Characterisation of ERK Distribution and Activity in Rat Pheochromocytoma Cells

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Matthew Edgar MacCormick
Institute of Molecular Biosciences
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ABSTRACT

Nerve growth factor (NGF) binds to the NGF receptor, TrkA, at the tips of nerve cell axons, sending a signal that prevents programmed cell death and causes survival, growth, and differentiation of the nerve cell. Both NGF and TrkA have been demonstrated to be retrogradely transported from axon tips to nerve cell bodies, however the mechanism of this transport, and its function, is strongly debated. Using a recently developed cell fractionation protocol in conjunction with *in vitro* reactions using an ATP regenerating system, our lab has isolated small vesicles containing NGF bound to activated TrkA. These vesicles may provide a vehicle for retrograde transport of the NGF signal and initiation of signal transduction in the cell body.

ERK1 is a serine/threonine kinase that is activated by NGF-activated TrkA. Prolonged ERK1 activity is characteristic of cells stimulated by NGF. The purpose of the experiments in this thesis was to characterise the intracellular distribution and activity of ERK1 before and after NGF stimulation, in rat pheochromocytoma (PC12) cells, which are a good model for nerve cells. We have found that ERK1 activity is redistributed between cell compartments after NGF stimulation of PC12 cells. ERK1 activity increased in sedimentable fractions that emerged from mechanically permeabilised cells after NGF treatment and *in vitro* reactions with ATP. Importantly, the results from glycerol velocity gradient experiments showed that ERK1 was not associated with membranes. Instead ERK1 was found in a rapidly sedimenting particle whose sedimentation was not affected by detergent solubilisation. These results suggest that ERK1 is recruited into a protein complex, after activation, which may be an important step in signal transduction. Formation of this complex is likely to be downstream of signalling vesicles containing NGF bound TrkA.

LIST OF ABBREVIATIONS

AMP	Adenosine monophosphate
APS	Ammonium persulphate
ATP	Adenosine triphosphate
BB	Bud buffer
BDNF	Brain-Derived Neurotrophic Factor
BSA	Bovine serum albumin
ChAT	Choline acetyltransferase
CNS	Central nervous system
COS	Transformed African Green Monkey kidney cell line
CRE	Cyclic AMP response element
CREB	Cyclic AMP response element binding protein
ddH_2O	Double-distilled water
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECL	Enhanced chemi-luminescence
EDTA	Ethylenediamine tetraacetic acid
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
ERK	Extracellular regulated kinase
FGF	Fibroblast growth factor
GAP	GTPase activating protein
GDP	Guanosine diphosphate
GST	Glutathione S-transferase
GTP	Guanosine triphosphate
Grb2	Growth factor receptor binding protein 2
IEG	Immediate early gene
IP.	Immunoprecipitation
kDa	Kilo Dalton
LRG	Late response gene
MAPK	Mitogen activated protein kinase

MEK	MAPK or ERK Kinase
MOPS	3-(N-Morpholino)propanesulphonic acid
mRNA	messenger ribonucleic acid
m-Sos	Mammalian Son-of-Sevenless
NGF	Nerve growth factor
NIH 3T3	Contact inhibited NIH Swiss mouse embryo cell line
NT	Neurotrophin
$p75^{NTR}$	p75 neurotrophin receptor
PBS	Phosphate buffered saline
PC12	Adrenal Pheochromocytoma rat cell line
PCD	Programmed cell death
PEE	PBS with EDTA and EGTA
PGB	PBS with glucose and BSA
PI-3 kinase	Phosphatidylinositol-3' kinase
PKC	Protein kinase C
PLC-γ1	Phospholipase C gamma 1
PMSF	Phenylmethylsulphonyl fluoride
PNS	Peripheral nervous system
PtdIns	Phosphatidylinositol
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SH	Src homology
SRF	Serum response factor
SY5Y	SK-N-SH-SY5Y human neuroblastoma cell line
TCA	Trichloroacetic acid
TBS	Tris buffer saline
TEMED	N,N,N',N' tetramethyl ethylene diamine
TH	Tyrosine hydroxylase
Tris	Tris(hydroxymethyl)aminoethane
Triton X-100	t-Octylphenoxypolyethoxyethanol
Trk	Tyrosine receptor kinase
Tween 20	Polyoxyethylenesorbitan monolaurate
Tween 40	Polyoxyethylenesorbitan monopalmitate
v/v	Volume/volume
w/v	Weight/volume

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CHAPTER 1 : INTRODUCTION

1.1 OVERVIEW OF PROGRAMMED CELL DEATH

Programmed cell death (PCD) is a term used by biologists to signify a genetic command that is activated in the cells of a particular area of an organism, telling the cells to die at a set time, and in a controlled manner. It was a term first introduced to describe degeneration of intersegmental muscles of the silkmoth that occurs during metamorphosis (Lockshin and Williams, 1964; Lockshin and Williams, 1965). The best-studied example is the death of specific cells during the embryonic development of the nematode *Caenorhabditis elegans* (Sternberg, 1991). In fact, of the 1090 cells required to produce the adult, 131 undergo programmed cell death, also the same 131 cells die in every developing animal. After the cells begin to degenerate, neighbouring cells phagocytose them. This whole process, from cell birth to cell death and degradation, usually takes only one hour (Ellis *et al.*, 1991). The fate of these cells does not appear to be influenced by surrounding cells. Neither killing neighbouring cells with a laser microbeam, nor genetic mutation to prevent phagocytosis prevents cell death (Hedgecock *et al.*, 1983; Sulston and Horvitz, 1977).

PCD in the nervous system of vertebrates is just as dramatic as in *C. elegans*. Up to 85% of certain populations of developing neurons undergo PCD (Ellis *et al.*, 1991). In the chick embryo the number of neurons that die depends on the size of the target area that the neurons innervate. If a limb bud from the embryo is removed, a greater than normal number of nerve cells die; conversely, surgically grafting another limb onto the embryo causes more neurons to survive (Hollyday and Hamburger, 1976).

In mammals Nerve Growth Factor (NGF) and related polypeptides control PCD. Understanding the intracellular signals triggered by these growth factors will help elucidate the mechanism by which they control PCD in the nervous system. Ultimately the control of PCD would benefit human health. Both inhibition and stimulation of PCD can be desirable; stimulation of PCD would provide a means to inhibit neural cancers such as childhood

neuroblastoma; inhibition of PCD may prevent degeneration of neural tissue such as in old age dementia.

Programmed cell death and apoptosis are not exactly the same. The term apoptosis was first used by Kerr *et al.* (1972), to describe events occurring to cells degenerating under pathological conditions. Apoptosis was a morphological description of dying cells' distinguishing characteristics, such as condensation of the chromatin, cytoplasmic blebbing, and internucleosomal cleavage of DNA. Not all of these characteristics are necessarily shown by the various kinds of cells that undergo programmed cell death during development (Schwartz *et al.*, 1993). However, over the years the terms apoptosis and programmed cell death have tended to become synonymous.

There are many external signals that can be given to cells grown in cultures that will cause apoptosis (eg. heat stress, ultraviolet light, osmotic stress, oxidative stress); therefore, it is possible that there are also a multitude of signalling pathways triggered by these external signals.

Most of these stresses are unlikely to be present during development. There are many examples in which PCD is necessary for healthy development; for example, intersegmental muscle degeneration during insect metamorphosis, tail resorbtion in maturing amphibians, elimination of tissue between digits of the developing amniote limb, and general heart and kidney morphogenesis. PCD is also required in the developing nervous system for establishment of correct synaptic patterns and axonal pathways (Oppenheim, 1989; Oppenheim, 1991; Oppenheim *et al.*, 1992; Johnson and Deckwerth, 1993).

1.2 NGF IS IMPORTANT FOR NEURON SURVIVAL

NGF was discovered 45 years ago by Levi-Montalcini and Hamburger (Levi-Montalcini, 1987b), and has since been found to belong to a family of neurotrophins which include Brain-Derived Neurotrophic Factor (BDNF), Neurotrophin (NT) -3, NT-4, and NT-5.

The term nerve growth factor was originally used because the protein's effect on sensory and sympathetic ganglia *in vivo* and *in vitro* was to cause a marked increase in size (Cohen *et al.*, 1954). These ganglia displayed increased numbers of neurons and neurite outgrowth, and it was later discovered that the increase in neurons was due to increased survival: less neurons were undergoing apoptosis during development (Cowan, 1973).

