbc induced stress fibre formation, whilst overexpression of FGD1 in Swiss 3T3 fibroblasts induced filopodia, stimulated G1 cycle progression and activation of the JNK and p70 S6 kinases which are typical responses to Rho and Cdc42 overexpression respectively (Olson *et al.*, 1996; Zheng *et al.*, 1996; Nagata *et al.*, 1998). There is also recent evidence suggesting that different GEFs for the same GTPase modulate its downstream events differentially. Both Dbl and

FDG1 are GEFs for Cdc42 but Dbl stimulated PAK1 activation to a higher degree than it could stimulate JNK activation, whereas FGD1 stimulated JNK activation but not PAK1 (Zhou *et al.*, 1998). However, despite this wealth of information on Rho GEFs, the processes that regulate them and those that they regulate have not yet been elucidated.

The guanine dissociation inhibitors (GDIs) bind to the GDP-bound form of Rho GTPases and stabilise the GDP-conformation by inhibiting the dissociation of GDP (Fukumoto *et al.*, 1990; Ueda *et al.*, 1990). However, later studies have shown that Rho GDIs could also associate with GTP-bound Rho, Rac and Cdc42 and inhibit the intrinsic GAP-stimulated GTPase activity by stabilisation of Mg^{++} in the nucleotide binding pocket (Hart *et al.*, 1992; Chuang *et al.*, 1993; Scheffzek *et al.*, 2000). In addition to this, Rho GDIs play an important role in the subcellular localisation of the Rho GTPases. Indeed, the Rho proteins are found in the cytoplasm when coupled to GDIs, but upon activation they are released from the GDI complex and are translocated to the membranes (Takai *et al.*, 1995). To date, three types of GDIs have been identified: (1) Rho GDI (Rho GDIα), the first identified, is ubiquitously expressed (Fukumoto *et al.*, 1990; Ueda *et al.*, 1990); (2) D4-GDI (Rho GDIβ) predominantly found in haematopoietic tissues (Lelias *et al.*, 1993; Scherle *et al.*, 1993); and (3) Rho GDIγ, mainly expressed in the brain and pancreas (Adra *et al.*, 1997). There is still little knowledge of the physiological functions of Rho GDIs. They have been shown to inhibit some downstream functions of Rho (Nishiyama *et al.*, 1994; Coso *et al.*, 1995) and more recently, D4-GDI was found to be a substrate for caspase-3 (Na *et al.*, 1996). However, not all Rho-GDIs are cleaved by caspase-3 (Essmann *et al.*, 2000). The cleavage of Rho-GDI (or perhaps even other GDIs) by caspases during apoptosis may therefore change the activity and the signalling by Rho-family GTP binding proteins.

1.5.2.2.2 Downstream effectors

To better understand the mechanisms underlying the various biological functions of the Rho GTPases, a lot of effort has been put towards identifying their downstream effectors (Van Aelst and D'Souza-Schorey, 1997). So far, several effectors have been identified for each member of the Rho GTPases (Figure 1.8)

Yeast two-hybrid system screens, combined with a ligand overlay assay with [^{35}S]GTPγS-Rho (Watanabe *et al.*, 1996), led to the identification of a number of Rho targets including PKN (Amano *et al.*, 1996), rhophilin (Watanabe *et al.*, 1996) and rhotekin (Reid *et al.*, 1996). Although both rhophilin and rhotekin share the same binding motif for Rho as PKN, they do not have a catalytic domain suggesting that they will have different activities. More recently, a kinase called p160ROCK (Rho-associated coiled-coil containing kinase) was isolated (Ishizaki *et al.*, 1996). Isoenzymes of p160ROCK such as ROKα and Rho kinase have also been identified as well as the myosin-binding subunit (MBS) of myosin phosphate (Leung *et al.*, 1995; Kimura *et al.*, 1996; Matsui *et al.*, 1996). In addition to these proteins which were shown to bind GTP-Rho, other molecules were found to be activated by GTP-Rho in cell lysates. They include PI3K, PI-4-phosphate 5-kinase and phospholipase D (Zhang *et al.*, 1993; Chong *et al.*, 1994; Malcolm *et al.*, 1994; Kuribara *et al.*, 1995).

Several putative effector molecules for Rac and Cdc42 have been identified and, curiously, many of these molecules are specific for Rac or Cdc42 or are able to associate with both (Figure 1.8). The realisation that all PAK proteins as well as p120ACK, a specific target of Cdc42 (Manser *et al.*, 1993), share an homologous putative interactive site for Cdc42/Rac (Manser *et al.*, 1994), led to the identification of a minimal conserved 16 amino acid motif important for such interactions: the CRIB domain (Burbelo *et al.*, 1995). A GeneBank™ data base search for proteins containing this CRIB motif identified over 25 proteins including the human MLK2/3, WASP (Wiskott-Aldrich syndrome protein) and MSE55 proteins. The WASP does not contain a kinase domain and rather functions as a scaffold protein linking Cdc42 to the actin cytoskeleton (Aspenstrom *et al.*, 1996; Symons *et al.*, 1996). Furthermore, patients with Wiskott-Aldrich syndrome have many immunological defects, including thrombocytopenia with small platelets, eczema and T- and B-lymphocyte defects [for review see (Kirchhausen and Rosen, 1996; Featherstone, 1997; Ochs, 1998)]. These defects have been linked to mutations within the X-linked recessive gene *WASP* and also to defects in cellular actin cytoarchitecture (Kenney *et al.*, 1986; Derry *et al.*, 1994; Kolluri *et al.*, 1995; Kwan *et al.*, 1995; Villa *et al.*, 1995).

In addition to the CRIB containing proteins, other proteins have been identified that interact with Cdc42 and/or Rac. For instance, POR1 (Partner Of Rac), interacts with Rac in a GTP-dependent manner and was found to be important in Rac-induction of membrane ruffles (Van Aelst *et al.*, 1996). Similarly, POSH (Plenty Of SH3s), also isolated through a yeast two-hybrid screen, was shown to act as a scaffold protein mediating Rac-induced activation of the JNK pathway and cell death (Tapon *et al.*, 1998). In addition, the regulatory subunit of PI3K, p85, was shown to bind GTP-bound Cdc42 and Rac1, suggesting that PI3K may be a downstream effector (Zheng *et al.*, 1994; Tolias *et al.*, 1995). Furthermore, the 70-kDa S6 kinase was shown to complex and to be activated by Rac1 and Cdc42 (Chou and Blenis, 1996) and $p67^{Phox}$ was identified as the target for Rac-mediated activation of the NADPH oxidase (Diekmann *et al.*, 1994).

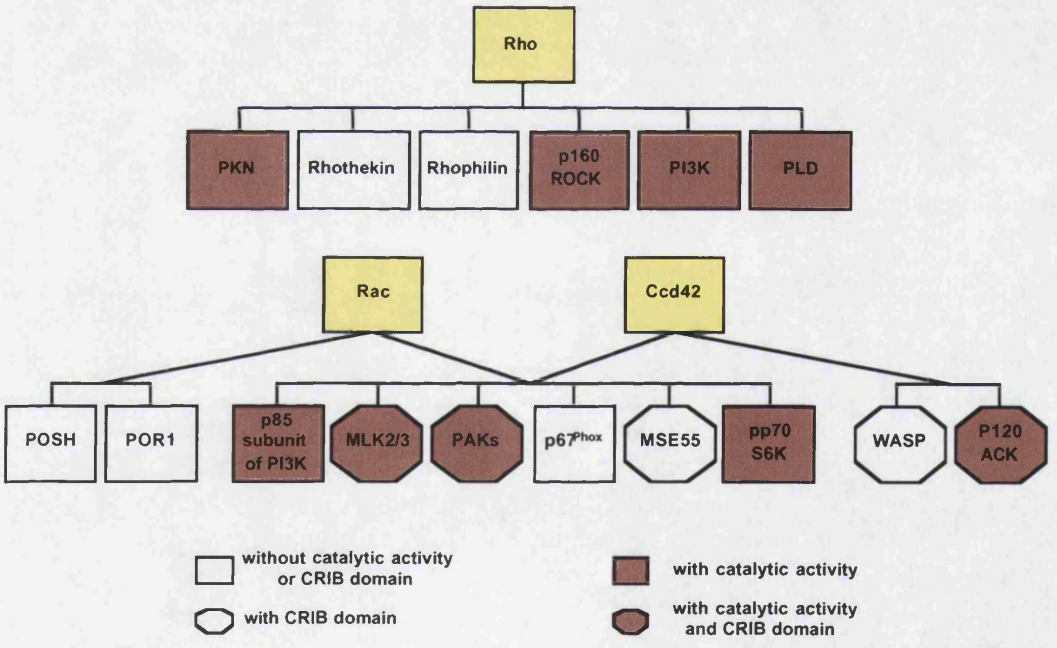


Figure 1.8 Mammalian targets of Rho, Rac and Cdc42.

The kinases PKN and the non-kinases Rhothekin and Rhophilin contain an homologous Rho-binding motif. Rac and Cdc42 share many common targets which may or may not contain a CRIB domain and/or catalytic activity. In addition, they also have specific binding partners.

1.5.2.3 Biological functions

The Rho GTPases have been shown to regulate a variety of cellular functions including cytokinesis (Kishi *et al.*, 1993; Drechsel *et al.*, 1997), activation of NADPH oxidase (Abo *et al.*, 1992; Diekmann *et al.*, 1994; Knaus *et al.*, 1995), vascular contraction (Seasholtz *et al.*, 1999), cell adhesion and motility (Stasia *et al.*, 1991; Takaishi *et al.*, 1993; Keely *et al.*, 1997), cell polarity (Eaton *et al.*, 1996; Eaton, 1997), transformation (Perona *et al.*, 1993; Khosravi-Far *et al.*, 1995; Qiu *et al.*, 1995), cell invasion (Michiels *et al.*, 1995; Keely *et al.*, 1997; Banyard *et al.*, 2000), vesicle formation and transport (Norman *et al.*, 1996), endocytosis (Lamaze *et al.*, 1996), nuclear signalling (Hill *et al.*, 1995; Perona *et al.*, 1997) and apoptosis (Esteve *et al.*, 1995; Jimenez *et al.*, 1995; Moorman *et al.*, 1996; Na *et al.*, 1996; Chuang *et al.*, 1997; Bazenet *et al.*, 1998; Subauste *et al.*, 2000).

1.5.2.3.1 Actin Rearrangements

Rho GTPases are best known as key regulators of cytoskeletal organisation. However, Rho, Rac, and Cdc42 regulate distinct cytoskeletal events in fibroblasts: lysophosphatidic acid activation of Rho induces the assembly of focal adhesions stress fibres (Ridley *et al.*, 1992); stimulation of Rac by platelet-derived growth factor and insulin induces the formation of membrane ruffles and lamellipodia (Ridley *et al.*, 1992); and activation of Cdc42 by bradykinin induces the formation of filopodia (Kozma *et al.*, 1995; Nobes and Hall, 1995). In addition, these three small GTPases were found to be organised in a cascade whereby activation of Cdc42 leads to activation of Rac and subsequently to activation of Rho (Nobes and Hall, 1995). Rho GTPases would therefore be expected to be involved in biological functions that require a co-ordinated rearrangement of the actin cytoskeleton and this has proven to be the case. A good example of this is in neurite outgrowth and retraction. Indeed, neurite extension and retraction is thought to be mediated by actin polymerisation and depolymerisation in growth cones (the tip of a growing neurite which bears filopodia and lamellipodia). This neurite flexibility of growth and retraction is thought to be crucial for the development of the nervous system. Rho, Rac and Cdc42 mediate these responses in a competing manner. Rho is involved in thrombin-, serum and LPA-induced neurite retraction and cell

rounding in N1E-115 neuroblastoma and PC12 cells (Nishiki *et al.*, 1990; Jalink and Moolenaar, 1992) probably by mediating contraction of the cortical actin-myosin system (Jalink *et al.*, 1994); whereas Rac and/or Cdc42 are involved in neurite extension by mediating filopodia and lamellipodia formation in growth cones (Kozma *et al.*, 1997). In addition, they have also been shown to play a role in phagocytosis (Adam *et al.*, 1996; Cox *et al.*, 1997; Caron and Hall, 1998) and gastrulation in *Drosophila* (Barrett *et al.*, 1997).

1.5.2.3.2 JNK and p38 MAP kinase pathways and transcriptional activation

Members of the Rho subfamily play important roles in linking cell surface receptors to MAP kinases, thereby transducing extracellular signals to regulate intracellular events, such as the transcription of new genes required for the execution of several cellular processes [for review see (Vojtek and Cooper, 1995)]. These processes include stress and inflammatory responses, differentiation, cell growth and apoptosis to name only a few (Denhardt, 1996; Clerk *et al.*, 1998). The stress and inflammatory response pathways involves the JNKs and p38 kinase (Kyriakis *et al.*, 1994; Lee *et al.*, 1994). Several independent groups have shown that Cdc42 and Rac (but not Rho) mediate the activation of JNK and p38 kinase. Overexpression of constitutively active mutants of Rac and Cdc42 in COS-7 fibroblasts led to a 5- to 10-fold increase in the immunoprecipitated JNK and p38 in vitro activities, with little or no effect on Ras dependent MAP kinase (ERK) activity (Coso *et al.*, 1995; Teramoto *et al.*, 1996a). Similar results were obtained in COS-1 cells (Bagrodia *et al.*, 1995), HeLa , NIH-3T3 (Minden *et al.*, 1995) and 293 human embryonic kidney cells (Teramoto *et al.*, 1996b), suggesting that the activation of JNK and p38 kinase pathways by Cdc42 and Rac can occur in most mammalian cells.

Small GTP-binding proteins have been shown to induce transcriptional activity in a variety of cases. For instance, a C-terminal region of the polycystic kidney disease I (PKD1) gene was recently shown to induce c-Jun/AP-1 transcriptional activation through JNK activity and this induction was blocked by co-expression of dominant negative forms of Rac and Cdc42 (Arnould *et al.*, 1998). These findings indicate that small GTP-binding proteins mediate PKD1-induced

JNK/AP-1 activation. Furthermore, in primary rat hepatocytes, the hyperosmotic-glucose-, TNF-α- and hepatocyte growth factor-induced stimulation of JNK1 activity and phosphorylation of c-Jun were inhibited by dominant negative mutants of Ras, Cdc42 and Rac (Auer *et al.*, 1998). In addition, Rho GTPases were shown to regulate the *c-fos* SRE (Hill *et al.*, 1995), and constitutively active Rho kinase was shown to stimulate the transcriptional activity of *c-fos* SRE (Chihara *et al.*, 1997). Finally, overexpression of RhoA, Cdc42 and Rac1 can also efficiently activate NF-κB (Sulciner *et al.*, 1996; Perona *et al.*, 1997). This induction was blocked by co-expression of the IκBα inhibitory subunit and TNF-α activation of NF-κB was inhibited by dominant negative mutants of Cdc42 and RhoA but not of Rac1 (Perona *et al.*, 1997). The above studies support a model in which the Rho GTPases mediate the activation of JNK and p38 kinase, leading to the translocation of these kinases to the nucleus where they phosphorylate different transcription activators.

1.5.2.3.3 Regulation of apoptosis

The first observation suggesting the involvement of the Rho GTPases in apoptosis came from the finding that overexpression of Rho proteins enhanced apoptosis seen in serum deprived murine NIH3T3 fibroblasts (Jimenez *et al.*, 1995). This activation of apoptosis was shown to be linked to the production of ceramides (Esteve *et al.*, 1995) which are lipids thought to mediate apoptosis through the inhibition of cell growth (Obeid and Hannun, 1995). However, because ceramide added to the cells was only able to induce apoptosis in RhoA-overexpressing cells, it led to the hypothesis that the Rho GTPases control two complementary signals (Lacal, 1997): (1) one pathway which generates ceramide and is involved in the progression towards apoptosis; and (2) one which controls the JNK pathway and makes the cells competent to interpret the ceramide-dependent signal (Verheij *et al.*, 1996). Indeed, stimulation of the Fas receptor or treatment with synthetic ceramides in Jurkat cells leads to a Ras- and Rac-dependent activation of JNK and p38 kinase (Brenner *et al.*, 1997). Furthermore, as stated above, there is increasing evidence that Cdc42 and Rac can mediate the activation of JNK and, since JNK has been implicated in the induction of

apoptosis in a number of cell systems, it is likely that these small GTPases can function as the upstream mediators of the death stimulus.

1.5.2.3.4 Role in disease

Any direct changes within a Rho GTPase, such as a mutation or even changes in any of their regulators, are likely to disturb their normal biological actions. To date however, no mutations within these GTPases have been directly identified as the major cause of a particular disease whilst changes/mutations within their direct regulators have. For instance, mutations within some of their GEFs, such as Dbl, have been implicated in cancer; FGD1 and Vav have been linked to developmental malformations; and Tiam-1 was shown to be important in tumour invasiveness. Also, some of their direct downstream effectors have been implicated in diseases. For example, WASP is implicated in a familial immunological disorder; the PKD1 protein plays a role in autosomal dominant polycystic kidney disease (Arnould *et al.*, 1998); and the myotonic dystrophy kinase-related Cdc42-binding kinase (MRCK) interacts with Cdc42 in the regulation of actin rearrangements (Leung *et al.*, 1998). Interestingly, Cdc42 and Rac have been shown to be upregulated in neuronal populations in patients with Alzheimer's Disease (AD), when compared to age-matched controls, and this increase in Cdc42 and Rac1 correlated with early cytoskeletal changes (Zhu *et al.*, 2000). This study suggests that these small GTPases may contribute to the pathogenic process and neurodegeneration in AD given the involvement of Cdc42 and Rac in many of the cellular processes known to be disturbed in AD such as cytoskeletal organisation, oxidative balance and oncogenic signalling [for review see (Anderton *et al.*, 1998; Terry, 1998; Raina *et al.*, 2000; Smith *et al.*, 2000)].

1.5.3 MAPK cascades

Signal transduction networks allow cells to respond to signals from the extracellular environment by executing an appropriate response. MAPK cascades play an essential role in a variety of biological processes such as cell differentiation, cytokinesis, cell movement and cell death. These kinase cascades are usually organised in a three-kinase layer made up of a MAPK, a MAPKK and a MAPKKK, where each downstream kinase serves as a substrate for the upstream

kinase. So far, in mammals, three MAPK modules have been identified and they include the ERK1/2/3 cascades which regulate growth and differentiation, p38 MAPK and JNK cascades which regulate stress responses such as inflammation and apoptosis [for review see (Robinson and Cobb, 1997; Ip and Davis, 1998; Lewis *et al.*, 1998)]. The different MAPK modules can usually signal independently of each other, however individual components may also participate in more than one signalling pathway.

Protein kinases have a catalytic or kinase domain consisting of 250-300 amino acid residues. The kinase domains are divided into 12 subdomains which are conserved in over 95% of the members of this superfamily of proteins [reviewed in (Hanks and Quinn, 1991)]. The three main functions of the kinase domain are binding and orientation of the ATP/GTP phosphate donor in complex with Mg^{++} or Mn^{++}; binding and orientation of the protein substrate; and transfer of the γ-phosphate from ATP/GTP to the acceptor hydroxyl residue (serine, threonine or tyrosine) of the substrate. The JNKs are activated by phosphorylation on threonine and tyrosine of the Thr-X-Tyr activation motif by either MKK4 (Sanchez *et al.*, 1994) or MKK7 (Tournier *et al.*, 1997). These kinases are in turn activated by a MAPKKK. Presently, eleven different MAPKKKs have been shown to be upstream activators of the JNK pathway (Widmann *et al.*, 1999): MEKK1 (Lange-Carter *et al.*, 1993), MEKK2 (Blank *et al.*, 1996), MEKK3 (Blank *et al.*, 1996), MTK1/MKK4 (Gerwins *et al.*, 1997; Takekawa *et al.*, 1997), Tpl-2/Cot (Aoki *et al.*, 1991; Salmeron *et al.*, 1996), MLK2/MST (Dorow *et al.*, 1995; Hirai *et al.*, 1997), MLK3/SPRK/PTK-1 (Gallo *et al.*, 1994; Ing *et al.*, 1994; Rana *et al.*, 1996), MUK/DLK/ZPK (Holzman *et al.*, 1994; Reddy and Pleasure, 1994; Hirai *et al.*, 1996), TAK1 (Yamaguchi *et al.*, 1995), ASK1/MAPKKK5 (Wang *et al.*, 1996; Ichijo *et al.*, 1997) and ASK2/MAPKKK6 (Wang *et al.*, 1998d). In addition, in certain MAPK modules there appears to be a group of kinases that functions upstream of the MAPKKKs (Kyriakis, 1999; Widmann *et al.*, 1999). Some of these MAPKKKKs can activate JNK and they include PAK1, 2, 3 and 4 (Manser *et al.*, 1994; Knaus *et al.*, 1995; Abo *et al.*, 1998), GCK (germinal centre kinase) (Katz *et al.*, 1994), HPK1 (haematopoietic protein kinase) (Kiefer *et al.*, 1996), NIK (Nck-interacting kinase) (Su *et al.*, 1997) and MST1 (mammalian Ste20) (Creasy and Chernoff, 1995).

For the purpose of this thesis, because the PAKs were the most upstream kinases of the JNK pathway that were also a target for Cdc42 and Rac, they appeared as likely candidates for the mediation of a signal from these GTPases to JNK.

1.5.3.1 PAKs

The p21-activated kinases (PAKs) were the first mammalian members of the Ste20-like family of serine/threonine kinases to be identified (Knaus *et al.*, 1995). PAK1 was the first kinase shown to be a target for Cdc42 and Rac1 (Manser *et al.*, 1994) and consequently drew much attention. To date, six mammalian isoforms of the PAKs have been identified: p65PAK/PAK1 or rat α-PAK (68 kDa), PAK2 or rat γ-PAK (62 kDa), PAK3 or rat β-PAK β (65 kDa), PAK4 (68 kDa), PAK5 and PAK6 (Bagrodia *et al.*, 1995; Knaus *et al.*, 1995; Manser *et al.*, 1995; Martin *et al.*, 1995a; Brown *et al.*, 1996; Abo *et al.*, 1998; Wagner *et al.*, 1999; Yang and Sun, 2000). In addition, *Drosophila* (DPAK) (Harden *et al.*, 1996), *C. elegans* (CePAK) (Chen *et al.*, 1996a) and *Xenopus* (X-PAK1) (Faure *et al.*, 1997) homologues have also been identified. PAK1 and PAK3 are expressed mainly in the brain, PAK2 seems to be ubiquitously expressed in all tissues, PAK4, although detected in all of the tissues tested so far, seems to be most highly expressed in prostate, testis and colon (Abo *et al.*, 1998) and PAK5 and PAK6 expression patterns remain to be characterised.

1.5.3.1.1 Structure

PAK1, 2 and 3 are very similar in structure and contain an N-terminal regulatory domain and a C-terminal kinase domain. They also show 73% overall sequence similarity and approximately 92% sequence homology within the kinase domain (Sells *et al.*, 1997). PAK4, 5 and 6 form another subgroup of this growing family of kinases and except for the kinase domain which shares about 50% sequence homology with the other PAKs and the CRIB domain, they are entirely different from the other PAKs (Abo *et al.*, 1998; Wagner *et al.*, 1999; Yang and Sun, 2000). In this investigation only PAK1 and PAK2 were studied and therefore from hereon the structure of this particular subgroup of the PAKs only, will be described.

The CRIB domain (Burbelo *et al.*, 1995) localises to the N-terminal regulatory domain and is required for the interaction with Cdc42 and Rac1. In addition to the CRIB domain, the regulatory domain of the PAKs contains two to four proline-rich regions which are potential binding sites for SH3 domains (Bagrodia *et al.*, 1995; Bokoch *et al.*, 1996; Galisteo *et al.*, 1996). The PAKs also have a conserved stretch of acidic residues C-terminal of the CRIB domain (Figure 1.9). However, its function is still unknown.

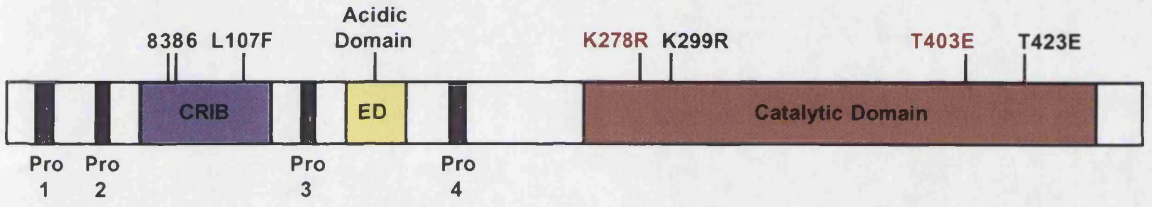


Figure 1.9 General structure of PAK1-3.

The PAK N-terminal regulatory domain consists of two to four proline rich domains (Pro) (PAK1=3, PAK2=2, PAK3=4), a Cdc42-Rac1 interactive binding domain (CRIB) and an acidic region (ED). Certain mutations within the CRIB domain (e.g. at positions 83/86 and 107 in PAK1) block Cdc42/Rac1 binding and result in a moderate activation of PAK or abolish the need for GTPase activation and result in a potent activation. A mutation within the kinase domain at the conserved threonine at position 423/403 (in PAK1 and PAK2 respectively) to glutamate renders PAK constitutively active, whilst a lysine to arginine mutation at position 299/278 renders PAK1 and 2, respectively, catalytically inactive.

1.5.3.1.2 Regulation

The PAKs have been shown to be activated via distinct mechanisms:
(1) Binding of GTP-bound Cdc42/Rac1 to the CRIB domain of PAK is thought to induce a conformational change in PAK releasing it from the autoinhibition of the C-terminal domain, leading to autophosphorylation and enabling the phosphorylation of exogenous substrates (Manser *et al.*, 1994; Manser *et al.*, 1995; Martin *et al.*, 1995a). In PAK1, upon binding of GTP-Cdc42, six serines in the regulatory N-terminal region and a single threonine in the catalytic domain are phosphorylated (Manser *et al.*, 1997).

(2) Adapter proteins, such as Nck, can mediate membrane localisation and thereby activation of PAK1. Nck interacts with the first proline rich region of PAK1 and is thought to mediate the association of PAK1 with specific lipids, such as sphingosine or phosphatidic acid (the formation or metabolism of which can be induced upon membrane receptor stimulation), which stimulate PAK1 activity. Additionally, the fusion of a membrane targeting sequence to PAK1 induced its translocation to the membrane and increased PAK1 activity (Lu *et al.*, 1997a). Furthermore, this stimulation was only partially dependent on PAK binding to an activated GTPase (Bokoch *et al.*, 1998). It is therefore possible that once PAK is recruited to the membrane via GTPases or an adaptor molecule such as Nck, its activity can be further regulated by direct interaction with lipids in the membrane. (3) PAK2, but not the other PAKs, has been shown to be activated through cleavage by caspases in Jurkat cells undergoing apoptosis (Lee *et al.*, 1997; Rudel and Bokoch, 1997). This cleavage produces an intact 28 kDa N-terminal domain and a constitutively active 34 kDa C-terminal catalytic domain. Walter *et al.* (Walter *et al.*, 1998) have shown that autophosphorylation of the regulatory and catalytic domains is required for activation of PAK2 following caspase cleavage.

1.5.3.1.3 Biological functions

Like the Rho GTPases, the PAKs are involved in a variety of cellular processes such as cytoskeletal organisation, neurite outgrowth, NADPH oxidase regulation, MAPK signalling (p38, JNK and ERK), cell cycle, apoptosis and survival.

The first evidence for the involvement of PAK in the regulation of the cytoskeleton came from studies in which microinjection of activated mutants of PAK1 into Swiss 3T3 fibroblasts induced the formation of lamellipodia and membrane ruffles like those induced by Rac1 and Cdc42 (Sells *et al.*, 1997). PAK1, when targeted to the membrane of PC12 cells, has also been shown to induce neurite outgrowth (Daniels *et al.*, 1998). This was shown to be independent of the catalytic domain and of binding to Cdc42 and/or Rac1, but dependent on the regulatory domain (Manser *et al.*, 1998).

When activated forms of both PAK1 and PAK3 were transfected into COS cells, they were shown to activate the JNK but not the ERK pathways (Bagrodia *et al.*, 1995; Polverino *et al.*, 1995; Zhang *et al.*, 1995; Brown *et al.*, 1996). In addition, JNK stimulation by Cdc42 and Rac1 could be blocked by overexpression of kinase inactive forms of PAK1, suggesting that JNK activation by Cdc42 and Rac1 is mediated by the PAKs (Zhang *et al.*, 1995). However, some groups have shown that the PAKs are not necessary for JNK activation, but rather inhibit JNK activation by Cdc42 (Teramoto *et al.*, 1996a). The PAKs have also been shown to phosphorylate upstream components of the signalling pathway, such as MEK1, leading to ERK activation (Frost *et al.*, 1996; Tang *et al.*, 1997). Hence, at present the roles of the PAKs in MAPK pathways are rather controversial.

As explained above, PAK2, unlike PAK1 and PAK3, is proteolytic cleaved by caspase-3 in Jurkat T-cells undergoing Fas-induced apoptosis (Lee *et al.*, 1997; Rudel and Bokoch, 1997). Overexpression of the constitutively active C-terminal fragment of PAK2 triggered apoptosis in CHO and HeLa cells (Lee *et al.*, 1997). In addition, dominant negative forms of PAK2 blocked Fas-induced apoptosis in CHO cells stably expressing a CD4-Fas chimera. These studies provided direct evidence that PAK2 plays a crucial role in the morphological changes occurring during apoptosis. The fact that the PAKs have been seen to mediate JNK activation, as described above, suggested that their role in mediating cell death could also occur via the JNK pathway. Indeed, Rudel *et al.* (Rudel *et al.*, 1998) showed that PAK2 activity is required for activation of the JNK pathway and apoptosis by Fas receptor in Jurkat T cells and that PAK2 activation was dependent on caspase activity.

In addition to playing a role in apoptosis, some PAKs, have also been shown to be anti-apoptotic and therefore involved in survival. The first evidence came from a study carried out in *Xenopus* oocytes whereby microinjection of a kinase dead mutant of *Xenopus* PAK1 (X-PAK1), facilitated the escape from the G_2 cell cycle arrest and rapidly induced apoptosis of these oocytes (Faure *et al.*, 1997). More recently, overexpression of a constitutively active mutant of PAK1 blocked IL-3 withdrawal-induced apoptosis (Schurmann *et al.*, 2000). The survival function of PAK1 seems to be mediated by phosphorylation of Bad on Ser112 and

Ser136 (Schurmann *et al.*, 2000), which reduces its interaction with Bcl-2 or Bcl-x_L and increases its association with 14-3-3, thereby inhibiting the pro-apoptotic effects of Bad (see section 1.4.2.2). A recent study by Tang *et al.* (Tang *et al.*, 2000) has shown that activated Akt stimulated PAK1, whereas a dominant negative Akt inhibited Ras activation of PAK1, placing PAK1 downstream of Akt. Hence, at present, the current model for the survival promoting function of PAK1, is a Ras→ PI3K→ Akt→ PAK1→ Bad pathway, whereby PAK1 promotes survival by inhibiting apoptosis. The above mentioned studies demonstrate that different members of the PAK family can either have pro- or anti-apoptotic functions.

1.5.3.1.4 Role in disease

Because the PAKs are involved in so many biological processes such as those illustrated above, they are likely to be involved in many pathologies such as cancers (either through regulation of the cell cycle or through decreased apoptosis), autoimmune disorders, developmental abnormalities, neurodegenerative diseases and perhaps many others. An interesting observation is that point mutations in the PAK3 gene have been linked to patients with an X-linked mental retardation syndrome (MRX), although the exact contribution of this has not yet been described (Allen *et al.*, 1998; Bienvenu *et al.*, 2000). Another interesting observation is that p35/Cdk5 kinase activity has been implicated in the pathology of Alzheimer's disease (Lee *et al.*, 2000) and this neuron specific kinase has been shown to hyperphosphorylate PAK1 thereby inhibiting it (Nikolic *et al.*, 1998). It has been suggested that this transient regulation of PAK1 might have implications at the level of neurite outgrowth and neuronal migration during development (Nikolic *et al.*, 1998). It is therefore tempting to speculate that PAK1 may also have implications in the disruption of the neuronal cytoskeleton observed in patients with Alzheimer's disease.

1.5.3.2 ASK1

Apoptosis signal-regulating kinase 1 (ASK1) was first identified using a degenerate polymerase chain reaction (PCR)-based strategy designed to identify serine-threonine kinases (Ichijo *et al.*, 1997).

1.5.3.2.1 Structure

ASK1 is a ubiquitously expressed, 155 kDa protein composed of various functional domains (Figure 1.10). In addition to its kinase domain, ASK1 was found to contain a motif for the binding of an FK506-binding protein (FKBP), of which the function is still unknown.

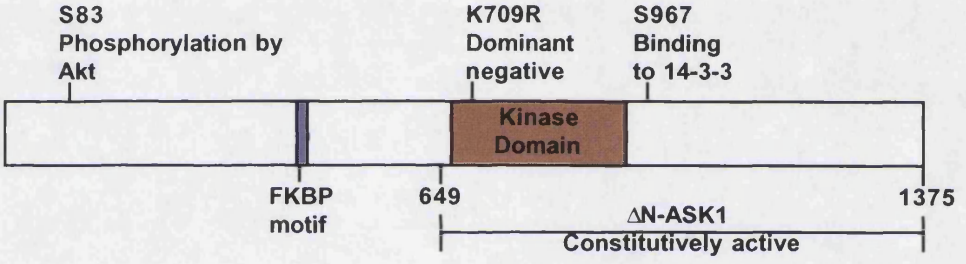


Figure 1.10 General structure of ASK1

The kinase domain of ASK1 has sequence similarity to members of the MAPKKK family (e.g. 30% similar to MEKK1, 30.4% similar to Ste11). A mutation on the lysine residue at position 709 to arginine (K709R) renders ASK1 constitutively inactive. Deletion of the first 648 N-terminal amino acids (ΔN-ASK1) renders ASK1 constitutively active. A consensus Akt phosphorylation site is found at serine 83 (RGRGS^{83}V). The RSIS^{967}LP motif is a recognition motif for 14-3-3, where serine 967 seems to play a crucial role.

1.5.3.2.2 Biological functions and regulation

Activation of JNK and p38 kinase pathways and induction of apoptosis

ASK1 has been found to activate the JNK and p38 signalling pathways. In COS-7 cells, ASK1 expression induced 7.6 and 5.0 fold activation of JNK and p38 MAPKs, respectively (Ichijo *et al.*, 1997). In addition, overexpression of ASK1 induced apoptotic cell death in mink lung epithelial (Mv1Lu) cell lines and endogenous ASK1 was activated in cells treated with TNF-α (Ichijo *et al.*, 1997; Nishitoh *et al.*, 1998; Saitoh *et al.*, 1998), serum withdrawal (Saitoh *et al.*, 1998),

anti-Fas antibody (Chang *et al.*, 1998) and treatment with anti-cancer drugs such as paclitaxel and docetaxel (Wang *et al.*, 1998c). Furthermore, TNF-α and Fas-induced apoptosis were inhibited by the dominant negative ASK1 (Ichijo *et al.*, 1997; Chang *et al.*, 1998). Altogether, these results demonstrate that ASK1 is required for cytokine- and stress-induced apoptosis.

Recently $p21^{Cip1/WAF1}$, a cell cycle inhibitory protein and an inhibitor of apoptosis (Guadagno and Newport, 1996; Polyak *et al.*, 1996; Gorospe *et al.*, 1997), was shown to bind ASK1 and to inhibit ASK1-induced JNK pathway activation and apoptosis (Asada *et al.*, 1999), suggesting that ASK1 induction of apoptosis is mediated by the JNK pathway. Furthermore, microtubule damaging agents such as paclitaxel can induce JNK activation (Wang *et al.*, 1998c) and phosphorylation of Bcl-2 (Yamamoto *et al.*, 1999) and this can be blocked by a combination of dominant negative ASK1, dnMKK7 and dnJNK1. This suggests that an ASK1/MKK7/JNK pathway mediates apoptosis through the phosphorylation and inactivation of Bcl-2. Interestingly, recent findings have demonstrated that ASK1-induced apoptosis of Mv1Lu epithelial cells and MEF fibroblasts requires caspase activation, which was a consequence of the induction of the mitochondrial release of cytochrome c to the cytosol with a concomitant activation of caspase-9 (Hatai *et al.*, 2000). ASK1-induction of apoptosis in those cells seems therefore to occur via a mitochondria-dependent caspase activation.

ASK1 has also been shown to be activated by Fas ligation, through an interaction with the Fas receptor-associated protein, Daxx (Chang *et al.*, 1998). In addition, the *Fas ligand* gene expression was shown to be upregulated by NGF withdrawal in PC12 cells and this was mediated by JNK (Le-Niculescu *et al.*, 1999). These results suggest that there might be a positive feedback loop whereby an initial stimulus that induces JNK pathway activation might be amplified through Fas expression, ASK1 activation and consequent reactivation of JNK.

Finally, very recently, ASK1, MKK4 and JNK3 have been found in complex with β-arrestin 2, a newly discovered MAPK scaffold protein (McDonald *et al.*, 2000). It is interesting to note that β-arrestin 2 only binds to the brain specific,

JNK3 isoform, and not to JNK1 or JNK2, raising questions about the physiological function of this scaffold molecule and ASK1 in the nervous system.

ROS-induced ASK1 dimerisation and activation

Reactive oxygen species (ROS) are produced in response to TNF-α treatment (Meier *et al.*, 1989; Zimmerman *et al.*, 1989; Larrick and Wright, 1990; Lo and Cruz, 1995). ROS have been shown to activate ASK1 and TNF-α-induced activation of ASK1 was blocked by antioxidants, suggesting that this is mediated by ROS (Gotoh and Cooper, 1998). Furthermore, thioredoxin (Trx), a reduction/oxidation regulatory protein that functions as an antioxidant, was found to bind the N-terminal part of ASK1 when in the reduced form and to inhibit TNF-α-induced apoptosis and activation of ASK1 (Saitoh *et al.*, 1998). Finally, ASK1 activity is stimulated by TNF-α via members of the TRAF (TNF receptor associated factor) family (Ichijo *et al.*, 1997; Nishitoh *et al.*, 1998; Hoeflich *et al.*, 1999) and this activation required the oxidation and subsequent dissociation of Trx from ASK1 (Liu *et al.*, 2000). Interestingly, Gotoh and co-workers (Gotoh and Cooper, 1998) found that ASK1 formed dimers upon treatment with TNF-α or hydrogen peroxide and showed that synthetic dimerisation of ASK1 was sufficient for its activation, providing the first evidence that dimerisation is important for the activation of a kinase that regulates stress-induced MAPK pathways. Altogether, these findings suggest a way in which the intracellular redox status, influenced by a variety of cytotoxic stresses, meets the apoptotic pathway and may also explain why antioxidants inhibit apoptosis.

Suppression of ASK1-induced apoptosis by 14-3-3 proteins

ASK1 has been shown to interact with 14-3-3 proteins, which regulate both Raf-1 (Campbell *et al.*, 1998) and Bad functions (Datta *et al.*, 1997; del Peso *et al.*, 1997). This interaction required phosphorylation of ASK1 on Ser967 and was independent of ASK1 catalytic activity. In addition, ASK1-induced apoptosis in HeLa cells was blocked by overexpression of 14-3-3, suggesting a new mechanism for the suppression of apoptosis (Zhang *et al.*, 1999). This study suggests that the 14-3-3 inhibition of apoptosis might be linked to an upstream kinase which phosphorylates ASK1 on Ser967, thus placing ASK1 under the control of a

survival pathway. In agreement with this hypothesis, a recent study has demonstrated that ASK1 is phosphorylated on serine 83 by Akt and this correlated with a decrease in ASK1 kinase activity (Kim *et al.*, 2001).