The biological importance of NGF has been established by pre- and postnatal injection of NGF antibodies into rats and mice. Addition of NGF antibodies in the early postnatal period results in virtually complete destruction of sympathetic ganglia (Levi-Montalcini and Angeletti, 1968), while injecting mouse embryos with NGF antibodies a few days before birth causes almost complete destruction of the adrenal medulla (Aloe and Levi-Montalcini, 1979). Embryonic sensory and sympathetic ganglia, cultured *in vitro*, do not survive unless the culture medium is supplied with NGF (Thoenen and Barde, 1980). NGF is also able to rescue neurons from injury or age related degeneration, thus providing a potential therapy for neurodegenerative diseases (Gage *et al.*, 1988).

Biologically active NGF is a 26 kDa homodimer of 13 kDa polypeptides that bind non-covalently, but remain stable even at physiologically low concentrations (Bothwell and Shooter, 1977). Crystal structure studies show each 13 kDa polypeptide has four surface loop regions (McDonald *et al.*, 1991). It is thought that these loop regions provide specificity for the neurotrophins to their receptors, as they vary between the different neurotrophin family members (BDNF, NT-3, -4, and –5) (Ebendal, 1992).

1.3 NGF RECEPTORS

In the early seventies work with ^{125}I-NGF showed populations of NGF receptors with saturable NGF binding (Banerjee *et al.*, 1973; Herrup and Shooter, 1975). Several years later there were found to be two distinct populations of NGF receptors with different binding affinities. Purification of these receptors revealed proteins of 75-80 kDa and 130-140 kDa (Puma *et al.*, 1983). The 75-80 kDa receptor, now known as p75^{NTR} (p75 neurotrophin receptor), was the first to be cloned and was found to have low binding affinity (higher rate of

association and dissociation) to NGF (Chao *et al.*, 1986; Woodruff and Neet, 1986; Schechter and Bothwell, 1981). The 130-140 kDa receptor was also cloned and was discovered to be the 140 kDa product of the protooncogene *trk* (tyrosine receptor kinase), $p140^{trk}$, and has a high binding affinity (slower rate of association and dissociation) (Meakin and Shooter, 1992; Kaplan *et al.*, 1991a; Kaplan *et al.*, 1991b; Martin-Zanca *et al.*, 1990; Woodruff and Neet, 1986; Schechter and Bothwell, 1981).

Subsequent cDNA library screens found two homologues of *trk*, *trkB* (Klein *et al.*, 1989), and *trkC* (Lamballe *et al.*, 1991). Trk, (now known as TrkA), shows the strongest affinity for NGF, while TrkB is activated by Brain Derived Neurotrophic Factor (BDNF) and Neurotrophin 4/5 (NT-4/5), and TrkC by NT-3 (Berkemeier *et al.*, 1991; Lamballe *et al.*, 1991; Ip *et al.*, 1992).

1.4 THE ROLE OF $P75^{NTR}$

Originally characterised as a NGF receptor, $p75^{NTR}$ binds actually binds all of the neurotrophins with similar affinities (Kaplan and Stephens, 1994). It was initially thought that $p75^{NTR}$ functioned only in association with TrkA, but recently it has come to be regarded as a signalling receptor in its own right.

Trk function is affected by $p75^{NTR}$ in several ways. TrkA will only bind NGF when $p75^{NTR}$ is present on the cell membrane; with the deletion of the $p75^{NTR}$ genotype, TrkA will also bind NT-3, which usually only binds to TrkB and TrkC (Benedetti *et al.*, 1993). $p75^{NTR}$ also increases the binding affinity of NGF for TrkA by increasing the rate of association of NGF to TrkA (Mahadeo *et al.*, 1994). This may be achieved by increasing the local concentration of NGF around the TrkA receptors, in effect temporary storage for NGF before it is bound by TrkA (Barker and Shooter, 1994). Since competition for NGF drives selection for survival, this could aid cells expressing $p75^{NTR}$ by sequestering NGF.

The importance of $p75^{NTR}$ as a receptor has been overshadowed by TrkA, largely because it has been shown that cell mutants that express $p75^{NTR}$ but not TrkA do not respond to NGF

(Green *et al.*, 1986), whereas expression of exogenous TrkA restores NGF responsiveness (Loeb *et al.*, 1991). Also, the production of *trkA-/-* mice causes massive neuronal loss and death soon after birth, while *$p75^{NTR}$ -/-* mice show much less dramatic effects that do not result in premature death or any gross defects in the nervous system (Lee *et al.*, 1992; Lee *et al.*, 1994; Smeyne *et al.*, 1994).

More recently, evidence has accumulated showing that $p75^{NTR}$ has important functions of its own. In glioma cells that express $p75^{NTR}$ but not TrkA, NGF activation of $p75^{NTR}$ causes an increase in intracellular ceramide levels (Dobrowsky *et al.*, 1994). Ceramide is a sphingolipid metabolite that is involved in a number of cellular responses, including stress activated signalling pathways (Hannun, 1996), which can lead to apoptosis (Verheij *et al.*, 1996). PC12 (pheochromocytoma, see section 1.6) cells expressing TrkA and $p75^{NTR}$ do not respond to NGF with increased ceramide levels, but if BDNF is used as a stimulus for $p75^{NTR}$ only, ceramide levels do increase. Therefore the presence of TrkA seems to be able to inhibit ceramide activation by $p75^{NTR}$, preventing apoptosis and stimulating survival and differentiation (Dobrowsky *et al.*, 1994). $p75^{NTR}$ reinforces the effect of TrkA activation by NGF by increasing the rate of association of NGF with TrkA. Therefore it is likely that there is a critical balance in cells expressing both TrkA and $p75^{NTR}$; if there is not enough NGF to stimulate TrkA signalling pathways, apoptosis is triggered by $p75^{NTR}$-stimulated signalling pathways. This is supported by recent results from Bamji *et al.* (1998). They show BDNF activates $p75^{NTR}$ in cultured mouse sympathetic neurons, without activating TrkA, and causes apoptosis. Also, *BDNF-/-* mice retain a higher than normal number of sympathetic neurons, while *$p75^{NTR}$ -/-* mice have twice the normal number of sympathetic neurons during the first three weeks after birth (Bamji *et al.*, 1998), which is the normal period of developmental death (Hendry, 1977a). These results suggest that during development, neurons exposed to the wrong neurotrophin undergo apoptosis, and $p75^{NTR}$ plays a central role in triggering this apoptosis.

1.5 *TrkA* GENE EXPRESSION AND REGULATON IN THE NERVOUS SYSTEM

NGF is not a neurotrophic factor for all neurons, only those expressing TrkA. The identification of NGF-responsive neural populations is critical in understanding the role of NGF in the development and maintenance of these populations with their targets (other nerves, organs, and muscle). In recent years, with the identification of TrkA as the high affinity NGF receptor, it has also been found that *trkA* expression is closely correlated with NGF responsiveness in both the peripheral nervous system (PNS) and central nervous system (CNS) (Longo *et al.*, 1993).

In the PNS, *trkA* mRNA is present in trigeminal sensory ganglia, dorsal root ganglia, and paravertebral sympathetic ganglia (Grimes *et al.*, 1993). NGF has been shown to be present in the target of each of these populations, and therefore in contact with their neurites, and NGF responsiveness has been demonstrated both *in vitro* and *in vivo* (Levi-Montalcini, 1987a; Longo *et al.*, 1993). NGF binding to TrkA has also been found in muscle during motor neuron synapse formation (Raivich *et al.*, 1985).

In the CNS, NGF mRNA is found in a number of areas, (hippocampus, neocortex, olfactory bulb), all of which are innervated by basal forebrain and striatal cholinergic neurons (Longo *et al.*, 1993). In fact, injection of NGF into the hippocampus results in its retrograde transport to the innervating cholinergic neurons, suggesting the hippocampus is a supply of NGF for these neurons. This is supported by a low ratio of NGF protein to mRNA, in the hippocampus and olfactory bulb, compared with the basal forebrain (Large *et al.*, 1986). Also, production of choline acetyltransferase (ChAT) in the basal forebrain did not begin until after retrograde transport of NGF.

ChAT is an enzyme, produced by cholinergic neurons and localised to these cells, that catalyses the synthesis of acetylcholine. Intraventricular administration of NGF causes a selective increase in ChAT activity in postnatal adult rats in basal forebrain and its projection areas, and striatum (Hagg *et al.*, 1989; Mobley *et al.*, 1986; Mobley *et al.*, 1985).

Several observations suggest NGF acts via TrkA to induce its effects, and *trkA* mRNA marks neurons with functional NGF receptors. There is a strong correlation between localisation of ChAT and *trkA* mRNA, while NGF treatment increases *ChAT* mRNA to 2-4 times the normal level in septum and striatum (Holtzman *et al.*, 1992). TrkA has also been cross-linked to NGF in striatum (Ip *et al.*, 1993), and NGF rapidly induces tyrosine phosphorylation of trkA in foetal basal forebrain cultures (Knusel *et al.*, 1992).

If neurotrophins are required for neural differentiation during development, they must be produced at the correct stage and following the expression of their appropriate receptors. Davies *et al.* (1987) have studied the effects of NGF actions on mouse trigeminal sensory neurons prior to contact with their target and source of NGF, the maxillary process. They show *trkA* mRNA is present in the neurons before they contact the target, and that NGF synthesis begins around the time of contact.

Birren *et al.* (1992) have a possible answer to how TrkA expression may be initially induced during development. They used an immortalised sympathoadrenal progenitor cell line that contains little or no *trkA* mRNA and does not respond to NGF. Depolarisation of the cells induced by 40 mM KCl results in TrkA expression and NGF responsiveness. This suggests spontaneous or induced depolarisation of sympathetic neuroblasts initiates *trkA* gene expression. This supports the theory that large numbers of neural cells undergo apoptosis in the absence of depolarising stimulation by other neural cells. Thus neurons are selected both by their ability to make the right connections, (neurotrophin reversal of apoptosis), and the actual function of those connections, (depolarisation-induced reversal of apoptosis).

NGF also regulates the expression of TrkA. Li *et al.* (1995) have shown that addition of NGF to developing basal forebrain increases both *trkA* and *ChAT* mRNA *in vivo*, while anti-NGF infusions suppresses expression of both genes. NGF infusion also increases the size of developing basal forebrain cholinergic neurons, and NGF antibodies inhibit the normal developmental increase.

1.6 PC12 CELLS

PC12 cells, a cell line derived from a solid pheochromocytoma tumour of the adrenal medulla, are a produced by X-ray irradiation in New England Deconess Hospital strain white rats by Greene & Tischler (Greene and Tischler, 1976). This cell line has been used extensively to study the effects of NGF.

Previously, experiments relied on cells prepared from dorsal root or sympathetic ganglia, which require NGF for survival *in vivo* and *in vitro* (Thoenen and Barde, 1980). Current research of this type uses mouse, rat, and chick sensory and sympathetic ganglia. Survival of these dissected neurons in culture requires the continual presence of NGF; otherwise apoptosis occurs (Edwards and Tolkovsky, 1994). The advantage of dissected ganglia is that they provide a more physiological model than tumour cell lines.

However, an advantage of using PC12 cells is that they do not require the presence of NGF for survival, but they do respond to NGF in a similar way to sympathetic neurons. Also, PC12 cells and similar cell lines (NIH 3T3, SY5Y), are relatively cheap and easy to maintain, can be produced in large quantities for biochemical studies, and can be maintained indefinitely in cell culture. Although these tumour cell lines only provide a guide to the physiological responses of neurons to neurotrophins, it is important to note that most of the signalling pathways discussed below, mainly using PC12 cells as a model system, respond to NGF stimulation in a similar way in dissected sympathetic neurons (Edwards and Tolkovsky, 1994; Nobes *et al.*, 1996; Creedon *et al.*, 1996; Virdee and Tolkovsky, 1995; Riccio *et al.*, 1997; Johanson *et al.*, 1995).

PC12 cells, like sympathetic neurons, store and secrete catecholamines (dopamine and nor-epinephrine but not epinephrine) in synaptic vesicles (Schweitzer and Kelly, 1985), and both are derived from a common primitive stem cell of neural crest origin (Thoenen and Barde, 1980). PC12 cells grown in media without NGF have a near round appearance and grow in clumps. By several days exposure to NGF, cell multiplication ceases, and neuronal processes appear. The number, length, and density of processes increase over the next 14 days, and resemble sympathetic neural processes. At least 80% of cells respond to NGF and grow

neural processes. Removal of NGF from the cells causes them to retract neuronal processes and resume multiplication in 3 days (Greene and Tischler, 1976).

NGF's action on TrkA has been extensively studied in cultured cells. In terms of gene expression, there is initial down-regulation of TrkA protein on NGF stimulation in PC12 cells, but by seven days exposure to NGF there is an increase in *trkA* mRNA and matched increases in TrkA receptors. In relation to neurons, this positive regulation may help neural cells already stimulated by NGF to remain alive by increasing responsiveness to NGF (Zhou *et al.*, 1995).

1.7 ACTIVATION OF TrkA – STIMULATION OF MULTIPLE DOWNSTREAM PATHWAYS

Activation of a series of molecular events, the signal transduction cascade, starts with ligand binding to its receptor. NGF binding to TrkA causes formation of a TrkA dimer. It is thought that the TrkA monomers each phosphorylate their dimeric partner in the intracellular domain. Phosphorylated tyrosine residues serve as binding sites for other proteins allowing activation of a number of signal transduction events including a kinase cascade (Jing *et al.*, 1992). *In vitro* studies have shown that TrkA autophoshorylates at multiple sites in the cytoplasmic domain, at tyrosines 490, 670, 674, 675 and 785 (Stephens *et al.*, 1994; Cunningham *et al.*, 1997). Some of the functions of specific phosphorylated tyrosines have been determined. Phosphotyrosine 490 is required for binding and activation of Shc (oncogenic SH2 [src homology 2] domain-containing protein) (Obermeier *et al.*, 1993b). Phosphotyrosine 785 is

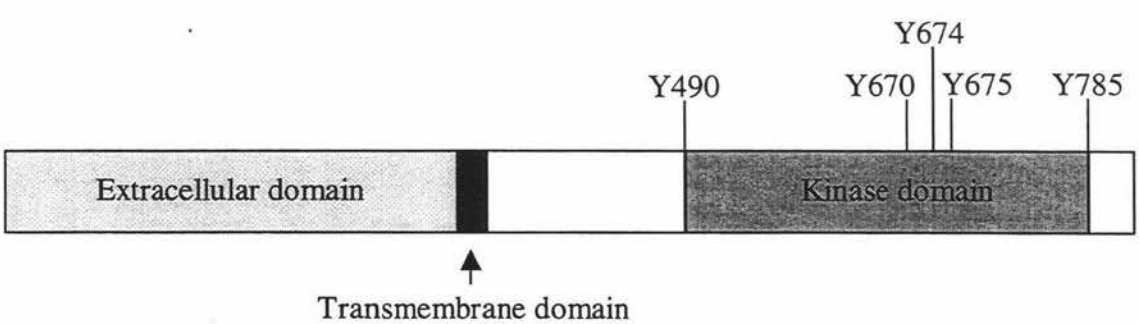


Figure 1: Tyrosine (Y) sites that become phosphorylated in response to NGF binding to TrkA.

required for binding and activation of PLC-γ1 (Phospholipase C-γ1) (Stephens *et al.*, 1994; Obermeier *et al.*, 1993a). The other three phosphotyrosine sites, 670, 674, and 675 recently have been shown to be required for autophosphorylation of p785 and hence PLC-γ1 activation. A mutation in any of these three tyrosines adversely affects general receptor autophosphorylation, suggesting these sites are required for an initial 'activation loop' to activate the dimerized receptors after NGF binding (Cunningham *et al.*, 1997).

Within minutes of NGF binding, TrkA interacts with two identified intracellular substrates, PLC-γ1, PI-3 kinase (Vetter *et al.*, 1991; Soltoff *et al.*, 1992), and possibly ERK1 (Loeb *et al.*, 1992). Each of these proteins is discussed below in the context of their specific signal transduction pathway.

1.8 PI-3 KINASE AND AKT

Phosphatidylinositol-3' kinase (PI-3-kinase) is a dimeric protein, with 85 and 110 kDa subunits, that phosphorylates phosphatidylinositol (PtdIns) lipids to create PtdIns 3,4 P_2, and PtdIns 3,4,5 P_3 (Carpenter *et al.*, 1990). These lipid second messengers are important for the downstream activation of other kinases, such as Akt, also known as protein kinase B (PKB) (see Figure 2). Phosphorylation of tyrosine 490 of the intracellular domain of TrkA is required for PI-3 kinase activation (Baxter *et al.*, 1995), suggesting PI-3 kinase binds at this site, which is also a site for Shc binding (Soltoff *et al.*, 1992). However, it is questionable whether PI-3 kinase binds directly to TrkA, as others have been unable to confirm the results of Soltoff *et al.* (1992). As PI-3 kinase has not been found to bind to Shc, it is possible there is another intermediate protein involved in its activation.