1.5.3.2.3 Role in disease

The main biological function of ASK1 described so far is its involvement in apoptosis, ASK1 misregulation is very likely to play a role wherever apoptosis is involved (as described in sections 1.2.2 and 1.3.2). Interestingly, ASK1 expression and JNK and p38 kinase phosphorylations were induced in an *in vivo* rat model of spinal cord injury, suggesting a possible involvement of ASK1-JNK and p38 kinase pathways in neuronal apoptosis after spinal cord injury (Nakahara *et al.*, 1999).

1.5.3.3 MLK3

MLK3 (mixed lineage kinase 3), also called PTK1 (protein tyrosine kinase 1) or SPRK (*src* homology 3 (SH3) domain-containing proline-rich kinase) was first identified in human cells by three different groups (Ezoe *et al.*, 1994; Gallo *et al.*, 1994; Ing *et al.*, 1994). MLK3 belongs to the MLK family of proteins which include MLK1 (Dorow *et al.*, 1993), MLK2/MST (Dorow *et al.*, 1993; Katoh *et al.*, 1995), DLK/ZPK/MUK (Holzman *et al.*, 1994; Reddy and Pleasure, 1994; Hirai *et al.*, 1996) and LZK (Sakuma *et al.*, 1997). Based on structural differences the MLK family can be divided into two subgroups: the MLK group (MLK1, MLK2 and MLK3) and the DLK group (DLK and LZK - which do not contain an SH3 domain or a CRIB domain).

1.5.3.3.1 Structure

The MLK family is so called because of its kinase domain having characteristics of both serine/threonine and tyrosine kinases. MLK3 is a ubiquitously expressed 95 kDa protein and its kinase domain is ≈75% identical to those of MLK1 and MLK2 (Gallo *et al.*, 1994).

Starting from the N-terminus and moving on towards the C-terminus, MLK3 contains the following motifs/domains (Figure 1.10): a glycine rich sequence which is unique to this family of proteins; an SH3 motif, which may

modulate ligand binding specificity (short proline-rich regions); a kinase domain; a double leucine-zipper-basic region which is thought to promote homo- or heterodimerisation of MLK proteins through hydrophobic interactions; a CRIB domain which is also found in MLK2; and a proline-rich region (24% proline) (Ezoe *et al.*, 1994; Gallo *et al.*, 1994; Ing *et al.*, 1994).

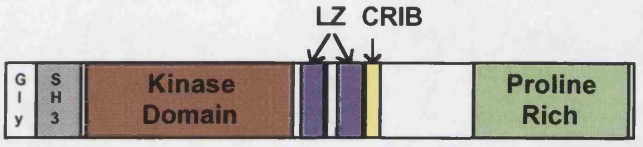


Figure 1.11 General structure of MLK3

Glycine rich region, SH3 domain, kinase domain, leucine zippers (LZ), CRIB domain and proline rich region are shown.

1.5.3.3.2 Function and Regulation

MLK3 has been identified as an upstream activator of the JNK pathway (Rana *et al.*, 1996; Tibbles *et al.*, 1996). Overexpression of MLK3 activated JNK via phosphorylation and activation of SEK1 (SAPK/ERK kinase 1) on serine and threonine residues (Rana *et al.*, 1996; Tibbles *et al.*, 1996), or via MKK7 (Whitmarsh *et al.*, 1998). MLK3 has also been shown to activate the p38 kinase pathway via MKK3/6 (Tibbles *et al.*, 1996) and the ERK pathway (Hartkamp *et al.*, 1999). Interestingly, putative scaffold proteins, JIP-1 and JIP-2 have recently been identified and shown to interact in a specific manner with members of the MLK family such as MLK3 and DLK, with MKK7 and with JNK, thereby linking these kinase-signalling components (Whitmarsh *et al.*, 1998; Yasuda *et al.*, 1999).

MLK3 has been shown to directly bind GTP-bound Cdc42 and Rac1 (Burbelo *et al.*, 1995; Teramoto *et al.*, 1996a) via its CRIB domain (Bock *et al.*, 2000) and co-expression of activated Cdc42 with MLK3 has been shown to increase JNK activity (Teramoto *et al.*, 1996a; Bock *et al.*, 2000). In addition, a dominant negative mutant of MLK3 blocked Cdc42 and Rac1-activation of JNK (Teramoto *et al.*, 1996a), suggesting that MLK3 mediates the activation of the JNK pathway by Cdc42 and Rac1.

Activation of MLK3 seems to occur by autophosphorylation which is mediated by a homodimerisation mechanism, in which the leucine zipper domains are crucial (Leung and Lassam, 1998). Furthermore, co-expression of Cdc42 with MLK3 increased the dimerisation of MLK3, suggesting that Cdc42 may induce the dimerisation and consequent activation of MLK3 (Leung and Lassam, 1998). However, a very recent study has examined the leucine zipper in more detail and found that the zipper dimerisation is not necessary for the full activation of MLK3 by Cdc42. Instead, it is required for the interaction and phosphorylation of SEK1 on both Ser254 and Thr258 and consequent activation of the JNK pathway (Vacratsis and Gallo, 2000).

1.6 Sympathetic and sympathetic-like neurons as a model of neuronal cell death

Although the phenomenon and function of developmental neuronal cell death would be best studied *in vivo*, studies aimed at elucidating the molecular mechanisms of neuronal cell death are more easily performed *in vitro*. This has the advantage that the cells under study can be isolated in an almost pure state and their environment can be rigorously controlled. As mentioned earlier, during development, one of the major factors determining neuronal survival is the ability to obtain sufficient trophic factor. The death occurring in the cells that do not obtain enough trophic factor can be studied *in vitro* by culturing particular neuronal cells and depriving them of their obligate trophic factor.

1.6.1 Superior cervical ganglion neurons

Sympathetic neurons depend on the availability of NGF for survival from approximately embryonic day 16 to 1 week postnatally (Coughlin and Collins, 1985). Indeed, removal of endogenous NGF with blocking antibodies, dramatically reduces the number of sympathetic neurons (Levi-Montalcini and Booker, 1960), whilst addition of exogenous NGF increased their survival in neonatal rats (Hendry and Campbell, 1976). Furthermore, deletion of the NGF gene (Crowley *et al.*, 1994) or of its receptor, TrkA (Smeyne *et al.*, 1994), caused a dramatic loss in the number of sympathetic neurons. After the period of target-regulated death, the neurons mature and are no longer dependent on trophic factors for survival.

NGF-dependent sympathetic neurons are isolated from the superior cervical ganglia of embryonic day 21 or postnatal day one rats and cultured in the presence of NGF for 5-7 days. Removal of NGF and addition of anti-NGF antibodies from the neurons at 5 to 7 days in culture results in the death of these neurons. This death has certain morphological features that are commonly associated with apoptosis such as chromatin condensation, detachment from the substrate and cytoplasmic shrinkage in addition to the loss of neurites (Deckwerth and Johnson, 1993; Edwards and Tolkovsky, 1994), thereby mimicking the effects that occur in an *in vivo* scenario. Soon after NGF withdrawal, the levels of TrkA phosphorylation and MAPK are reduced to basal levels (Franklin *et al.*, 1995; Virdee and Tolkovsky, 1995; Creedon *et al.*, 1996) and reactive oxygen species are generated (Greenlund *et al.*, 1995). The levels of glucose uptake decrease within an hour of NGF deprivation and at 12 hours the rate of protein and RNA synthesis have dropped dramatically [reviewed in (Johnson *et al.*, 1996)]. The cells atrophy following this decrease in protein synthesis, as the protein degradation rates seem to be kept constant (Franklin and Johnson, 1998). NGF deprivation has also been shown to decrease the efflux of purine 2-3 fold within 10 hours (Tolkovsky and Buckmaster, 1989). At 18 hours after NGF deprivation DNA fragmentation is detected (Edwards *et al.*, 1991) and by 18-24 hours the nuclei have begun to shrink and condense, with most neurons being dead by 48 hours (Martin *et al.*, 1988; Deckwerth and Johnson, 1993; Edwards and Tolkovsky, 1994). An important point concerning the mechanisms of trophic factor withdrawal-induced cell death is that, in its early stages, it is dependent on the synthesis of new proteins. Indeed, inhibitors of protein and RNA synthesis block NGF withdrawal-induced cell death (Martin *et al.*, 1988). Some of these events have also been observed in sympathetic neurons undergoing apoptosis in vivo during development (Wright *et al.*, 1983) and after injection of anti-NGF antibodies into new-born mice (Levi-Montalcini *et al.*, 1969), suggesting that apoptosis occurring in culture corresponds to the physiological and normally occurring developmental cell death. This demonstrates the great advantage of using these neurons as a model of neuronal developmental cell death. In addition, these neurons die in a reproducible manner with most of the neurons having undergone apoptosis by 48-72 hours

(Martin *et al.*, 1988). Furthermore, they can be established in culture in an almost pure fashion through the use of pre-plating techniques and the use of anti-mitotic compounds. The main disadvantage of using this neuronal cell death system is that only about 10,000-20,000 cells are obtained per animal (in rodent species), making it a difficult model in which to perform biochemical experiments. These cells are also extremely difficult to transfect and until now the only way of introducing foreign genes into these cells was through intracellular injection of expression plasmid [for example, see (Garcia *et al.*, 1992; Ham *et al.*, 1995)]. More recently, adenoviral vectors have been used as a very efficient method (Slack and Miller, 1996).

1.6.2 PC12 cells

An alternative model to SCG neurons is the PC12 clone of a rat pheochromocytoma cell line. PC12 cells, when treated with NGF, stop dividing and acquire a neuron-like phenotype (Greene and Tischler, 1976). Similar to SCG neurons, when differentiated, these cells also depend on NGF for survival. PC12 cell differentiation can also be induced upon treatment with cAMP analogues which, like NGF, promote neurite outgrowth and survival of PC12 cells and SCG neurons (Rydel and Greene, 1988; Rukenstein *et al.*, 1991). PC12 cells die by apoptosis when deprived of NGF and by 30 hours about 50% of the cells are dead (Philpott *et al.*, 1996). Most of the biochemical events underlying PC12 cell death, including the dependence on *de novo* protein synthesis, resemble those of sympathetic neurons (Mesner *et al.*, 1992). In contrast to SCG neurons, these cells can be obtained in large numbers and are therefore a useful tool for the performance of more complex biochemical studies, where a large amount of protein is required, such as Western blots. These cells therefore provide a good model for studying neuronal differentiation and trophic factor withdrawal-induced apoptosis.

1.7 Thesis aims

It is clear from the information gathered above that apoptosis plays an important role in the nervous system, not only at developmental stages but also at a pathologic level such as in neurodegenerative diseases. Apoptosis should therefore be a plausible target for therapeutic intervention in human neurodegenerative disease. Induction of apoptosis has been shown to be mediated by the JNK pathway in a number of different neurons. The work presented in this thesis aimed at identifying and characterising upstream regulators of JNK in the context of neuronal apoptosis. It has been described that in most cases apoptosis is initiated upon the stimulation of certain cell surface receptors by binding of specific ligands, or even, like in neurons, by the lack of essential survival factors which are required for the maintenance of cell survival. Because the Rho-like GTPases had been shown to be activated upon cell surface receptor-stimulation and also to be mediators of JNK activation, this work started by looking at the effect of these GTPases on the death of primary sympathetic neurons deprived of NGF (Chapter 3). However, because these small GTP-binding proteins can mediate a variety of cellular responses and because the intent was to block the apoptotic response only, it was important to identify the specific effector mediating apoptosis. Therefore, once the role of Cdc42 and Rac on neuronal apoptosis and JNK activation was established, the aim moved towards identifying the kinase downstream of these small GTPases that mediates neuronal cell death via the activation of JNK (Chapters 4, 5, 6 and 7). The understanding of the mechanisms of developmental neuronal cell death might therefore provide important clues for the development of therapies for diseases in which neuronal cell death occurs.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals, equipment and suppliers

ABI

ABI Prism 310 Genetic Analyser.

Agar Scientific

Number 5 Dumont forceps.

Amersham

ECL chemiluminescence reagent, [γ^{32}P]-ATP (185 Tbq/mmol), Hybond ECL nitrocellulose membrane, Hyperfilm-MP, protein A sepharose, rainbow protein markers.

Becton-Dickison and Co.

Falcon tissue culture flasks and dishes.

Bio 101

Geneclean kit.

Bio-Rad

Gel dryer, Mini-Protean II electrophoresis cell, Trans-blot semi-dry transfer cell acrylamide:bis-acrylamide (30:1), ammonium persulfate (APS), β-mercaptoethanol, Bradford protein assay kit, ethylenediamine tetra-acetic acid (EDTA), N'N'N'N'tetramethylethylene-diamine (TEMED), Glycine (electrophoresis purity reagent), imaging densitometer (GS-670), sodium dodecyl sulphate (SDS), Tris - electrophoresis purity reagent, Tween-20.

Boehringer Mannheim

anti-NGF antibody, TUNEL assay kit.

Carl Zeiss

Axiovert 100 and 135M inverted fluorescence microscope.

Campden Instruments

Horizontal electrode pipette puller (773).

Citifluor

Citifluor.

Costar

0.22 μM spin-X columns.

Difco Laboratories

Bacto-agar, bacto-tryptone, yeast extract.

Eppendorf

Micromanipulator (5171), transjector (5246), heater (TRZ 3700), CO_2 controller (CTI 3700) , environmental chamber.

Fisons Scientific Equipment

Boric acid.

Gibco-BRL

Horizon agarose gel electrophoresis tanks, agarose (electrophoresis grade), CsCl optical grade, DTT, Elongase enzyme mix kit, First strand buffer, L-glutamine, 1 Kb DNA size markers, L15 medium, dNTP mix, Oligo $(dT)_{12\text{-}18}$, Poly-ethylene glycol (PEG) ligation buffer (5x), penicillin/streptomycin (10,000 u/ml; 10,000 μg/ml respectively), random primers, SuperScript II - Reverse transcriptase,

trypsin/EDTA solution (0.5 g/l trypsin; 0.2 g/l EDTA), ultrapure phenol:chloroform.

ICN Biomedicals

BSA Path-o-cyte 4 (366 g/l).

Improvision

Openlab software.

Intracel

1.2 mm outer diameter x 0.8 mm inner diameter x 10 cm glass capillaries.

John Weiss

Dissection scissors and forceps.

Kopf

Vertical pipette puller (720).

Marvel

Non-fat milk powder.

Merck Ltd. BDH

13 mm glass coverslips, microscope slides, chloroform, ethylenediaminetetra-acetic acid disodium salt (EDTA), ethanol - AnalaR grade, Glacial acetic acid - AnalaR grade, glycerol, isoamyl alcohol - AnalaR grade, isopropanol - AnalaR grade, KCl, KH_2PO_4, $MgCl_2$, $MnCl_2$, methanol - AnalaR grade, NaCl, Na_2HPO_4, NaOH, Nonidet P40 (NP-40).

MJ Research

Peltier Thermal Cycler.

Molecular Probes

70 kDa Texas Red Dextran neutral.

New England Biolabs

*Eco*RI, *Bam*HI, T4 DNA ligase, Lambda DNA *Hind* III digest.

Nikon

Microphot FXA fluorescence microscope.

Novex

Colloidal Blue staining kit, DryEase mini-cellophane.

PAA Laboratories

Foetal calf serum (FCS).

Polaroid

Film 667 (ASA 3000), Instant Camera System (MP4+).

Promega

2.5 S NGF.

Qiagen

QIAprep Spin Miniprep kit, Rneasy kit.

Sigma

Ampicillin, aprotinin, bovine serum albumin (BSA), bromophenol blue, $CaCl_2$, chloramphenicol, dimethyl sulphoxide (DMSO), tissue culture grade, Dulbecco's Modified Eagle's Medium-DMEM (4.5 g/l glucose, 0.11 g/l sodium pyruvate, without glutamine), ethidium bromide, ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N',-tetraacetic acid (EGTA), 5'-fluoro-2-deoxyuridine, goat serum, guinea pig IgG - reagent grade, Hoechst 33342, insulin, potassium acetate, laminin (from basement membrane of Engelbreth-Holm-Swarm mouse sarcoma, leupeptin,

lysozyme, $MgSO_4$, NaF, Na_3VO_4, 3-[N-Morpholino] propanesulfonic acid (MOPS), paraformaldehyde (PFA), poly-L-lysine MW = 70,000-150,000 (PC12 cells), poly-L-lysine MW > 300,000 (SCG neurons), pepstatin A, phenyl methyl sulphonyl fluoride (PMSF), Ca^{+}/Mg^{2+} free phosphate buffered saline (CMF-PBS), Dulbecco's PBS, piperazine-N,N'-bis(2-ethansulfulfonic acid) (PIPES), Ponceau-S, progesterone, putrescine, RbCl, selenium, sodium acetate, L-thyroxine, transferrin, tri-iodo-thyronine, triton X-100, trypsin, type III (12,700 u/mg), uridine, xylene cyanol FF.

Spectroline

UV trans-illuminator (TVL-312A).

Upstate Biotechnology

Collagen type 1 - rat tail.

Whatman Scientific

3MM paper.

Worthington Biochemicals

Collagenase, type II (from *Clostridium histolyticum*).

Xillix Technologies

Microimager digital camera (Model 1400).

2.1.2 Antibodies

5 prime → 3 prime, Inc

Rabbit anti-chloramphenicol acetyl transferase (CAT)

Amersham

Goat anti-rabbit horseradish peroxidase conjugated IgG

Boehringer Manheim

Mouse (clone 9E10) anti-Myc epitope tag (EQKLISEEDL)

Mouse (clone 27/21) anti-NGF

Jackson Immunoresearch Laboratories

Donkey anti-guinea pig Ig, TRITC conjugated

Goat anti-mouse Ig, FITC conjugated

Goat anti-rabbit Ig, FITC conjugated

Kinetek

Rabbit anti-Ste20 (subdomain VI)

Santa Cruz Biotechnologies

Rabbit anti-PAK1 (C-19)

Rabbit anti-PAK2 (N-19)

Rabbit anti-MLK3 (C-20)

Sigma

Mouse M2 anti-FLAG (DYKDDDDK)

UBI

Rabbit anti-mouse Fas

Other antibodies

The anti c-Jun antibody was raised against a GST-c-Jun protein, encompassing amino acids 1-58 (Lallemand *et al.*, 1997) and the anti phospho-c-Jun antibody was raised against a phospho-peptide, encompassing amino acids 57-68 of c-Jun, with phospho-serine 63 (Lallemand *et al.*, 1998). Both antibodies were kind gifts of Dr. D. Lallemand, Institut Pasteur, Paris, France.

2.1.3 Bacterial strains

DH5α F-psid80d, *lac*ZM15, *end*A1, *rec*A1, *hsd*R17, (r_k-m_k-)*sup*E44, *thi*-1_k-*gyr*A96, *rel*A1 (*lac*ZYA-*arg*F)U69

2.1.4 Plasmids

pGEX V12Ras, pGEX V14RhoA, pGEX N17Cdc42, pGEX N17Rac1, pGEX N19RhoA, pRK5 (V12Cdc42, V12Rac1, Cdc42L61, Cdc42L61C40, Cdc42L61A37) were kind gifts of Prof. Alan Hall, LMCB-MRC, UCL, London, UK.

PcD-FLAG-Δ169 and pcD-Bcl-2 were described by Ham *et al.* (Ham *et al.*, 1995).

PMT-2-SEK-*AL* was constructed by Yan *et al.* (Yan *et al.*, 1994)

CMV6 (PAK1, PAK1 L107F, PAK1 K299R, PAK1 T423E, PAK2, PAK2 T403E, PAK2 K278R) were kind gifts of Jonathan Chernoff, Fox Chase Center, Philadelphia, USA.

pcD-FLAG (MLK3, MLK3 K144E) were described by Tibbles *et al.* (Tibbles *et al.*, 1996).

PcD-FLAG (MLK3 CRIB- and MLK3 K144E CRIB-) were constructed by M. Reeder, Fox Chase Center, Philadelphia, USA (Mota *et al.*, submmited).

pcDNA3 (ASK1, ΔN-ASK1, KR-ASK1) were described by Ichijo *et al.* (Ichijo *et al.*, 1997)

c-jun CAT was described by (Eilers *et al.*, 1998).

PMT-SM-MEKK1 (Myc epitope-tagged MEKK1 C-terminus) was constructed by M. Olson (Olson *et al.*, 1995).

2.1.5 Stock solutions, media and buffers

All solutions were made up in MilliQ deionised water unless specified otherwise.

Ampicillin	100 mg/ml
Chloramphenicol	34 mg/ml in 100% ethanol
Collagen	10 μg/ml in CMF-PBS

Collagenase	0.2% w/v in Dulbecco's PBS
DMEM/FCS	10% heat inactivated FCS 100 units/ml penicillin 100 µg/ml streptomycin 2 mM glutamine in DMEM (with 4.5 g/l glucose, 0.11 g/l pyruvate)
5 x DNA loading buffer	0.2% w/v bromophenol blue 0.2% w/v xylene cyanol 25% glycerol 50 mM EDTA, pH 8.0
LB Medium (Luria-Bertani)	1% w/v bacto-tryptone 0.5% w/v yeast extract 1% w/v NaCl
LB Agar	LB containing 1.5% w/v bacto-agar
Lysis buffer (plasmid preps.)	50 mM glucose 25 mM Tris-HCl, pH 8.0 10 mM EDTA, pH 8.0
Lysozyme	10 mg/ml in 10 mM Tris-HCl, pH 8.0
MLK3 kinase buffer	50 mM Tris, pH 7.4 10 mM $MgCl_2$ 1 mM EGTA

MLK3 lysis buffer	1.5 mM $MgCl_2$
	1% Triton X-100
	50 mM Hepes, pH 7.5
	1 mM EGTA
	150 mM NaCl
	10% glycerol
	1 mM PMSF
	1 μg/ml leupeptin
	1 μg/ml pepstatin
	1 μg/ml aprotinin
	1 mM Na_3VO_4
	10 mM NaF
	10 mM β-glycero phosphate
Neutralising solution	Potassium acetate, pH 5.5
	(3 M with respect to potassium,
	5 M with respect to acetate)
NP-40 lysis buffer	50 mM Tris-HCl, pH 7.4
	250 mM NaCl
	0.1% v/v NP-40
	5 mM EDTA
	50 mM NaF
	1 mM Na_3VO_4
	1mM PMSF
	10 μg/ml aprotinin
	1 μg/ml leupeptin
	10 μg/ml TPCK

2 x PAK kinase buffer	100 mM Hepes, pH 7.3 10 mM $MgCl_2$ 10 mM $MnCl_2$ 2 mM DTT 0.1% v/v Triton X-100 100 μM ATP
PAN	10 mM PIPES, pH 7.0 100 mM NaCl 20 μg/ml aprotinin
3% PFA	3% PFA w/v in CMF-PBS 0.1 mM $CaCl_2$ 0.1 mM $MgCl_2$ pH 7.4
1 x PBS	2.5 mM KCl 8.1 mM Na_2HPO_4, pH 7.4 1.5 mM KH_2PO_4 140 mM NaCl
1x PBS-T	PBS containing 0.1% v/v Tween-20
PMT (PBS-Milk-Tween)	PBS-T containing 5% non-fat dried milk
Ponceau-S solution	0.2% Ponceau-S 3% trichloroacetic acid 3% sulfosalicylic acid

RFI	100 mM RbCl
	50 mM $MnCl_2$
	30 mM K Acetate
	10 mM $CaCl_2$
	15% v/v glycerol
	pH adjusted to 5.8 with acetic acid
RFII	10 mM MOPS
	10 mM RbCl
	75 mM $CaCl_2$
	15% v/v glycerol
	pH adjusted to 6.8 with NaOH
2 x sample buffer	62.5 mM Tris-HCl, pH 6.8
	4% w/v SDS
	20% v/v glycerol
	2% v/v β-mercaptoethanol
	0.02% w/v bromophenol blue
SATO mix	45% v/v BSA Patho-o-cyte 4
	2.82 µg/ml progesterone
	1.76 µg/ml selenium
	0.73 mg/ml putrescine
	18.2 µg/ml L-thyroxine
	15.3 mg/ml tri-iodo-thyronine
SATO medium	2.1% SATO mix
	100 units/ml penicillin
	100 µg/ml streptomycin
	0.0125% w/v transferrin
	1.25 mM glutamine
	in DMEM

SDS-base	1% w/v SDS 0.2 N NaOH
SDS resolving gel	10% or 12.5% acrylamide:bis-acrylamide 0.1% w/v SDS 0.06% v/v TEMED 0.03% v/v APS in 0.375 M Tris-HCl, pH 8.8
SDS stacking gel	5% acrylamide:bisacrylamide 0.1% w/v SDS 0.06% v/v TEMED 0.03% v/v APS in 0.125 M Tris-HCl, pH 6.8
SDS running buffer	192 mM glycine 25 mM Tris base 0.1% w/v SDS pH 8.3
1 x TE	10 mM Tris-HCl, pH 8.0 1 mM EDTA
1 x TBE	90 mM Tris 90 mM boric acid 2 mM EDTA, pH 8.0
Trypsin	0.025 % w/v in Dulbecco's PBS

2.2 Methods

2.2.1 Molecular biology

2.2.1.1 General

The following are techniques that were used routinely and have therefore been described here to avoid repetition.

2.2.1.1.1 Phenol:chloroform extraction

Phenol:chloroform:isoamyl alcohol (25:24:1) extractions were used to purify nucleic acids of proteins from bacterial cell lysates and also whenever required to inactivate and remove enzymes during cloning procedures. An equal volume of phenol:chloroform:isoamyl alcohol was added to the nucleic acid/protein solution and the contents were mixed thoroughly for a few seconds. The mix was then centrifuged at 12,000 x g at room temperature (RT), for 2 minutes. The upper aqueous phase containing the nucleic acids was then transferred to a new tube. In order to achieve a higher DNA purity, a second phenol:chloroform extraction can be carried out as described above. To remove any traces of phenol, a final extraction with an equal volume of chloroform:isoamyl alcohol (24:1) extraction was performed. The double-stranded DNA was then precipitated as described in the next paragraph.

2.2.1.1.2 Ethanol or Isopropanol precipitation

This method was used to concentrate DNA. DNA was precipitated with 2 volumes of 100% ethanol and 1/10 volume of 3 M sodium acetate pH 5.2 for 5 minutes at RT and centrifuged for 10 minutes at 12,000 x g at 4°C. The DNA pellet was then washed with 70% ethanol, centrifuged at 12,000 x g for 10 minutes at 4°C and allowed to air dry before being resuspended in TE or H_2O.

2.2.1.2 Restriction endonuclease digestion

Restriction endonuclease digestions were carried out to analyse plasmid DNA and to prepare specific DNA fragments for cloning. DNA (0.2-2.0 μg) was digested in the presence of an excess amount of restriction enzyme, the

appropriate volume of enzyme buffer and made up to a final volume of 10-20 µl with H_2O. The reaction mix was then incubated at 37°C for 2 hours. DNA fragments were resolved on agarose gels as described in section 2.2.1.3.

2.2.1.3 Agarose gel electrophoresis

To analyse the digested DNA, 1/5 volume of 5 x DNA loading buffer was added to the restriction digest mix and the DNA fragments were then resolved on an agarose gel. Agarose gels (0.7-1-2%) were prepared in 1 x TBE buffer with 0.5 µg/ml ethidium bromide. The digested DNA was loaded in 1x DNA loading buffer and electrophoresis was carried out in 1x TBE buffer. 1Kb DNA size markers were also loaded into adjacent lanes. The DNA was visualised on a UV trans-illuminator and photographed using a Polaroid camera with a Polaroid film (ASA 3000).

2.2.1.4 Recovery of DNA from agarose gel

DNA fragments over 500 bp in length were purified from gel slices using the "GeneClean II Kit" from BIO 101 Inc. This kit contains a silica matrix (glassmilk) that binds single- and double-stranded DNA, but not contaminants, resulting in very pure DNA. This kit was used following the manufacturer's instructions.

2.2.1.5 Ligation of DNA

The plasmid vectors necessary for the expression of small GTPase proteins in sympathetic neurons were constructed by digesting pRK5 (vector) and pGEX containing V12Ras, V14RhoA, N17Rac1 or N17Cdc42 (inserts) with *Eco*RI and *Bam*HI for 2 hours at 37°C. The DNA fragments were then purified from agarose gels as described above. The DNA was then quantified by running a known volume on a 1% mini-agarose gel alongside 500 ng of Lambda DNA digested with *Hind* III. The intensity of the DNA to be quantified was compared to each fragment of the Lambda *Hind* III digest. The inserts were then ligated into pRK5 by incubating them at a 1:3 molar ratio of vector to insert at 16°C overnight with T4 DNA ligase in a PEG ligation buffer. Competent *E. Coli* bacteria were transformed with the ligation mix as described in section 2.2.1.7. The colonies of

transformed bacteria were then screened for the presence of the plasmid of interest as described in section 2.2.1.8.

2.2.1.6 Preparation of competent *E. coli*

Competent bacteria were prepared from either a single bacterial colony or from a bacterial glycerol stock and grown in standard LB medium together with 10 mM $MgSO_4$ overnight at 37°C with shaking. This initial culture (5 ml) was diluted into 120 ml of warm LB containing 10 mM $MgSO_4$ and incubated at 37°C with shaking until the optical density at 600 nm had reached an absorbency of 0.2. The culture was then transferred into pre-chilled 50 ml tubes and allowed to cool on ice for 15 minutes before being centrifuged at 2,500 x g for 10 minutes at 4°C. The pellet was resuspended in 10 ml of RF1 buffer, vortexed gently, incubated on ice for 15 minutes and then centrifuged down at 2,500 x g for 10 minutes at 4°C. The bacterial pellet was resuspended in 2.4 ml of RF2 buffer by gentle mixing and again incubated on ice for 15 minutes. The competent cells were then distributed in 100 µl aliquots, flash frozen in an ethanol-dry ice bath and stored at -70°C.

2.2.1.7 Transformation of competent bacteria

Transformation of bacteria was carried out by thawing 100 µl competent cells on ice and by adding 50 ng of DNA. The bacteria were incubated on ice for 15 minutes, heat-shocked for 90 seconds at 42°C and then cooled on ice for 2 minutes. Nine hundred microlitres of pre-warmed LB medium was added to the cells to allow them to grow and express their antibiotic resistance gene. After 45 minutes at 37°C, 10-200 µl were spread onto LB agar petri dishes containing 100 µg/ml of ampicillin. The petri dishes were allowed to dry before being inverted and incubated overnight at 37°C.

2.2.1.8 Screening for the presence of recombinant plasmid

The transformed bacteria were picked and inoculated in 5 ml of LB medium containing 100 µg/ml ampicillin, then incubated overnight at 37°C with vigorous shaking. The plasmid DNA was harvested as described in section 2.2.1.9.1, then submitted to restriction digestion with the appropriate enzyme(s)

(section 2.2.1.2) before being run on an agarose gel against a DNA ladder (section 2.2.1.3).

2.2.1.9 Plasmid preparation

2.2.1.9.1 Small scale (Miniprep)

A single bacterial colony was cultured in LB medium containing ampicillin (100 µg/ml) and incubated overnight with vigorous shaking at 37°C. The bacteria were harvested from 1.5 ml of the starter culture by centrifugation at 600 x g for 5 minutes. The supernatant was discarded and the pellet was resuspended by vigorous vortexing in 100 µl of TE. The cells were lysed by addition of 200 µl of SDS-base then gently mixed by inversion and incubated on ice for 5 minutes. Following addition of 150 µl of ice cold 5 M potassium acetate, pH 5.5, the solutions were mixed by gentle inversion and incubated on ice for 5 minutes. The flocculent white precipitate containing proteins, chromosomal DNA and cellular debris was removed by centrifugation at 5,000 x g for 5 minutes at 4°C. The supernatant, containing the plasmid DNA, was then transferred to a fresh 1.5 ml centrifuge tube. The remaining protein contamination was removed by a double phenol:chloroform:isoamyl alcohol extraction followed by a wash with chloroform as explained in section 2.2.1.1.1. The DNA was precipitated as described in section 2.2.1.1.2 and resuspended in 30 µl TE. Five to ten microlitres were digested with an appropriate restriction enzyme before being submitted to electrophoresis on a TBE-agarose gel. The DNA was stored at -20°C.

For sequencing reactions the plasmid DNA was prepared using the Qiagen high-salt silica based system (QIAprep) which was carried out exactly according to the manufacturer's instructions.

2.2.1.9.2 Large scale (Maxiprep)

A single colony was inoculated in 5 ml of LB containing ampicillin (100 µg/ml) and incubated overnight with shaking at 37°C. The following day 400 ml LB medium containing ampicillin was inoculated with 4 ml of the starter culture and incubated at 37°C with shaking until the optical density at 600 nm reached an absorbency of 0.9-1.0. At this point chloramphenicol was added to 170 µg/ml for

amplification of the plasmid and the incubation was continued at 37°C with agitation for at least 16 hours.

The bacteria were harvested by centrifugation at 3,000 x g for 15 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in 10 ml of ice cold lysis buffer. One millilitre of freshly prepared lysozyme solution was added, thoroughly mixed by inversion and incubated at RT for 5 minutes. Twenty millilitres of SDS-base was added, mixed and incubated at RT for 10 minutes until the solution became clear. Fifteen millilitres of neutralising solution was added to the bacterial lysate and incubated on ice for 10 minutes. To remove bacterial debris and proteins, the lysate was centrifuged at 10,000 x g for 15 minutes at 4°C and the resulting clear supernatant was filtered through sterile gauze. The plasmid DNA was precipitated by the addition of 0.6 volumes of isopropanol. After 20 minutes at RT, the DNA was centrifuged down at 7,500 x g for 15 minutes at RT. The resulting pellet was allowed to air dry and resuspended in 4 ml of TE.

The plasmid DNA was further purified through two sequential CsCl-ethidium bromide gradients (Radloff *et al.*, 1967). Solid CsCl salt (4.25 g) and 200 µl of a 10 mg/ml ethidium bromide solution were added to the DNA and mixed well. The solution was transferred to a 5.5 ml quick-seal ultracentrifuge tube, the volume and weight adjusted with either TE or a 1 g/ml CsCl solution and then spun at 500,000 x g at 20°C for at least 16 hours in a Beckman NVT-90 near vertical rotor. The lower of the two bands, containing the supercoiled DNA, was harvested with a 2 ml syringe and submitted to a second CsCl-ethidium bromide gradient, run as before, for further purification. To extract the intercalating ethidium bromide from the DNA, an equal volume of NaCl-saturated isopropanol was added to the aqueous DNA solution and vigorously mixed. The upper phase (containing ethidium bromide) was removed and the extraction was repeated until the bottom phase (containing the DNA) was completely clear of ethidium bromide. To dilute the CsCl salt present in the DNA solution, 3 volumes of H_2O were added and then the DNA was precipitated as described in section 2.2.1.1.2. The DNA was resuspended in H_2O or TE to yield a concentration of more than 1 mg/ml. The DNA concentration was estimated by reading the

Primers were designed based on the human MLK3 sequence for a 120 base pair glycine rich region which is highly specific for MLK3 (Sakuma *et al.*, 1997).

absorbency of a diluted sample at 260 nm using a UV spectrophotometer. The DNA concentration was then calculated by applying the following formula: [nucleic acid] = (A_{260}) x (dilution) x (Σ), where A_{260} is the absorbency at 260 nm and Σ = 50 µg/ml for double stranded DNA. The DNA was considered significantly pure if the A_{260}/A_{280} ratio was between 1.5 and 2.0.

2.2.1.10 Preparation of total RNA from SCG neurons

SCG neurons culture medium was transferred to a centrifuge tube to collect the detached, apoptotic cells. The cells remaining on the dish were harvested by rinsing them in a small volume of ice-cold CMF-PBS. The adherent and floating cells were pooled and spun at 1,800 x g for 10 minutes at 4°C, rinsed twice with CMF-PBS and total RNA was prepared using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions.

2.2.1.11 Reverse transcriptase-polymerase chain reaction (RT-PCR)

First strand cDNA was prepared from the total RNA extracts from SCG neurons using Superscript™ II RNase Reverse Transcriptase. Briefly, 2 µg total SCG RNA was incubated with 0.5 µg of Oligo $(dT)_{12-18}$ Primer and 3 µg Random Primers in a total volume of 20 µl for 10 minutes at 70°C. The reaction mix was placed on ice for 1-2 minutes before addition of 4 µl First Strand Buffer, 2 µl 0.1 M DTT and 1 µl 10 mM dNTP mix. The contents of the tube were mixed gently and incubated at 42°C for 2 minutes. Two hundred units of SuperScript II were added and the reaction mix incubated for 50 minutes at 42°C. Heating at 70°C for 15 minutes inactivated the reaction. The PCR reactions were carried out using Elongase Enzyme Mix kit following the manufacturer's recommendations.
Forward primer: 5'-GTCATGGAATGGCAGTGG-3';
Reverse primer: 5'- GGCTGTAGTCGAACAGG-3'. The PCR products were analysed on a 1% agarose gel.

2.2.1.12 DNA sequencing

All DNA sequencing was carried out by Cesare Spadoni (Eisai London Research Laboratories, UCL, UK) using an automated system based on a high

viscosity polymer (ABI Prism 310 Genetic Analyser). Plasmid DNA to be sequenced was prepared either by CsCl gradient or by Qiagen minipreps as described in section 2.2.1.9.1.

2.2.2 Cell Biology

2.2.2.1 Cell Culture

2.2.2.1.1 Primary superior cervical neurons

Sympathetic neurons were isolated from superior cervical ganglia of newborn Sprague-Dawley rats. Sixteen to twenty ganglia were removed and desheethed using fine forceps and were then enzymatically dissociated for 30 minutes in 5 ml 0.025% trypsin at 37°C. Five millilitres 0.2% collagenase was then added and the incubation continued for 30 minutes at 37°C. The digestion was stopped by addition of 5 ml of SCG culture medium (DMEM/FCS plus 100 ng/ml NGF) and the cells were centrifuged at 900 x g for 10 minutes at RT. The supernatant was discarded and the cells were triturated in SCG culture medium through a 19 gauge needle until completely dissociated into single neurons. After a 2 hour pre-plating in tissue culture dishes, to remove adherent, non-neuronal cells, the neurons were gently removed and centrifuged at 900 x g for 10 minutes and then resuspended in 50 µl of culture medium per ganglion. For all experiments, between 2,000-10,000 cells were plated onto poly-L-lysine (1 mg/ml) and laminin (20 µg/ml) coated 13 mm glass coverslips and were allowed to attach by incubation at 37°C, 10% CO_2 for 2 hours. Finally, the cells were flooded with culture medium containing 20 µM fluorodeoxyuridine and uridine to limit the growth of non-neuronal cells. The neurons were kept for 5-7 days in culture at 37°C, 10% CO_2, before being used for experiments. The cells were re-fed after 2, 5 and 7 days in culture. For the NGF withdrawal experiments, the cells were washed once with DMEM and then re-fed with culture medium lacking NGF and containing 50 ng/ml of anti-NGF antibody.

2.2.2.1.2 PC12 cells

Undifferentiated PC12 cells were grown in SATO medium to which 10 μg/ml insulin and 2% FCS was added, in collagen-coated tissue culture 75 cm^2 flasks. The flasks were coated for 2 hours and then washed twice with DULBECCO'S PBS. Stock cultures were kept at 37°C, 8% CO_2 and passaged every week by treatment with 2.5 ml of trypsin/EDTA, per 75 cm^2 flask, for 5 minutes at 37°C. The activity of trypsin was blocked by addition of 10 ml SATO medium. Following centrifugation at 1,000 x g for 10 minutes, the cells were resuspended in 10 ml of SATO medium, using a 10 ml syringe and a 19 gauge needle, before being counted and plated at a density of 2 x 10^6 cells per 75 cm^2 flask. The culture medium was changed every 3 to 4 days. To differentiate the cells, PC12 cells were plated in 9 cm dishes at a density of 2 x 10^6 cells per dish and fed with SATO medium supplemented with 100 ng/ml NGF. In this case the dishes were first coated with poly-L-lysine (1 mg/ml) for 1 hour, washed twice with water and then coated with collagen as above. The cells were allowed to differentiate for 7 days prior to experimental manipulation. In NGF deprivation experiments, differentiated PC12 cells were gently washed once with medium lacking NGF and then refed in this medium supplemented with a neutralising anti-NGF antibody (100 ng/ml). Both attached and detached (apoptotic) cells were collected for protein extraction at various times.

2.2.2.1.3 Jurkat cells

Jurkat cells were cultured in tissue culture flasks or dishes and maintained at 37°C 10% CO_2 in DMEM/FCS. The cells were passaged every 3-4 days by diluting them at a 1:6 to 1:10 ratio into new growth medium. Apoptosis of Jurkat cells was induced by treatment with 50 ng/ml anti-Fas IgM for determined times.

2.2.2.2 Microinjection

Microinjection of sympathetic neurons (5-7 days in culture) was carried out using a Zeiss Axiovert 135M microscope fitted with a heated stage and CO_2 chamber and using an Eppendorf transjector and micromanipulator. DNA expression vectors were injected directly into the nucleus in 0.5 x CMF-PBS at a DNA concentration of 0.05-0.6 mg/ml with needles pulled from glass capillaries

using a Campden Instruments horizontal electrode or using a Kopf Instruments vertical pipette puller.

2.2.2.3 Viability assays

2.2.2.3.1 Survival assay

To detect the injected cells, neutral 70 KDa Texas Red dextran was co-injected with the DNA at a final concentration of 5 mg/ml. Four to twenty-four hours after injection the neurons were refed with the appropriate SCG culture medium and the number of Texas Red dextran-positive cells that had survived injection was assessed as 100% survival value. Typically, over 80% of the injected cells survived injection. After determined periods of time, the neuronal viability was assessed by phase morphology. Viable cells retained Texas Red-dextran within the nucleus. Apoptotic neurons had shrunken cell bodies and distorted/fragmented or condensed nuclei. In addition, the Texas Red-dextran was no longer exclusive to the nucleus. The number of Texas Red dextran-positive cells with normal morphology was counted and the percentage of viable cells calculated. On average 200 cells were injected per experiment.

2.2.2.3.2 TUNEL (TdT-mediated dUTP nick end) labelling

TUNEL labelling of cells was carried out using the Boehringer Mannheim Fluorescein *In Situ* Cell Death Detection Kit, to assess the number of apoptotic neurons, usually at 16 hours post-treatment (microinjection, NGF withdrawal). Neurons were fixed in 3% PFA for 20 minutes, washed in CMF-PBS, permeabilised with 0.5% Triton X-100 for 5 minutes, washed in CMF-PBS, and incubated with the TUNEL reaction mixture for 1 hour at 37°C. The cells were washed in CMF-PBS and stained for guinea pig IgG (GP-IgG) to detect the injected cells as described below.

2.2.2.4 Immunocytochemistry

To identify the injected cells, purified GP-IgG was added to the injection mix at a final concentration of 5 mg/ml. In the case of the c-Jun chloramphenicol acetyl transferase (CAT) reporter gene assay, sympathetic neurons were also

injected with 0.05 mg/ml *jun* CAT in the presence or absence of a DNA construct of interest. The injected neurons, after appropriate times, were fixed either by treatment with methanol/acetone (50/50) for 20 minutes at -20°C (myc tag staining) or by treatment with 3% PFA for 10-30 minutes at RT (FLAG tag, phospho-c-Jun, c-Jun, and CAT staining). The cells fixed in 3% PFA were washed in CMF-PBS, permeabilised with 0.5% Triton X-100 in CMF-PBS for 5 minutes and washed again in CMF-PBS. The cells were blocked with 50% normal goat serum in 0.5% BSA in CMF-PBS for 30 minutes and then incubated with the appropriate primary antibody [9E10 (1:300), M2 (1:1500), anti-phospho-Jun (1:20,000), anti-c-Jun (1:100) anti-CAT (1:100)], diluted to the suitable concentration in 1% BSA in CMF-PBS for 1 hour. The cells were washed in CMF-PBS and incubated with both a rhodamine-conjugated goat anti-GP-IgG antibody and a fluorescein isothiocyanate (FITC)-conjugated secondary antibody both diluted at 1:100 for 1 hour at RT. The cells were washed in CMF-PBS, stained for 5 minutes in 1 μg/ml Hoechst dye and washed in H_2O. The coverslips were mounted in Citifluor on glass slides. The cells were then examined on a Nikon FXA fluorescence microscope and the proportion of injected cells that stained positive was calculated. Only the cells showing a clear increase over the background staining were scored positive. When examining the nuclear morphology, the ratio of injected cells that had pyknotic (fragmented or condensed) nuclei was assessed.

2.2.3 Biochemistry

2.2.3.1 Preparation of cell lysate

Conditioned medium was removed and centrifuged at 2,000 x g for 10 minutes at 4°C to collect the detached/dead cells. Cells remaining on the dish were rinsed twice with CMF-PBS and lysed with cold NP-40 lysis buffer (for Western blot analysis) or MLK3 lysis buffer (for MLK3 kinase assay). The cells were scraped, the lysate was added to the pellet of the detached cells and incubated on ice for 10 minutes. The cell debris was removed by centrifugation at 5,000 x g for 15 minutes at 4°C.

2.2.4 Statistical analysis

Data were analysed by a paired two-tailed Student's *t*-test.

2.2.3.2 Protein estimation

Protein estimation of cellular lysates was determined by the Bradford method (Bradford, 1976) against a BSA protein standard ranging from 0 to 20 μg/ml as according to the manufacturer's instructions.

2.2.3.3 Immunoprecipitation

PAK1 was immunoprecipitated from differentiated PC12 cells maintained in the presence or absence of NGF using the C-19 anti-PAK1 antibody (Santa Cruz). MLK3 was immunoprecipitated from either PC12 cells or SCG neurons maintained in the presence or absence of NGF using the C-20 anti-MLK3 antibody (Santa Cruz). One microgram of anti-PAK1 antibody or 3 μg of anti-MLK3 antibody was added to the lysate of $2x10^6$ cells (or 20 μg of total cellular protein from SCG neurons) together with 15 μl of protein A-agarose beads previously equilibrated in the respective lysis buffer. The mixture was then incubated end over end for 1 hour. The beads were centrifuged down at 5,000 x g for 2 min at 4°C and washed twice with lysis buffer.

2.2.3.4 In vitro kinase assay

Following immunoprecipitation, the beads (in the case of PAK1) were resuspended in 50 μl PAN buffer and 10 μl were incubated with 10 μl of 2 x PAK kinase buffer containing 10 μCi of [γ-^{32}P]ATP and 15 μg of Myelin Basic Protein (MBP), as an exogenous substrate, for 10 min at 30°C. In the case of MLK3, the beads were resuspended in 10 μl of MLK3 kinase buffer and incubated for 10 minutes on ice with 15 μg MBP. The reaction was started with the addition of 10 μCi [γ-^{32}P] ATP and incubation was carried out for 20 minutes at 30°C. In both cases, the reactions were stopped by addition of 20 μl of 2X sample buffer. The samples were heated to 98°C for 3 minutes and then separated on a 12.5% sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel as described below. The gel was fixed, by staining with Colloidal Blue for 3 hours and destained in H_2O overnight, dried and the labelled proteins were revealed by

autoradiography. Finally, the auto-radiographs were scanned with an imaging densitometer.

2.2.3.5 Polyacrylamide gel electrophoresis of proteins

One dimensional SDS-PAGE was carried out as described by Laemmli (Laemmli, 1970). Cellular lysates were prepared as described in section 2.2.3.1. Sample buffer was added to the extracts which were boiled for 3 minutes and resolved on a SDS-PAGE gel of 10% or 12.5% acrylamide:bis-acrylamide (30:1). Following electrophoresis the gels were either immunoblotted as described in section 2.2.3.6 or fixed and stained with a Colloidal Blue Stain Kit (Novex) according to the manufacturer's instructions. Following a 3 hour staining period, the gels were detained overnight with H_2O. For long term storage or analysis of the in vitro kinase assays, the gels were dried.

2.2.3.6 Immunoblotting of proteins

Following electrophoresis the proteins were transferred onto Hybond-ECL nitrocellulose membrane by semi-dry electroblotting. The proteins were transferred for 45 minutes at 25 V at RT. Protein loading and transfer was checked by staining the membrane for 5 minutes in 0.2% w/v Ponceau-S solution followed by washing in H_2O. The membrane was blocked for 1 hour with PMT. Following overnight incubation at 4°C with the primary antibody diluted in PMT, the membrane was washed 6 x 10 minutes with PBS-T and then incubated with a 1:2,000 dilution of anti-rabbit horse radish peroxidase (HRP) conjugated antibody in PMT for 1 hour at RT. After a 6 x 10 minutes wash with PBS-T, the membrane was treated with ECL reagent for 1 minute and exposed to auto-radiography film. In the case of peptide competition assays, the primary antibody was pre-incubated with a 10-fold excess of the corresponding peptide antigen for 1 hour at 4°C with rotation, prior to being added to the membrane.

3. The importance of Cdc42 in the induction of apoptosis of sympathetic neurons

3.1 Introduction

The importance of the c-Jun transcription factor in the induction of neuronal apoptosis has been shown in studies where NGF withdrawal-induced death of sympathetic neurons could be blocked by antibodies against c-Jun or by a dominant negative c-Jun (Estus *et al.*, 1994; Ham *et al.*, 1995). These studies suggested that pathways regulating the level of c-Jun or its phosphorylation would therefore be important in neuronal apoptosis. Indeed, one pathway that was found to be activated in many different types of neurons undergoing cell death was the signalling cascade leading to JNK activation (Xia *et al.*, 1995; Virdee *et al.*, 1997; Eilers *et al.*, 1998; Maroney *et al.*, 1998; Watson *et al.*, 1998). It was therefore of interest to identify the upstream regulators of this pathway which are involved in mediating neuronal cell death.

At the start of this investigation, several independent groups had shown that the protein kinase cascade that controls the activation of JNK could be regulated by the small GTP-binding proteins Rac and Cdc42. For instance, Coso *et al.* (Coso *et al.*, 1995) and Minden *et al.* (Minden *et al.*, 1995), by using transient transfection assays with various mutants of Cdc42, Rac1 and Rho together with an epitope-tagged JNK cDNA clone as a reporter, were able to show that JNK activation in COS-7, NIH 3T3 and HeLa cells is dependent on Cdc42 and Rac1 but not on RhoA. Similar observations were also reported in COS-1 and human embryonic kidney (HEK) cells (Bagrodia *et al.*, 1995; Teramoto *et al.*, 1996b). These studies suggest that Cdc42 and/or Rac1 may be important mediators of cell signals leading to apoptotic responses and they prompted this investigation on their role in the induction of sympathetic neuron death.

Mutations on a number of conserved amino acids can change the intrinsic and GAP-stimulated GTPase activity, or the guanine nucleotide binding affinity of the Rho-like GTPases. The mutation of glycine to valine in position 12 (G12→V,

based on the Ras numbering) results in a decreased intrinsic GTPase activity or in an insensitivity to GAPs (Figure 1.6). These mutants therefore remain mainly in the GTP-bound, active form in cells, or they have an enhanced exchange rate (Barbacid, 1987; Bourne *et al.*, 1991). In the GTP-bound state, these proteins are believed to continuously interact with a number of different downstream effectors which are likely to mediate their actions [for review see (Boettner and Van Aelst, 1999)]. The serine/threonine to asparagine (S17→N) mutation results in a conformation that favours binding of GDP rather than GTP, thereby maintaining the GTPase in an inactive state. These mutants are thought to function as dominant negative forms as the continuous interaction with regulatory proteins such as the GEFs results in a decrease in the GEF concentration and thus reduces the activation of their respective endogenous GTPases (Feig and Cooper, 1988; Farnsworth and Feig, 1991; Ridley *et al.*, 1992).

To study the role of the small Rho GTPases in neuronal apoptosis, both constitutively active (G12→V) and dominant negative (S17→N) forms of these proteins were overexpressed in SCG neurons. As discussed in Chapter 1 these neurons, isolated from 1 day old rats, are post-mitotic and extremely difficult to transfect by conventional methods such as calcium-phosphate or liposomes. Alternative ways of introducing foreign genes into these cells is by using an adenoviral system or by microinjection. Throughout this investigation, microinjection was used as the means of expressing foreign genes in sympathetic neurons. Professor Alan Hall (MRC-LMCB, UCL, UK), kindly provided the GST-myc tagged fusion proteins of activated forms of Rac1, Cdc42, RhoA and Ras and their respective dominant negative forms, which were subcloned into a mammalian expression vector (pRK5) as described in section 2.2.1.

In this Chapter the experiments represented on Figures 3.6 (A and B), 3.7 and 3.8 were kindly carried out by Dr. C. Bazenet (Eisai London Research Laboratories, UCL, UK).

3.2 Immunocytochemical analysis of injected cells

To examine the expression of V14RhoA,, V12 (Ras, Cdc42 and Rac1) and N17 (Cdc42 and Rac1) in sympathetic neurons,. GP-IgG was co-injected with the DNA of interest at a final concentration of 5 mg/ml. Four to sixteen hours later, the cells were fixed and stained as described in Chapter 2. Over 80% of the injected cells clearly expressed the GTPases as detected by the anti-c-myc antibody (9E10) (data not shown). Figure 3.1 illustrates the different expression patterns of the various small GTPase mutants tested. Expression of all GTPases were observed in the cytoplasm. However, their precise intracellular localisation cannot be determined by this type of study.

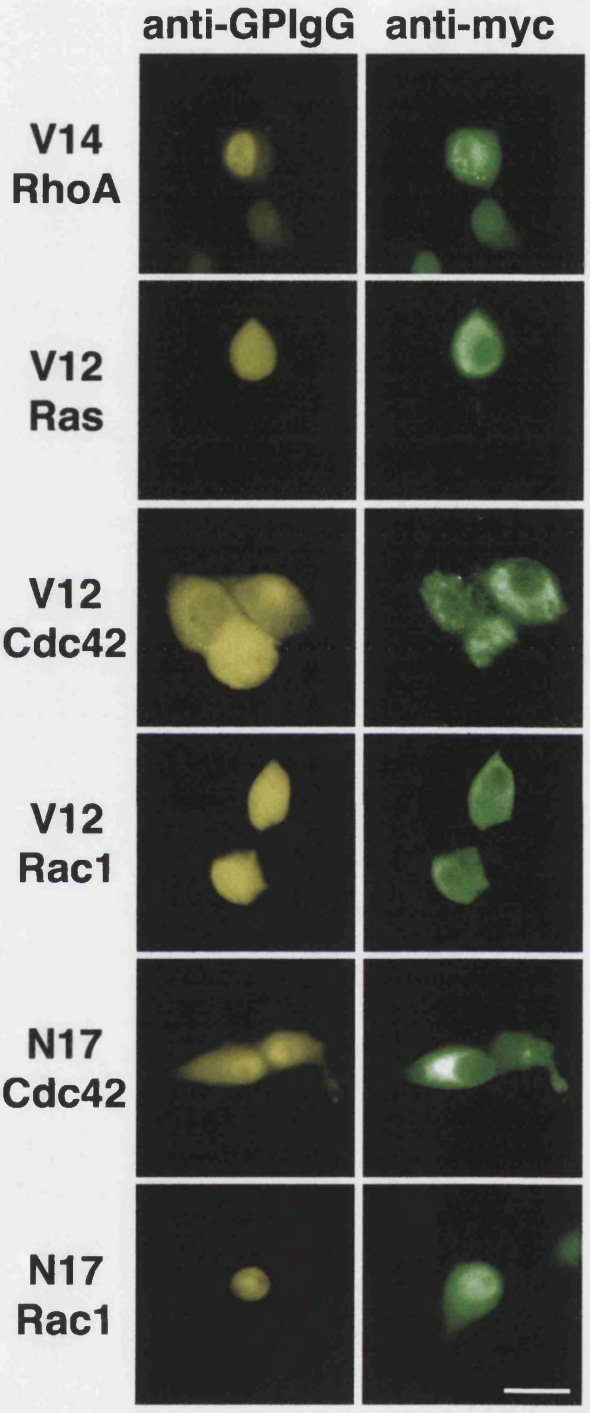


Figure 3.1 Expression of RhoA, Ras, Rac and Cdc42 in sympathetic neurons.
Sympathetic neurons, cultured for 5-7 days in the presence of NGF, were microinjected with 0.1 mg/ml DNA and 5 mg/ml guinea pig IgG to mark the injected cells. Four to sixteen hours after injection, the cells were stained with an anti-guinea pig IgG and an anti-c-myc antibody to detect specific expression of the exogenous proteins in the injected cells. Bar = 30 μm.

3.3 Cdc42 and Rac1 induce apoptosis in SCG neurons

In order to study the role of the Rho-subfamily of GTPases in sympathetic neurons, the activated forms of Ras, RhoA, Rac1 and Cdc42 (V12 mutants and V14 mutant for RhoA) and the empty expression vector were microinjected into SCG neurons, cultured for 5-7 days in the presence of NGF, at the indicated concentrations (Figures 3.2 and 3.3). Expression of the activated forms of either Ras or RhoA had no significant effect on the survival of the neurons (Figure 3.2) whereas expression of activated Cdc42 and Rac1 reduced the survival of the neurons by 60% after 72 hours in the presence of NGF (Figure 3.3A). The difference in response obtained for different GTPases demonstrates that the effect of Cdc42 and Rac1 in inducing neuronal death is a specific one. In addition, dominant negative mutants of Cdc42 and Rac1 (N17 mutants) had no effect on the survival of SCG neurons maintained in the presence of NGF (Figure 3.5).

To characterise the death induced by Rac1 and Cdc42, TUNEL analysis was carried out on cells microinjected with activated Rac1 and Cdc42 as well as Bax, a strong inducer of apoptotic death in this cell system (Vekrellis *et al.*, 1997). TUNEL staining was performed 16 hours after injection. About 20% of Bax injected cells and 15% of V12Cdc42 and V12Rac1 injected cells were stained positive for TUNEL compared to 2% of the cells injected with the empty vector (Figure 3.3B). The nuclear morphology was examined by Hoechst staining. Sixteen to twenty four hours after injection nuclei of cells expressing V12Cdc42 and V12Rac1 started to exhibit a pyknotic/condensed morphology, typical of apoptotic cells (Figure 3.3C), supporting the TUNEL staining results. Taken together, these data show that expression of constitutively activated forms of Cdc42 and Rac1 induce neuronal apoptosis.

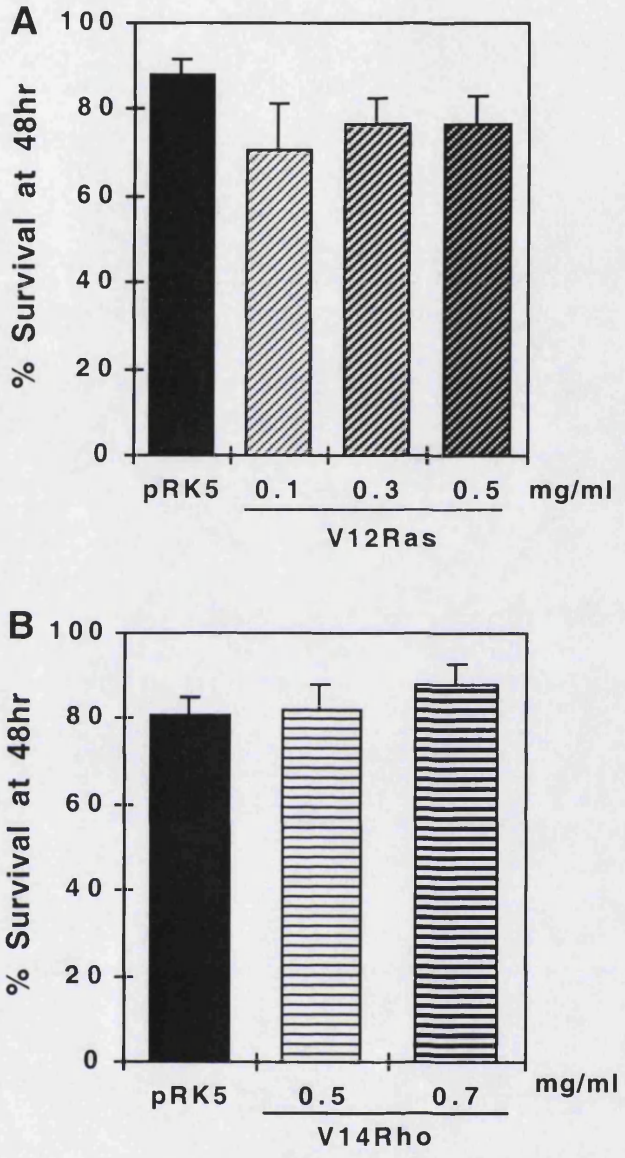


Figure 3.2 Activated Ras and RhoA do not induce apoptosis in SCG neurons.

Sympathetic neurons, cultured for 5-7 days in the presence of NGF, were microinjected with pRK5 empty vector [0.5 mg/ml (A), 0.7 mg/ml (B)], V12 Ras (A) or V14 RhoA (B) at the indicated concentrations and 5 mg/ml 70 kDa Texas Red dextran to mark the injected cells. Forty-eight hours after injection the percentage of surviving cells was assessed by phase microscopy. The results are the mean ± SEM of 10 (A & B) independent experiments.

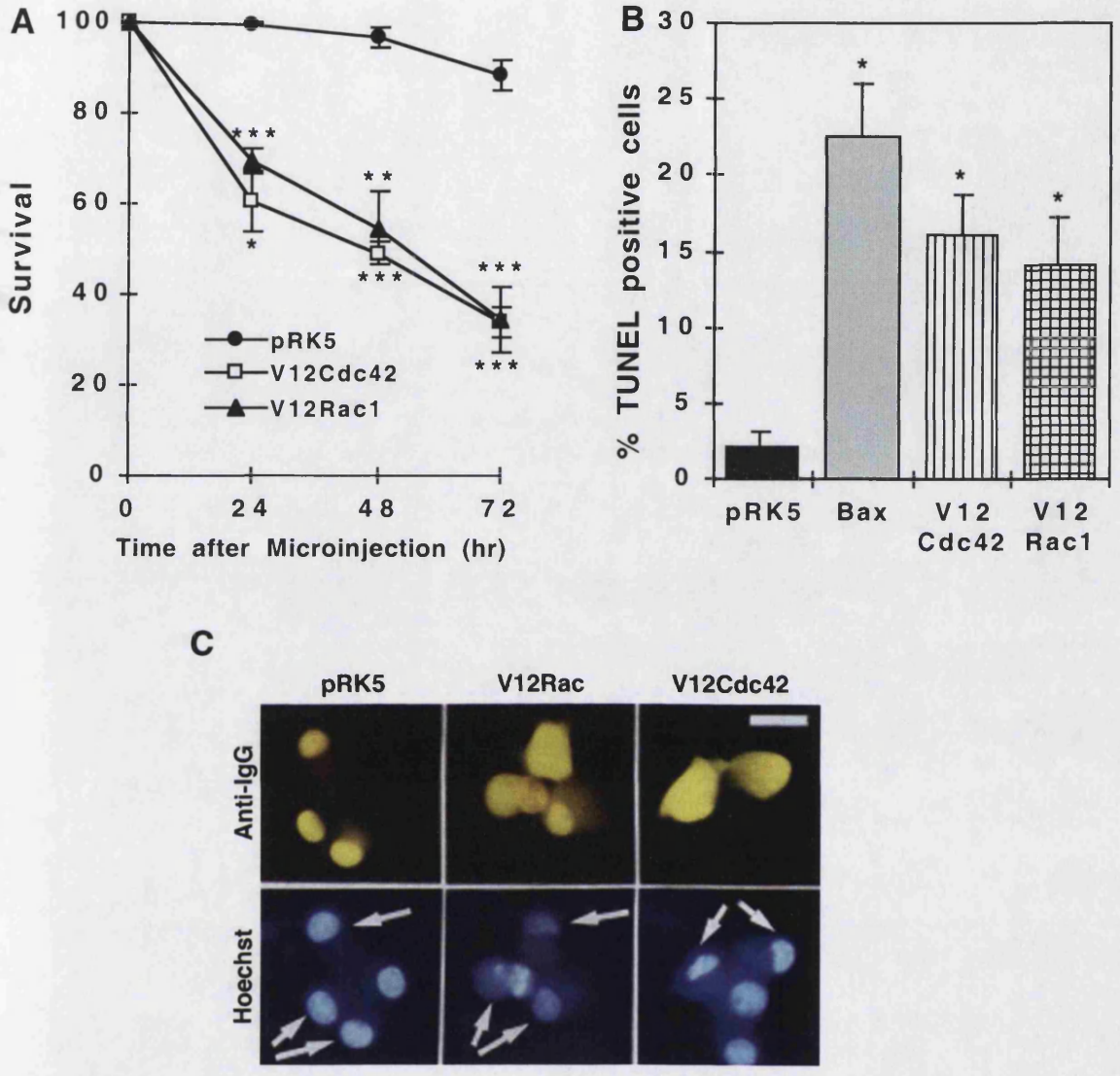


Figure 3.3 Activated Cdc42 and Rac1 induce apoptosis in SCG neurons.

(A) SCG neurons, cultured for 5-7 days in the presence of NGF, were injected with 0.1 mg/ml pRK5, V12Cdc42 or V12Rac1. Twenty-four, 48 and 72 hours later, the percentage of surviving cells was determined. The results are the mean ± SEM of 3 independent experiments. * $p<0.05$; ** $p<0.01$; *** $p<0.005$ (compared to the empty vector control at 24, 48 and 72 hours respectively).

(B) SCG neurons, cultured for 5-7 days in the presence of NGF, were microinjected with 0.3 mg/ml V12Cdc42, V12Rac1, pRK5 or 0.05 mg/ml Bax. TUNEL analysis was performed 16 hours later. The results are the mean ± SEM of 3 independent experiments. * $p<0.05$ (compared to the empty vector control).

(C) Nuclear morphology of SCG neurons microinjected with 0.3 mg/ml of pRK5, V12Rac1 or V12Cdc42. The cells were stained with an anti-GP-IgG antibody and Hoechst dye 16-24 hours after injection. The arrows point to nuclei of injected neurons. Bar = 30 μm.

3.4 Cdc42 and Rac1 are required for NGF withdrawal-induced apoptosis

To determine whether NGF withdrawal-induced apoptosis in SCG neurons caused activation of a Cdc42- and Rac1-mediated apoptotic response, their respective dominant negative mutants were microinjected into sympathetic neurons. Four hours after injection, NGF was removed and the percentage of surviving cells was assessed 48 hours later by phase microscopy. Both N17Cdc42 and N17Rac1 protected neurons from NGF withdrawal-induced apoptosis in a dose-dependent manner (Figure 3.4). These results suggest that not only are Cdc42 and Rac1 able to induce apoptosis, they are also required for cell death induced by NGF deprivation in SCG neurons.

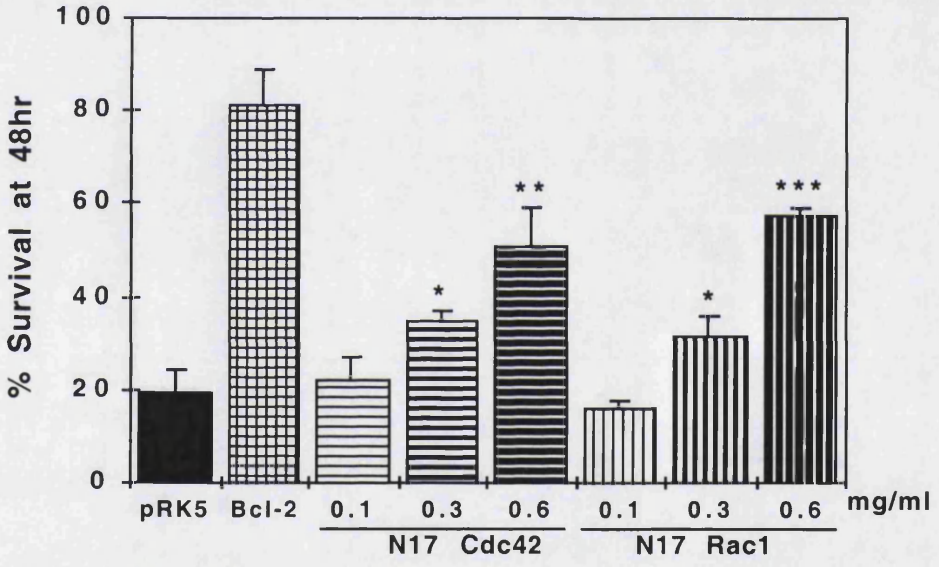


Figure 3.4 Dominant negative mutants of Cdc42 and Rac1 protect sympathetic neurons against NGF withdrawal-induced death.

SCG neurons were microinjected with increasing concentrations of N17Cdc42 and N17Rac1, 1.0 mg/ml pRK5 (negative control) or 0.05 mg/ml Bcl-2 (positive control). Twenty-four hours after injection the cells were withdrawn from NGF and left for an additional 48 hours before assessing survival by phase microscopy. The results are the mean ± SEM of 4 independent experiments. * $p<0.05$; ** $p<0.01$; *** $p<0.005$ (compared to the empty vector control).

3.5 Cdc42 lies upstream of Rac1

In order to examine the relationship between Cdc42 and Rac1 in the induction of neuronal death, V12Cdc42 and V12Rac1 were microinjected with N17Rac1 and N17Cdc42 respectively, into SCG neurons in the presence of NGF. The percentage of survival was assessed 48 hours later by phase microscopy. N17Rac1 blocked V12Cdc42-induced death whereas N17Cdc42 had no significant effect on neuronal death following microinjection with V12Rac1 (Figures 3.5A and B). This suggests that Cdc42 lies upstream of Rac1 in SCG neurons, a result which is consistent with studies in other cell types (Kozma *et al.*, 1995; Nobes and Hall, 1995). Based on this finding the research efforts of this thesis were concentrated on the mechanism of Cdc42-induced apoptosis.

3.6 Activation of Cdc42 results in an increase in the level of c-Jun and of its phosphorylation

To determine whether V12Cdc42 would induce an increase in the level of c-Jun protein and of its phosphorylation, as happens upon NGF withdrawal (Ham *et al.*, 1995), sympathetic neurons were microinjected with either V12Cdc42 or the empty expression vector, pRK5, and stained 16 hours later with a specific c-Jun antibody (Lallemand *et al.*, 1997). About 20% of the neurons injected with the empty vector stained above background for c-Jun, which represents no increase in staining when compared to uninjected cells (data not shown). In contrast, neurons injected with V12Cdc42 showed a significant, 2-fold increase in the level of c-Jun expression when compared to the empty expression vector (Figure 3.6A). Furthermore, when these cells were stained with an anti-phospho-c-Jun antibody, specific for phosphorylation on serine 63 (Lallemand *et al.*, 1998), the empty vector-injected cells did not show an increase in the level of nuclear phospho-c-Jun staining as compared to the background, whereas V12Cdc42 induced a significant increase in the number of cells clearly expressing nuclear phospho-c-Jun above the background (Figure 3.6B). These findings suggest that activation of Cdc42 in sympathetic neurons results in an increase in the level of c-Jun and of its

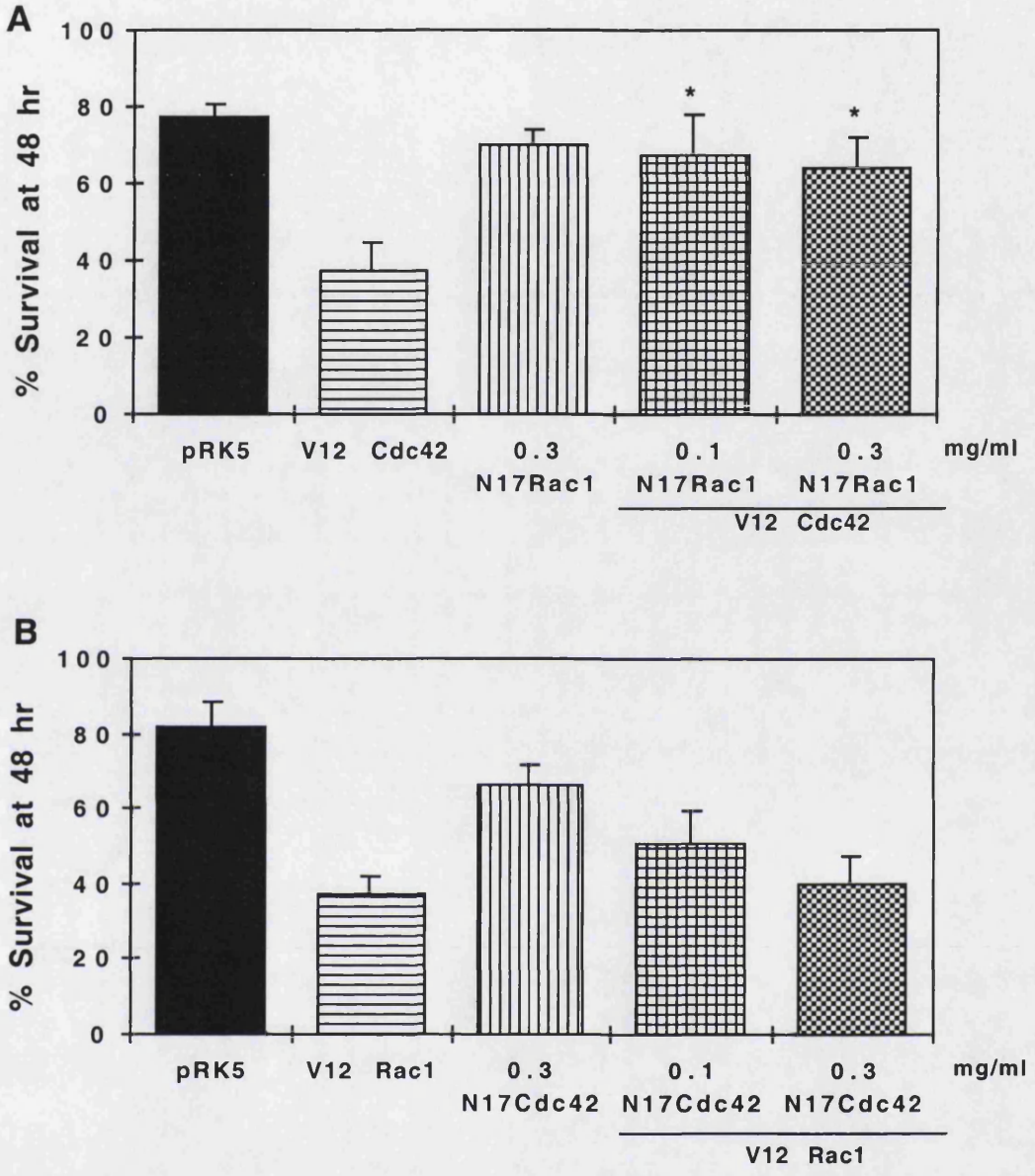


Figure 3.5 Cdc42 is upstream of Rac1.

(A) SCG neurons, cultured for 5-7 days in the presence of NGF, were co-injected with 0.1 mg/ml V12Cdc42 and increasing concentrations of N17Rac1 or 0.4 mg/ml pRK5. Forty-eight hours later, the percentage of surviving cells was assessed by phase microscopy.

(B) SCG neurons, cultured for 5-7 days in the presence of NGF, were co-injected with 0.1 mg/ml V12Rac1 and increasing concentrations of N17Cdc42 or 0.4 mg/ml pRK5. Forty-eight hours later, the percentage of surviving cells was assessed.

The results are the mean ± SEM of 3 (A) or 4 (B) independent experiments. * $p<0.05$ [compared to V12Cdc42 (A)]. No significant differences were observed between V12Rac1 and V12Rac1 + 0.1/0.3 mg/ml N17Cdc42 (B).

phosphorylation, corroborating with the findings of Perona *et al.* (Perona *et al.*, 1997) which showed that the Rho family of GTPases efficiently induce the transcriptional activity of c-Jun. In addition, these results also suggest that Cdc42 activation in SCG neurons results in the activation of JNK as previously observed in other cell types (Coso *et al.*, 1995; Minden *et al.*, 1995). To determine whether Cdc42 was necessary for induction of c-Jun, N17Cdc42 or an empty expression vector were microinjected into SCG neurons and NGF was withdrawn 4 hours later. The cells were stained for c-Jun 24 hours after NGF deprivation and were then examined by immunofluorescence. About 60% of the cells injected with the empty vector expressed high levels of c-Jun whereas only about 25% of the cells injected with N17Cdc42 were scored positive for c-Jun staining (Figure 3.6C). The fact that N17Cdc42 was able to decrease c-Jun levels suggests that Cdc42 is required for c-Jun induction after NGF withdrawal.

Figure 3.6 Activation of Cdc42 results in an increase in the level of c-Jun protein and its phosphorylation.

Left panel: quantitation of the experiments described below. Right panel: examples of typical c-Jun or phospho-c-Jun stainings observed in the experiments described below. Bar = 30 μm.

(A) Cdc42 induces an increase in the level of c-Jun.

V12Cdc42 or pRK5 (0.3 mg/ml) were microinjected into SCG neurons, which cultured for 5-7 days and maintained in the presence of NGF. Twenty-four hours after injection, the cells were fixed, permeabilised, and stained with Hoechst to reveal the nuclei, a rhodamine-conjugated anti-GP-IgG antibody to detect the injected cells and an anti-c-Jun antibody. Only the cells in which c-Jun staining was clearly above background were scored as positive.

(B) Cdc42 induces an increase in the level of phosphorylated c-Jun.

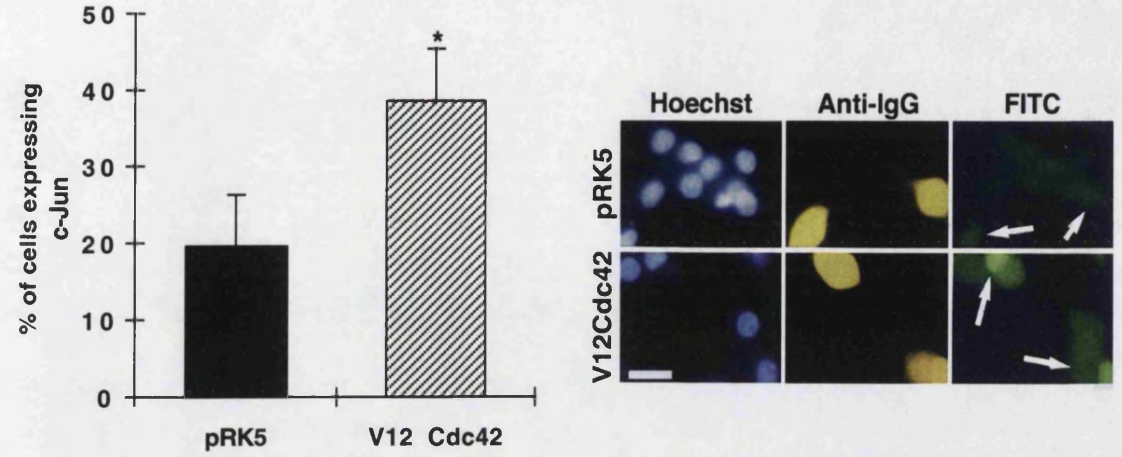
V12Cdc42 or pRK5 (0.3 mg/ml) were microinjected into SCG neurons, that were cultured for 5-7 days in the presence of NGF. Twenty-four hours after injection, the percentage of cells expressing phospho-c-Jun was assessed. Only the cells in which phospho-c-Jun staining was clearly above background were scored positive.