Interest in PI-3-kinase has grown since it was shown that its inhibition by specific inhibitors prevents NGF-mediated cell survival in serum starved PC12 cells (Yao and Cooper, 1995b). It is also required for insulin-like growth factor 1 promoted survival of cerebellar neurons (Dudek *et al.*, 1997). The cause of this cell survival seems to be activation of Akt by PI-3-kinase (Dudek *et al.*, 1997; Kauffmann-Zeh *et al.*, 1997). Akt activity is stimulated by PtdIns 3,4 P_2, which binds to the pleckstrin homology (PH) domain of Akt (Datta *et al.*, 1996;

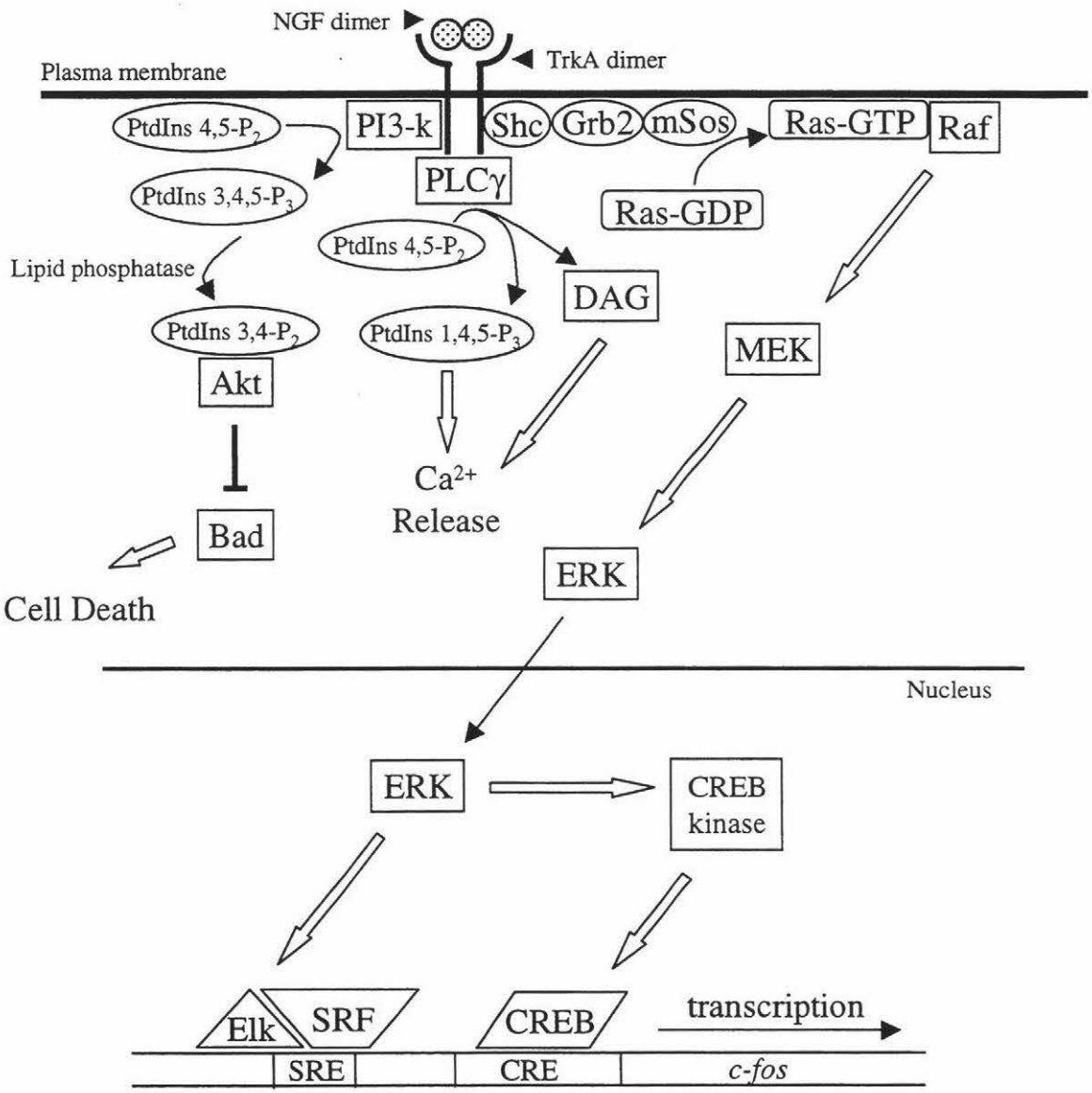


Figure 2: Multiple downstream pathways stimulated by TrkA activation.

Franke *et al.*, 1997). Recruitment of Akt to membranes causes Akt's phosphorylation and activation in a mechanism that may involve another kinase (Hemmings, 1997).

At present one downstream target of Akt, Bad, may be linked to cell survival. Bad is a member of the Bcl-2 family of proteins, a group that is central to the regulation of cell death in eukaryotes (Merry and Korsmeyer, 1997). Bad is normally thought to promote cell death by interacting with other Bcl-2 family members, but if it is phosphorylated it is sequestered in the cytosol, bound to the protein 14-3-3, leading to cell survival (Zha *et al.*, 1996). It has been shown that Akt phosphorylates Bad, blocking Bad-mediated cell death (Datta *et al.*, 1997). This suggests activation of the PI-3-kinase/Akt pathway by TrkA may be a key in the promotion of cell survival by NGF.

As yet it is not certain where PI-3-kinase and Akt are placed with regard to other receptor activated pathways. Rodriguez-Viciana *et al.* (1994), showed Ras, a GTPase involved in downstream signalling from TrkA, binds directly to the p110 subunit of PI-3-kinase, and the dominant negative Ras mutant N17 inhibits production of 3' phosphoinositides in PC12 cells. Conversely transfection of Ras into COS cells (Transformed African Green Monkey kidney cells) increases levels of these lipids. Taken together these results suggest Ras is upstream of, and regulates the activation of PI-3-kinase. However Hu et al. (1995), show dominant negative Ras blocks PI-3-kinase-induced transcription of the *fos* gene, even in the presence of of constitutively active p110, which shows high specific activity as a PI-3-kinase. This places PI-3-kinase upstream of Ras, conflicting with the results of Rodriguez-Viciana *et al.* (1994).

1.9 PLC-γ1

Phospholipase C-γ (PLC-γ) catalyses the hydrolysis of PtdIns 4,5 P_2 to diacylglycerol and Ins1,4,5 P_3. Diacylglycerol activates Protein Kinase C and Ins 1,4,5 P_3 activates release of calcium from intracellular stores (Rhee and Choi, 1992). These effects cause a change in intracellular pH, cytoskeletal rearrangements, and gene induction (Kaplan and Stephens, 1994). With the discovery of other members of this protein family PLC-γ is now called PLC-γ1.

PLC-γ1 binds directly to activated TrkA (Obermeier *et al.*, 1993a; Loeb *et al.*, 1994). Removal of the PLC-γ1 binding site on TrkA by mutation of Y785 suggests PLC-γ1 activation is not required for neuronal differentiation (Loeb *et al.*, 1994). PLC-γ1 is however, required for induction of peripherin, a neuron-specific intermediate filament protein that is induced 3-5 fold by NGF in PC12 cells (Leonard *et al.*, 1988; Loeb *et al.*, 1994). Following previous experiments, it was shown that mutation of either the PLC-γ1 binding site on TrkA, (Y785), or the Shc/PI-3-kinase binding site (Y490), does not noticeably affect neurite outgrowth in NGF stimulated PC12 cells (Stephens *et al.*, 1994). However, cells expressing TrkA with mutations in both Y490 and Y785 fail to extend neurites in response to NGF. This suggests there is some overlap and redundancy in TrkA-activated signalling pathways.