(C) Cdc42 is required for c-Jun induction in SCG neurons after NGF withdrawal.

SCG neurons, cultured for 5-7 days in the presence of NGF, were microinjected with 0.6 mg/ml N17Cdc42 or pRK5. The neurons were withdrawn from NGF 4-6 hours after injection. Twenty-four hours later, the percentage of cells expressing nuclear c-Jun was assessed.

The results are the mean ± SEM of 6 (A & C) or 4 (B) independent experiments. * $p<0.05$; *** $p<0.005$ (Student's *t*-test).

A


50
40
30
20
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% of cells expressing c-Jun
*
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V12 Cdc42
Hoechst
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pRK5
V12Cdc42

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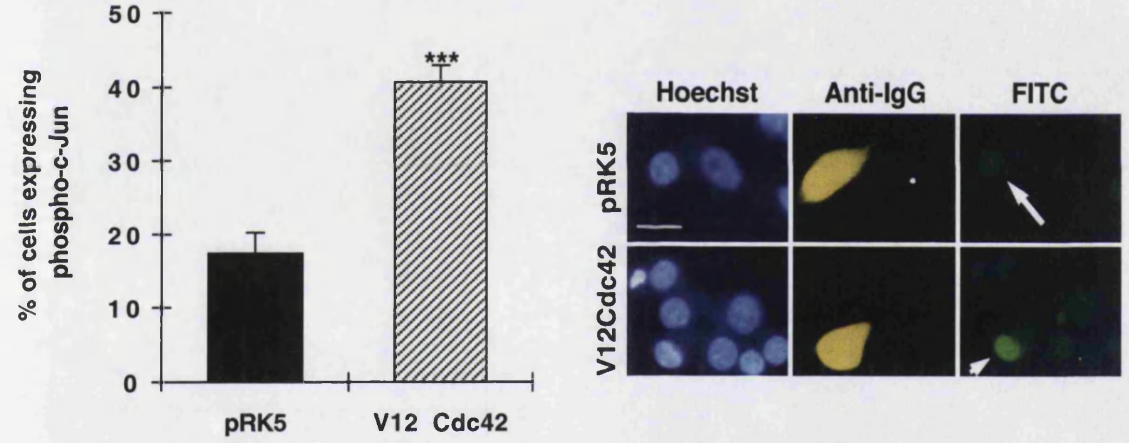
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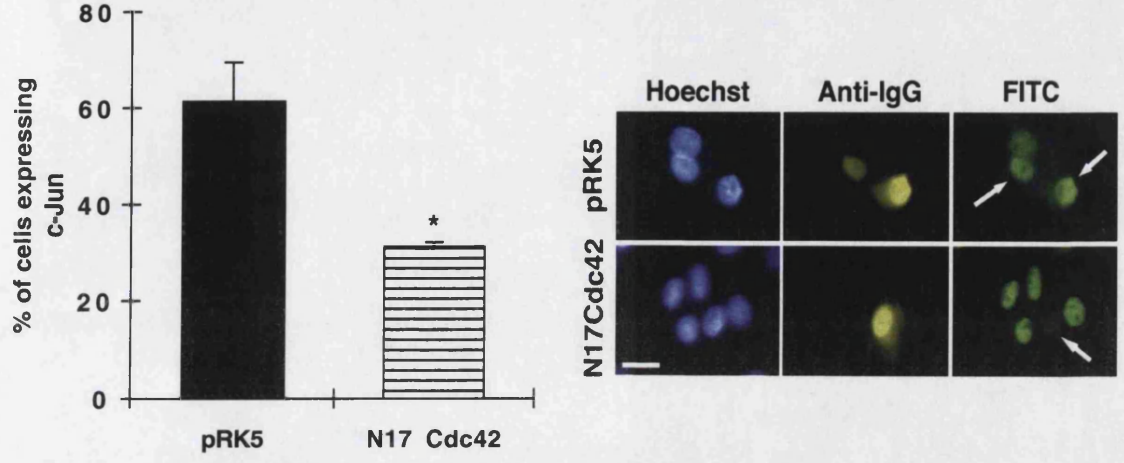
80
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% of cells expressing c-Jun
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pRK5
N17 Cdc42
Hoechst
Anti-IgG
FITC
pRK5
N17Cdc42

3.7 Cdc42-induced apoptosis requires AP-1 activity

In order to determine whether AP-1 activity was required for Cdc42-induced apoptosis, sympathetic neurons were co-injected with V12Cdc42 and FLAG-Δ169, a mutant of c-Jun that lacks the amino-terminal transactivation domain and functions as a dominant negative of AP-1 activity (Ham *et al.*, 1995). This dominant negative mutant was able to fully protect the neurons from Cdc42-induced apoptosis in the presence of NGF (Figure 3.7), suggesting that AP-1 activity is essential for Cdc42-induced apoptosis in SCG neurons.

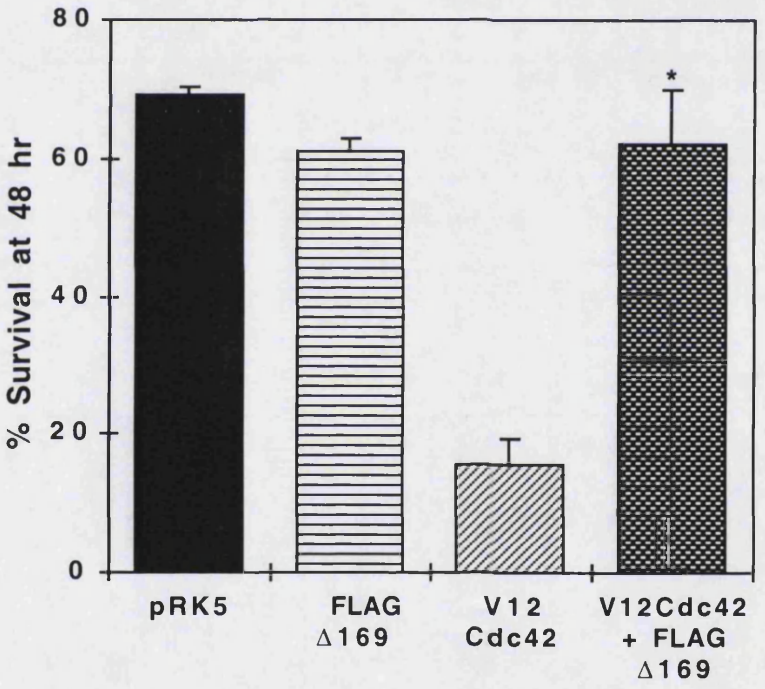


Figure 3.7 Cdc42-induced apoptosis requires AP-1 activity.

Sympathetic neurons were microinjected with 0.5 mg/ml of pRK5, 0.1 mg/ml V12Cdc42, 0.4 mg/ml pcDN-FLAG-Δ169 or 0.1 mg/ml V12Cdc42 + 0.4 mg/ml FLAG-Δ169. The cells were maintained in the presence of NGF and the percentage of surviving cells was assessed 48 hours later by phase microscopy. The results are the mean ± SEM of 3 independent experiments. * $p<0.05$ (compared to V12Cdc42).

3.8 The dominant negative mutant of SEK1/MKK4 (SEK-AL) does not block Cdc42-induced death in sympathetic neurons

In many cell types, including differentiated PC12 cells, the MAPKK SEK1/MKK4 has been identified as the mediator of JNK activation by MEKK1 (Sanchez *et al.*, 1994; Yan *et al.*, 1994; Xia *et al.*, 1995; Le-Niculescu *et al.*, 1999). In addition, in sympathetic neurons, microinjection of MEKK1 was shown to mediate an increase in the levels of c-Jun and c-Jun phosphorylation and to induce apoptosis (Eilers *et al.*, 1998). Furthermore, MEKK1-induced c-Jun expression, c-Jun phosphorylation and apoptosis could be blocked by co-expression of a dominant negative mutant of SEK1 (SEK-AL), suggesting that MEKK1 activates the JNK pathway and induces apoptosis in SCG neurons in a SEK1-dependent manner (Eilers *et al.*, 1998). It was therefore of interest to check whether SEK1 would mediate apoptosis induced by Cdc42. Sympathetic neurons were co-injected with V12Cdc42 and SEK-AL and the survival assessed 48 hours later by phase microscopy. The dominant negative mutant of SEK1 failed to protect the neurons from V12Cdc42-induced death, demonstrating that SEK1 is not essential for the mediation of apoptosis by Cdc42 (Figure 3.8). In the study by Eilers *et al.* (Eilers *et al.*, 1998), SEK-AL could not prevent NGF withdrawal-induction of c-Jun, c-Jun phosphorylation or apoptosis, suggesting that in SCG neurons SEK1 is not required for the activation of the JNK pathway and apoptosis induced by NGF deprivation. This is in contrast to the study by Xia *et al.* (Xia *et al.*, 1995) which found that overexpression of a dominant negative SEK1 mutant could block NGF withdrawal-induced apoptosis in differentiated PC12 cells. Taken together these results do not completely exclude the involvement of SEK1 in mediating Cdc42- and/or NGF withdrawal-induced activation of JNK, as it is not clear whether SEK1 is activated at all and suggests that there might be other MAPKKs, downstream of Cdc42, which are responsible for the relay of the apoptotic signal towards JNK.

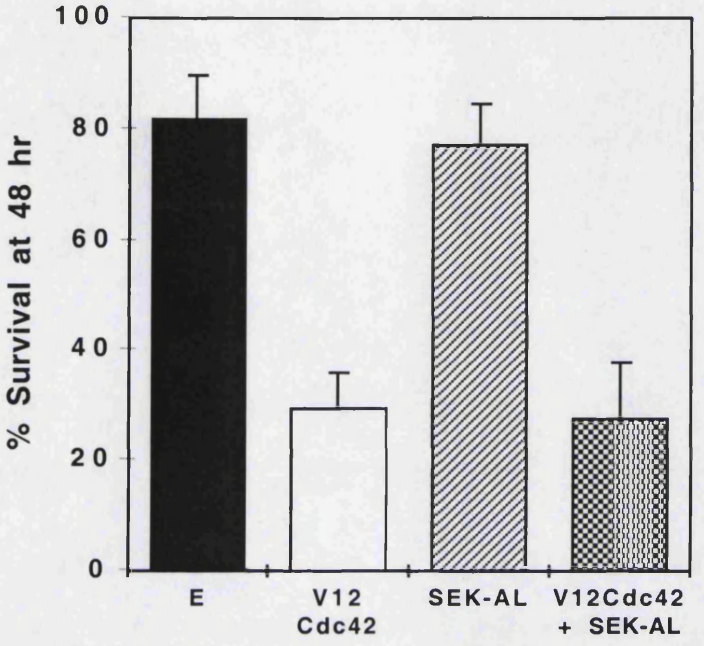


Figure 3.8 Cdc42-induced death is not mediated by SEK1.

Sympathetic neurons were microinjected with 0.5 mg/ml of pRK5, 0.1 mg/ml V12Cdc42, 0.4 mg/ml pcDN-SEK-AL or 0.1 mg/ml V12Cdc42 + 0.4 mg/ml SEK-AL. The cells were maintained in the presence of NGF and the percentage of surviving cells was assessed 48 hours later by phase microscopy. The results are the mean ± SEM of 3 independent experiments. No significance difference was observed between V12Cdc42 and V12Cdc42 + SEK-AL.

3.9 The broad spectrum caspase inhibitor zVAD-fmk inhibits Cdc42-induced apoptosis in SCG neurons

Benzyloxycarbonyl-Val-Ala-Asp-(O-methyl)-fluoromethyl-ketone (zVAD-fmk) is a broad, irreversible inhibitor of caspases shown to inhibit apoptosis in various cell types (Fearnhead *et al.*, 1995; Zhu *et al.*, 1995; Slee *et al.*, 1996), including a number of neuronal systems such as PC12 cells (Troy *et al.*, 1996), cerebellar granule neurons (Armstrong *et al.*, 1997; Taylor *et al.*, 1997) and sympathetic neurons (McCarthy *et al.*, 1997). In order to determine whether activated Cdc42 would mediate apoptosis via the activation of downstream caspases, SCG neurons, pre-treated for 3-4 hours with 100 μM zVAD-fmk, were microinjected with V12Cdc42 or pRK5 and maintained in the presence of NGF. The cells were scored for survival 48 hours after injection (Figure 3.9A). About 90% of cells injected with pRK5 were alive after 48 hours of treatment with the caspase inhibitor, indicating that at this concentration zVAD-fmk does not have a toxic effect. About 80% of the neurons injected with V12Cdc42 were dead after 48 hours. This death was prevented with pre-treatment with zVAD-fmk. Because the survival obtained with zVAD-fmk in V12Cdc42-injected neurons did not reach levels similar to the empty vector control, it raised the question of whether this constitutively activated form of Cdc42 generates a too potent death signal for it to be completely blocked by non-toxic amounts of caspase inhibitor. To check whether this was the case, WTCdc42 was microinjected into sympathetic neurons in independent experiments (Figure 3.9B). Similar to the experiment in Figure 3.9A, zVAD-fmk had no effect on the survival of the empty vector injected cells, which had survival rates of about 80%. Only about 30% of the neurons microinjected with WTCdc42 were still alive after 48 hours whilst about 80% of the neurons that had been treated with 100 μM zVAD-fmk had survived. This experiment demonstrated that zVAD-fmk could inhibit most of the death induced by Cdc42. Taken together, these results suggest that the induction of neuronal cell death by Cdc42 is mediated by the activation of caspases. The exact mechanism of how Cdc42 induces caspase activation remains to be investigated.

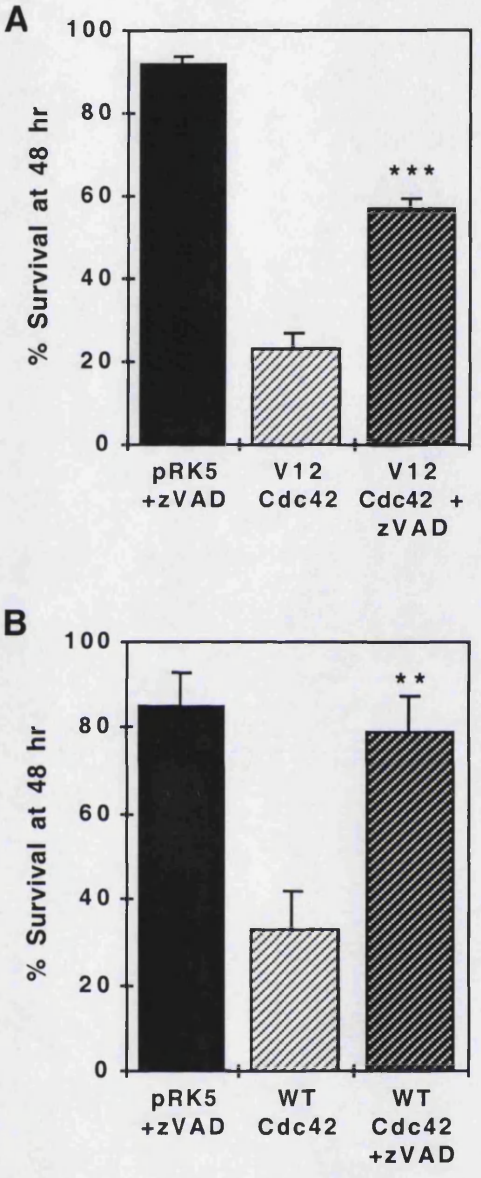


Figure 3.9 zVAD-fmk can protect sympathetic neurons from V12Cdc42-induced death.

SCG neurons were microinjected with 0.1 mg/ml of pRK5 or V12Cdc42 (A) or 0.2 mg/ml pRK5 or WTCdc42 (B). Cells were pre-treated with 100 μM zVAD-fmk for 3 hours prior to microinjection. The cells were maintained in the presence of NGF following injection and the percentage of surviving cells was determined 48 hours later. The results are the mean ± SEM of 3 (A & B) independent experiments. ** $p<0.01$; *** $p<0.005$ [compared to V12Cdc42 (A) or WTCdc42 (B)].

3.10 Discussion

In this Chapter the role of the small GTP-binding proteins in neuronal cell death was investigated. It was demonstrated that activated forms of Cdc42 and Rac1 can induce apoptosis of sympathetic neurons in the presence of NGF. However, neither RhoA nor Ras had an effect on the survival of the neurons, even when expressed at high concentrations, strongly suggesting that the effect observed with both Cdc42 and Rac1 is specific and not just a result of overexpressing this type of proteins in neurons. This is in agreement with the observation that in many systems Rho, in contrast to Cdc42 and Rac, is unable to activate JNK (Minden *et al.*, 1995; Olson *et al.*, 1995), which is required for the induction of apoptosis in the SCG neuronal cell system used in this study (Eilers *et al.*, 1998; Harding *et al.*, 2000). In addition, overexpression of activated RhoA did not induce cell death in Jurkat T cells, while Cdc42 and Rac1 did (Chuang *et al.*, 1997). Intracellular loading of activated Ras protein has been shown to promote the survival of SCG neurons in the absence of NGF, while Fab fragments of neutralising antibodies to p21Ras blocked the survival effect of NGF, LIF, CNT and cAMP (Nobes and Tolkovsky, 1995). In addition, overexpression of a dominant negative mutant of Ras (N17Ras), caused massive apoptosis in SCG neurons in the presence of NGF (Xue *et al.*, 2000). These findings show that Ras is primarily involved in the survival as opposed to the death pathway in SCG neurons and corroborates with the inability of Ras to induce death. The main evidence for the requirement of Cdc42 and Rac1 in the execution of apoptosis upon NGF deprivation in SCG neurons was demonstrated by the fact that dominant negative mutants of Cdc42 and Rac1 could rescue these cells from NGF withdrawal. These results show that NGF withdrawal-induced apoptosis is a result of downstream signalling by active Cdc42 and Rac1 and that in these neurons Cdc42 and Rac1 play an important physiological role. In agreement with previous studies, Cdc42 was shown to lie upstream of Rac1 (Kozma *et al.*, 1995; Nobes and Hall, 1995). Taking this into account, the studies that followed were performed with Cdc42 only.

In many cell types including neurons, fibroblasts and cells of the myeloid lineage, induction of apoptosis by various stresses is followed by an activation of the JNK pathway (Westwick *et al.*, 1995; Xia *et al.*, 1995; Cahill *et al.*, 1996; Chen *et al.*, 1996b; Cosulich and Clarke, 1996; Frisch *et al.*, 1996; Verheij *et al.*, 1996; Wilson *et al.*, 1996; Zanke *et al.*, 1996). When overexpressed in SCG neurons, constitutively activated Cdc42 induced an increase in both the levels of c-Jun and of phosphorylated c-Jun. Furthermore, a dominant negative mutant of Cdc42 prevented the increase in the amount of c-Jun protein observed upon NGF withdrawal. Taken together, these results provide evidence that in sympathetic neurons JNK activation and c-Jun expression are regulated by Cdc42. The results are in agreement with previous studies in which the small GTP-binding proteins Cdc42 and Rac1 were able to activate the kinase cascade that regulates JNK (Bagrodia *et al.*, 1995; Coso *et al.*, 1995; Minden *et al.*, 1995; Zhang *et al.*, 1995). A c-Jun dominant negative mutant, FLAG-Δ169, blocked apoptosis induced by V12Cdc42, further demonstrating that the apoptotic response to Cdc42 is elicited by proteins that are upregulated or controlled by the AP-1 transcription factor which is in agreement with Minden *et al.* (Minden *et al.*, 1995) and Clarke *et al.* (Clarke *et al.*, 1998) who showed that Rac1 and Cdc42 stimulated c-Jun's transcriptional activity. These findings are corroborated by the study by Chuang *et al.* (Chuang *et al.*, 1997) who found Cdc42 and Rac1 to be important mediators of apoptosis and JNK activation in Jurkat cells. A dominant negative mutant of SEK1 did not block V12Cdc42-induced death in sympathetic neurons. This, together with the findings of Eilers *et al.* (Eilers *et al.*, 1998), shows that SEK1 is not essential for V12Cdc42- and NGF withdrawal-induced death of SCG neurons and suggests that perhaps other kinases, together with or instead of SEK1, are activated upon NGF withdrawal or activation of Cdc42 and are critical for the mediation of apoptosis and JNK activation.

In the last few years, it has become evident that caspases play an important role in regulating apoptosis [reviewed by (Thornberry and Molineaux, 1995; Takahashi, 1999)] including in many neuronal cell types deprived of growth factors (Gagliardini *et al.*, 1994; Martinou *et al.*, 1995; Milligan *et al.*, 1995; Troy *et al.*, 1996; McCarthy *et al.*, 1997). Recently, two novel GDIs have been identified: D4-

GDI, which is expressed only in haemapoietic tissues (Lelias *et al.*, 1993; Scherle *et al.*, 1993) and Rho GDIγ, which is expressed in brain and pancreas (Adra *et al.*, 1997). In vitro studies have shown that D4-GDI can function as a GDI for RhoA, Rac and Cdc42 (Adra *et al.*, 1993) and that Rho GDIγ functions as a GDI for Cdc42, RhoA, B and G (Zalcman *et al.*, 1996; Adra *et al.*, 1997). Very little is known about the biological function of the Rho GDIs in vivo, but they have been shown to inhibit the downstream functions of Rho-GTPases (Nishiyama *et al.*, 1994; Coso *et al.*, 1995). In addition, D4-GDI has been found to be an important substrate of CPP32/caspase- 3 in Jurkat cells undergoing apoptosis (Na *et al.*, 1996). The cleavage of Rho-GDI (or perhaps even other GDIs) by caspases during apoptosis may therefore change the activity and the signalling by Rho-family GTP binding proteins. The result that zVAD-fmk protected the neurons from Cdc42-induced death provides a direct evidence that caspases are involved in the mediation of cell death by Cdc42. It is therefore tempting to speculate that, in sympathetic neurons, activation of caspases could lead to cleavage and thereby inactivation of Rho-GDIs specific for Cdc42/Rac thereby further prolonging the activated state of these small GTPases and therefore amplifying this death signalling pathway (positive feedback mechanism). Interestingly, in NGF withdrawal-induced death of differentiated PC12 cells, Rho-GDI itself was not found to be cleaved (C. Bazenet, unpublished observations). This, however, does not exclude the possibility that other GDIs could still be cleaved.

Overall, the requirement for Cdc42 in neuronal apoptosis suggests that Cdc42 is a key component of the cell death machinery in sympathetic neurons and that activation of Cdc42 is one of the early events in the death signalling pathway. The identification of the downstream effector(s) of Cdc42 mediating the activation of the JNK cascade and of apoptosis is therefore an important step towards understanding the underlying signalling events of neuronal cell death. The observation that a MEKK1→SEK1 pathway is not essential for NGF withdrawal and Cdc42 induced-deaths [this Chapter and (Eilers *et al.*, 1998)], suggests that there are other MAPKKKs and MAPKKs that are both activated by Cdc42 and/or NGF withdrawal and are crucial elements of the signalling to JNK activation and

induction of neuronal cell death. The remaining Chapters focus on trying to identify this/these crucial kinase(s).

4. Mutational analysis of Cdc42

4.1 Introduction

One of the ways that has been used to look at downstream pathways of Ras family members is through the use of effector mutants. For instance, an approach that was used to dissect the role of various putative targets of Ras, was the introduction of single amino acid substitutions in the effector domain of Ras, thereby abrogating the interaction with specific targets and inhibiting specific biological outcomes (White *et al.*, 1995). A similar approach has also been taken by Lamarche *et al.* (Lamarche *et al.*, 1996) to determine which of the downstream effectors of Rac1 and Cdc42 are responsible for the induction of actin polymerization and G1 cell cycle progression. Lamarche *et al.* (Lamarche *et al.*, 1996) introduced single point mutations in the effector domain of the constitutively activated Cdc42L61 and Rac1L61 mutants, thereby selectively abolishing the binding to and/or activation of particular downstream effectors. In this study, the same mutants were used to determine which of the downstream effectors of Cdc42 mediates JNK pathway activation and induction of cell death in rat sympathetic neurons, because of their different abilities to bind to CRIB containing proteins and activate the JNK pathway. Interestingly, in Lamarche's study (Lamarche *et al.*, 1996), the ability of Cdc42 to activate JNK required Cdc42 to be able to interact with CRIB containing proteins. In agreement with this, various CRIB containing proteins, such as the PAKs and MLK3, have been shown to activate the JNK pathway (Brown *et al.*, 1996; Tibbles *et al.*, 1996). The experiments presented in this Chapter aimed at identifying a link between Cdc42, CRIB containing proteins, JNK activation and cell death.

In this study, three Cdc42 mutants were used: Cdc42L61, Cdc42L61C40 and Cdc42L61A37 (Figure 1.6). The Q61→L mutation, as with the G12→V mutation, results in a decrease in the intrinsic GTPase activity, thereby rendering it constitutively active (Barbacid, 1987; Bourne *et al.*, 1991). The Y40→C amino acid substitution in the constitutively active Cdc42L61 mutant prevents the interaction

with CRIB containing proteins including WASP, MSE55, the *C. elegans* protein F09F7.5 and PAK1, and could therefore affect the recruitment of other CRIB containing proteins such as MLKs or other PAK isoforms (Lamarche *et al.*, 1996). At the time of this study, the Y40→C mutant had not been shown to interact with any CRIB containing proteins. This mutant is unable to activate the serine/threonine kinase activity of PAK1 and the JNK cascade, but does not affect Cdc42-dependent actin reorganisation as evidenced by its induction of filopodium formation in Swiss 3T3 fibroblasts (Lamarche *et al.*, 1996). On the other hand, the F37→A amino acid substitution does not affect the ability of Cdc42 to activate the JNK cascade. However, this mutant is unable to activate Rac and Cdc42L61A37-mediated induction of filopodia is not accompanied by the normal Rac-mediated lamellipodia formation (Lamarche *et al.*, 1996).

4.2 Effect of the various Cdc42 mutants on the survival of SCG neurons in the presence of NGF

To determine which of the downstream effectors of Cdc42 mediates induction of cell death in rat sympathetic neurons, SCG neurons, cultured for 5-7 days in the presence of NGF, were microinjected with 0.3 mg/ml of either V12Cdc42 (positive control), Cdc42L61, Cdc42L61C40, Cdc42L61A37 or of the empty expression vector pRK5, together with 5 mg/ml 70 kDa Texas Red Dextran to detect injected cells. The cells were refed with fresh NGF-containing medium and the percentage of injected viable neurons was determined 48 hours later. Injection of all mutants into primary rat sympathetic neurons significantly decreased their viability (Figure 4.1). No significant differences between the L61 and the V12 mutant, that was used in the previous studies [Chapter 3 and (Bazenet *et al.*, 1998)], were observed.

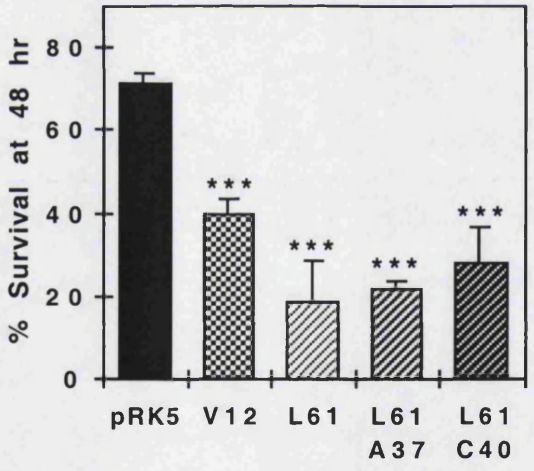


Figure 4.1 Cdc42 mutants decrease the survival of SCG neurons in the presence of NGF.

Sympathetic neurons, cultured for 5-7 days, were microinjected with 0.3 mg/ml of the empty pRK5 expression vector, V12Cdc42, Cdc42 L61, Cdc42 L61A37 or Cdc42 L61C40 together with 5.0 mg/ml of Texas Red-dextran to identify the injected cells. The percentage of surviving cells was assessed 48 hours later by phase microscopy. The results shown are the mean ± SEM of 4 independent experiments. *** $p<0.005$ (compared to the pRK5 empty vector control).

To characterise the death induced by the various mutants, sympathetic neurons were injected with 0.3 mg/ml of each mutant and maintained in the presence of NGF. Twenty-four hours later, the nuclear morphology was assessed by Hoechst staining and the percentage of pyknotic nuclei was determined. Over 50% of the cells injected with either one of the mutants displayed pyknotic nuclei as opposed to less than 20% for the empty vector control (Figure 4.2).

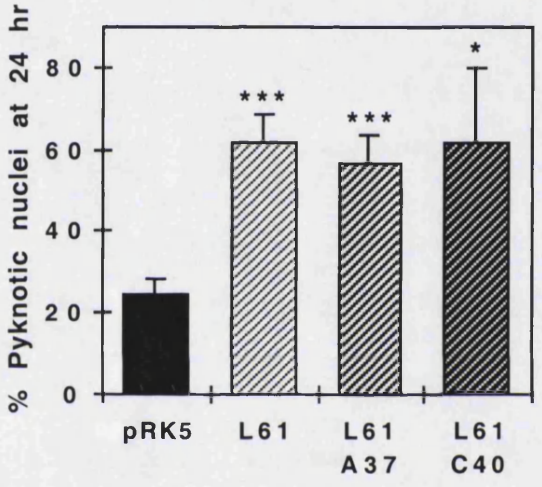


Figure 4.2 Effect of various Cdc42 mutants on the nuclear morphology of SCG neurons.

Morphology of cells injected with pRK5, or pRK5 containing Cdc42 L61, Cdc42 L61A37 or Cdc42 L61C40. SCG neurons, cultured for 5-7 days in the presence of NGF, were injected with 0.3 mg/ml of either DNA together with 2.5 mg/ml guinea-pig IgG to detect the injected cells and stained with Hoechst dye 24 hours later. The results are the mean ± SEM of 3 independent experiments. Only the cells overexpressing the Cdc42 mutants display clearly pyknotic nuclei. * $p<0.05$; *** $p<0.005$ (compared to the pRK5 empty vector control).

In other cells systems, the impairment of Cdc42 in binding to CRIB containing proteins resulted in Cdc42 not being able to activate the JNK cascade (Lamarche *et al.*, 1996). The results presented above suggest that Cdc42's inability to bind to CRIB containing proteins and to activate the JNK cascade do not affect its ability to induce neuronal apoptosis. These results are therefore surprising as

activation of the JNK pathway has been shown to be required for apoptosis of SCG neurons (Eilers *et al.*, 1998; Harding *et al.*, 2000).

4.3 Activation of JNK by Cdc42 effector mutants

In light of the above results it was important to examine the ability of each mutant to activate the JNK cascade in sympathetic neurons. To determine whether the levels of phospho-c-Jun would increase in the cells overexpressing the various mutants, SCG neurons were microinjected with 0.3 mg/ml of either Cdc42 effector mutant or pRK5, together with GP-IgG to detect the injected cells. After 24 hours, the cells were fixed and stained with a phospho-c-Jun antibody, specific for phosphorylated serine 63 (Eilers *et al.*, 1998) and the percentage of injected cells expressing phospho-c-Jun was determined (Figure 4.3). All mutants induced a significant ≈2-fold increase in the level of phospho-c-Jun compared to the empty vector. These results correlate with the survival results shown in Figures 4.1 and 4.2, suggesting that Cdc42 activation of the JNK pathway induces cell death, as already demonstrated in Chapter 3. However, although the increase in the levels of phosphorylated c-Jun was expected for the L61 and the L61A37 mutants, it was somewhat surprising to observe a similar increase with the L61C40 mutant which did not activate JNK in fibroblasts (Lamarche *et al.*, 1996).

Taken together, these results demonstrate that the effects on the activation of the JNK pathway, obtained with these mutants in SCG neurons, differ from those seen in fibroblasts (Lamarche *et al.*, 1996). On the other hand, they also show a strong correlation between cell death and activation of the JNK pathway.

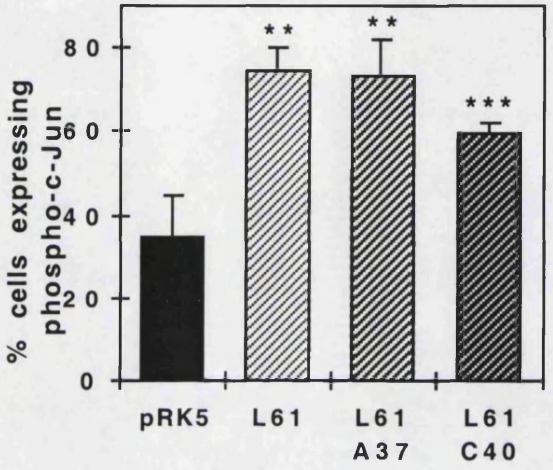


Figure 4.3 Increase in the level of phosphorylated c-Jun induced by Cdc42 mutants.

Sympathetic neurons, cultured for 5-7 days in the presence of NGF, were injected with 0.3 mg/ml of the empty pRK5 expression vector, Cdc42 L61, Cdc42 L61A37 or Cdc42 L61C40 together with 2.5 mg/ml of guinea-pig IgG to mark the injected cells. Twenty-four hours after injection, the percentage of surviving cells expressing phospho-c-Jun was determined. The results are the mean ± SEM of 3 independent experiments. ** $p<0.01$; *** $p<0.005$ (compared to the pRK5 empty vector control).

4.4 Discussion

Recently, Cdc42 has been identified as an initiator of apoptosis in Jurkat T lymphocytes (Chuang *et al.*, 1997) and in rat sympathetic neurons [Chapter 3 & (Bazenet *et al.*, 1998)]. Induction of cell death by Cdc42 led to the activation of the JNK pathway in both cell types. In addition, the JNK pathway has been shown to play a crucial role in the induction of apoptosis, by a variety of means, in a number of cell types including SCG neurons and cerebellar granule neurons (Eilers *et al.*, 1998; Watson *et al.*, 1998; Harding *et al.*, 2000). Therefore, identification of the upstream regulators of this cascade is a necessary step in understanding the mechanisms underlying neuronal apoptotic cell death. Possible mediators of activation of the JNK cascade by Cdc42 include the p21-activated kinase (PAK1) (Manser *et al.*, 1994; Bagrodia *et al.*, 1995; Zhang *et al.*, 1995; Brown *et al.*, 1996; Frost *et al.*, 1996; Lamarche *et al.*, 1996), the mixed lineage kinases, MLK2 (Nagata *et al.*, 1998) and MLK3 (Rana *et al.*, 1996; Teramoto *et al.*, 1996a; Tibbles *et al.*, 1996) as well as MEKK1 and MEKK4 (Fanger *et al.*, 1997; Gerwins *et al.*, 1997).

In this Chapter, the role of CRIB containing proteins in linking Cdc42 to the JNK cascade and in inducing apoptosis in SCG neurons was investigated using various Cdc42 mutants. Overexpression of either of the mutants, in rat sympathetic neurons, induced apoptosis (Figures 4.1 and 4.2). This result suggested that binding of CRIB containing proteins and activation of the JNK pathway was not required for the induction of apoptosis by Cdc42. However, all mutants induced an increase in the level of phosphorylated c-Jun (Figure 4.3) comparable to that induced by the activated mutant of Cdc42 (Figure 3.6B). Furthermore, the equivalent Rac1 L61C40 mutant behaved in a similar way and induced both neuronal cell death and an increase in the level of phospho-c-Jun (data not shown). There are two possible explanations for these results: (1) CRIB containing proteins are not important for JNK activation; or (2) these mutants behave differently in neurons and they can still bind CRIB domain containing proteins. The first hypothesis would be supported by the report by Peter *et al.* (Peter *et al.*, 1996) suggesting that activation of the MAPK pathway mediated by

mating pheromones does not require the interaction between Cdc42 and Ste20, the yeast homologue of PAK. Also, Westwick *et al.* (Westwick *et al.*, 1997) showed that an effector mutant of Rac1, that fails to bind PAK, is still a very potent JNK activator. The second hypothesis is supported by the recent findings of Manser *et al.* (Manser *et al.*, 1998) who showed that, in COS cells, the Y40→C effector mutant was capable of increasing PAK kinase activity but mainly when co-transfected with the PAK-interacting exchange factor (PIX). Moreover, PAK4, which shares 7 out of the 8 conserved amino acids in the CRIB domain with PAK1, was found to interact with the Y40→C Cdc42 mutant (Abo *et al.*, 1998), suggesting that this point mutation is not sufficient to abrogate the interaction of Cdc42 with all PAK isoforms.

Unfortunately, the uncertainties, that were later raised about the specificity of these point mutations in abrogating specific protein interactions, could not be tested, either because of lack of tools at the time (e.g. specific antibodies, efficient transfection system) or due to the limitations of this neuronal system. Also, in the study by Lamarche *et al.* (Lamarche *et al.*, 1996), only PAK1 and JNK1 activation were examined. It is possible that other PAK and/or JNK isoforms can be stimulated by the L61C40 mutant. Indeed, in sympathetic neurons, all mutants induced a significant increase in the level of phosphorylated c-Jun, suggesting that if JNK1 activity is not induced by the L61C40 mutant, perhaps other JNK isoforms are.

Overall, these results did not rule out a role for the PAKs and/or other CRIB containing proteins in neuronal apoptosis, implicating redundant pathways leading to both activation of JNK and induction of neuronal apoptosis. In the following Chapters, the role of potential downstream effectors of Cdc42 in the induction of apoptosis and the JNK pathway is examined directly.

5. The role of PAKs in neuronal apoptosis

5.1 Introduction

As discussed earlier, there are many putative mediators of JNK activation by Cdc42. At the time of this investigation, many studies in the literature suggested that the PAKs were indeed some of the kinases linking Cdc42 to JNK activation (Manser *et al.*, 1994; Bagrodia *et al.*, 1995; Zhang *et al.*, 1995; Brown *et al.*, 1996; Frost *et al.*, 1996; Lamarche *et al.*, 1996). This work was focused on PAK1 because it is highly expressed in neuronal tissues (Manser *et al.*, 1994) and on PAK2 because of its demonstrated contribution to the execution of apoptosis (Lee *et al.*, 1997; Rudel and Bokoch, 1997).

To examine the role of PAKs in neuronal apoptosis, wild type and mutant PAK1 (L107F, K299R) and PAK2 (T403E, K278R) were used in this investigation. The L107F-PAK1 mutant contains a substitution in the Cdc42-binding domain, which abolishes the need for activation by Cdc42 and makes it constitutively active. In addition this mutant has been shown to activate the JNK/SAPK when expressed in COS-7 cells pathway (Brown *et al.*, 1996). T403E-PAK2 contains a threonine to glutamate mutation within the kinase domain, which renders it constitutively active. Both K299R-PAK1 and K278R-PAK2 are catalytically inactive mutants in which a critical lysine in the kinase subdomain II has been replaced by an arginine (Sells *et al.*, 1997) (see Figure 1.9).

5.2 PAK1 and PAK2 are expressed in rat sympathetic neurons

PAK1 has been found to be expressed mainly in the brain (Manser *et al.*, 1994) whereas PAK2 seems to be ubiquitous (Martin *et al.*, 1995a). If these two kinases were to play an important role in apoptosis of rat sympathetic neurons it was crucial to confirm that they were expressed in these cells. Thirty micrograms of total cellular protein, extracted from SCG neurons or from differentiated PC12 cells, were resolved on a 12.5% SDS-PAGE, transferred onto nitro-cellulose and probed with either an anti-Ste20 (subdomain VI - cross reacts with rat PAK1-Kinetek) or an anti-PAK2 (N-19 - Santa Cruz) polyclonal antibody. Figure 5.1 shows that both PAK1 (68 kDa) and PAK2 (62 kDa) are indeed expressed in SCG neurons and in differentiated PC12 cells.

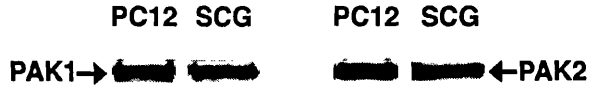


Figure 5.1 PAK1 and PAK2 are expressed in sympathetic neurons.

Cell extracts were prepared from SCG neurons and differentiated PC12 cells, cultured for 7 days in the presence of NGF. Thirty micrograms of proteins were resolved on a 12.5 % SDS-PAGE polyacrylamide gel, transferred onto nitrocellulose and probed with a polyclonal antibody to either PAK1 or PAK2.

5.3 PAKs decrease the viability of primary rat sympathetic neurons

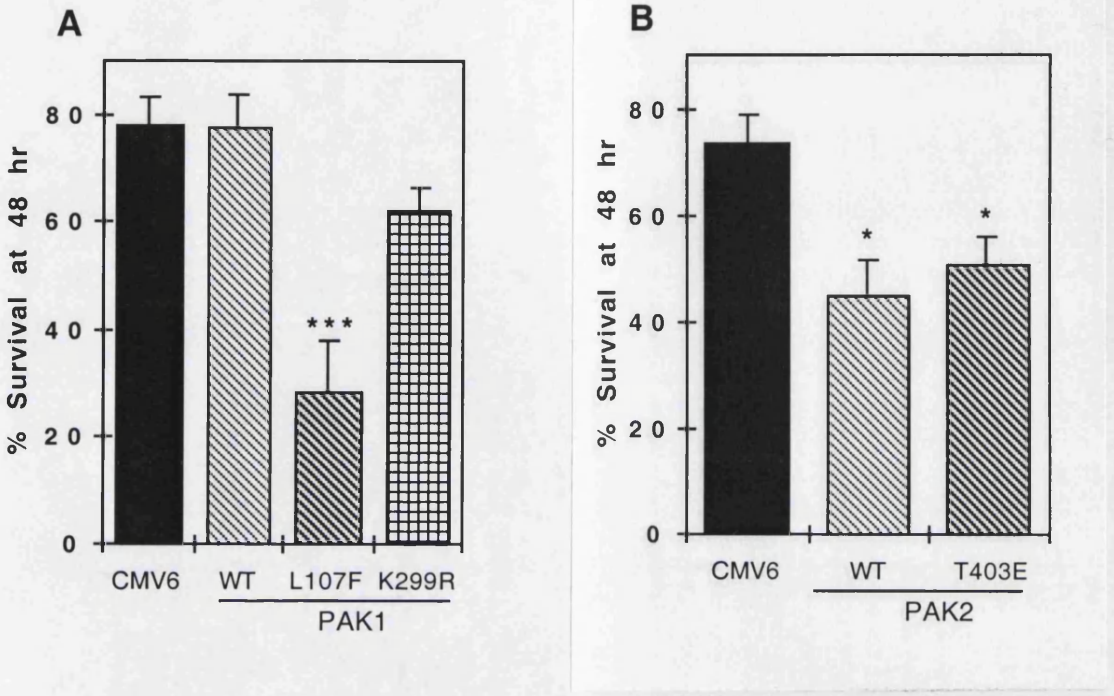
To determine whether the PAKs are involved at all in the induction of neuronal apoptosis, SCG neurons were microinjected with 0.3 mg/ml of the empty vector, CMV6, WT-PAK1 and the PAK1 mutants: L107F and K299R. Similarly, the effect of WT, T403E and K278R-PAK2 on the survival of SCG neurons in the presence of NGF was examined. The results show that overexpression of activated mutants of either PAK isoform significantly decreased the viability of the neurons (Figures 5.2A and B) whereas their dominant negative forms had no effect on the survival of the SCG neurons in the presence of NGF (Figure 5.2A and data not shown). In contrast to the outcome of WT-PAK1 overexpression, which had no effect on the viability of the SCG neurons, WT-PAK2 decreased neuronal viability quite significantly suggesting that there is no need for further activation of PAK2 for death to occur. Both WT and the constitutively active T403E-PAK2 mutant were equally efficient in reducing cell viability. Surprisingly, examination of the nuclear morphology of the cells, microinjected with the activated mutants of either PAKs, showed that the majority of the nuclei were not pyknotic (Figure 5.2C). This suggests that activation of PAK1 and PAK2 probably leads to changes in the cytoskeleton integrity that causes cells to detach. However, the possibility that the activated PAK-injected cells also undergo nuclear changes but detach prior to these events remains.

Figure 5.2 Constitutively activated PAK induces cell death in the presence of NGF.

Sympathetic neurons, cultured for 5-7 days in the presence of NGF, were microinjected with (A) 0.3 mg/ml empty CMV6 expression vector or CMV6 containing WT-PAK, L107F-PAK1 or K299R-PAK1 or (B) 0.3 mg/ml empty CMV6 vector or CMV6 containing WT-PAK2 or T403E-PAK2. The percentage of surviving cells was determined 48 hours later.

Only the activated mutants of PAK1 can induce cell death, whereas both WT and activated PAK2 were effective at killing SCG neurons. (C &D) Nuclear morphology of the neurons microinjected with CMV6 alone, L107F-PAK1, Bax, WT-PAK2 or T403E-PAK2. Sympathetic neurons, cultured for 5-7 days in the presence of NGF, were microinjected with 0.3 mg/ml CMV6, L107F-PAK1, WT-PAK2 or T403E-PAK2 or 0.05 mg/ml pcDNA1 Bax together with 2.5 mg/ml guinea-pig IgG to detect the injected cells. The cells were stained with Hoechst 16-24 hours after injection.

The results are the mean ± SEM of 4 (A) or 3 (B, C & D) independent experiments. *** $p<0.005$ (compared to the CMV6 empty vector control).


A
% Survival at 48 hr
0
20
40
60
80
***
CMV6
WT
L107F
K299R
PAK1
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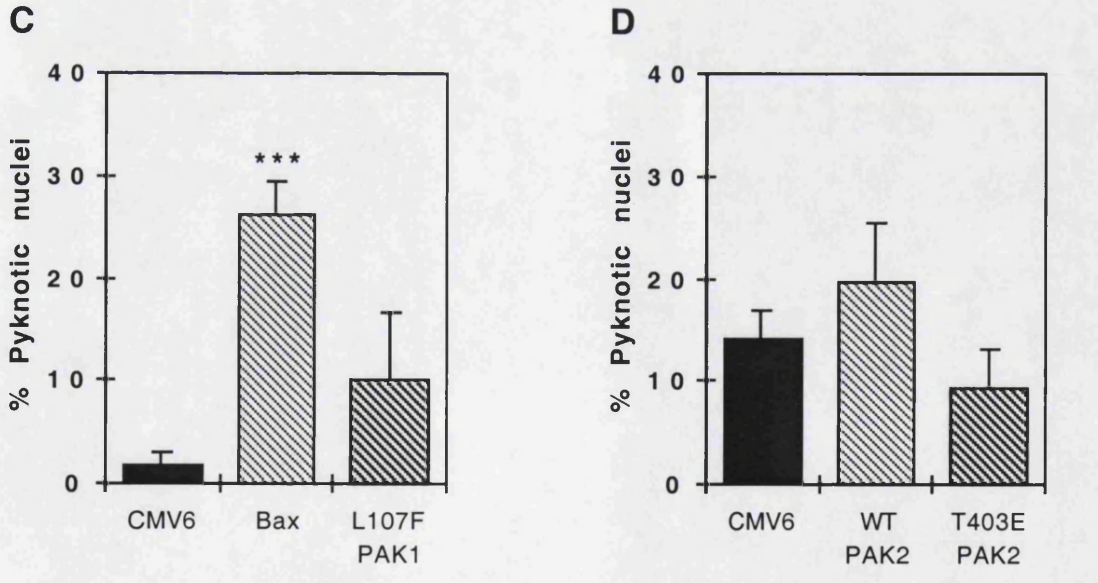
CMV6
WT
T403E
PAK2


C
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PAK2
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PAK2

5.4 PAKs are not required for NGF withdrawal- and Cdc42-induced death of SCG neurons

To assess the requirement for the PAKs in NGF withdrawal-induced death, SCG neurons were microinjected with 0.3 mg/ml K299R-PAK1, CMV6 or 0.05 mg/ml Bcl-2 (as a positive control) or, in independent experiments, 0.3 mg/ml K278R-PAK2, CMV6 or 0.6 mg/ml N17Cdc42 (also as a positive control) and then withdrawn from NGF. Forty-eight hours later, the percentage of survival was assessed by phase microscopy. Both the dominant negative PAK1 and PAK2, when compared to their respective positive controls, failed to protect the neurons from dying from NGF deprivation (Figures 5.3A and B). These results demonstrate that neither PAK1 nor PAK2 kinase activity is required for NGF withdrawal-induced death of primary rat sympathetic neurons.

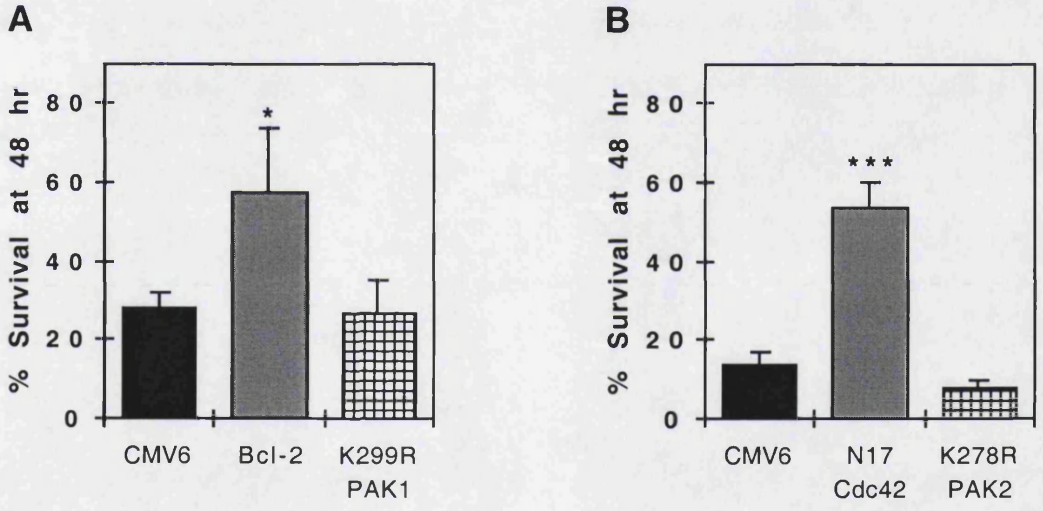


Figure 5.3 Effect of dominant negative mutants of PAK1 and PAK2 on the survival of SCG neurons in the absence of NGF.

SCG neurons were microinjected with (A) 0.3 mg/ml CMV6 or K299R-PAK1 or 0.05 mg/ml pcDNA1 Bcl-2 or (B) 0.3 mg/ml CMV6 or K278R-PAK2 or 0.6 mg/ml N17Cdc42, together with Texas Red-dextran to identify the injected cells. Cells were withdrawn from NGF 4 hours after injection. The percentage of surviving cells was assessed 48 hours later by phase microscopy. The results are the mean ± SEM of 3 (A & B) independent experiments. * $p<0.05$; *** $p<0.005$ (compared to the CMV6 empty vector control).

The role of PAK1 in Cdc42-induced death was also examined. Sympathetic neurons were co-injected with 0.1 mg/ml V12Cdc42 and increasing concentrations of K299R-PAK1. The percentage of survival was assessed by phase microscopy 48 hours later. The dominant negative mutant of PAK1 failed to rescue SCG neurons from V12Cdc42 -induced death (Figure 5.4), suggesting that Cdc42-induced death does not require PAK1 catalytic activity.

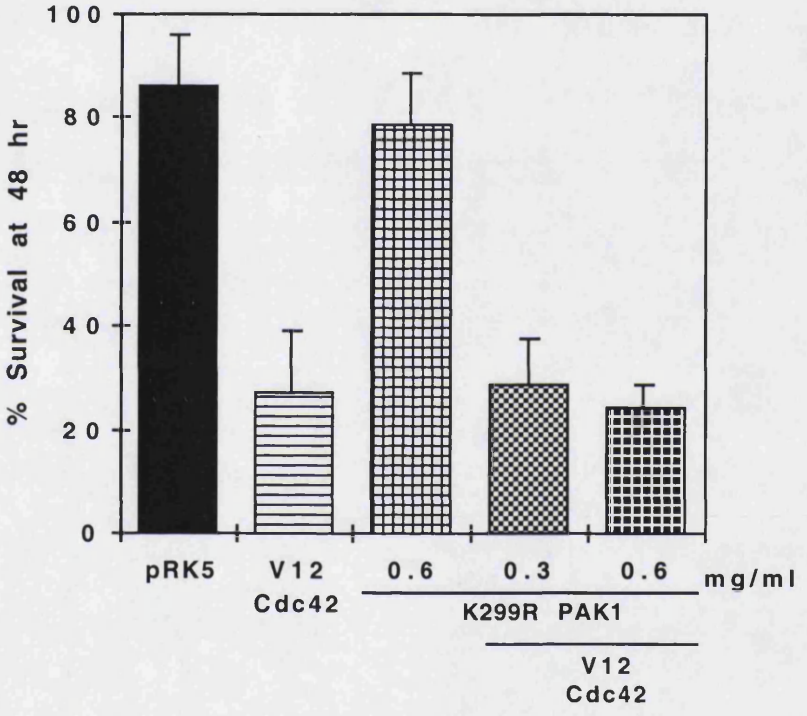


Figure 5.4 V12Cdc42-induced death of SCG neurons is not rescued by a dominant negative mutant of PAK1.

Sympathetic neurons, cultured for 5-7 days in the presence of NGF, were microinjected with 0.7 mg/ml pRK5, 0.1 mg/ml pRK5 V12Cdc42, 0.6 mg/ml CMV6 PAK1 K299R or 0.1 mg/ml pRK5 V12Cdc42 and 0.3 or 0.6 mg/ml CMV6 PAK1 K299R. The percentage of surviving cells was determined 48 hours later. The results are the mean ± SEM of 3 independent experiments. No significant differences were observed between V12Cdc42 and V12Cdc42 + 0.3/0.6 mg/ml K299R PAK1.

5.5 Differential activation of JNK or of the c-jun promoter by the PAK isoforms

To determine whether PAK1 and PAK2-induced neuronal cell death correlates with an increase in JNK activity, the effect of activated PAKs on the phosphorylation of c-Jun in rat primary sympathetic neurons was investigated. Neurons, microinjected with 0.5 mg/ml CMV6 or L107F-PAK, were stained with an anti-phospho-c-Jun antibody. Activation of PAK1 did not induce any change in the phosphorylation of c-Jun on serine 63 (Figure 5.5A). Contrary to what has been described in COS-7 cells and in yeast (Brown *et al.*, 1996), PAK1 does not appear to be an upstream regulator of the JNK pathway in SCG neurons. However, microinjection of 0.3 mg/ml of either WT or activated PAK2 produced a significant increase in the number of cells expressing phosphorylated c-Jun (Figure 5.5B). Injection of the control K278R-PAK2 mutant had no significant effect on the level of c-Jun phosphorylation (data not shown).

These results were confirmed by the effect of PAKs on the activation of the *c-jun* promoter. L107F-PAK1, WT-PAK2 or PAK2-T403E (0.3 mg/ml) were microinjected into SCG neurons together with a c-*jun*-Chloramphenicol Acetyl Transferase (CAT) reporter gene (0.035 mg/ml). The c-*jun*-CAT reporter gene contains the *c-jun* sequences from -1600 to +170 cloned upstream of the bacterial CAT gene (van Dam *et al.*, 1995). The injected cells were refed with NGF-containing medium and stained for the expression of the CAT protein 20 hours later. The percentage of cells expressing CAT was very low in the presence of NGF, correlating with low activation of *c-jun* in the presence of NGF, whereas NGF deprivation induced a 3-4 fold increase over background [Figures 5.6A, B and C; (Eilers *et al.*, 1998)]. Co-injection of an active PAK1 did not alter the number of cells expressing CAT whereas co-expression of a kinase active PAK2 resulted in a 2-3 fold increase. However, expression of the kinase inactive mutants of either PAKs could not block the activation of the *c-jun* promoter that occurs after NGF deprivation. These results support the observation that PAK kinase activity is not required for NGF withdrawal-induction of the apoptotic pathway. Also, these data illustrate the specificity of responses elicited by the two different PAK isoforms in

that only PAK2 was capable of activating the JNK pathway in sympathetic neurons.

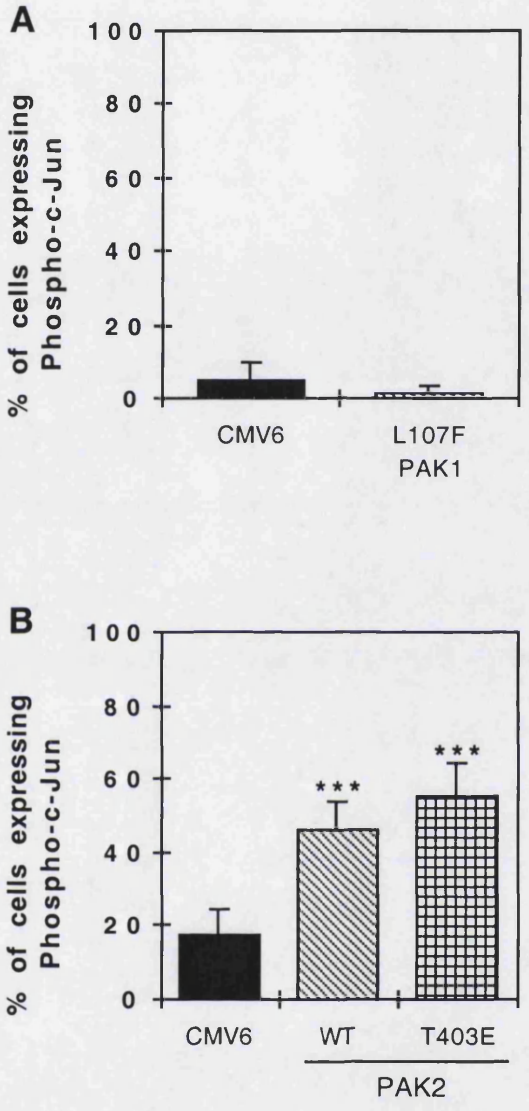


Figure 5.5 Differential effect of PAK1 and PAK2 on c-Jun phosphorylation.

Sympathetic neurons were microinjected with (A) 0.6 mg/ml of CMV6 or L107F-PAK or (B) 0.3 mg/ml of WT-PAK2 or T403E-PAK2. Twenty-four hours after injection, the percentage of cells expressing phospho-c-Jun was determined. Only the injected cells in which phospho-c-Jun staining was clearly above background were scored as positive. The results are the mean ± SEM of 3 (A & B) independent experiments. *** $p<0.005$ (compared to the CMV6 empty vector control).

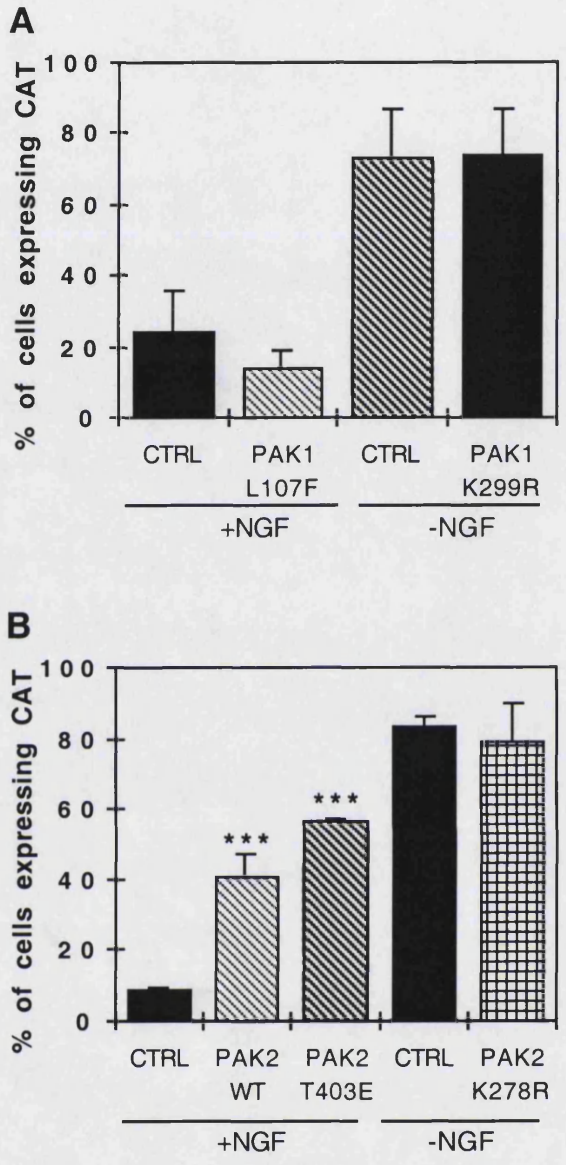
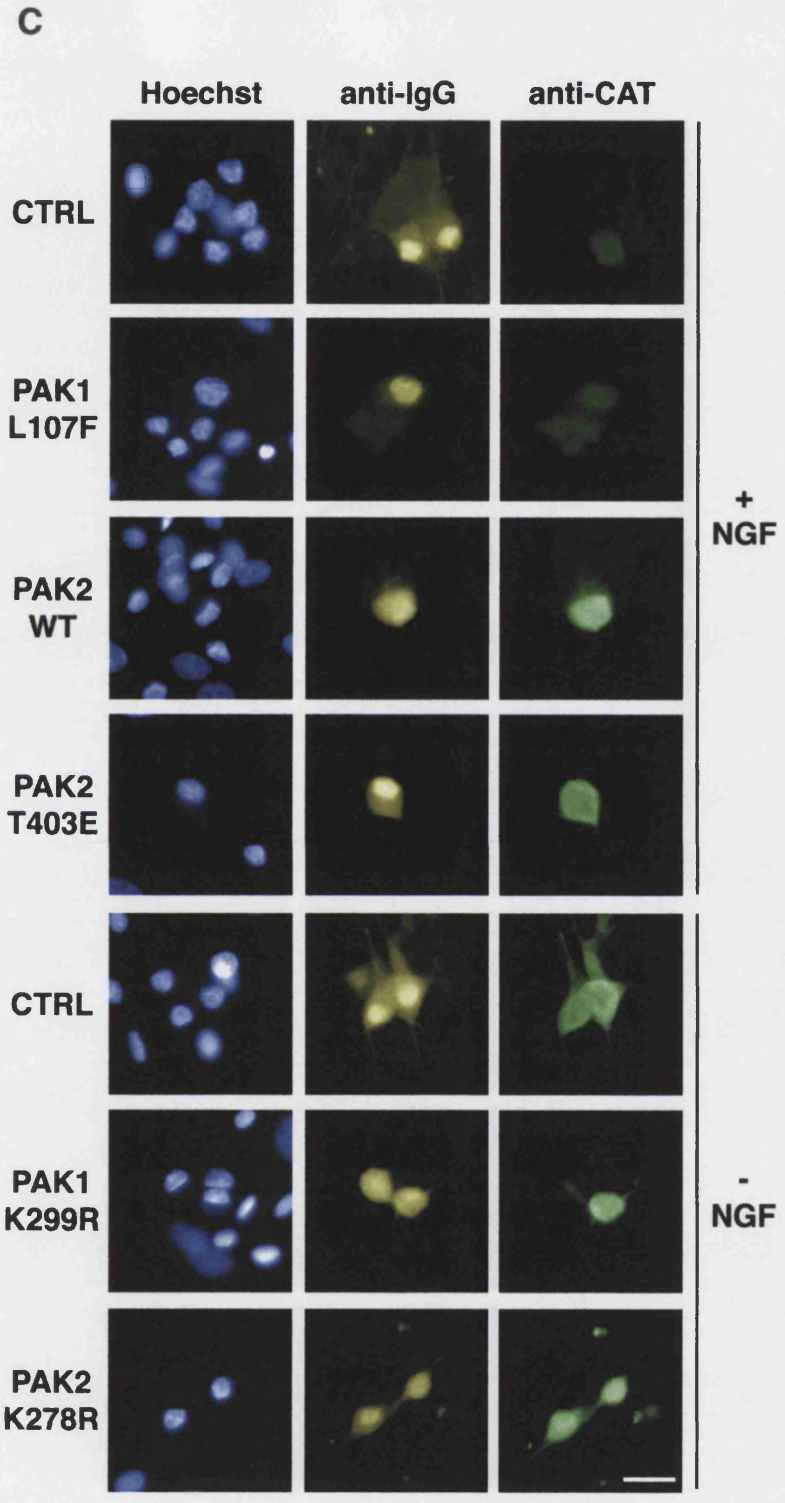


Figure 5.6 Differential effect of PAK1 and PAK2 on *c-jun* activation.

Sympathetic neurons were microinjected with 0.035 mg/ml *c-jun* CAT alone (CTRL) or together with 0.3 mg/ml L107F-PAK1 or 0.6 mg/ml K299R-PAK (A) or with 0.3 mg/ml WT-PAK2, T403E-PAK2 or K278R-PAK2 (B). The cells were maintained in the presence of NGF or withdrawn from NGF as indicated for 24 hours and then stained with Hoechst, an anti-GP-IgG and an anti-CAT antibody. The results are the mean ± SEM of 3 (A & B) independent experiments. *** $p<0.005$ (compared to the respective controls).

(C) Typical examples of the immunofluorescent staining observed in this assay. Bar = 30 μm.


C
Hoechst
anti-IgG
anti-CAT
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L107F
PAK2
WT
PAK2
T403E
CTRL
PAK1
K299R
PAK2
K278R
+
NGF
-
NGF

5.6 PAK1 activity does not increase upon NGF withdrawal

To determine whether PAK1 is activated at all upon NGF withdrawal-induced apoptosis, its activity was measured in PC12 cells in an immunokinase assay. NGF-differentiated PC12 cells behave in many ways like sympathetic neurons (Greene and Tischler, 1976) and offer a model in which it is easier to perform biochemical analysis. PC12 cells were differentiated for 7 days and either maintained in the presence of NGF or withdrawn from NGF as described in material and methods. After 4, 8, 16 and 24 hours, the cells were lysed and PAK1 was immunoprecipitated with a specific antibody raised against the C-terminal region of PAK1 (Santa Cruz). The kinase activity of the resulting immunoprecipitates was assayed against the myelin basic protein. No differences were observed in PAK1 kinase activity, when assessed in the presence or in the absence of NGF (Figure 5.7). However, the basal levels of PAK1 activity were high.

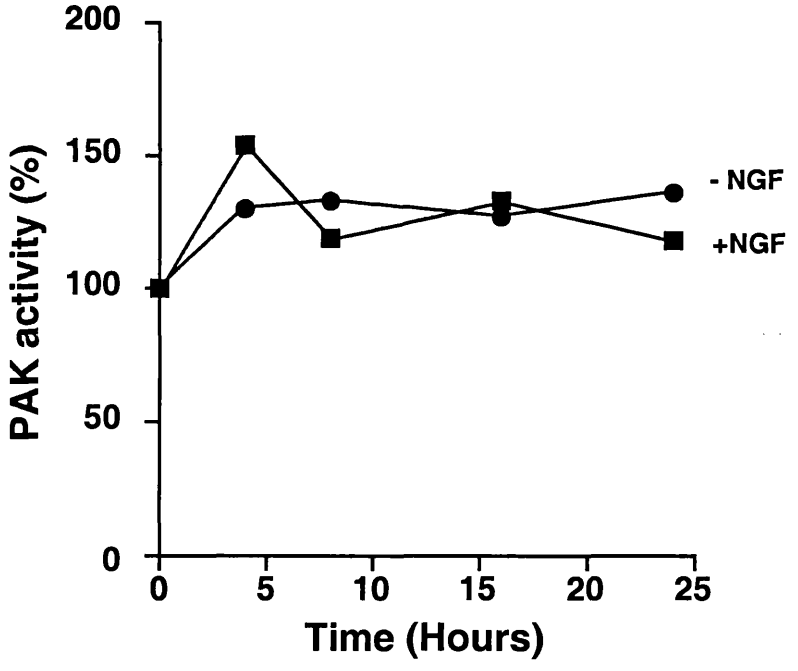


Figure 5.7 PAK1 kinase activity in PC12 cells deprived of NGF.

PC12 cells were differentiated in the presence of NGF for 7 days and then refed with fresh medium supplemented with either 100 ng/ml of NGF or with an anti-NGF antibody. After 4, 8, 16 and 24 hours, PAK1 was immunoprecipitated from PC12 cells using a C-terminus antibody and the kinase assays were performed. These results are the mean of 2 independent experiments.

5.7 Analysis of PAK2 activation

In apoptotic Jurkat cells, caspase-3 cleaves PAK2 after aspartate 212, resulting in the release of its regulatory N-terminus and activation of its kinase (Rudel and Bokoch, 1997). It was therefore of interest to determine whether PAK2 was activated by a similar mechanism in apoptotic PC12 cells and SCG neurons. Differentiated PC12 cells and SCG neurons were withdrawn from NGF for 4, 8, 24 and 48 hours (PC12 cells) or for 24 hours (SCG neurons). Cells were then lysed and 30 μg protein was separated by SDS-PAGE and transferred onto nitrocellulose. The membranes were incubated with an anti-PAK2 antibody (Santa Cruz) raised against the N-terminal region of the protein. As a positive control, extracts from Jurkat cells, treated or not with an anti-Fas antibody for 4 hours, were examined. Figure 5.8 shows that there is no detectable cleavage of PAK2 in either PC12 cells or sympathetic neurons undergoing apoptosis compared to the control extracts from Jurkat cells, in which a reactive band appears at 28 kDa (N-terminal cleavage product). These results suggest that PAK2 is not activated by proteolytic cleavage in both PC12 cells and SCG neurons following NGF deprivation. It is still unclear whether PAK2 is activated at all in these neuronal systems.

Finally, the irreversible caspase inhibitor zVAD-fmk failed to rescue sympathetic neurons from PAK2-induced death (Figure 5.9), suggesting that the mechanism by which PAK2 kills SCG neurons is not an apoptotic one, although it activates JNK.

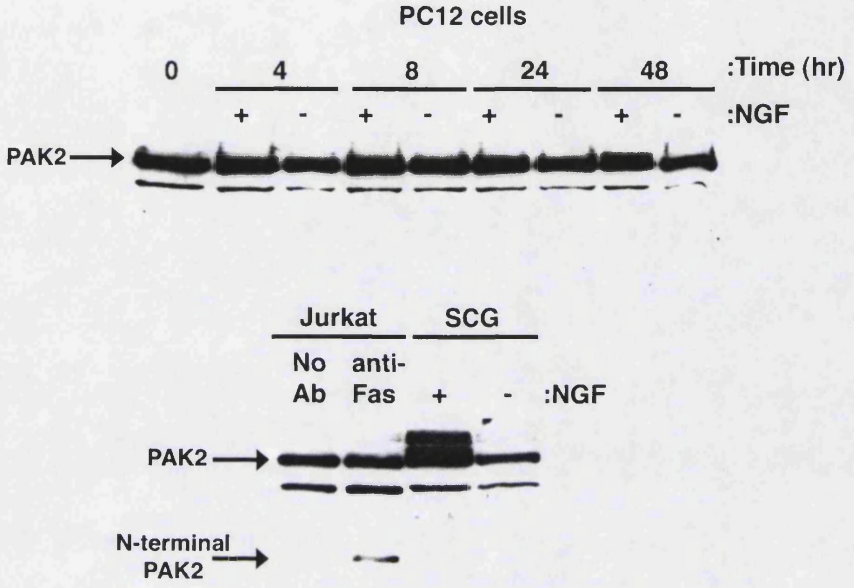


Figure 5.8 Proteolytic cleavage of PAK2.

PC12 cells were differentiated for 7 days and withdrawn from NGF for 4, 8, 24 and 48 hours. SCG neurons were cultured for 7 days and withdrawn from NGF for 24 hours. Apoptosis of Jurkat cells was induced by addition of 50 ng/ml of an anti-Fas antibody for 4 hours. Cells were lysed and 30 µg protein from total cell lysates of PC12 cells, SCG neurons and Jurkat cells were subjected to an anti-N-terminus PAK2 Western blot. The arrows indicate either the full length PAK2 or to the 28 kDa cleavage product.

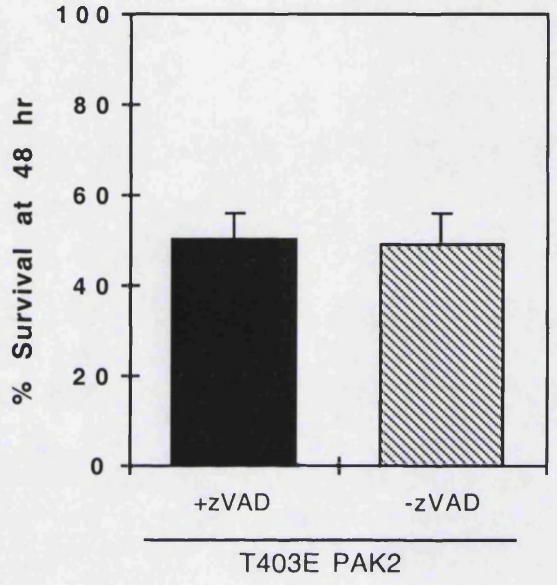


Figure 5.9 The caspase inhibitor zVAD-fmk does not rescue SCG neurons from PAK2-induced death.

Sympathetic neurons, cultured for 5-7 days in the presence of NGF, were microinjected with 0.3 mg/ml T403E-PAK2, together with 5.0 mg/ml of Texas Red-dextran to mark the injected cells in either the presence or absence of 100 μM zVAD-fmk. The percentage of surviving cells was assessed 48 hours later by phase microscopy. The results are the mean ± SEM of 5 independent experiments.

5.8 Discussion

In this Chapter. the role of PAK1 and PAK2 in linking Cdc42 to the JNK cascade and in inducing apoptosis in SCG neurons was investigated. In summary, it has been demonstrated that neither PAK1 nor PAK2 is required for Cdc42- and NGF-induced activation of the JNK pathway and apoptosis. Indeed, neither PAK1 nor PAK2 dominant negative mutants blocked Cdc42-induced apoptosis and NGF withdrawal-induced activation of JNK and apoptosis. In addition, no activation of PAK1 or cleavage-activation of PAK2 in differentiated PC12 cells and SCG neurons undergoing apoptosis was detected, corroborating the suggestion that the catalytic activities of PAK1 and PAK2 are not involved in neuronal apoptosis.

It was also demonstrated that the PAKs do not induce neuronal apoptosis. Indeed, despite the specific induction of death by constitutively active forms of both PAK isoforms, none of the nuclear changes, typical of apoptosis, were observed in the injected cells. In addition, activated PAK1 was unable to elevate the level of phosphorylated nuclear c-Jun or to activate *c-jun*, which is crucial for NGF withdrawal-induced apoptosis in sympathetic neurons (Eilers *et al.*, 1998; Harding *et al.*, 2000). This is in agreement with recent findings in which activated PAK1 failed to enhance JNK activity in COS-1 and NIH 3T3 cells (Teramoto *et al.*, 1996a; Westwick *et al.*, 1997; Tapon *et al.*, 1998). In contrast, PAK2 produced an increase in both the levels of activation of *c-jun* and phosphorylation of the c-Jun protein. However, it was observed that caspase activity was not required for PAK2-induction of cell death, which suggests either that PAK2 does not induce apoptosis as, in SCG neurons, zVAD-fmk blocks apoptosis induced by NGF withdrawal (McCarthy *et al.*, 1997) or that PAK2 acts downstream of caspase activation. This latter hypothesis would suggest a positive feedback loop whereby PAK2 would activate JNK and induce cell death. However, no cleavage activation of PAK2 was observed.

But how else could PAKs reduce cell viability? One possibility is that the PAKs cause cells to detach and that this event occurs either instead of or prior to nuclear changes. Consistent with this hypothesis, Lee *et al.* (Lee *et al.*, 1997) found that PAK2-induced apoptosis of HeLa and CHO cells occurred after the cells had

detached from the plates. PAK1 may also play a role in cytoskeletal changes that would cause the cells to detach. In this regard, it has been reported that PAK1 could promote morphological changes, including membrane ruffling and increased lamellipodia formation, at growth cones and shafts of NGF-induced neurites in PC12 cells (Obermeier *et al.*, 1998), although these changes were independent of PAK's catalytic activity. However, it is not known which cytoskeletal changes could cause cells to detach.

In conclusion, despite having demonstrated that neither PAK1 nor PAK2 is required for NGF withdrawal-induced apoptosis, their involvement in some of the cytoskeletal events occurring during cell death cannot be completely ruled out.

The studies presented in the next two chapters aimed at identifying a kinase which would link Cdc42 to JNK and be required for NGF withdrawal-induced death of SCG neurons. Studies on the role of ASK1 and MLK3 were started simultaneously and preliminary experiments suggested that either ASK1 or MLK3 could be the kinase we intended to identify. The results obtained on ASK1 are presented first.

6. The function of ASK1 in the induction of apoptosis of sympathetic neurons

6.1 Introduction

The results presented in Chapter 3 and in Eilers *et al.* (Eilers *et al.*, 1998), demonstrate that a MEKK1→SEK1 pathway is not required for NGF withdrawal and Cdc42 induced-neuronal cell death. The results also suggest that another MAPKKK, which is activated by Cdc42 and/or NGF withdrawal, is important in the signalling to JNK activation and induction of neuronal cell death.

Recently, a new MAPKKK, the Apoptosis Signal-regulating Kinase 1, ASK1, has been identified which activates both the JNK and p38 MAP kinase pathways and plays pivotal roles in the mechanisms of stress- and cytokine-induced apoptosis [(Ichijo *et al.*, 1997; Chang *et al.*, 1998; Saitoh *et al.*, 1998) and see section 1.6.3.2]. However, because the role of ASK1 in neuronal cell death had never been investigated, it prompted the study of its involvement in apoptosis in both PC12 cells and SCG neurons. The results presented were obtained in sympathetic neurons. Similar results were obtained by our collaborators Kanamoto, T., Takeda, K., Miyazono, K. and Ichijo, H. in PC12 cells. The work that resulted from this collaboration has recently been published (Kanamoto *et al.*, 2000).