1.10 THE MAPK PATHWAY

Binding of proteins to the activated TrkA receptor activates a succession of tyrosine and serine/threonine kinases in a kinase cascade. This cascade is called the MAPK (mitogen activated protein kinase) pathway, because it can be activated by a number of mitogens (growth stimulators) in many cell types, such as insulin, epidermal growth factor (EGF), fibroblast growth factor (FGF), and NGF. TrkA activation of the MAPK pathway leads to a number of events including activation of transcription factors and translation. Recently a number of kinase cascades have been shown to be present in the same cell type, some with opposite effects, leading to the question how different kinase cascades interact in either a positive or negative manner.

1.10.1 SH2/SH3/PH Domains

The importance of binding modules (protein units that bind to other proteins) was first noted with the discovery that the Rous sarcoma virus oncogene, *src*, has two *src*-homology (SH) domains distinct from the tyrosine kinase domain (SH1), that are found in many other

proteins (Cohen *et al.*, 1995). SH2 and SH3 domains were the first to be identified (Koch *et al.*, 1991).

SH2 domains will form stable associations with tyrosine phosphorylated, but not unphosphorylated receptor tyrosine kinases (Cohen *et al.*, 1995). Three amino acids on the carboxyl side of the tyrosine residue provide specificity for binding of different SH2 domains. There are two broad categories: group 1 SH2s prefer a consensus sequence of pTyr-hydrophilic-hydrophilic-hydrophobic; group 2 SH2s prefer pTyr-hydrophobic-X-hydrophobic (Songyang *et al.*, 1993).

SH3 domains appear to have a role in localising proteins to the plasma membrane or to the cytoskeleton. For example PLC-γ binds to cytoskeletal microfilaments using the SH3 domain, and Grb2 requires both its SH3 domains, but not the SH2 domain, to localise to membrane ruffles (Bar-Sagi *et al.*, 1993). All high affinity SH3 ligands so far identified contain a PXXP motif (Cohen *et al.*, 1995).

A third motif, Pleckstrin homology domains, were recognised later (Mayer *et al.*, 1993; Haslam *et al.*, 1993). Pleckstrin homology domains consist of a region of amino acids of about 100 residues in length, and two of these domains were first identified in the Pleckstrin protein, which is the major protein kinase C substrate in platelets.

A multitude of proteins have been found to have SH2 domains and bind to growth factor receptors, including PLCγ, PI-3K, Shc, Growth Factor Receptor Binding Protein 2 (Grb2), and GTPase activating protein (GAP) (Cohen *et al.*, 1995).

1.10.2 Shc, Grb2, and Sos Bind First

An early event in NGF activation of TrkA is the association of the receptor and Shc (Stephens *et al.*, 1994). The mammalian Shc gene encodes three overlapping proteins of 46, 52, and 66 kDa, with a carboxy-terminal SH2 domain that binds to activated growth factor receptors.

These Shc proteins also become tyrosine phosphorylated when activated by growth factor receptors, including TrkA and EGFR (EGF receptor) (Pelicci *et al.*, 1992; Rozakis-Adcock *et al.*, 1992; Ohmichi *et al.*, 1994). Shc is expressed in all tissues, except in the brain, where a neural-specific form is found (N-Shc) of 52 and 64 kDa (Nakamura *et al.*, 1996). Overexpression of Shc causes neurite extension in PC12 cells, but this effect is blocked by expression of the dominant negative Ras mutant, Ras N17, indicating Shc is upstream of Ras (Rozakis-Adcock *et al.*, 1992).

After binding to TrkA, Shc is phosphorylated and then associates with Grb2 (Rozakis-Adcock *et al.*, 1992; Ohmichi *et al.*, 1994). Grb2 is an adapter protein, with an SH3-SH2-SH3 structure, that lies upstream of Ras (Clark *et al.*, 1992). Mammalian Son-of-Sevenless (m-Sos) is a guanine nucleotide exchange factor that binds to the SH3 domain of Grb2 through its C-terminal domain (Rozakis-Adcock *et al.*, 1992; Chardin *et al.*, 1993).

Shc seems to associate more strongly with TrkA than EGFR, and although EGFR can be immunoprecipitated with Shc, Grb2, and m-Sos, TrkA immunoprecipitates only with Shc, not Grb2 or m-Sos. Addition of low concentrations of a phosphopeptide Trk-Y490P, which binds to the SH2 domain of Shc, has little effect on NGF-stimulated Ras activation but does prevent association of Shc with TrkA. Higher concentrations of Trk-Y490P inhibit tyrosine phosphorylation of Shc and formation of Shc complexes with Grb2, resulting in strong inhibition of Ras activation. This suggests the association between Shc and Grb2 is more important than the association between TrkA and Shc for Ras activation (Basu *et al.*, 1994).

m-Sos is a guanine nucleotide exchange factor that stimulates GDP dissociation from Ras, allowing GTP to bind, thus forming an activated Ras-GTP complex (Johnson and Vaillancourt, 1994). m-Sos becomes phosphorylated with either EGF (Rozakis-Adcock *et al.*, 1993; Chardin *et al.*, 1993) or NGF stimulation (Suen *et al.*, 1993; Chardin *et al.*, 1993). Grb2 and m-Sos appear to be constitutively bound together. Mutations in either of the SH3 domains of Grb2 prevents m-Sos binding (Li *et al.*, 1993). There is evidence that MEK and MAPKs play an inhibitory role by phosphorylating m-Sos and reducing m-Sos activity, thus providing negative feedback (Rozakis-Adcock *et al.*, 1995; Waters *et al.*, 1995), although it is not known whether this plays a role in regulation of TrkA signal transduction pathways.

1.10.3 Ras

Ras belongs to a superfamily of small GTPases that are involved in a large variety of cell signalling pathways (Macara *et al.*, 1996). It is a 21 kDa farnesylated protein associated with the inner surface of the plasma membrane through its lipid tail (Papageorge *et al.*, 1982; Casey, 1995).

Ras is a GTP-binding protein, and its activity is regulated by bound guanine nucleotides. GDP-bound Ras is inactive, while the GTP-bound form is active. Expression of constitutively active Ras leads to the differentiation of PC12 cells (Satoh *et al.*, 1987). NGF stimulation of PC12 cells stimulates the activity of guanine nucleotide exchange and increases Ras-GTP by nearly 10 fold within two minutes (Muroya *et al.*, 1992). Ras activation, measured by exchange of GTP for GDP, is maximal at about 10 minutes (Li *et al.*, 1992). TrkA activity is critical for Ras activation, as treatment of cells with K252a, an inhibitor of Trk kinase activity, prevents Ras activation (Ng and Shooter, 1993). Ras-GAP (GTPase activating protein) activity also increases, again maximal at 10 minutes, although it is not phosphorylated nor associated with the Trk receptor (Li *et al.*, 1992). GAP is a negative regulator of Ras in PC12 cells (Yao and Cooper, 1995a), indicating NGF stimulation of Ras is tightly regulated.

The importance of Ras in NGF signalling was first noticed when oncogenic and proto-oncogenic human Ras was microinjected into PC12 cells grown in media without NGF. Oncogenic Ras caused the cells to extend neurites while proto-oncogenic Ras did not (Bar-Sagi and Feramisco, 1985). Soon after it was found that Anti-Ras antibody, injected into PC12 cells immediately before stimulation of the cells by NGF, prevented the injected cells from producing neurites. Anti-Ras antibody inhibited neurite formation and caused neurite regression up to 36 hours after NGF treatment (Hagag *et al.*, 1986). These two results suggested active Ras was critical for neuritogenesis.

The concentration of NGF affects the response of Ras. At low NGF concentrations Ras activation slowly increases with time, whereas high concentrations of NGF cause rapid activation of Ras within 5 minutes (Ng and Shooter, 1993).

1.10.4 Ras Association with Raf Leads to Raf Activation

Raf is a serine/threonine kinase consisting of two functional domains, an amino terminal regulatory domain and a carboxy terminal kinase domain. There are three identified isozymes of Raf in mammals; c-Raf-1, A-Raf-1, and B-Raf (Heidecker *et al.*, 1992), however, c-Raf-1 has been the most extensively researched (below, Raf or Raf-1 refers to c-Raf-1 unless otherwise indicated).

Early work on Ras-Raf interactions using inducible dominant negative Ras in PC12 cells (Ras N17) suggested Ras N17, induced in NGF-stimulated cells, prevented phosphorylation of Raf (Wood *et al.*, 1992). Ras and the N-terminal region of Raf-1 associate directly *in vitro* which requires GTP-bound Ras and not GDP-bound Ras (Vojtek *et al.*, 1993; Warne *et al.*, 1993). A single amino acid change in Raf-1 (Arg^{89} to Leu) is enough to prevent the interaction with Ras (Fabian *et al.*, 1994).