In this study wild type (ASK1-WT), constitutively active (ASK1-ΔN) and dominant negative (ASK1-KR) forms of ASK1 were used. The N-terminal regulatory domain has been suggested to function as an autoinhibitory domain which keeps the catalytic domain in an inactive conformation (Chang *et al.*, 1998). Therefore, deletion of the first 648 N-terminal amino acids renders ASK1 constitutively active by changing the kinase domain into an active shape (Saitoh *et al.*, 1998). A mutation on the lysine residue at position 709 to arginine renders ASK1 constitutively inactive which acts as a dominant negative [(Saitoh *et al.*, 1998) and see Figure 1.10].

6.2 Activated ASK1 induces apoptosis

To investigate whether ASK1 plays a role in neuronal cell death, the effect of ASK1-WT, ASK1-ΔN and ASK1-KR in rat primary sympathetic neurons was examined. SCG neurons, cultured for 5-7 days in the presence of NGF, were microinjected with 0.3 mg/ml of an empty expression vector (control) or the different ASK1 forms. The percentage of survival was assessed by phase microscopy 48 hours after injection. Both ASK1-WT and ASK1-ΔN significantly decreased the viability of the SCG neurons, whereas the empty vector and the kinase inactive mutant ASK1-KR had no effect (Figure 6.1A). This suggests that the catalytic activity of ASK1 is necessary for the induction of cell death. Surprisingly, ASK1 does not seem to require further activation as ASK1-WT induced death to levels similar to the constitutively active ASK1 mutant. These results suggest that overexpression of ASK1 by itself is sufficient to activate it. Consistent with this is the fact that overexpression of ASK1 led to a high level of basal activity in the absence of stimulation (Ichijo *et al.*, 1997). Also, the recent findings of Gotoh and Cooper (Gotoh and Cooper, 1998), suggesting ASK1 homodimerisation as a mechanism of its activation, could provide an explanation for the above results. Similar results have been obtained by our collaborators in PC12 cells (Kanamoto *et al.*, 2000). Indeed, they found that differentiated PC12 cells that had been transfected by recombinant adenovirus expressing ASK1-ΔN had a reduced viability when compared to β-galactosidase infected cells.

A great proportion of the ASK1 injected cells displayed pyknotic nuclei, which is a typical feature of apoptotic cell death (Figure 6.1B). Similarly, the ASK1-ΔN infected PC12 cells displayed morphological features of apoptosis such as membrane blebbing, pyknosis and cell body condensation and genomic DNA fragmentation could be observed at 24 hours post-infection (Kanamoto *et al.*, 2000).

Taken together, these results demonstrate that ASK1 can induce apoptosis in neurons and that its kinase activity is required for the induction of cell death.

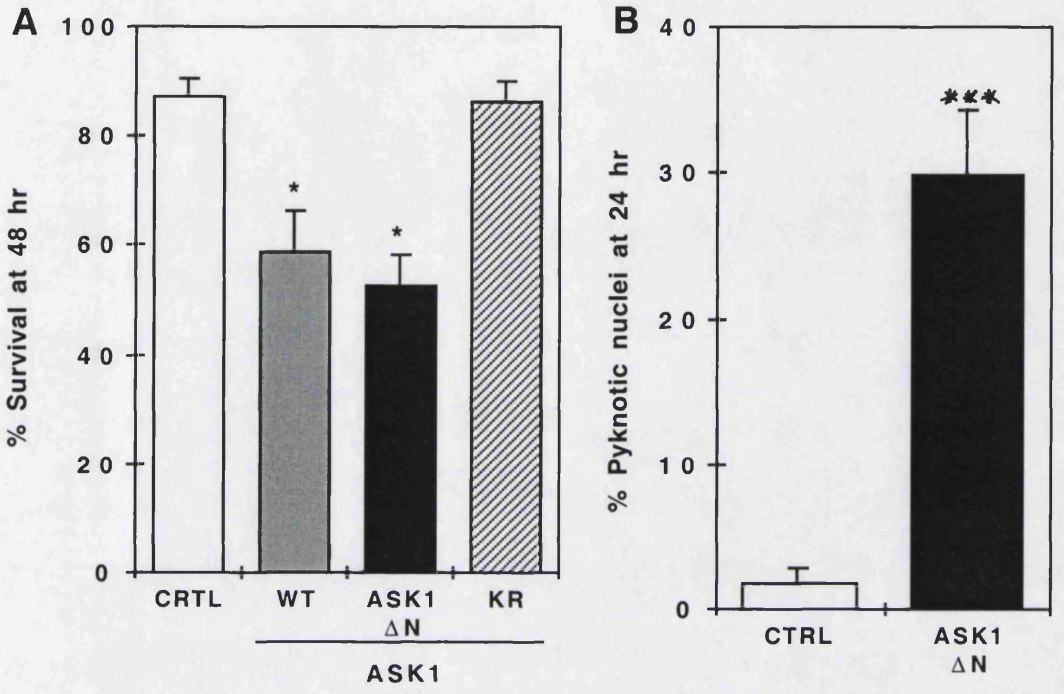


Figure 6.1 Induction of neuronal cell death by ASK1-ΔN in SCG neurons

(A) SCG neurons, cultured for 5-7 days in the presence of NGF, were microinjected with 0.3 mg/ml empty pcDNA1 expression vector (CTRL), ASK1-WT, ASK1-ΔN or ASK1-KR, together with 70 kDa Texas Red Dextran to detect the injected cells. Forty-eight hours later, the percentage of surviving cells was assessed by phase microscopy.

(B) Sympathetic neurons, cultured for 5-7 days in the presence of NGF, were microinjected with 0.3 mg/ml of empty vector (CTRL) or ASK1-ΔN together with 2.5 mg/ml of guinea-pig IgG to identify the injected cells. The cells were stained with Hoechst 16-24 hours post injection.

The results are the mean ± SEM of 3 (A & B) independent experiments. * $p<0.05$; *** $p<0.005$ (compared to the respective controls).

6.3 ASK1-induced apoptosis in SCG neurons is caspase dependent

Because members of the caspase family have been shown to be crucial mediators of neuronal apoptosis including in SCG neurons (Troy *et al.*, 1996; Armstrong *et al.*, 1997; McCarthy *et al.*, 1997; Taylor *et al.*, 1997), the requirement for caspase activation in ASK1-induced death was investigated using zVAD-fmk. SCG neurons, pre-treated for 3-4 hours with 100 μM zVAD-fmk, were

microinjected with 0.3 mg/ml ASK1-ΔN. The cells were scored for survival 48 hours after injection. About 60% of the neurons injected with ASK1-ΔN in the presence of NGF were dead after 48 hours and this death was significantly inhibited in the presence of zVAD-fmk (Figure 6.2). This result suggests that caspase activity is required for ASK1-induced apoptosis.

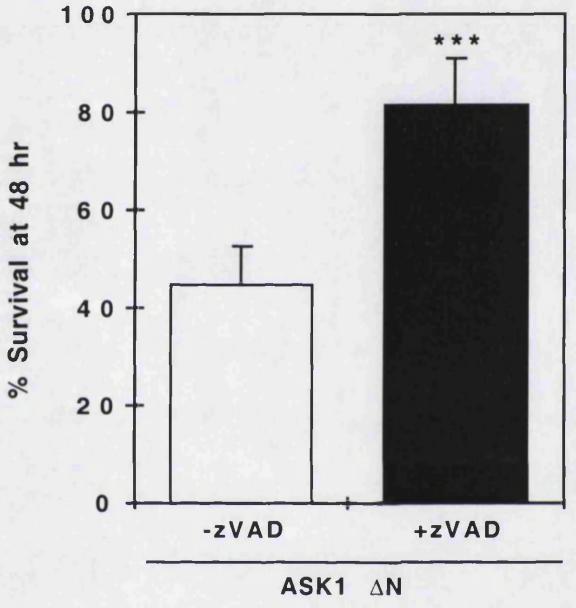


Figure 6.2 The caspase inhibitor zVAD-fmk protects SCG neurons from ASK1-induced death.

Sympathetic neurons, cultured for 5-7 days in the presence of NGF, were microinjected with 0.3 mg/ml of ASK1-ΔN, together with 5.0 mg/ml of Texas Red-dextran in either the presence or absence of 100 μM zVAD-fmk. The percentage of surviving cells was assessed 48 hours later by phase microscopy. The results are the mean ± SEM of 3 independent experiments. *** $p<0.005$ (Student's *t*-test).

6.4 ASK1 is an important component of NGF withdrawal-induced death

To assess the role of ASK1 in NGF withdrawal-induced death, increasing concentrations of dominant negative ASK1-KR DNA or 0.3 mg/ml empty vector (negative control) or 0.05 mg/ml Bcl-2 (positive control) were microinjected into SCG neurons. Four hours after injection, the cells were deprived of NGF and the percentage of surviving cells was assessed 48 hours later by phase microscopy. ASK1-KR was able to protect SCG neurons from NGF withdrawal-induced death in a dose-dependent manner, although not as efficiently as the Bcl-2 positive control (Figure 6.3A). The effect of ASK1-KR on the nuclear morphology of the injected cells was also examined. Sympathetic neurons were microinjected with 0.3 mg/ml of either an empty expression vector or ASK1-KR. The cells were withdrawn from NGF and stained with Hoechst 24 hours later. Controls of uninjected cells, maintained in the presence or absence of NGF, were included. Figure 6.3B shows that ASK1-KR significantly reduced the number of cells displaying pyknotic nuclei after NGF withdrawal, suggesting that ASK1 activity is indeed necessary for the induction of apoptosis by NGF withdrawal. Similar results were obtained by our collaborators in differentiated PC12 cells, where overexpression of a similar kinase dead mutant of ASK1 (ASK1-KM) considerably reduced the percentage of apoptotic cells after NGF deprivation (Kanamoto *et al.*, 2000). ASK1-KR has been tested against other MAPKKKs such as MEKK1, MLK3, TAK1 and Tpl-2 and was found to be specific for ASK1 only (H. Ichijo, unpublished observations). Taken together, these findings demonstrate that ASK1 is a crucial element of neuronal apoptosis by neurotrophic factor deprivation.

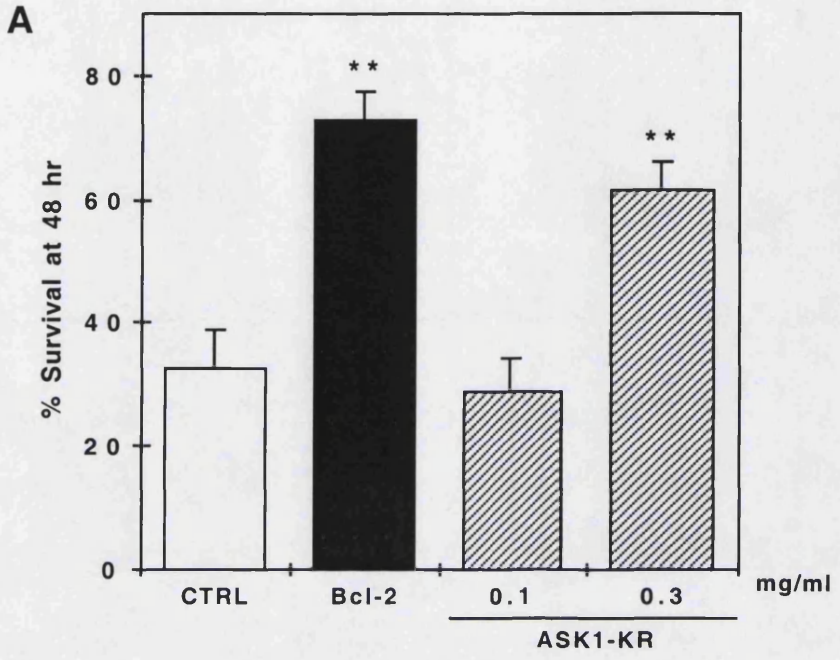
In addition, Kanamoto *et al.* (Kanamoto *et al.*, 2000) measured ASK1 activity in differentiated PC12 cells and found that it peaked at 3 hours after NGF withdrawal. These results suggest that ASK1 catalytic activity can be regulated after induction of cell death by neurotrophin withdrawal.

Figure 6.3 ASK1-KR prevents NGF withdrawal-induced cell death in SCG neurons.

(A) SCG neurons, cultured for 5-7 days in the presence of NGF, were microinjected with 70 kDa Texas Red Dextran and either ASK1-KR (0.1 or 0.3 mg/ml), 0.3 mg/ml pcDNA1 (CTRL) or 0.05 mg/ml of Bcl-2. Twenty-four hours later, the cells were deprived of NGF and the number of injected cells was scored (100% value). The percentage of surviving cells was assessed after 48 hours by phase microscopy.

(B) SCG neurons were injected with 0.3 mg/ml of the control empty expression vector or ASK1-KR and withdrawn from NGF. Uninjected cells, maintained in the presence or absence of NGF, were included as controls. After 24 hours, the nuclear morphology was visualised by Hoechst staining.

The results are the mean ± SEM of 3 (A & B) independent experiments. ** $p<0.01$ (compared to the controls).


A
% Survival at 48 hr
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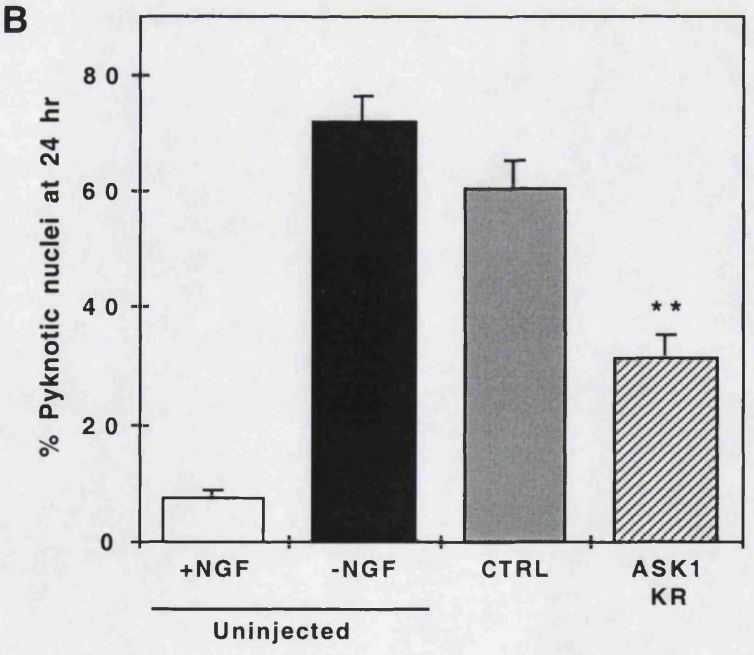
mg/ml
ASK1-KR


B
% Pyknotic nuclei at 24 hr
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60
40
20
0
**
+NGF
-NGF
CTRL
ASK1
KR
Uninjected

6.5 ASK1 is crucial for the activation of the JNK pathway after NGF withdrawal in SCG neurons

Because ASK1 had been shown to activate the JNK pathway in a variety of cell types (Wang *et al.*, 1996; Ichijo *et al.*, 1997; Chang *et al.*, 1998), it was important to determine whether ASK1-induction of cell death was mediated by the activation of the JNK pathway in neurons. The effect of activated ASK1-ΔN and dominant negative ASK1-KR on the level of phosphorylated c-Jun in SCG neurons was therefore examined in the presence and absence of NGF respectively. Cells, microinjected with either the ASK1 mutants or an empty expression vector, were stained with a specific anti-phospho-c-Jun antibody (Lallemand *et al.*, 1998). Figure 6.3A shows that cells injected with the empty vector control did not display a significant increase in phospho-c-Jun staining when compared to the uninjected cells, whereas overexpression of ASK1-ΔN induced a 4.3 fold increase in the level of nuclear phosphorylated c-Jun, suggesting that ASK1 is able to induce c-Jun phosphorylation. Expression of the ASK1-KR significantly reduced the increase in phospho-c-Jun levels after NGF deprivation (Figure 6.4A), whereas it had no effect on the level of phospho-c-Jun when cells were maintained in the presence of NGF (Figure 6.6B). Moreover, overexpression of ASK1 induced an activation of JNK in PC12 cells (Kanamoto *et al.*, 2000). This suggests that ASK1 activity is crucial for the induction of phosphorylation of c-Jun after NGF withdrawal.

It was also examined whether ASK1 activated the *c-jun* promoter in SCG neurons by using the c-jun-CAT reporter gene system (see Chapter 5). SCG neurons were co-injected with ASK1-ΔN and the c-jun-CAT reporter gene together with GP-IgG as a marker for the injected cells. Following injection cells were refed with NGF-containing medium and stained with an anti-CAT antibody 20 hours later. ASK1-ΔN induced a 2 fold increase in the percentage of cells expressing CAT (Figure 6.4B). The relatively weak CAT-inducing activity of ASK1-ΔN may represent a slight underscoring because of the decreased viability of ASK1-ΔN-injected cells. Thus, *c-jun* appears to be activated by ASK1 in SCG neurons.

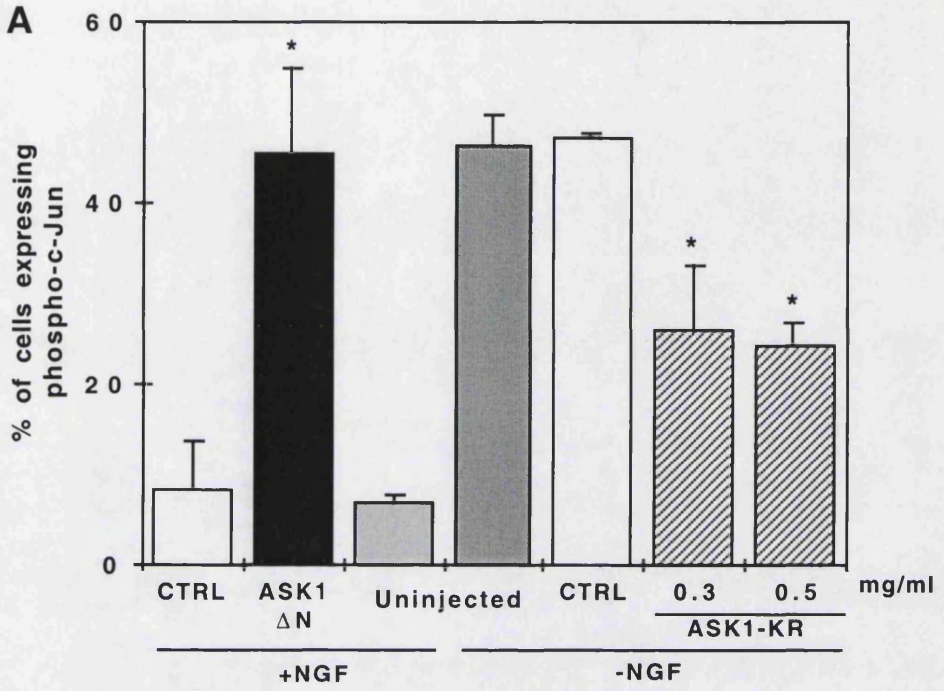
To determine whether ASK1 is required for the activation of the c-Jun transcriptional pathway, increasing concentrations of ASK1-KR were microinjected into SCG neurons together with the *c-jun*-CAT reporter construct. Four hours after injection, the cells were deprived of NGF and stained for the expression of the CAT protein 20 hours later. Overexpression of ASK1-KR blocked the activation of the *c-jun* promoter that occurs after NGF deprivation in a dose-dependent manner (Figure 6.4B). These results demonstrate not only that ASK1 is capable of activating the *c-jun* transcriptional pathway in neuronal cells but also that it is required for the activation of this pathway by NGF withdrawal.

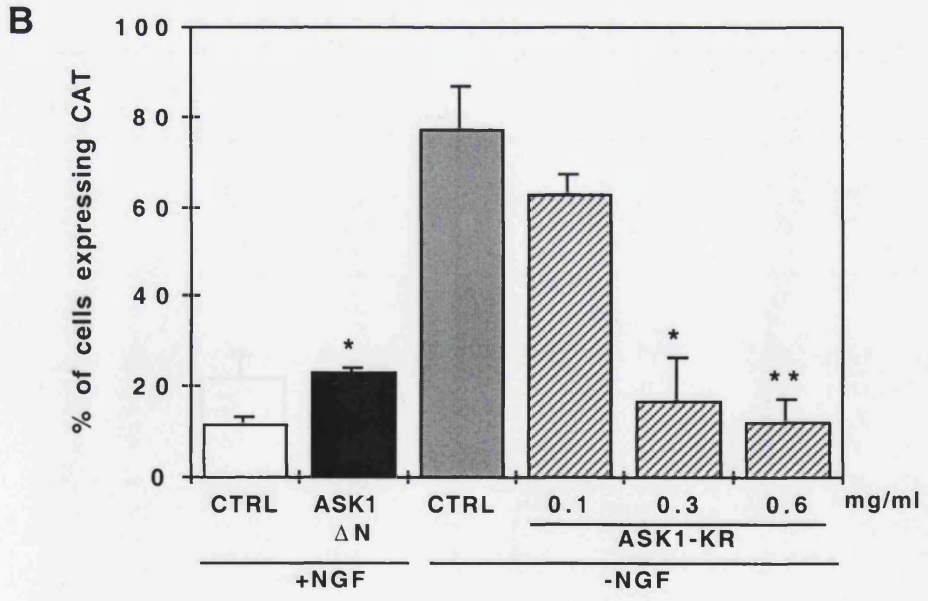
In order to determine whether AP-1 activity is required for ASK1-induced apoptosis, SCG neurons were co-injected with 0.3 mg/ml of ASK1-ΔN and increasing concentrations of FLAGΔ169. Cell survival was assessed by phase microscopy 48 hours after refeeding the cells with medium supplemented with NGF. This dominant negative c-Jun mutant was able to block ASK1-induced apoptosis (Figure 6.5), suggesting that AP-1 activity is essential for ASK1-induced apoptosis in SCG neurons.

Taken together, these results demonstrate that the ASK1 catalytic activity is important for both the induction of c-Jun phosphorylation and the activation of the c-jun promoter in SCG neurons deprived of NGF. These data also suggest that the death signal induced by NGF withdrawal is mediated at least in part by the ASK1-JNK-c-Jun transcriptional pathway in neurons.

Figure 6.4 ASK1-dependent activation of the JNK pathway

SCG neurons were microinjected with (A) either 0.3 mg/ml empty expression vector (CTRL) or ASK1-ΔN or (B) 0.05 mg/ml of the c-jun-CAT reporter gene was microinjected alone (CTRL) or with 0.3 mg/ml ASK1-ΔN, together with 5 mg/ml GP-IgG to detect the injected cells and maintained in the presence of NGF. In the NGF withdrawal experiments SCG neurons were microinjected with (A) 0.5 mg/ml control empty vector (CTRL) or various concentrations of ASK1-KR or (B) 0.05 mg/ml of the c-jun-CAT reporter gene alone or with increasing concentrations of ASK1-KR, together with 5 mg/ml GP-IgG to mark the injected cells and withdrawn from NGF. Twenty-four hours after injection, the percentage of cells expressing phospho-c-Jun (A) or c-jun CAT (B) was determined. Only the injected cells in which staining was clearly above background were scored as positive. The results are the mean ± SEM of 4 (A) or 3 (B) independent experiments. * $p<0.05$; ** $p<0.01$ (compared to the respective controls).


A
60
40
20
0
% of cells expressing phospho-c-Jun
*
*
*
CTRL
ASK1 ΔN
Uninjected
CTRL
0.3
0.5
mg/ml
ASK1-KR
+NGF
-NGF


B
100
80
60
40
20
0
% of cells expressing CAT
*
*
**
CTRL
ASK1 ΔN
CTRL
0.1
0.3
0.6
mg/ml
ASK1-KR
+NGF
-NGF

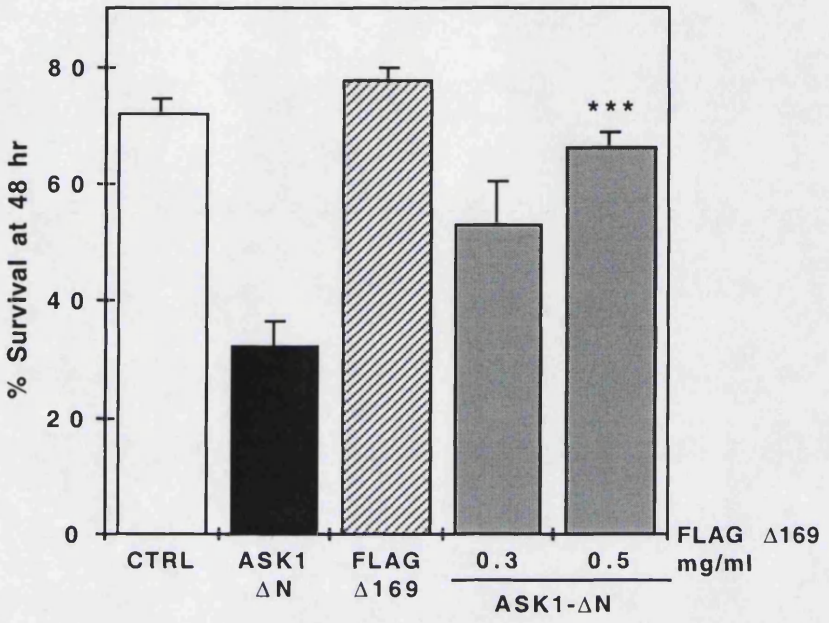


Figure 6.5 FLAGΔ169 blocks ASK1-induced apoptosis.

SCG neurons were microinjected with either 0.8 mg/ml empty pCDNA1 expression vector (CTRL), 0.3 mg/ml of ASK1-ΔN, 0.5 mg/ml of FLAGΔ169 or 0.3 mg/ml ASK1-ΔN together with 0.3/0.5 mg/ml FLAGΔ169 and maintained in the presence of NGF. The percentage of surviving cells was assessed 48 hours later by phase microscopy. The results are the mean ± SEM of 3 (A & B) independent experiments. *** $p<0.005$ (compared to ASK1-ΔN).

6.6 ASK1 is required for Cdc42-induced death

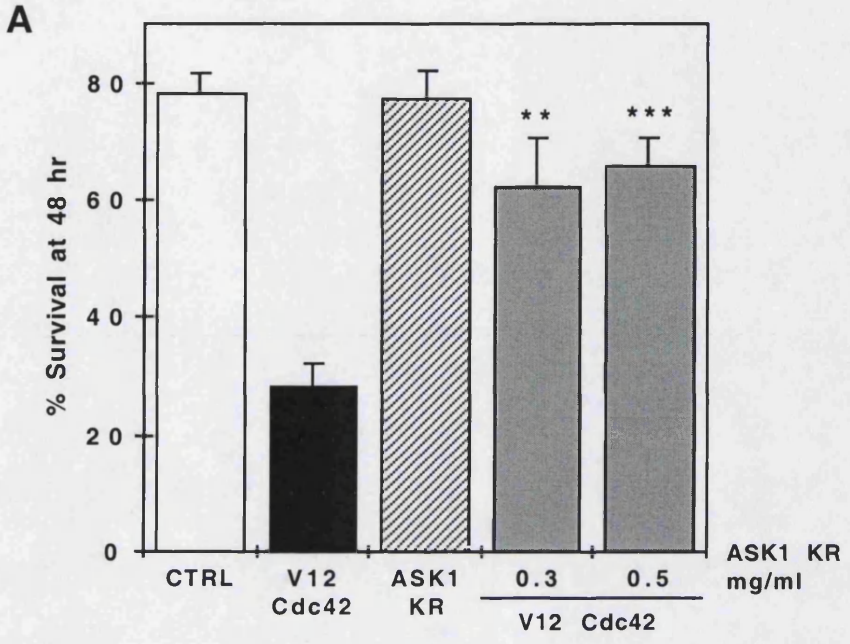
To determine whether ASK1 and Cdc42 lie on the same death signalling pathway, sympathetic neurons were co-injected with V12Cdc42 and the kinase inactive mutant ASK1-KR. The cells were maintained in the presence of NGF and the percentage of survival was determined 48 hours after injection. The dominant negative ASK1 was able to inhibit cell death induced by Cdc42 overexpression (Figure 6.6A). To check whether this inhibition correlated with a decrease in JNK pathway activation, SCG neurons were injected as described above and stained with a specific anti-phospho-c-Jun antibody 24 hours later. ASK1-KR significantly blocked the induction of c-Jun phosphorylation by V12Cdc42 (Figure 6.6B). These results suggest that ASK1 kinase activity is required for both Cdc42-induced death and c-Jun phosphorylation and that ASK1 may therefore lie downstream of Cdc42 in SCG neurons.

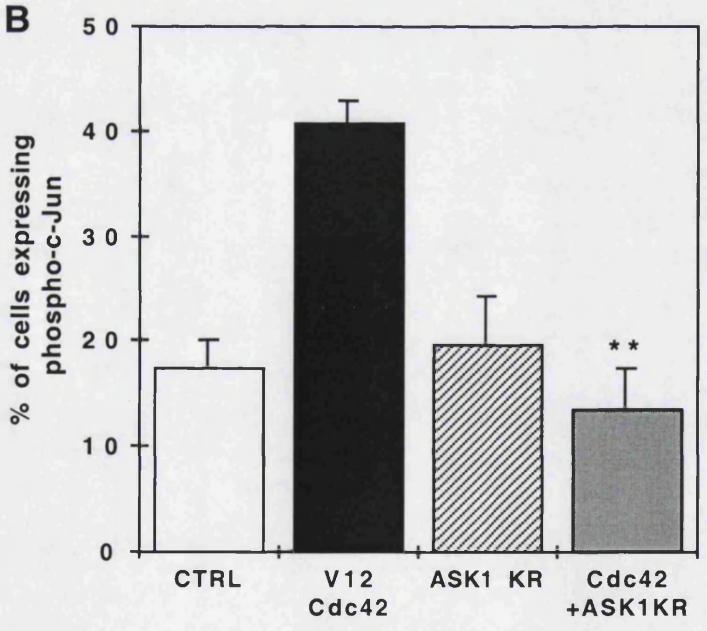
Figure 6.6 Effect of a dominant-negative ASK1 on Cdc42-induced cell death.

(A) Cdc42-induced apoptosis requires ASK1 activity. SCG neurons were microinjected with 0.6 mg/ml pRK5 empty expression vector (CRTL), 0.1 mg/ml V12Cdc42, 0.5 mg/ml ASK1-KR or 0.1 mg/ml V12Cdc42 and 0.3/0.5 mg/ml ASK1-KR. 70 kDa Texas Red Dextran was included to mark the injected cells. The cells were maintained in the presence of NGF and the percentage of surviving cells was assessed by phase microscopy 48 hours after injection.

(B) ASK1-KR blocks Cdc42-induced increase of c-Jun phosphorylation. SCG neurons were co-injected with 0.5 mg/ml of ASK1-KR and 0.1 mg/ml of V12Cdc42 together with 5 mg/ml GP-IgG to detect the injected cells and maintained in the presence of NGF. The cells were stained 24 hours later with a specific anti-phospho-c-Jun antibody.

The results are the mean ± SEM of 3 (A & B) independent experiments. ** $p<0.01$; *** $p<0.005$ (compared to V12Cdc42).


A
% Survival at 48 hr
80
60
40
20
0
**
***
CTRL
V12 Cdc42
ASK1 KR
0.3
0.5
V12 Cdc42
ASK1 KR mg/ml


B
% of cells expressing phospho-c-Jun
50
40
30
20
10
0
**
CTRL
V12 Cdc42
ASK1 KR
Cdc42 +ASK1KR

6.7 Discussion

In this chapter the role of ASK1 in apoptosis of sympathetic neurons was investigated. The aim was to identify a mediator of neuronal apoptosis induced by NGF withdrawal that also lies on the Cdc42-JNK pathway.

ASK1 overexpression induced apoptosis of rat sympathetic neurons. The same result was observed in differentiated PC12 cells (Kanamoto *et al.*, 2000). In addition, ASK1-induced death required caspase activity, which is consistent with the very recent findings of Hatai *et al.* (Hatai *et al.*, 2000) who showed that ASK1-induced apoptosis of Mv1Lu mink lung epithelial cells and mouse embryonic fibroblasts is executed by mitochondria-dependent caspase activation. Furthermore, Kanamoto *et al.* (Kanamoto *et al.*, 2000) found that ASK1 activity peaked at 3 hours after NGF withdrawal in differentiated PC12 cells. These results are consistent with a model in which ASK1 activation precedes the peak of JNK activity, which, depending on the report, has been shown to occur between 6 and 16 hours after NGF withdrawal (Xia *et al.*, 1995; Eilers *et al.*, 1998). Consistent with the proposed model, activated ASK1 induced an increase in the level of phosphorylated c-Jun and of its transcriptional activity in SCG neurons maintained in the presence of NGF. Furthermore, overexpression of ASK1 induced a 5-fold activation of JNK in PC12 cells (Kanamoto *et al.*, 2000). These results demonstrate that ASK1 is capable of activating JNK and of inducing c-Jun's transcriptional activity in neurons which is necessary for NGF withdrawal-induced apoptosis in sympathetic neurons (Eilers *et al.*, 1998; Harding *et al.*, 2000).

It was also demonstrated that ASK1 activity was crucial for NGF withdrawal- and Cdc42-induction of the JNK cascade and apoptosis. However, in COS-7 cells, MEKK1 but not ASK1 is likely to be involved in Cdc42-induced JNK activation (Berestetskaya *et al.*, 1998), suggesting that the downstream mediators of Cdc42 signalling may be cell specific.

Even though SEK1 has been shown to be activated by ASK1 (Ichijo *et al.*, 1997), a dominant negative mutant of SEK1, SEK-AL, did not block ASK1-induced death in SCG neurons (data not shown). This is in agreement with previous studies showing that SEK-AL could not block NGF withdrawal- or Cdc42-induced

death in SCG neurons, whilst it blocked MEKK1-induced death [(Eilers *et al.*, 1998) and Chapter 3]. Although the involvement of both MEKK1 and SEK1 in neuronal apoptosis cannot be completely ruled out, it appears that there exists an additional JNKK that is activated by Cdc42 or by ASK1. In this regard, a recently identified JNKK, termed MKK7, might be the target of ASK1 in SCG neurons (Holland *et al.*, 1997; Lu *et al.*, 1997b; Moriguchi *et al.*, 1997; Tournier *et al.*, 1997; Foltz *et al.*, 1998; Tournier *et al.*, 1999). Interestingly, the scaffold proteins, JIP-1 and JIP-2 (Dickens *et al.*, 1997; Whitmarsh *et al.*, 1998; Yasuda *et al.*, 1999), have been shown to interact in a specific manner with members of the MLK family of MAPKKKs such as MLK3, MKK7 and JNK (Whitmarsh *et al.*, 1998). Furthermore, a small amount of ASK1 has also been reported to be present in JIP complexes (Yasuda *et al.*, 1999) suggesting that MAPKKs that bind the JIPs are potential downstream targets of ASK1 in mediating JNK activation. Because the p38 kinase pathway is not activated in SCG neurons after NGF deprivation (Eilers *et al.*, 1998), ASK1 probably initiates cell death in neurons by regulating the JNK pathway only. This hypothesis is supported by the fact that a dominant negative mutant of c-Jun, FLAGΔ169, blocked ASK1-induction of cell death in SCG neurons. It seems therefore, that following NGF withdrawal, ASK1 may induce neuronal apoptosis through the JNK-c-Jun pathway.

In conclusion, it is demonstrated in this study that ASK1 is a crucial element of NGF withdrawal-induced activation of the Cdc42-c-Jun pathway and neuronal apoptosis.

7. Evidence for a role of MLK3 in neuronal apoptosis

7.1 Introduction

As previously mentioned, the investigation on the role of MLK3 in neuronal apoptosis was conducted in parallel with the study on the role of ASK1 presented in Chapter 6. In contrast to ASK1, at the start of this study, MLK3 had not been shown to play a role in apoptosis. However, there were strong reasons for considering MLK3 as an putative mediator of NGF withdrawal and Cdc42-induced apoptosis in sympathetic neurons. MLK3 contains a CRIB domain through which it binds Cdc42 (Ezoe *et al.*, 1994; Gallo *et al.*, 1994; Ing *et al.*, 1994; Bock *et al.*, 2000) and it has been shown to be regulated/activated by Cdc42 (Teramoto *et al.*, 1996a; Bock *et al.*, 2000). In addition, MLK3 was shown to activate the JNK pathway (Rana *et al.*, 1996; Tibbles *et al.*, 1996) and also to be part of the JIP scaffolding complex (Whitmarsh *et al.*, 1998; Yasuda *et al.*, 1999). Furthermore, a recent patent showed that the target of CEP-1347, a very potent neuroprotective drug and an inhibitor of the JNK pathway in both neuronal and non-neuronal cells (Glicksman *et al.*, 1998; Maroney *et al.*, 1998; Maroney *et al.*, 1999b; Kujime *et al.*, 2000; Wagner *et al.*, 2000) is MLK3 (Maroney *et al.*, 1999a). These studies strongly suggested that MLK3 may play an important role in the mechanisms of stress-induced activation of the JNK pathway and they prompted the study described in this Chapter on its role in sympathetic neuronal death.

To investigate the role of MLK3 in neuronal cell death, in addition to WT MLK3, the following MLK3 mutants were used: K144E, CRIB(-) and CRIB(-)K144E. The K144→E mutation in the ATP binding site inactivates MLK3 thereby acting as a kinase dead (KD) or dominant negative mutant (Tibbles *et al.*, 1996). In order to abrogate binding of MLK3 to the small GTP binding proteins, additional mutations, S493→P, P495→A and H500→L, were introduced in the consensus CRIB sequence. These amino acids were replaced in both wild type (WT) and KD MLK3 to generate the corresponding CRIB minus mutants (Mota *et al.*, submmited).

7.2 MLK3 is expressed in sympathetic neurons

Recently, MLK3 protein has been shown to be expressed in the ependyma, choroid plexus and meninges of normal adult rat brain, however it could not be detected in neurons in immunofluorescence studies (Merritt *et al.*, 1999). To determine whether MLK3 is expressed in rat sympathetic neurons both its mRNA and protein levels were examined. Primers were designed based on the human MLK3 sequence for a 120 base pair glycine rich region which is highly specific for MLK3 (amino acids 15 to 54) (Sakuma *et al.*, 1997), as there was no published rat MLK3 sequence available. RNA, extracted from either differentiated PC12 cells or purified cultures of sympathetic neurons (kindly prepared by Cesare Spadoni, EISAI London Research Laboratories, UCL, UK), was then amplified by RT-PCR. The purity of the cells was checked using primers corresponding to S100β protein, a marker for Schwann cells (Kligman and Hilt, 1988). S100β mRNA was not detected in RNA extracts from differentiated PC12 cells and was detected at very low levels in extracts from purified cultures of SCG neurons when compared to extracts from standard SCG neuron cultures (data not shown), suggesting that the purified SCG cultures contained few Schwann cells. A single PCR product of 120 bases was obtained from both PC12 cells and purified SCG neurons with the MLK3 primers (Figure 7.1A). Sequencing of these products confirmed that they were derived from MLK3. The identified rat sequence shares over 75% homology with the human sequence. In addition, the intensity of the 120 bp PCR product was similar for both purified and standard SCG extracts (data not shown), suggesting that MLK3 is expressed in both sympathetic neurons and Schwann cells. To determine the presence of MLK3 protein in neuronal cells, protein extracts from Jurkat cells (positive control for the antibody), PC12 cells and SCG neurons were prepared. Western blotting using the MLK3 antibody detected a 95 kDa band in the extracts from all cell types (Figure 7.1B), demonstrating, contrary to Merritt *et al.* (Merritt *et al.*, 1999), that MLK3 is indeed expressed in neurons. The immunodetection of MLK3 could be competed by a 10-fold excess by weight of the peptide antigen confirming the specificity of this antibody (Figure 7.1B).