Raf is normally cytosolic but it has been observed to co-localise to the plasma membrane with oncogenic Ras (Traverse *et al.*, 1993), suggesting activation of Raf occurs at the plasma membrane. To confirm this, Leevers *et al.* (1994) fused the membrane localisation signal of Ras to the carboxy terminus of Raf. This membrane-localised Raf is constitutively active causing neurite outgrowth in PC12 cells, even when Ras N17 is co-expressed. This indicates that Ras targets Raf to the plasma membrane but is not required for Raf activation, or once Raf is active, as Ras was not detected in active preparations of detergent-solubilised Raf.

Recently, Wartmann *et al.* (1997) have found that Raf hyperphosphorylation coincides with a decrease in membrane-associated Raf, and inhibition of hyperphosphorylation restores the amount of membrane-bound Raf to normal. They propose a model in which mitogen-stimulated Raf is recruited by GTP-Ras to the plasma membrane, where Raf becomes

activated. The majority of Raf becomes hyperphosphorylated (perhaps by a number of different kinases), and this decreases its affinity for the plasma membrane as a consequence of activating the MAPK cascade.

The Ras binding domain, residues 51-131, has recently been found sufficient and necessary for recruiting Raf to the plasma membrane. For full activation Raf requires phosphorylated tyrosine residues 340 and 341, an intact zinc finger (defined by the cysteine rich domain residues 139-184), and activated Ras (Roy *et al.*, 1997).

Although research is proceeding quickly to investigate the mechanism of Raf activation after Ras binding, most of this work has centred on c-Raf, but it is likely that B-Raf and not c-Raf is preferentially activated in PC12 cells stimulated by NGF (Jaiswal *et al.*, 1994). B-Raf is expressed mainly in neural tissues, and is larger than c-Raf (95 kDa compared to 74 kDa) (Stephens *et al.*, 1992; Heidecker *et al.*, 1992), although in human and mouse tissues B-Raf isoforms range from approximately 69 to 99 kDa (Eychene *et al.*, 1995; Barnier *et al.*, 1995; Cabrera *et al.*, 1996).

Interestingly, B-Raf is not strongly activated by the cytoplasmic kinase Src, whereas c-Raf is. However, B-Raf is more strongly activated by oncogenic Ras than c-Raf, suggesting B-Raf may be more strongly associated with the MAPK cascade (Marais *et al.*, 1997). In addition, when c-Raf and B-Raf are co-expressed in the same cells, such as PC12 cells, both can be inhibited by cAMP, but only B-Raf is resistant to cAMP inactivation in NGF stimulated cells (Erhardt *et al.*, 1995). The activation of the two isozymes may be very similar however, as they both associate with 14-3-3 and HSP90, chaperone proteins that have been found to bind constitutively to c-Raf, and appear to play a role in Raf activation (Yamamori *et al.*, 1995; Morrison and Cutler, 1997; Jaiswal *et al.*, 1996).

1.10.5 Raf Activates MEK

The first Raf found to activate MEK (MAPK or ERK Kinase) was c-Raf-1. MEK is a threonine/tyrosine kinase of 43.5 kDa, that is phosphorylated and activated on serine residues

by Raf and forms a stable complex with Raf *in vitro* (Huang *et al.*, 1993; Wu *et al.*, 1993; Dent *et al.*, 1992; Kyriakis *et al.*, 1992; Crews *et al.*, 1992). To date six isoforms of MEK have been identified, but only MEK1 and MEK2 are involved in the Ras/ERK pathway (Robinson and Cobb, 1997). Both MEKs 1 and 2 contain proline rich sequences, which appear to confer specific activation by Raf (Catling *et al.*, 1995).

Interestingly, it has been shown that in NGF-stimulated PC12 cells MEK-1 can be co-purified with B-Raf but not c-Raf, although both are activated by Ras (Jaiswal *et al.*, 1994). As with the evidence of Marais *et al.*, (1997), this suggests B-Raf is a key transducer of the NGF signal in PC12 cells. Like Ras, MEK appears to be required for neuronal differentiation of PC12 cells; it will prevent differentiation if a dominant negative mutant is expressed in these cells (Cowley *et al.*, 1994).

1.10.6 MEK Activates ERK

Once activated MEK phosphorylates and activates ERK (Extracellular Regulated Kinase) by phosphorylating Thr-183 and Tyr-185 (Crews *et al.*, 1992; Zheng and Guan, 1993; Nakielny *et al.*, 1992). ERK is the most recent name for this kinase, but it has also been called MAP2 kinase, for its ability to phosphorylate Microtubule Associated Protein 2, and MAPK, or Mitogen Associated Protein Kinase, which is also a general term for this family of related kinases (Boulton *et al.*, 1990).

There have been six ERKs discovered so far, but only ERK1 and ERK2 have been extensively studied (Robinson and Cobb, 1997). ERKs 1 and 2 are 90% identical with molecular weights of 44 and 42 kDa respectively (Boulton *et al.*, 1991), and are predominantly found in the spinal cord and brain, as are their mRNAs. ERK3 is a 62 kDa protein kinase, 50% identical to ERKs 1 and 2, found in all tissues, and is a constitutively nuclear protein (Boulton *et al.*, 1991; Cheng *et al.*, 1996).

ERKs 1 and 2 decrease in activity if they are exposed to either CD45 (phosphotyrosine specific phosphatase) or phosphatase 2a (phosphoserine/phosphothreonine specific

phosphatase), indicating that they require phosphorylation on both tyrosine and serine/threonine residues for full activation (Anderson *et al.*, 1990; Boulton and Cobb, 1991). These enzymes undergo autophosphorylation and self-activation slowly *in vitro*, but it is unlikely this is important *in vivo* due to the presence of phosphatases (Seger *et al.*, 1991).

Following the discovery that MEK binds to and phosphorylates ERK, it was shown that activated ERK translocates to the nucleus while MEK remains in the cytoplasm (Chen *et al.*, 1992; Lenormand *et al.*, 1993; Zheng and Guan, 1994). MEK is excluded from the nucleus because it contains an N-terminal leucine rich sequence (residues 32 to 44), which acts as a nuclear export signal (Fukuda *et al.*, 1996). Serum stimulation or constitutively active mutants of Ras or MEK are sufficient to cause nuclear translocation of ERK (Fukuda *et al.*, 1997). When ERK is expressed at high levels in resting cells, it is found in the nucleus as well as the cytoplasm, but in serum stimulated cells kinase-inactive mutant ERK is also translocated to the nucleus (Lenormand *et al.*, 1993). This indicates that ERK does not have to be active to enter the nucleus. Although ERK binds to residues 1 to 32 of MEK, the nuclear export sequence of MEK is also required to prevent ERK entering the nucleus (Fukuda *et al.*, 1997). These results suggest MEK acts as a cytoplasmic anchor for ERK, and that activation of MEK releases ERK, whether ERK itself is activated or not.

To date, the major ERK of interest in NGF-stimulated cells has been ERK1, because it associates with TrkA, whereas ERK2 does not, and it is still active hours after NGF stimulation (Loeb *et al.*, 1992).

1.10.7 Prolonged Activation of ERK Leads to Differentiation

Some mitogens (growth factors and other agents that cause cell growth and multiplication), cause sustained activation of ERKs, whereas others do not. The effects of NGF and EGF on Ras and ERK1 were compared in PC12 cells (Qiu and Green, 1991). Both mitogens activate Ras and Erk 1, but for different lengths of time; Erk 1 loses activity after less than an hour of EGF treatment, but it is still active after six hours of exposure to NGF. Ras activity also remains high with prolonged NGF treatment, but it seems that NGF activates Ras in a biphasic manner. Activated Ras peaks at three minutes treatment, falls to 50% after 30

minutes, then rises again and remains at approximately 60% of peak activation after six hours of NGF treatment. In contrast, activation of Ras after EGF treatment also peaks at three minutes but drops dramatically thereafter, and is almost zero after two hours. Another difference is that B-Raf is resistant to cAMP inactivation (see section 1.10.4) in NGF- but not EGF-treated PC12 cells, although there is conflicting evidence on whether B-Raf remains active when exposed to cAMP (Peraldi *et al.*, 1995). Thus, it seems that although NGF and EGF stimulate the Ras/ERK pathway, only NGF causes prolonged Ras and ERK activity in PC12 cells, and this is associated with neuronal differentiation.