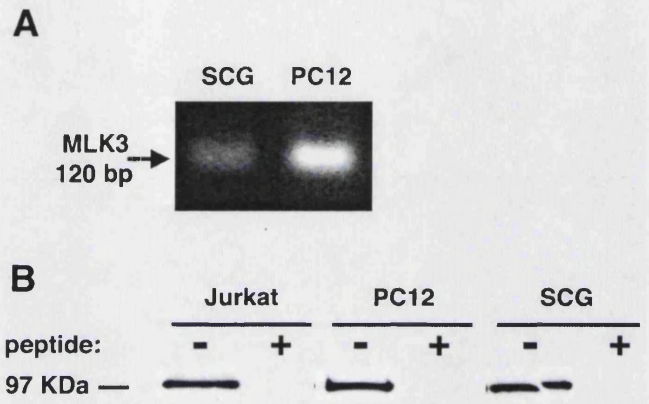


Figure 7.1 MLK3 is expressed in sympathetic neurons.

(A) MLK3 RNA is expressed in sympathetic neurons. Sympathetic neurons, cultured for 6 days in the presence of NGF, were lysed in RNA lysis buffer and total RNA was extracted. RT-PCR was performed on the total RNA and the products were analysed on a 0.7% agarose gel. The amplified 120 bp fragment was sequenced and is consistent with MLK3.

(B) MLK3 protein is expressed in sympathetic neurons and PC12 cells. Cell extracts were prepared from SCG neurons, cultured for 6 days in the presence of NGF, differentiated PC12 cells and Jurkat cells. Thirty micrograms of protein was resolved on a 12.5 % SDS-PAGE polyacrylamide gel, transferred onto nitrocellulose and subjected to an immunoblot with a polyclonal antibody to MLK3. The immunodetection of MLK3 could be competed by a 10-fold excess by weight of the peptide antigen confirming the specificity of this antibody.

7.3 Characterisation of the MLK3 mutants and expression in SCG neurons

Before investigating the role of MLK3 in neuronal cell death, the binding properties of the different MLK3 mutants to Cdc42 were verified by immune complex assay. These experiments were kindly performed by Melissa Reeder (Fox Chase Center, Philadelphia, USA). COS-7 cells were transiently transfected with myc-tagged Cdc42 along with 2 μg of the various FLAG-tagged MLK3 constructs. Forty-eight hours after transfection, the cells were harvested in lysis buffer and immunoprecipitates of the myc-tagged Cdc42 were analysed by Western blotting for the presence of bound MLK3. In addition, the FLAG-tagged MLK3 proteins were also immunoprecipitated to control for their level of expression. Figure 7.2A shows that Cdc42 can immunoprecipitate WT and KD MLK3 but not WT CRIB(-) or KDCRIB(-) demonstrating that the CRIB minus mutants fail to bind the activated Cdc42 and function as expected.

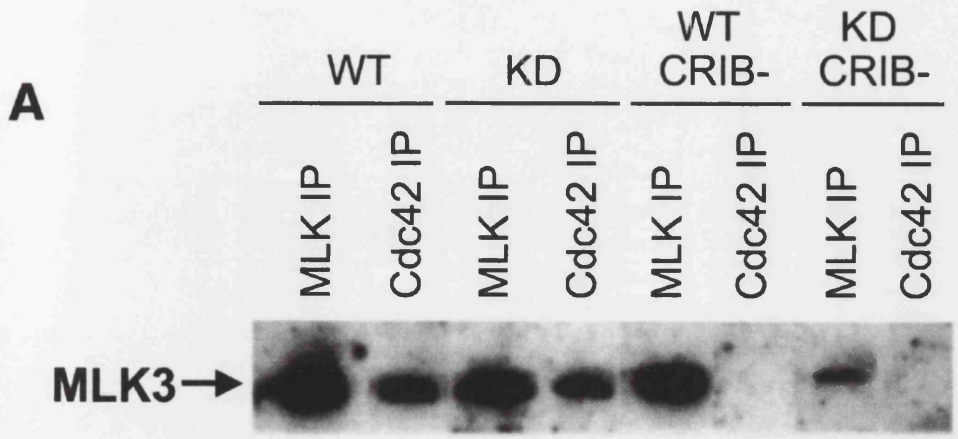
Next, it was important to determine whether the MLK3 mutants would be expressed in sympathetic neurons. Rat sympathetic neurons, cultured for 5-7 days in the presence of NGF, were microinjected with 0.3 mg/ml of an empty expression vector or of the various MLK3 mutants. Twenty-four hours after injection, the cells were stained with an anti-FLAG antibody to check the level of expression. In all cases, 80-100% of the injected cells consistently expressed the MLK3 construct. Interestingly, the kinase active forms [WT and CRIB(-)] of MLK3 were mainly expressed at the plasma membrane whereas the kinase dead mutants [KD and KD-CRIB(-)] were seen throughout the cytoplasm of the neurons (Figure 7.2B). It has been reported that the leucine zipper motifs of MLK3 are sufficient and necessary for its dimerisation which in turn is a pre-requisite for trans-autophosphorylation and autoactivation of MLK3 (Leung and Lassam, 1998). Therefore, overexpression of MLK3 mutants might be sufficient to drive their dimerisation as long as they contain the leucine zipper motifs. However, translocation of MLK3 from the cytoplasm to the plasma membrane occured only with a kinase active mutant. Dimerisation should still occur in the kinase dead mutants, suggesting that autophosphorylation is responsible for the translocation of MLK3 from the cytoplasm to the plasma membrane. Surprisingly, the

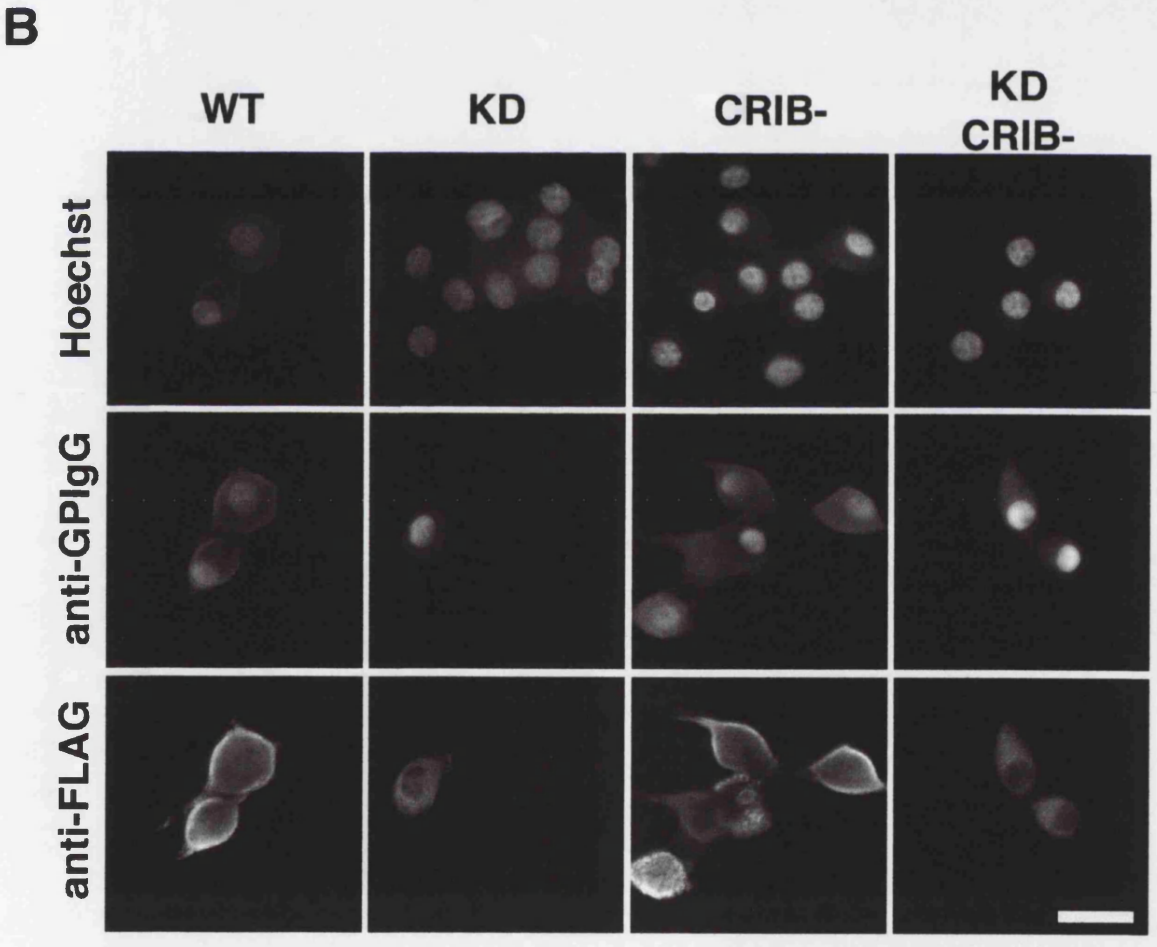
mutations in the CRIB domain had no effect on the subcellular localisation of MLK3. This suggests that trans-autophosphorylation recruits MLK3 to a membrane component other than or in addition to Cdc42. Curiously, co-expression of V12Cdc42 with MLK3 KD did not induce obvious changes in the subcellular localisation of MLK3 KD (data not shown), further suggesting that binding to Cdc42 alone is not sufficient to drive MLK3 to the plasma membrane, whilst MLK3 autophosphorylation seems to be required.

Figure 7.2 Characterisation of the MLK3 mutants and expression in SCG neurons.

(A) *In vivo* binding of MLK3 proteins to Cdc42. COS-7 cells were transiently transfected with 2 μg of various FLAG-tagged MLK3 constructs along with 0.5 μg myc-tagged Cdc42 (pRK5). Cells were harvested and lysed 48 hours post transfection The lysates were adjusted for equal protein and incubated with an anti-myc antibody to immunoprecipitate Cdc42 along with bound MLK3 proteins and with an anti-FLAG antibody to immunoprecipitate MLK3 protein. The immune complexes were then analysed by Western blotting to detect MLK3 protein.

(B) Subcellular localisation of the different MLK3 mutants in SCG neurons. Sympathetic neurons, cultured for 5-7 days in the presence of NGF, were microinjected with 0.3 mg/ml of plasmid DNA and 5 mg/ml of GP-IgG, to mark the injected cells. Twenty-four hours after injection, the cells were stained with an anti-FLAG antibody to detect MLK3 expression, with an anti-GP-IgG antibody to detect the injected cells and with Hoechst to visualise the nuclei. Photographs were taken using a Xillix digital camera and Openlab software. Bar = 30 μm.


A
WT
KD
WT CRIB-
KD CRIB-
MLK IP
Cdc42 IP
MLK IP
Cdc42 IP
MLK IP
Cdc42 IP
MLK IP
Cdc42 IP
MLK3


B
WT
KD
CRIB-
KD CRIB-
Hoechst
anti-GPIgG
anti-FLAG

7.4 MLK3 induces neuronal apoptosis

To investigate the role of MLK3 in neuronal cell death, the effect of different MLK3 mutants on sympathetic neurons was examined. SCG neurons, cultured for 5-7 days in the presence of NGF, were microinjected with 0.3 mg/ml of an empty expression vector or 0.1 or 0.3 mg/ml of the different MLK3 mutants. The percentage of survival was assessed by phase microscopy 48 hours after injection. Expression of the kinase active forms [WT and CRIB(-)] of MLK3 significantly decreased the survival of SCG neurons in a dose-dependent manner, whereas the empty vector and the kinase dead mutants [KD and KD-CRIB(-)] had no effect (Figure 7.3A). To further characterise the death induced by MLK3, the nuclear morphology of the injected neurons was examined by Hoechst staining. Twenty-four hours after injection, WT and WT CRIB(-) induced an increase in the percentage of pyknotic nuclei (Figure 7.3B) whereas neither of the KD mutants had an effect on the nuclear morphology of the injected cells. These results demonstrate that MLK3 can induce neuronal apoptosis in the presence of NGF and that its kinase activity is required for its pro-apoptotic effect.

To further confirm the apoptotic nature of MLK3-induced death, an inhibitor of caspases was examined for its ability to protect SCG neurons from MLK3-induced death. Sympathetic neurons were pre-treated with 100 μM zVAD-fmk for 2 hours or maintained in their culture medium prior to microinjection with WT MLK3. The percentage of surviving cells was assessed by phase microscopy 48 hours later. As a control SCG neurons were treated with zVAD-fmk and maintained in the presence or absence of NGF. zVAD-fmk had no toxic effect and rescued SCG neurons from NGF withdrawal (data not shown). In addition, zVAD-fmk could rescue neurons from MLK3-induced death to levels similar to the +NGF control suggesting that MLK3 can activate a caspase-dependent apoptotic pathway in SCG neurons (Figure 7.3C and data not shown).

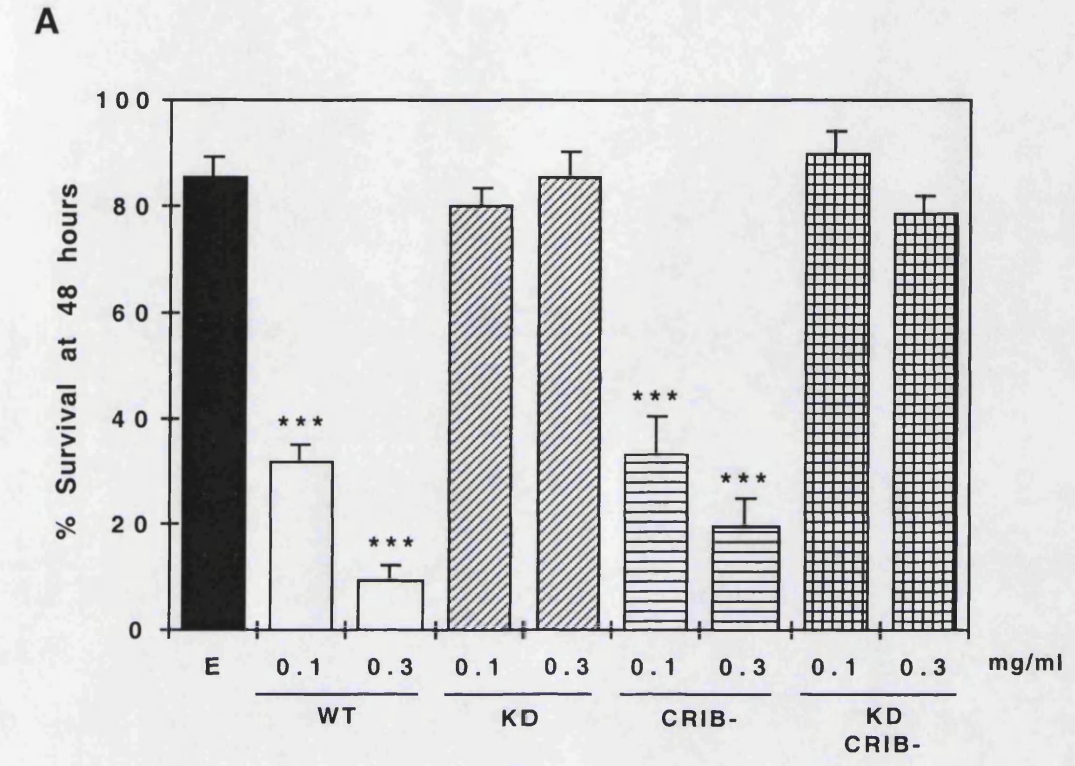
Figure 7.3 MLK3 induces neuronal apoptosis.

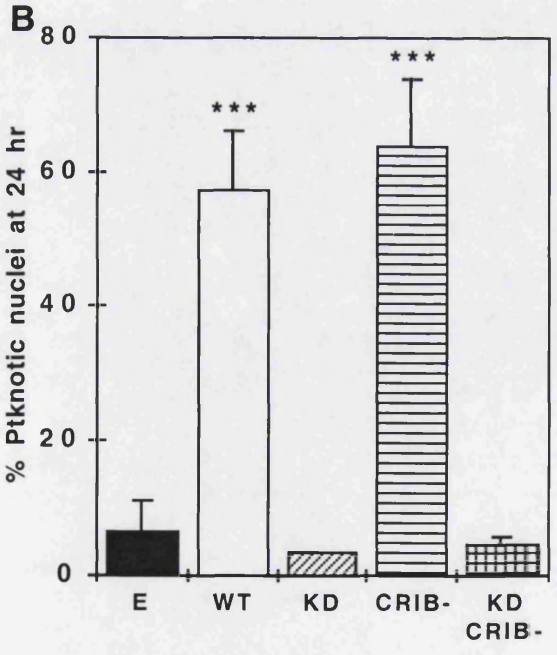
(A) Induction of neuronal cell death by MLK3 in SCG neurons. Rat sympathetic neurons, cultured for 5-7 days in the presence of NGF, were microinjected with 0.3 mg/ml of an empty expression vector, or with 0.1 or 0.3 mg/ml of the WT MLK3 or the different MLK3 mutants [KD, CRIB(-) and CRIB(-)KD], along with 70 kDa Texas Red Dextran to mark the injected cells. Forty-eight hours later, the percentage of surviving cells was assessed by phase microscopy.

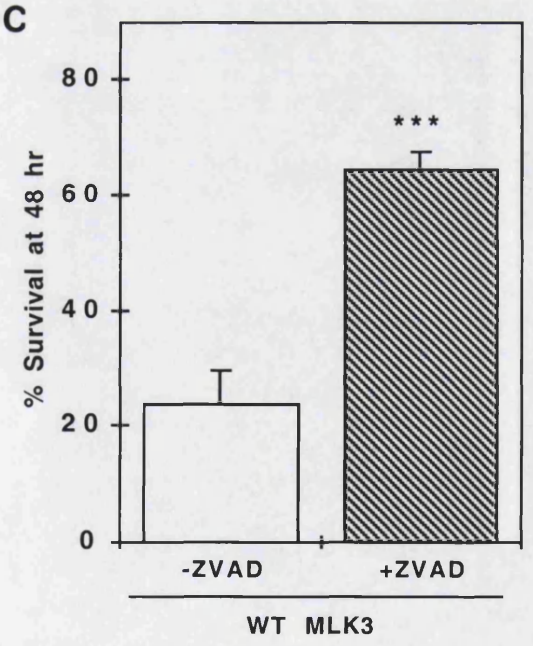
(B) MLK3 increases the number of pyknotic nuclei in SCG neurons. SCG neurons were injected with 0.3 mg/ml of a control empty expression vector or of the various mutants of MLK3. After 24 hours, the nuclear morphology was visualised by Hoechst staining.

(C) zVAD-fmk protects SCG neurons from MLK3-induced death. SCG neurons, cultured for 5-7 days in the presence of NGF, were pre-treated with 100 μM zVAD-fmk for 2 hours or left untreated prior to microinjection with 0.3 mg/ml WT-MLK3. The percentage of surviving cells was assessed 48 hours later by phase microscopy.

The results are the mean ± SEM of 3 (A, B & C) independent experiments. *** $p<0.005$ [compared to the respective empty vector controls (A & B) and -ZVAD (C)].


A
100
80
60
40
20
0
% Survival at 48 hours
***
***
***
***
E
0.1
0.3
0.1
0.3
0.1
0.3
0.1
0.3
mg/ml
WT
KD
CRIB-
KD
CRIB-


B
80
60
40
20
0
% Ptknotic nuclei at 24 hr
***
***
E
WT
KD
CRIB-
KD
CRIB-


C
80
60
40
20
0
% Survival at 48 hr
***
-ZVAD
+ZVAD
WT MLK3

7.5 MLK3 catalytic activity increases following NGF withdrawal

To examine whether induction of neuronal apoptosis by NGF withdrawal had any effect on the endogenous MLK3 kinase, MLK3 kinase activity was measured in an immune complex assay. MLK3 was immunoprecipitated from whole-cell extracts of SCG neurons and differentiated PC12 cells, using an antibody specific for MLK3 only. Following immunoprecipitation, MLK3 kinase activity was assayed using myelin basic protein as a substrate. The specificity of this antibody in binding to MLK3 has been demonstrated in Figure 7.1B, in which the bands corresponding in size to that of MLK3 could be competed away if the antibody was pre-incubated with the peptide used to generate it. MLK3 kinase activity was first measured in extracts prepared from differentiated PC12 cells which were deprived of NGF for 3, 5 and 7 hours. MLK3 kinase activity increased, reaching a maximum induction of ≈ 2.5 fold at 5 hours after NGF withdrawal (Figures 7.4A and B). This increase was not the result of disturbing the cells or due to feeding the cells with fresh medium, as MLK3 kinase activity did not increase in cells refed with fresh NGF-containing medium. Next, MLK3 kinase activity was measured in extracts prepared from sympathetic neurons that had been in culture for 5 days *in vitro*. In the presence of NGF, sympathetic neurons contained very high levels of MLK3 activity. NGF withdrawal led to a ≈ 2 fold increase in MLK3 kinase activity after 5 hours (Figure 7.4C). These results are in support of a model in which MLK3 activation would occur prior to the peak of JNK activity, which has been shown to occur between 3 and 16 hours after NGF withdrawal in both systems (Xia *et al.*, 1995; Virdee *et al.*, 1997; Eilers *et al.*, 1998), suggesting a role for MLK3 as a physiological activator of JNK in neurons.

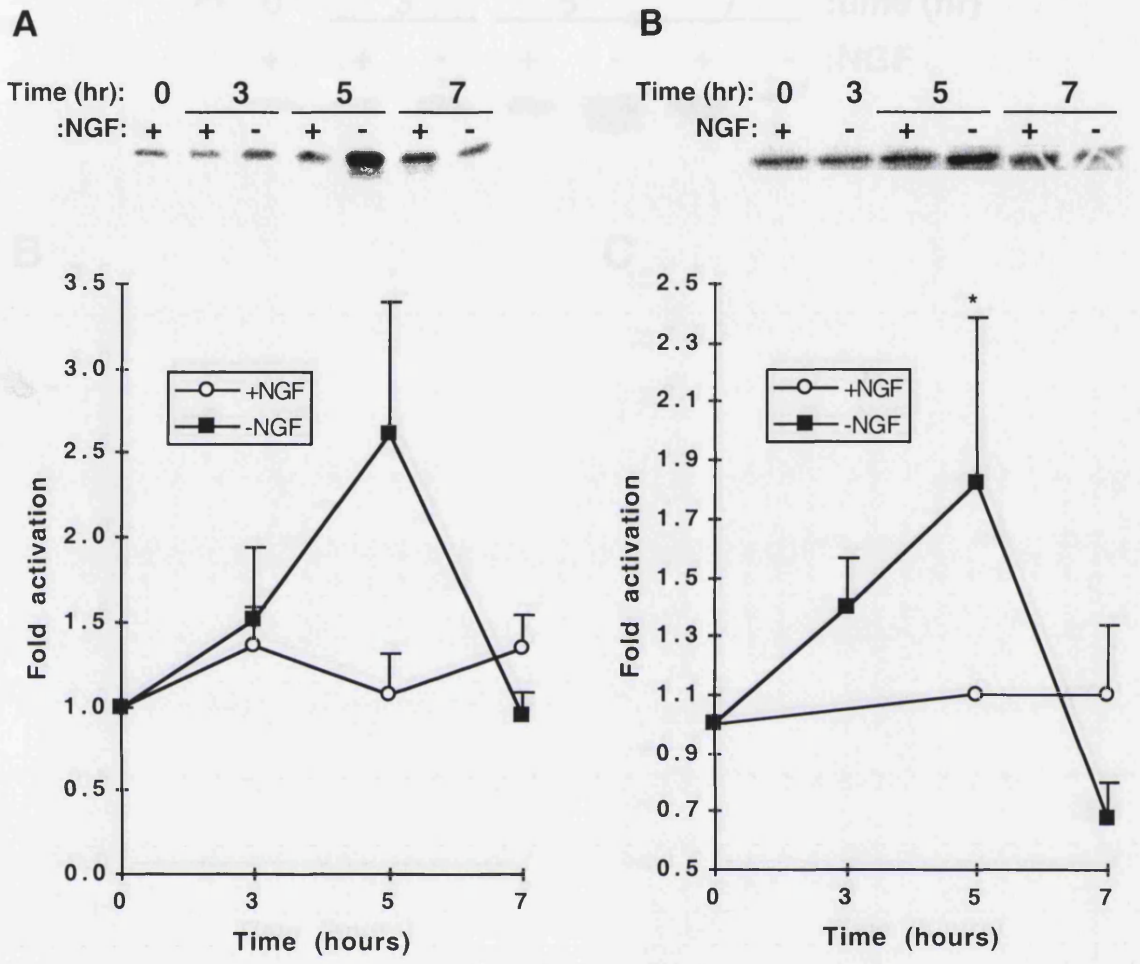


Figure 7.4 MLK3 kinase activity is increased following NGF withdrawal in PC12 cells and sympathetic neurons.

MLK3 kinase assays were performed in differentiated PC12 cells (A) and in SCG neurons (B) that had been maintained in the presence of NGF or withdrawn from NGF for 3, 5, and 7 hours. One hundred micrograms (PC12 cells) or 30 μg (SCG neurons) of lysate were used per immunoprecipitation. The products of the MLK3 kinase assay were separated on a 12.5% SDS-polyacrylamide gel, which was fixed and dried, and submitted to autoradiography. Relative MLK3 kinase activity was determined by using the phosphoimaging analysis system from Bio-Rad. The level of MLK3 kinase activity at time 0 was set as 1. The results shown are the mean ± SEM of 3 (A & B) independent experiments. * $p<0.05$ (compared to +NGF control at 5 hours after NGF withdrawal). Representative autoradiographs of MLK3 kinase assays in PC12 cells and SCG neurons are shown.

7.6 MLK3 activity is required for NGF withdrawal-induced death of sympathetic neurons

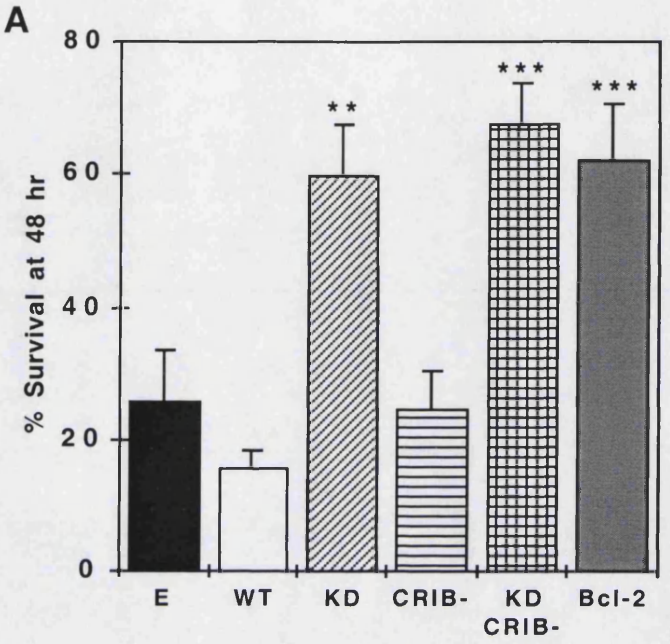
To examine whether MLK3 plays a physiological role in NGF withdrawal-induced neuronal cell death, each of the MLK3 constructs, as well as an empty expression vector control (negative control) and Bcl-2 (positive control) were microinjected into SCG neurons. The cells were withdrawn from NGF and the percentage of surviving cells was assessed by phase microscopy 48 hours later. Both kinase dead mutants [KD and KD-CRIB(-)] protected the neurons from NGF-withdrawal-induced death to levels similar to that obtained with Bcl-2 (Figure 7.5A). Neither WT forms of MLK3 [WT and WT-CRIB(-)] rescued the sympathetic neurons (Figure 7.5A). In addition, the effect of these mutants on the nuclear morphology of the injected neurons was observed by Hoechst staining at 24 hours post injection. The cells injected with the empty vector control had a higher proportion of pyknotic nuclei when compared to the empty vector, whereas the cells injected with the kinase dead mutants of MLK3 had a much lower percentage of condensed or fragmented nuclei (Figure 7.5B). These results confirm that MLK3 is involved in the mediation of apoptosis in SCG neurons and that its kinase activity seems to be an important requirement for the execution of neuronal cell death.

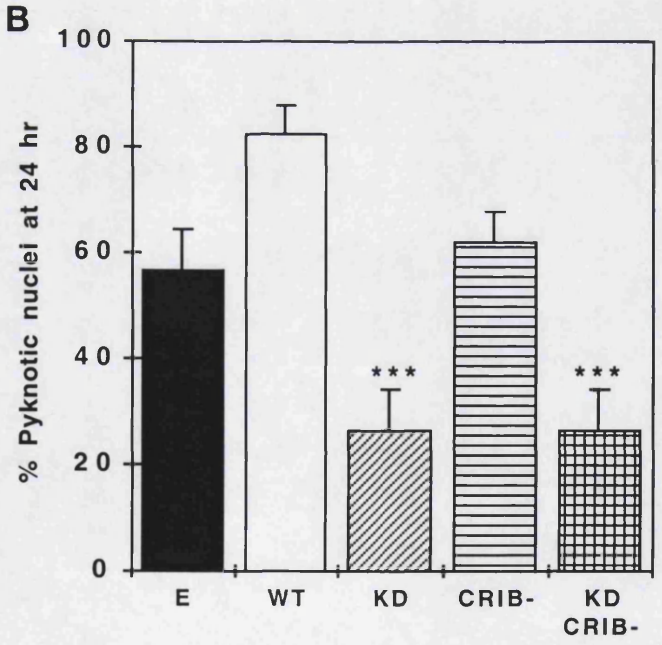
Figure 7.5 MLK3 is required for NGF-withdrawal-induced apoptosis.

(A) MLK3-KD mutants prevent NGF-withdrawal-induced cell death in SCG neurons. SCG neurons, cultured for 5-7 days in the presence of NGF, were microinjected with 70 kDa Texas Red Dextran and 0.3 mg/ml of the various MLK3 mutants, or of an empty vector or 0.05 mg/ml of Bcl-2. Four hours later, the cells were withdrawn from NGF and the number of injected cells was scored (100% value). The percentage of surviving cells was assessed by phase microscopy after 48 hours.

(B) MLK3-KD mutants decrease the number of pyknotic nuclei after NGF withdrawal-induced cell death in SCG neurons. SCG neurons were injected with 0.3 mg/ml of a control empty expression vector or the different MLK3 mutants and withdrawn from NGF. After 24 hours, the nuclear morphology was visualised by Hoechst staining.

The results are the mean ± SEM of 3 (A & B) independent experiments. ** $p<0.01$; *** $p<0.005$ (compared to the empty vector controls).


A
% Survival at 48 hr
80
60
40
20
0
**
***
***
E
WT
KD
CRIB-
KD CRIB-
Bcl-2


B
% Pyknotic nuclei at 24 hr
100
80
60
40
20
0
***
***
E
WT
KD
CRIB-
KD CRIB-

7.7 MLK3 activity is required for Cdc42 -induced neuronal death

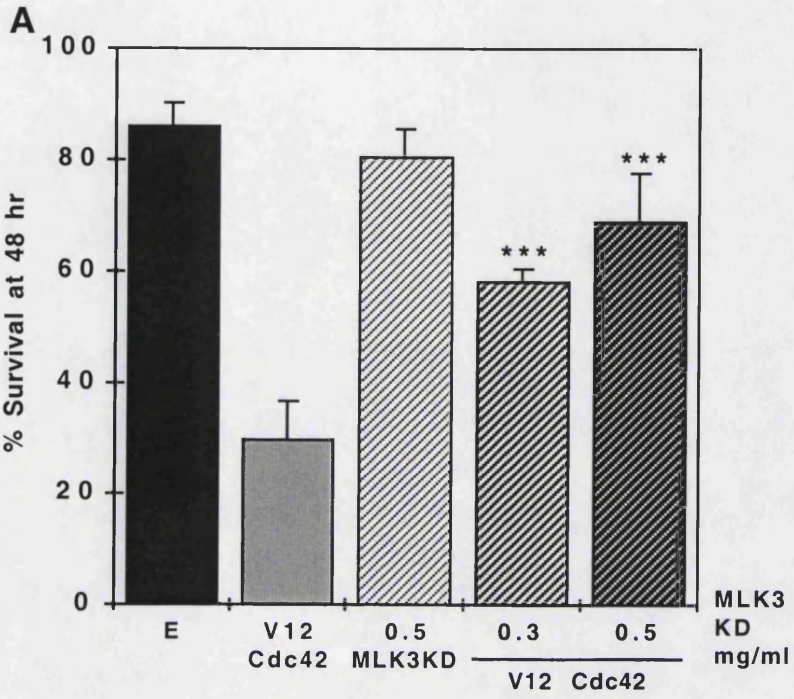
To examine whether MLK3 and Cdc42 lie on the same death signalling pathway, an activated mutant of Cdc42 (V12Cdc42) was co-injected with both the CRIB(+) or CRIB(-) kinase dead mutants of MLK3 into SCG neurons. The percentage of surviving cells was assessed 48 hours after injection by phase microscopy. Independent of their ability to bind Cdc42, the kinase inactive mutants of MLK3 efficiently blocked V12Cdc42-induced death (Figures 7.6A and B) suggesting that the kinase activity of MLK3, but not the binding to Cdc42, is crucial for the induction of apoptosis by Cdc42 and that MLK3 is a downstream mediator of Cdc42 in sympathetic neurons. These results, together with the data from section 7.6 demonstrate that blocking MLK3 activity is sufficient to inhibit both NGF withdrawal- and Cdc42-induced death.

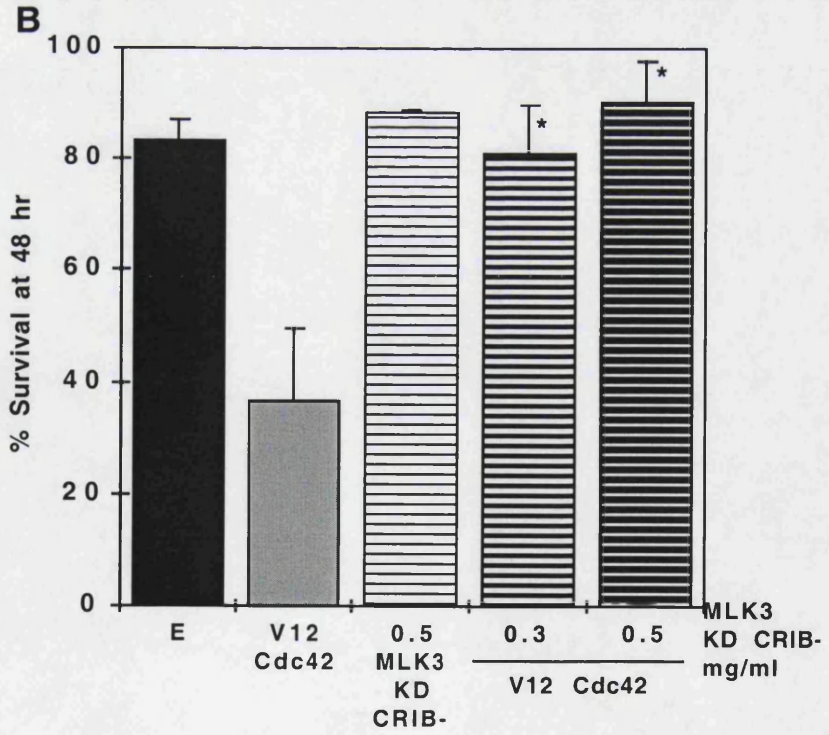
Figure 7.6 MLK3 is required for Cdc42-induced death.

(A) Cdc42-induced apoptosis requires MLK3 activity. Sympathetic neurons were injected with 0.3 mg/ml and 0.5 mg/ml MLK3-KD along with 0.1 mg/ml V12Cdc42 and 70 kDa Texas Red Dextran into SCG neurons. The cells were maintained in the presence of NGF and the percentage of surviving cells was assessed by phase microscopy 48 hours after injection.

(B) MLK3-induced apoptosis does not require binding to Cdc42. SCG neurons, cultured for 5-7 days in the presence of NGF, were microinjected with MLK3-CRIB(-)KD at the indicated concentrations and 0.1 mg/ml V12Cdc42. The percentage of surviving cells was assessed by phase microscopy 48 hours later.

The results are the mean ± SEM of 3 (A & B) independent experiments. * $p<0.05$; *** $p<0.005$ (compared to V12Cdc42).

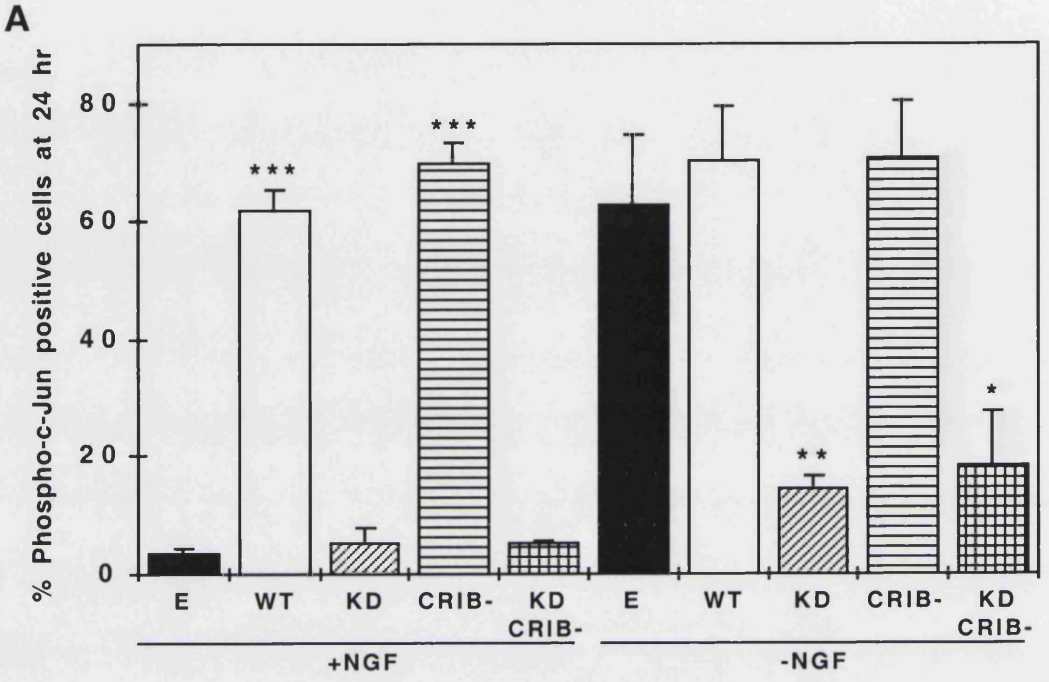

A
100
80
60
40
20
0
% Survival at 48 hr
***
***
E
V12 Cdc42
0.5 MLK3KD
0.3
0.5
V12 Cdc42
MLK3 KD mg/ml

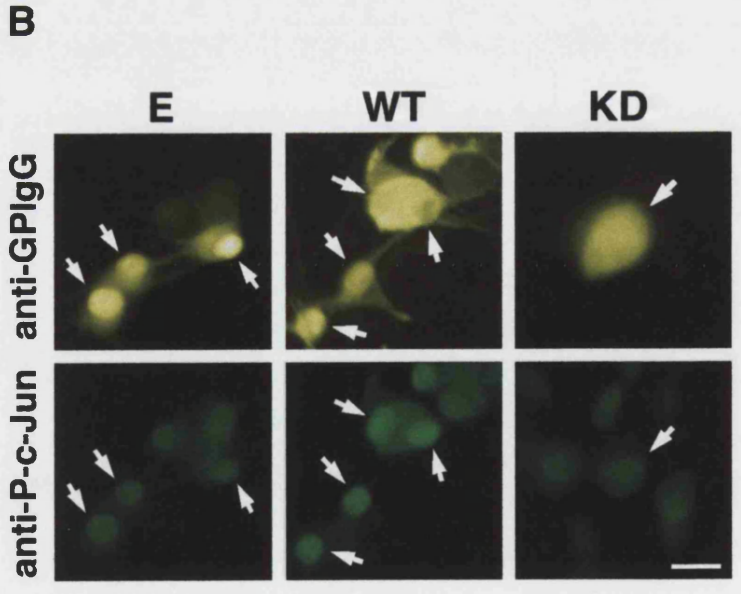

B
100
80
60
40
20
0
% Survival at 48 hr
*
*
E
V12 Cdc42
0.5 MLK3 KD CRIB-
0.3
0.5
V12 Cdc42
MLK3 KD CRIB- mg/ml

7.8 MLK3 is required for the activation of the JNK pathway in SCG neurons

MLK3 had previously been shown to activate the JNK pathway in non-neuronal cells (Teramoto *et al.*, 1996a; Tibbles *et al.*, 1996). To investigate whether this would be the case in sympathetic neurons, the effect of MLK3 on the phosphorylation of c-Jun in both the presence and absence of NGF was examined. SCG neurons, cultured for 5-7 days in the presence of NGF, were microinjected with 0.3 mg/ml empty vector or with each of the different MLK3 constructs. The cells were fixed, permeabilised and stained with a specific anti-phospho-c-Jun antibody (Lallemand *et al.*, 1998). Cells expressing either the control empty vector or the kinase dead mutants of MLK3 did not show any increase in the level of phosphorylated c-Jun compared to the non-injected cells (Figure 7.7A). However, overexpression of the kinase active forms of MLK3 induced a clear increase in the levels of phosphorylated nuclear c-Jun (Figure 7.7A). The effect of the dominant negative mutants of MLK3 in preventing the increase in the level of phosphorylated c-Jun that occurs after NGF withdrawal was also examined. Overexpression of either of the kinase dead mutants in sympathetic neurons blocked the increase in the level of phosphorylated c-Jun induced by NGF withdrawal, whereas the empty vector and the kinase active forms of MLK3 did not (Figure 7.7B). These results demonstrate that MLK3 is essential for the activation of the JNK pathway after NGF withdrawal in sympathetic neurons.

An accumulation of phosphorylated c-Jun in the nucleus should lead to activation of the c-Jun transcriptional pathway. To investigate whether MLK3-induced apoptosis requires activation of the c-Jun transcriptional pathway, sympathetic neurons were co-injected with WT-MLK3 and FLAG-Δ169, a dominant negative mutant of c-Jun (Ham *et al.*, 1995). Co-expression of FLAG-Δ169 with WT-MLK3 completely blocked MLK3-induced death (Figure 7.7C). Taken together, these results suggest that the MLK3 kinase activity is important for the induction of c-Jun phosphorylation and AP-1 transcriptional activity in sympathetic neurons deprived of NGF and that the death signal induced by MLK3 is mediated via the JNK pathway.


A
% Phospho-c-Jun positive cells at 24 hr
80
60
40
20
0
***
***
**
*
E
WT
KD
CRIB-
KD CRIB-
E
WT
KD
CRIB-
KD CRIB-
+NGF
-NGF


B
E
WT
KD
anti-GPIgG
anti-P-c-Jun

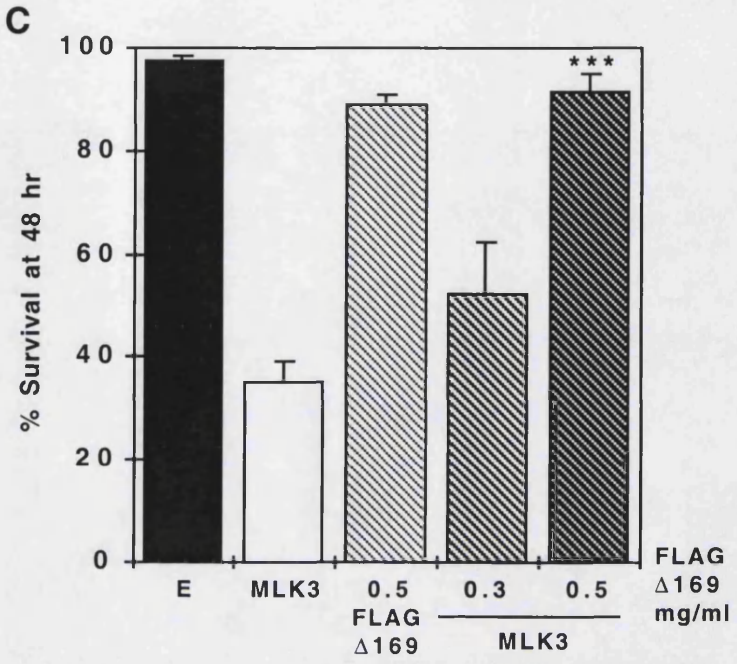


Figure 7.7 MLK3 activates the JNK pathway in neurons.

(A & B) MLK3-dependent phosphorylation of c-Jun in SCG neurons. SCG neurons were microinjected with either 0.3 mg/ml of an empty expression vector or WT MLK3 or the different mutants of MLK3, together with 5 mg/ml GP-IgG to detect the injected cells, and maintained in the presence of NGF or withdrawn from NGF as indicated. After 24 hours, the cells were fixed, permeabilised and stained with a specific anti phospho-c-Jun antibody, an anti-GP-IgG antibody and Hoechst . Only the cells in which phospho-c-Jun staining was clearly above background were scored as positive.

(B) Photographs were taken using a Xillix digital camera and Openlab software. Bar = 30 μm.

(C) FLAG-Δ169 blocks MLK3-induced apoptosis. FLAG-Δ169 at the indicated concentrations and 0.1 mg/ml of WT MLK3 were microinjected into SCG neurons, 5-7 days in culture, and maintained in the presence of NGF. The percentage of surviving cells was assessed by phase microscopy 48 hours later. The results are the mean ± SEM of 3 (A & C) independent experiments. * $p<0.05$; ** $p<0.01$; *** $p<0.005$ [compared to the respective empty vector controls (A &C)].

7.9 Relationship between MLK3 and ASK1

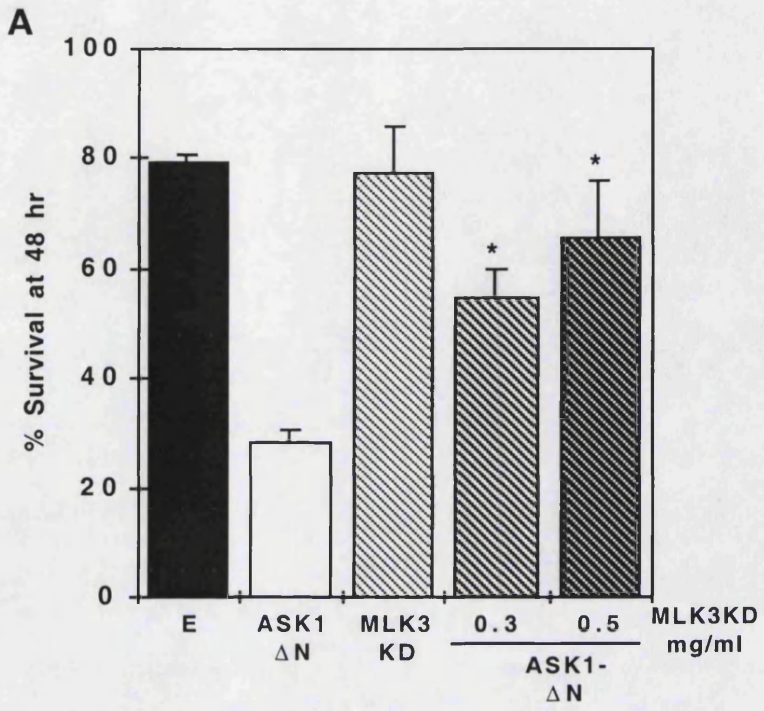
In Chapter 6 it was demonstrated that ASK1, like MLK3, is required for both NGF withdrawal and Cdc42-induced neuronal death. To determine the relationship between these two MAPKKKs, sympathetic neurons, cultured for 5-7 days in the presence of NGF, were co-injected with WT-MLK3 and dominant negative ASK1 (KR-ASK1) or with ΔN-ASK1 and dominant negative MLK3 (KD-MLK3) (Figures 7.8A and B). The percentage of viability was assessed 48 hours later by phase microscopy. Neurons overexpressing KR-ASK1 were not protected against MLK3-induced death, suggesting that ASK1 does not lie downstream of MLK3. In the converse experiment, the kinase dead mutant of MLK3 efficiently blocked ASK1-induced death, suggesting that MLK3 is downstream of ASK1. In separate experiments KD-MLK3 did not protect SCG neurons from MEKK1-induced death (data not shown), demonstrating that the effect obtained with this dominant negative mutant is specific for ASK1-induced death.

Figure 7.8 Relationship between MLK3 and ASK1

(A) Sympathetic neurons were injected with 0.6 mg/ml empty vector or 0.1 mg/ml ASK1-ΔN or MLK3-KD at the indicated concentrations and together with 0.1 mg/ml ASK1-ΔN and 70 kDa Texas Red Dextran to detect the injected cells. The cells were maintained in the presence of NGF and the percentage of surviving cells was assessed 48 hours after injection by phase microscopy.

(B) SCG neurons, cultured for 5-7 days in the presence of NGF, were microinjected with 0.6 mg/ml of an empty vector or 0.1 mg/ml MLK3-WT or 0.5 mg/ml ASK1-KR or 0.1 mg/ml MLK3-WT together with 0.5 mg/ml ASK1-KR. The cells were maintained in the presence of NGF and the percentage of surviving cells was assessed 48 hours after injection by phase microscopy.

The results are the mean ± SEM of 3 (A) or 4 (B) independent experiments. * $p<0.05$ [compared to ASK1-ΔN (A)]. No significant differences were observed between MLK3 injected neurons and neurons co-expressing MLK3 and ASK1-KR.


A
100
80
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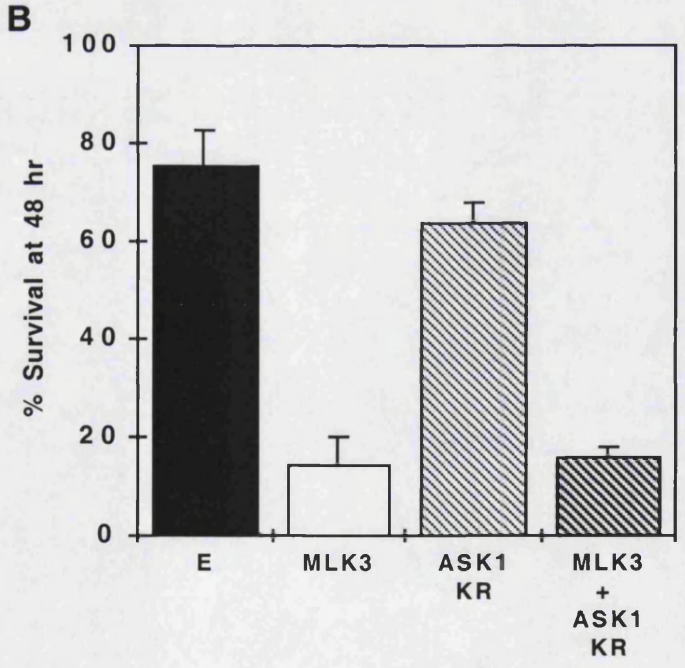
0
% Survival at 48 hr
*
*
E
ASK1 ΔN
MLK3 KD
0.3
0.5
MLK3KD mg/ml
ASK1-ΔN


B
100
80
60
40
20
0
% Survival at 48 hr
E
MLK3
ASK1 KR
MLK3 + ASK1 KR

7.10 Discussion

As stated throughout this thesis, the Cdc42-JNK-c-Jun pathway is a crucial component in the induction of apoptosis in sympathetic neurons by survival factor deprivation. In this Chapter the role of MLK3 was investigated in relation to the activation of the Cdc42-JNK-c-Jun pathway and in the context of NGF withdrawal. Here it was shown, by both immunoblotting and RT-PCR techniques, that MLK3 is expressed endogenously in sympathetic neurons. This is in contradiction to the findings of Merritt *et al.* (Merritt *et al.*, 1999) who failed to detect MLK3 in neurons of rat brain slices in immunofluorescence using the same antibody. Perhaps the reason Merritt *et al.* could not detect MLK3 above background by immunofluorescence studies is because either MLK3 is expressed at low levels in neurons or neuronal MLK3 may be in a conformation or in a complex with other protein(s), which does not expose the MLK3 epitope recognised by this antibody in such studies.

To examine the role of MLK3 in neuronal apoptosis, a series of MLK3 mutants were used. When overexpressed in SCG neurons, it was observed that the MLK3 mutants with an active kinase concentrated at the plasma membrane whilst the kinase dead mutants were mainly cytoplasmic. Recently, Leung and Lassam (Leung and Lassam, 1998) showed that the leucine zipper motifs of MLK3 are sufficient for its dimerisation. In addition, they demonstrated that dimerisation of MLK3 is a pre-requisite for its autophosphorylation and thereby activation. Furthermore, they found that Cdc42 led to an increase in MLK3 dimerisation, suggesting that recruitment of MLK3 to the plasma membrane by Cdc42 might increase the local concentration of MLK3 and therefore the chances of dimerisation. In light of these findings, dimerisation should still occur in the kinase dead mutants, as they contain an intact leucine zipper domain, and the translocation of the CRIB containing mutants to the membrane should be increased compared to the CRIB minus ones. However, neither of the KD mutants locate to the plasma membrane whereas the mutants with an intact kinase domain [CRIB(+) and CRIB(-)] do. These results suggest that autophosphorylation, but not interaction with Cdc42, is required for the

translocation of MLK3. However, the possibility that overexpression of MLK3 may override the necessity for upstream activators of dimerisation cannot be excluded.

MLK3 expression induced apoptosis in SCG neurons and its kinase activity is required for its pro-apoptotic effect. Indeed, in addition to reducing neuronal viability, MLK3 [WT and WT-CRIB(-)]-injected neurons clearly displayed pyknotic nuclei, whilst the KD mutants did not. In addition, zVAD-fmk was able to block MLK3-induced death, supporting the notion that MLK3 has a role in the induction of apoptosis in SCG neurons and that it requires the activation of caspases for the execution of apoptosis.

It was also demonstrated that blocking MLK3 activity by overexpression of MLK3 kinase dead mutants is sufficient to prevent neuronal apoptosis. Although KD-MLK3 may interfere with a related kinase, as MLK3 has been reported to form complexes with co-expressed MLK2 (Leung and Lassam, 1998), these observations strongly suggest a role of MLK3 in the induction of neuronal apoptosis.

Because evidence that MLK3 is important for the death of sympathetic neurons was obtained, it was important to investigate whether Cdc42 lies upstream of MLK3. The kinase dead mutants of MLK3 could block apoptosis induced by overexpression of Cdc42, suggesting that MLK3 activity is important for the induction of cell death by Cdc42 and that MLK3 is a downstream mediator of Cdc42-induced death in SCG neurons. In addition, the ability of the kinase dead MLK3 mutants in inhibiting Cdc42-induced death is not related to sequestration of Cdc42 in neurons, as MLK3-KD (CRIB-) could still block the pro-apoptotic effect of Cdc42. Consistent with this, Bock and colleagues showed that although Cdc42 activates MLK3, it is not necessary to maintain MLK3 in an activated state and that activation of MLK3 by Cdc42 requires an additional cellular component (Bock *et al.*, 2000).

More importantly, a rapid increase in MLK3 activity was observed in both differentiated PC12 cells and SCG neurons following NGF withdrawal. Even in the presence of NGF, high levels of MLK3 activity were detected when compared to the control of substrate alone. It is possible that immunoprecipitation may artificially increase MLK3 activity by aiding dimer formation. Nevertheless, an increase in MLK3 phosphorylating activity following NGF withdrawal was

detected. This increase peaked at 5 hours after NGF withdrawal for both PC12 cells and SCG neurons and precedes the reported increase in JNK activity in both systems (Xia *et al.*, 1995; Virdee *et al.*, 1997; Eilers *et al.*, 1998), suggesting that MLK3 may act as a physiological activator of the JNK pathway. In agreement with this, it was shown in this study that MLK3 induces an increase in the phosphorylation of c-Jun and also that MLK3 kinase activity is required for c-Jun phosphorylation following NGF withdrawal from SCG neurons, suggesting that MLK3 induces JNK activation. Furthermore, expression of FLAG-Δ169 increased the percentage of viable cells after MLK3-induced death. These results demonstrate that not only does MLK3 mediate activation of the JNK-c-Jun transcriptional pathway but also that, in dying SCG neurons, MLK3 activity is required for the activation of that pathway.

In light of these results it was important to examine the relationship between MLK3 and ASK1, as both kinases are crucial for activation of the JNK pathway and consequent induction of apoptosis following NGF withdrawal in sympathetic neurons (Chapter 6 and this Chapter). The results presented in this Chapter suggest that MLK3 lies downstream of ASK1 thereby explaining the requirement for both kinases in neuronal apoptosis. To support this finding are the observations that the peak ASK1 activity, at 3 hours after NGF withdrawal from PC12 cells (Kanamoto *et al.*, 2000), precedes that of MLK3, at 5 hours (this Chapter), which precedes that of JNK, at 6-16 hours (Xia *et al.*, 1995; Eilers *et al.*, 1998). However, there is still a possibility that the dominant negative MLK3 mutant might compete for a common downstream effector (such as MKK7) thereby indirectly blocking the effect of ASK1. The kinase dead mutant of MLK3 did not rescue SCG neurons from MEKK1-induced death (data not shown), suggesting that, if the above hypothesis is correct, MLK3-KR must be competing for a kinase other than SEK1/MKK4. In addition, ASK1 has been reported to be present in scaffolding complexes such as the JIPs but to a much lower extent than MLK3 (Dickens *et al.*, 1997; Yasuda *et al.*, 1999). The formation of such multiprotein complexes might be a convergence point of various pathways mediated by ASK1 and by MLK3.

In conclusion, it has been demonstrated that MLK3, or a closely related kinase, is a physiological element of NGF withdrawal-induced activation of the Cdc42-c-Jun pathway and neuronal apoptosis.

8. Discussion

Neuronal apoptosis is a crucial process involved not only in the regulation of development of the nervous system but also in pathological situations such as stroke, Alzheimer's disease, Parkinson's disease, spinal cord injury, traumatic brain injury and perhaps many others (Clarke, 1990; Oppenheim, 1991; Henderson, 1996; Katoh *et al.*, 1996; Li *et al.*, 1996; Linnik, 1996; Crowe *et al.*, 1997; Pettmann and Henderson, 1998). Therefore, understanding the mechanisms of neuronal cell death is of great importance and interest. Neuronal apoptosis or survival are normally regulated by the restricted availability of specific neurotrophins which bind to particular cell surface receptors. For instance, NGF can activate specific cellular pathways which are responsible for maintaining the neurons alive. Alternatively, suboptimal amounts of NGF can lead to activation or de-inhibition of death pathways.

At the start of this investigation, it was becoming apparent that the activation of the c-Jun transcriptional pathway played a very important role in the signalling of neuronal apoptosis in sympathetic neurons deprived of NGF (Estus *et al.*, 1994; Ham *et al.*, 1995). In addition, it was known that the JNKs could phosphorylate and activate c-Jun (Pulverer *et al.*, 1991; Derijard *et al.*, 1994; Kyriakis *et al.*, 1994; Karin *et al.*, 1997; Ip and Davis, 1998), suggesting that the JNKs could play a role in the induction of apoptosis. However, there was little knowledge of how the absence of NGF could trigger the activation of the JNK pathway. Identifying the early upstream regulators of JNK and apoptosis in neurons may therefore provide clues that can increase the understanding of the mechanisms of neurotrophin withdrawal-induced death. In addition, the discovery of new upstream regulators of neuronal apoptosis should reveal potential targets for the development of novel therapies for neurodegenerative diseases. Studies by Minden *et al.* (Minden *et al.*, 1995) and Coso *et al.* (Coso *et al.*, 1995) showed that constitutively activated mutants of Cdc42 and Rac were efficient activators of a pathway leading to both JNK and p38 activation. It was therefore of interest to determine whether the small GTP-binding proteins also play a role in

JNK activation and induction of apoptosis in neurons, a starting point for the investigation presented in this thesis.

It was shown that Cdc42/Rac1, ASK1 and MLK3 are crucial elements of apoptosis of rat sympathetic neurons deprived of NGF. It was also demonstrated that the induction of apoptosis by Cdc42, ASK1 and MLK3 is mediated via the activation of the JNK pathway, which is crucial for the induction of apoptosis in sympathetic neurons (Eilers *et al.*, 1998; Harding *et al.*, 2000). Work from colleagues (Neame *et al.*, 1998) has shown that the release of cytochrome c from the mitochondria is crucial for apoptosis of sympathetic neurons upon NGF withdrawal. Interestingly, release of cytochrome c can be blocked by FLAG-Δ169 (Whitfield *et al.*, in press), suggesting that the crucial induction of the Cytc→Caspase pathway is mediated by the JNK→c-Jun pathway in sympathetic neurons. The results presented in this thesis showed that Cdc42-, ASK1- and MLK3-induced death could be prevented by the broad caspase inhibitor zVAD-fmk, thereby providing evidence that Cdc42, MLK3 and ASK1 mediate apoptosis by stimulating the JNK→Cytc→Caspase pathway.

However, not all activators of the JNK pathway are such in neurons (e.g. PAK1) or induce neuronal cell death (e.g. PAK1 and PAK2) (Chapter 5), suggesting that the specific cellular components that are required for PAK1 and PAK2 to induce apoptosis and activate the JNK pathway may not be present in neurons. In support of this hypothesis are the observations that some regulators of the Rho GTPases are expressed only in particular cell types and are responsible for mediating specific responses [see Chapter 1 and for review see (Boivin *et al.*, 1996; Kaibuchi *et al.*, 1999; Scita *et al.*, 2000)]. For instance, GEFs for Cdc42, such as Dbl, can stimulate PAK activation, whilst others, such as FGD1, cannot (Zhou *et al.*, 1998). It has also been shown that binding to adapter proteins such as Nck (Lu *et al.*, 1997a) can trigger the activation of PAK. It is therefore possible that the GEF(s) that is(are) required for Cdc42 to activate PAK and/or a key regulatory protein necessary for PAK activation and its induction of the JNK pathway and trigger apoptosis may not be present in SCG neurons. This hypothesis could explain why no increase in the kinase activity of PAK1 was detected after NGF withdrawal from SCG neurons and also why PAK1 did not activate the JNK pathway (Chapter

5). PAK2, on the other hand, despite its ability to activate the JNK pathway, was not able to induce apoptosis (Chapter 5). It is possible that either a specific JNK isoform needs to be activated or that activation of JNK has to be above certain levels for the induction of apoptosis to occur. PAK2, like PAK1, may also require interaction and/or activation with specific proteins for it to be able to induce apoptosis in sympathetic neurons.

In this investigation it was also demonstrated that NGF withdrawal- and Cdc42-induction of apoptosis requires both ASK1 and MLK3 activities, placing Cdc42 upstream of ASK1 and MLK3 in the JNK apoptotic pathway in SCG neurons. How can the requirement for both MAPKKKs be explained? There are two possible explanations. (1) ASK1 may be required to induce the activation of MLK3 necessary for the activation of the JNK pathway. This hypothesis is supported by the findings that a dominant negative MLK3 blocked ASK1-induced death (Chapter 7), suggesting that ASK1 lies upstream of MLK3. The kinetics of ASK1 and MLK3 activation [(Kanamoto *et al.*, 2000) and Chapter 7], with the peak of ASK1 and MLK3 activities occurring at 3 and 5 hours, respectively, after NGF withdrawal, would also support the above hypothesis. (2) Both ASK1 and MLK3 are required to phosphorylate an essential common downstream effector on different sites. In support of this is the fact that MKK4/SEK1 requires phosphorylation on both its serine and threonine residues to be able to phosphorylate JNK (Vacratsis and Gallo, 2000). Even though MKK4/SEK1 is not required for NGF- withdrawal and ASK1-induced death of sympathetic neurons, it is plausible that MKK7 or another, as yet unidentified, MAPKK may be regulated in a similar manner. In addition, both MLK3 and ASK1 have been reported to be present in JIP scaffolding complexes (Whitmarsh *et al.*, 1998; Yasuda *et al.*, 1999). These complexes seem to facilitate the activation of JNK by MAPKKKs by aggregating specific components of the MAPK cascade to form a functional JNK signalling module. It is therefore possible that ASK1 and MLK3 compete for the formation of such multiprotein complexes, as they may interact with common downstream partners. The second hypothesis is based on the assumption that both dominant negative MLK3 and ASK1 behave in a different manner. The dominant negative MLK3 could be blocking ASK1-induced death by competing

for a common downstream effector. Indeed, MLK3 contains many protein interaction domains, such as proline-rich, SH3 and leucine-zipper domains (Ezoe *et al.*, 1994; Gallo *et al.*, 1994; Ing *et al.*, 1994). On the other hand, the kinase dead ASK1 would function by competing for ASK1 only or for its upstream regulators. Indeed, homodimerisation of ASK1 has been shown to induce its activation (Gotoh and Cooper, 1998) and it is possible that dominant negative ASK1 can dimerise with endogenous ASK1 and prevent it from being activated. In addition, the dominant negative ASK1 could not prevent JNK activation induced by other MAPKKKs such as MEKK1, MLK3, TAK1 and Tpl-2 (H. Ichijo, unpublished observations and data not shown) supporting the fact that it acts either upstream or at the level of ASK1. Determining the exact mode of action of both ASK1 and MLK3 dominant negative mutants may therefore provide important clues towards the understanding of the relationship between ASK1 and MLK3 in NGF withdrawal-induced apoptosis of sympathetic neurons.

But how then does Cdc42 activate both ASK1 and MLK3? It has been shown that Cdc42 induces the activation of MLK3 by binding to its CRIB motif (Burbelo *et al.*, 1995; Teramoto *et al.*, 1996a; Bock *et al.*, 2000). It has been suggested that activated Cdc42 recruits MLK3 to the plasma membrane and induces an increase in the local concentration of MLK3 thereby promoting its homodimerisation which is sufficient for autoactivation (Leung and Lassam, 1998). Alternatively, binding of MLK3 to Cdc42 could allow it to adopt a conformation that leads to its autophosphorylation. It has recently been shown that autophosphorylation is essential for MLK3 activation (Leung and Lassam, 2001). Taken together, these results suggest a mechanism by which binding to Cdc42 triggers the autophosphorylation and activation of MLK3. The results presented in Chapter 7 also show that Cdc42 binds to the CRIB domain of MLK3, as CRIB(-) mutants of MLK3 could not interact with Cdc42. However, a kinase dead CRIB(-) mutant of MLK3 blocked Cdc42-induced neuronal apoptosis suggesting that Cdc42 does not need to interact with MLK3 to be able to induce apoptosis. The possibility that a direct interaction of Cdc42 with MLK3 is required for the activation of MLK3 in neurons, however, cannot be ruled out, as overexpression of MLK3 may override

the need for upstream regulators and/or the KD CRIB(-) MLK3 may dimerise with the endogenous MLK3, bound to Cdc42, and thereby keep it inactive.

Alternatively, Cdc42 may indirectly activate MLK3 via ASK1. Indeed, the findings presented in Chapter 7 suggest that ASK1 lies upstream of MLK3 in the apoptotic pathway in sympathetic neurons. To date however, there are no reports suggesting a direct interaction between Cdc42 and ASK1. Perhaps Cdc42 interacts with ASK1 via an adaptor molecule which then recruits the necessary components required for the activation of ASK1.

How does NGF deprivation lead to the activation of the JNK pathway and apoptosis in sympathetic neurons? There are two possible mechanisms: (A) An "active" mechanism mediated via a death receptor, such as the p75NTR, and (B) a "passive" mechanism in which the lack NGF is able to "derepress" the death pathway.

(A) Members of the Rho subfamily of GTPases have been shown to play crucial roles in linking cell surface receptors to MAPK cascades [for review see (Vojtek and Cooper, 1995)]. One possibility is that Cdc42 binds p75NTR and NGF binding would abolish Cdc42 activation in a manner analogous to what happens to RhoA (Yamashita *et al.*, 1999). Cdc42 activation would then lead to the activation of ASK1/MLK3 and the JNK pathway as discussed above.

In addition, ASK1 may also be directly activated by p75NTR, as activation of ASK1 can be triggered upon stimulation of other death receptors. For instance, following Fas receptor stimulation, the adaptor protein Daxx displaces the inhibitory intramolecular interaction of ASK1, thereby causing ASK1 to open up into an active conformation (Chang *et al.*, 1998). In addition, TNF receptor stimulation can generate sufficient reactive oxygen species that induce the dissociation of ASK1's direct inhibitor, the anti-oxidant thioredoxin, leading to ASK1 activation (Saitoh *et al.*, 1998; Liu *et al.*, 2000). TNF receptor stimulation has also been shown to activate ASK1 (Ichijo *et al.*, 1997), and it has been demonstrated that ASK1 activation by TNF receptor requires the adaptor protein TRAF2 (Nishitoh *et al.*, 1998). Of relevant interest is the observation that TRAF2 binds to p75NTR in the absence of ligand and induces an increased apoptotic effect (Ye *et al.*, 1999). It is therefore possible that the TRAF2-p75NTR interaction leads

to the activation of ASK1 and the JNK pathway and consequent neuronal cell death.

(B) NGF promotes survival by binding to TrkA (Kaplan *et al.*, 1991a; Kaplan *et al.*, 1991b) and this event stimulates the activity of Ras (Segal and Greenberg, 1996; Kaplan and Miller, 1997), which is required for the survival of rat sympathetic neurons (Nobes and Tolkovsky, 1995; Nobes *et al.*, 1996; Markus *et al.*, 1997). Recently, it has been demonstrated that one way by which Ras exerts its survival effects in SCG neurons is by suppressing the level of p53 (Mazzoni *et al.*, 1999). Interestingly, a recent report has demonstrated that overexpression of p53 induces apoptosis through an increase in the levels of Cdc42, which is followed by a cytoplasm to plasma membrane translocation leading to the activation of Cdc42 (Thomas *et al.*, 2000). Therefore, the absence of NGF leading to the activation of the p53-mediated cell death pathway could be another mechanism resulting in the activation of Cdc42. However, in sympathetic neurons, p53 has been shown to be downstream of JNK (Aloyz *et al.*, 1998) and therefore p53-induction of *cdc42* may provide a positive feedback amplification mechanism for the apoptotic cascade rather than transducing the initial apoptotic signal.

The PI3K/Akt kinases have been shown to be important for the survival of sympathetic neurons (Philpott *et al.*, 1997; Crowder and Freeman, 1998). Recently Rac1 has been found to be phosphorylated by Akt at serine 71 within the Akt consensus motif, resulting in a change in its GTP-binding activity (Kwon *et al.*, 2000). Putative Akt phosphorylation consensus sequences have been found in all known Rho-like GTPases (Kwon *et al.*, 2000) and it is therefore likely that the activity of other GTPases such as Cdc42 may also be under the control of Akt and its survival pathway.

A recent study has also demonstrated that ASK1 is phosphorylated on serine 83 by Akt and this correlated with a decrease in ASK1 kinase activity (Kim *et al.*, 2001). Perhaps withdrawal of NGF leads to a change in Akt kinase activity resulting in the activation of Cdc42 and ASK1 and induction of apoptosis in sympathetic neurons. In contrast to Cdc42 and ASK1, MLK3 does not contain an Akt phosphorylation site and there are no reports to date suggesting that MLK3 is under the direct control of a survival pathway.

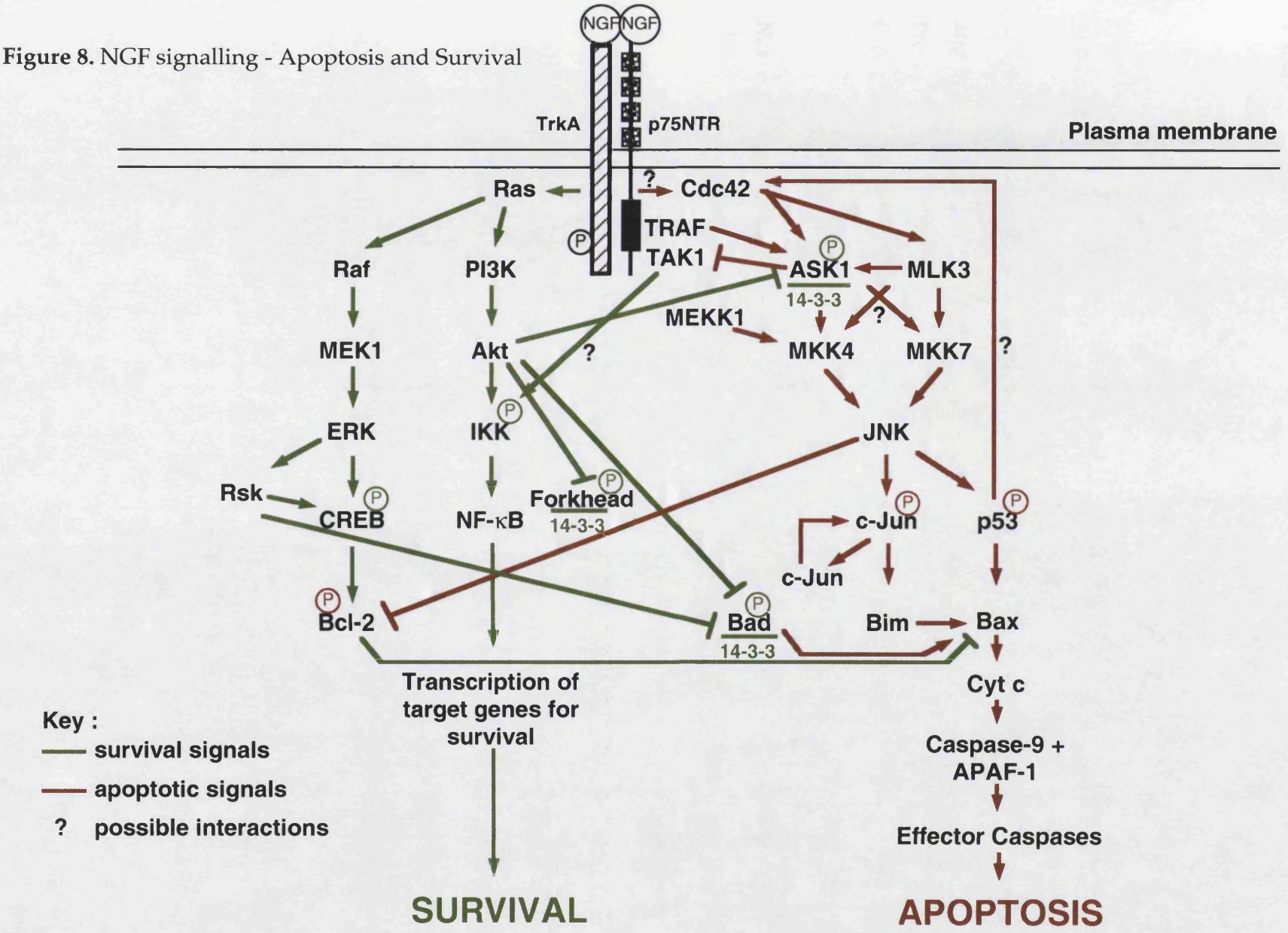
Recent publications have contributed to a clearer understanding of ASK1 regulation which may shed some light on how ASK1 may be "passively" activated following NGF withdrawal. It has been shown that inhibition of ASK1-induced cell death by the adaptor molecule 14-3-3, involves phosphorylation of ASK1 on serine 967 (Zhang *et al.*, 1999). This suggests that ASK1 may be under the control of an additional signal transduction pathway responsive to cell survival stimuli.

It is becoming more and more evident that the regulation/control of cell survival, death or cellular homeostasis is much more complex than it was initially thought. For instance, the knowledge of the level of complexity involved in activation of JNK by Rho-like GTPases has increased with the identification of two MAPKKs (SEK1/MKK4 and MKK7) (Lin *et al.*, 1995; Tournier *et al.*, 1999), many MAPKKKs (five MLKs, ASK1, TAK1 and Tpl-2) (Dorow *et al.*, 1993; Patriotis *et al.*, 1993; Gallo *et al.*, 1994; Hirose *et al.*, 1994; Holzman *et al.*, 1994; Katoh *et al.*, 1995; Ichijo *et al.*, 1997; Sakuma *et al.*, 1997), several MAPKKKKs (PAKs, GCK and HPK1) (Manser *et al.*, 1994; Pombo *et al.*, 1995; Hu *et al.*, 1996) and four scaffold proteins (JIP1-3 and b-arrestin 2) (Dickens *et al.*, 1997; Whitmarsh *et al.*, 1998; Ito *et al.*, 1999; Yasuda *et al.*, 1999; McDonald *et al.*, 2000). In addition, the interplay between survival and death pathways is crucial in deciding the fate of a cell. Figure 8 shows a simplified scheme of some of the current knowledge on how NGF signalling pathways may be regulated.

Finally, what is the physiological significance of the findings presented in this thesis in the context of neuronal apoptosis? It was demonstrated that inhibiting either ASK1 or MLK3 is sufficient to prevent death of rat sympathetic neurons. Would the same hold true in an *in vivo* disease model? The target of the Cephalon compound CEP-1347, a known inhibitor of the JNK pathway, has been recently identified as the MLKs, in particular MLK3 (Maroney *et al.*, 1999a). This compound blocks apoptosis of a number of neuronal cultures including sympathetic neurons (Maroney *et al.*, 1999b), motoneurons (Maroney *et al.*, 1998) and cholinergic neurons (Saporito *et al.*, 1998), among others. More importantly, CEP-1347 was equally successful in preventing neuronal cell death *in vivo*. Indeed, it reduced degeneration of motoneurons in an *in vivo* model of motor neuronal death (Glicksman *et al.*, 1998); rescued auditory hair cells and neurons

from noise-induced hearing loss in guinea pigs (Pirvola *et al.*, 2000); and protected dopaminergic neurons from MPTP-induced nigrostriatal degeneration (Saporito *et al.*, 2000). These studies suggest that inhibiting MLK3 might have beneficial effects in treating neurodegenerative diseases. It is therefore tempting to speculate that perhaps the same holds true for Cdc42 or ASK1. The strong correlation between the functionality of certain proteins in both *in vitro* and *in vivo* systems suggests that the study of neuronal apoptosis in primary neuronal cultures can be of high importance and relevance for the identification and/or validation of new targets for the treatment of neurodegenerative diseases.

Figure 8. NGF signalling - Apoptosis and Survival



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Bazenet, C.E., Mota, M.A. and Rubin, L.L. (1998) The small GTP-binding protein Cdc42 is required for nerve growth factor withdrawal-induced neuronal death. *Proc Natl Acad Sci U S A*, **95**, 3984-3989.

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