Other mitogens also show a differential Ras/ERK pathway response. Fibroblast growth factor (FGF) causes prolonged activation of the Ras/ERK pathway and neuronal differentiation (Togari *et al.*, 1985; Rydel and Greene, 1987). Insulin, like EGF, causes a mitogenic response, with transient activation of ERK and little or no nuclear translocation. However, over-expression of either the insulin or EGF receptor in PC12 cells causes prolonged MAPK activation and neuronal differentiation in response to their ligands (Dikic *et al.*, 1994; Traverse *et al.*, 1992).

These observations have lead to the theory that it is the length of time of the Ras/ERK pathway's activation, and ERK's subsequent translocation into the nucleus, that determines whether neuronal differentiation occurs (Marshall, 1995). This is not generally true for all cell types. In fibroblasts sustained ERK activation is associated with proliferation and not differentiation (Meloche *et al.*, 1992; Cowley *et al.*, 1994). Apparently sustained activation leads to gene expression that is distinct from that caused by transient activation, and the particular genes that are expressed in one cell type determines the cell's response.

1.11 GENE ACTIVATION BY NGF

The end result of the signal transduction events previously mentioned is a change in gene expression. A large number of genes are activated in response to NGF in PC12 cells, in excess of 60 have been identified (see Table 1), but little is known about the physiological action of most of them (Halegoua *et al.*, 1991).

Gene induction by NGF is generally divided into two sections, immediate early genes (IEG) and late response genes (LRG). IEGs are induced within minutes of NGF stimulation and their expression is likely to be a result of second messenger action, such as the Ras/ERK pathway. It is thought that the role of many IEGs is to produce products that induce expression of LRGs.

Production of tyrosine hydroxylase (TH) has been used as an indicator of NGF action (Thoenen *et al.*, 1971). However, in PC12 cells NGF stimulation causes a decrease in TH levels (Greene and Tischler, 1976). Other enzymes whose expression is increased with NGF stimulation are choline acetyltransferase, acetylcholinesterase, Na^+/K^+ ATPase, neuron-specific enolase, 2'-5' oligo-A synthetase, 2' phosphodiesterase and orthinine decarboxylase (Qiu *et al.*, 1991; Halegoua *et al.*, 1991). PC12 cells treated with NGF also change their electrical properties, due to expression of Ca^{++} channels and voltage dependent Na^+ channels (Garber *et al.*, 1989; Dichter *et al.*, 1977).

Immediate Early Genes	Late Response Genes
c-fos	MAPs
NGFI-A	Tyrosine hydroxylase
NGFI-B	Neurofilament-L
c-jun	Neurofilament –M
jun-B	Calcium channels
b-actin	Sodium channels
activity regulated cytoskeletal protein	Synapsin
collagenase 1	α and β tubulin
plasminogen activator inhibitor-1	Stromelysin-1
VH6/MKP-3	Acetylcholine esterase

Table 1: Selected early response and late response genes that are activated by NGF stimulation of cells.

The first of these IEGs to be discovered was *c-fos*, the proto-oncogenic form of *v-fos*, a transforming oncogene found in two murine retroviruses (Halegoua *et al.*, 1991). In PC12

cells *c-fos* transcription begins within five minutes, peaks at 15 to 30 minutes, and returns to basal levels after about one hr (Kruijer *et al.*, 1985). *c-fos* expression can be activated by multiple agents apart from NGF such as phorbol esters, calcium ionophores, and EGF, as well as second messengers like PKC, elevated cAMP, and Ras (Halegoua *et al.*, 1991; Sassone-Corsi *et al.*, 1989; Kruijer *et al.*, 1985; Milbrandt, 1986).

As well as being the first IEG, *c-fos* is also the best understood IEG activated by NGF. As *c-fos* is activated by a number of stimuli, including NGF, it is not likely to be a differentiation specific gene. However it is thought that activation of IEGs causes activation of LRGs, which are likely to be required for differentiation. For example, c-fos is thought to bind to the regulating element of the TH gene to induce TH production (Halegoua *et al.*, 1991).

The ERK pathway triggers c-fos production. After translocating to the nucleus, ERK phosphorylates and activates cyclic AMP response element binding protein (CREB) kinase, which in turn phosphorylates CREB, which can bind to three sites on the *c-fos* gene to activate transcription (Berkowitz *et al.*, 1989; Bonni *et al.*, 1995; Xing *et al.*, 1996). ERK also phosphorylates and activates the transcription factor Elk1 (Janknecht *et al.*, 1993). ERK1 and 2 are the only known activators of Elk1 (Gille *et al.*, 1995). Elk1 complexes with serum response factor (SRF) and binds to the serum response element (SRE) (Marais *et al.*, 1993), a promoter in many IEGs (Miranti *et al.*, 1995; Janknecht *et al.*, 1993). For full activation of transcription of the c-fos gene CREB, Elk1 and SRF binding is required (Bonni *et al.*, 1995).

Other IEGs include *NGFI-A, NGFI-B*. These genes, including c-fos, are thought to produce transcription factors as gene products, but are probably not differentiation-specific as they are induced by EGF as well as NGF in PC12 cells (Greenberg *et al.*, 1985; Bartel *et al.*, 1989; Wu *et al.*, 1989). Recently, four IEGs have been found to be activated by NGF and not EGF: *activity regulated cytoskeletal protein, collagenase 1, plasminogen activator inhibitor-1*, and *VH6/MKP-3* (Vician *et al.*, 1997).

Products of LRGs include neurofilament-M, neurofilament -L, acetylcholine esterase, and tyrosine hydroxylase (Halegoua *et al.*, 1991). There is also increased production of many cytoskeletal proteins involved in the production of microtubules. α- and β- tubulin, and their

mRNAs, increase about twofold during NGF induced neurite outgrowth. Production of MAPs (microtubule associated proteins) is also increased 3 to 15 fold ten days after NGF treatment (Aletta *et al.*, 1988; Brugg and Matus, 1988). One interesting LRG is the product of the Stromelysin-1 gene, a metalloproteinase, and one of the most highly induced NGF-responsive LRG products in PC12 cells (Matrisian *et al.*, 1986; Leonard *et al.*, 1987). Stromelysin-1 degrades components of the extracellular matrix associated with tissue remodelling, including axonal invasiveness (deSouza *et al.*, 1995). In PC12 cells, Stromelysin-1 mRNA levels increase with NGF- but not EGF- or PDGF-stimulation suggesting it is a differentiation-specific gene (Machida *et al.*, 1989). In fact, most of the LRGs mentioned are involved with the cytoskeleton and axon development, which is expected in differentiating cells, and holds with the theory that some IRGs lead to the activation of differentiation specific LRGs.

The above sections show that the pathways leading from NGF stimulation of TrkA are slowly being revealed, as are the subsequent nuclear events. These signal transduction pathways seem appropriate enough for ordinary cells that transfer signals a short distance across the cytoplasm from the cell membrane to the nucleus. However, the length of a nerve cell from the axon tip to the cell body can be several centimetres to a metre. This is a huge distance for a molecular signal to travel and has provided an intriguing area of research since the discovery of retrograde transport in the early seventies.

1.12 RETROGRADE TRANSPORT OF NGF

Hendry *et al.* (1974), were the first to demonstrate retrograde transport, by showing that ^{125}I-NGF injected into the anterior eye chamber or submaxillary gland of mice leads to accumulation of radioactivity in the superior cervical ganglion (Hendry *et al.*, 1974; Paravicini *et al.*, 1975). This retrograde transport of NGF takes place at a rate of about 2.5mm/hr (Hendry *et al.*, 1974), and is specific to the cells exposed to NGF (Hendry, 1977b).

These results raised questions about the purpose of this retrograde transport; it could be a degradation pathway for the eventual transport of NGF to lysosomes, or for the activation of

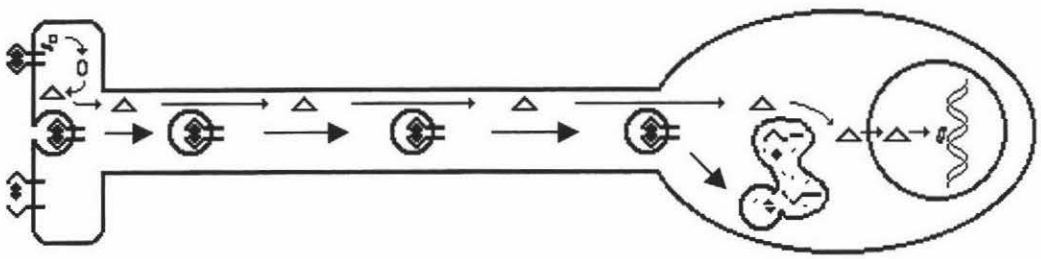
molecular pathways in the cell body. The second scenario seems more likely as tyrosine hydroxylase and dopamine-β-hydroxylase activity increased in cells receiving retrogradely transported NGF (Thoenen *et al.*, 1971).

It was initially thought that NGF might be the primary mediator for a physiological response in the cell (Marchisio *et al.*, 1980). Yet direct introduction of NGF into PC12 cells fails to cause neuronal differentiation (Heumann *et al.*, 1981), while injection of NGF antibodies into the cytoplasm does not prevent neurite outgrowth in response to NGF in the cell media (Heumann *et al.*, 1984). It has been suggested that a NGF-NGFR complex may be transported to the cell body in transport vesicles (Misko *et al.*, 1987; Thoenen and Barde, 1980; Halegoua *et al.*, 1991). This observation was supported by experiments in which axonal transport of NGF was disrupted by colchicine, a drug that interferes with microtubule assembly (Hendry *et al.*, 1974; Claude *et al.*, 1982).

Currently there are three models of retrograde transport of the NGF signal (Figure 3). One model (Figure 3a) proposes that NGF-activated TrkA triggers downstream kinases, which are then retrogradely transported down the nerve axon separately from NGF. In this model retrograde transport of NGF and TrkA is simply part of a degradation pathway and serves no further purpose. Evidence for this model, presented by (Johanson *et al.*, 1995), shows that PI3-K, ERK and MEK accumulate on the distal side of ligated rat sciatic nerve axons after NGF stimulation.

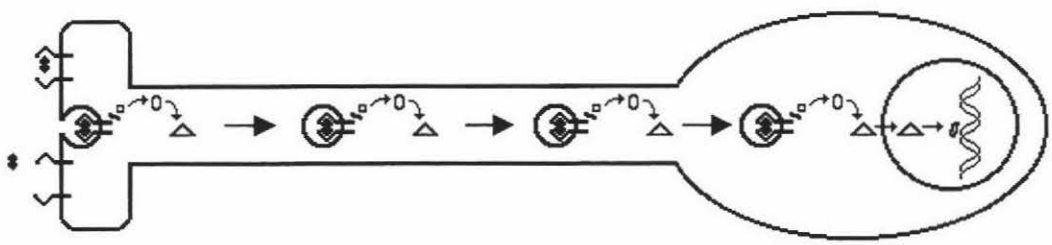
Recent data also supports the view that transport of NGF to the cell body is due to the transport of activated TrkA. TrkA retrograde transport is promoted by NGF stimulation and retrogradely transported TrkA is tyrosine phosphorylated, suggesting it is active (Ehlers *et al.*, 1995). Tyrosine phosphorylated TrkA has also been found along the length of axons and accumulates distal to ligation in rat sciatic nerves (Bhattacharyya *et al.*, 1997). Also, retrogradely transported NGF remains in the cell body with a half-life of around three hours, which is consistent with a retrograde signalling role (Ure and Campenot, 1997). It seems the mechanism for TrkA retrograde transport also requires NGF. Application of NGF to the distal terminals of rat sympathetic neurons in culture is sufficient to cause activation of CREB within the nucleus. But if NGF is physically prevented from entering nerve terminals by

3a



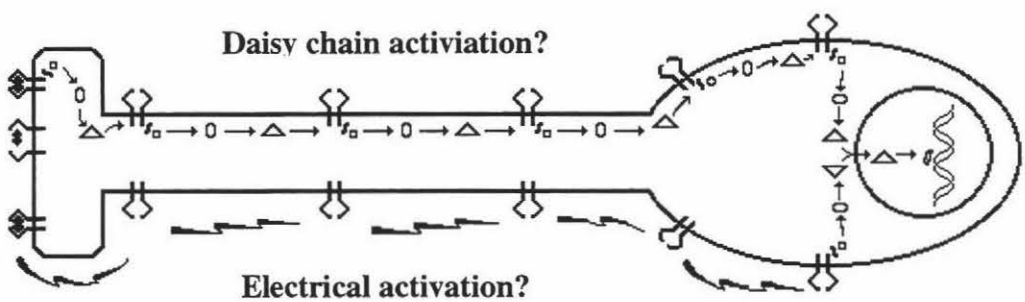
Degradation Pathway

3b



Activated Vesicle

3c



As 3b but including fast activation

Figure 3: Models for the retrograde transport of NGF activated TrkA. (a) After NGF binding to TrkA, the complex is retrogradely transported to the cell body for degradation. The NGF signal is carried to the cell body by downstream kinases. (b) Retrogradely transported signalling vesicles activate downstream signalling events in the axon and cell body. (c) Recent results indicate fast activation of TrkA in the cell body that can occur in addition to retrograde transport of NGF, TrkA, and downstream kinases. This might be through local signalling cascades causing 'daisy chain activation' of TrkA, or an electrical propagation signal.

attachment to microbeads, it is still able to activate TrkA in distal axons, but does not cause activation of CREB in cell bodies (Riccio *et al.*, 1997).

It is increasingly likely that transport of activated TrkA is via a NGF-TrkA transport vesicle. Firstly, TrkA has been shown to be down-regulated from the cell surface with exposure to NGF as would be expected to happen with endocytosis (Hosang and Shooter, 1987; Zhou *et al.*, 1995). Down-regulated TrkA and can be found in an active form in small organelles derived from clathrin-mediated endocytosis, and TrkA in these organelles is bound to NGF, tyrosine phosphorylated, and also bound to PLC-γ1 (Grimes *et al.*, 1996). *In vitro* reactions with ATP promote vesicle production, facilitating isolation and characterisation of transport vesicles containing active TrkA (Grimes *et al.*, 1997). These organelles are likely candidates for TrkA signalling vesicles – they are of a size consistent with them being transport vesicles, and contain TrkA that appears to be active and competent for signal transduction.

Thus, mounting evidence supports the second model (Figure 3b) in which NGF binds to TrkA and is internalised, forming vesicles with the extracellular domain of TrkA in the lumen of the vesicle bound to NGF. This means the intracellular domain has ready access to signal transduction proteins in the cytosol. This vesicle would then be retrogradely transported down the axon, triggering signalling pathways en route, to the cell body, where it can initiate signalling cascades that effect changes in gene expression in the nucleus.

Interestingly, there is some evidence of signal propagation considerably faster than accepted rates of vesicle retrograde transport of 2 to 5 μm/sec (Richardson and Riopelle, 1984; Ure and Campenot, 1997). Using adult male rats, Bhattaacharyya *et al.* (1997) report an increase in TrkB catalytic activity in sciatic nerves, 1 to 3 cm away from a BDNF injection site, after ten minutes. This equates to signal transduction at 16 μm/sec (it is difficult to determine whether they take into account movement of BDNF through capillaries). Senger and Campenot (1997) have studied TrkA activation in rat sympathetic neurons, with nerve terminals isolated from cell bodies by compartments in cell cultures. Their evidence suggests TrkA activation can occur ten times faster than observed by Bhattaacharyya *et al.* (1997). They report TrkA activation in cell bodies (isolated from their nerve terminals by a 1 cm deep barrier) as little as one minute after stimulation of nerve terminals with NGF. This was followed by

phosphorylation of other proteins in 5 to 15 minutes. Conversely, ^{125}I-NGF took one hour to travel the same distance. These results give rise to the possibility that some NGF-stimulated signals may occur without retrograde transport of signalling molecules; possibly by electrical propagation or a chain of TrkA molecules phosphorylating other TrkA molecules up the axon (Figure 3c). However, the results from Riccio *et al.* (1997) suggest these fast signals are insufficient to activate the transcription factor CREB in the cell body, as activation of CREB required internalisation of NGF. NGF coupled to large latex beads applied to neurite tips caused local activation of TrkA, but none in the cell body. Therefore, NGF stimulation of neurite tips is not sufficient for a full response, which appears to require retrograde transport of TrkA to the cell body.

Overall, there seem to be multiple possibilities for NGF signal transduction to the cell body from the cell surface. If results showing fast activation prove to be repeatable, it will be interesting to explore the difference between fast and slow retrograde signal transduction. In cultured neurons there is a biphasic activation of the MAPK cascade, with long term activation stabilising after about 30 minutes (Meloche *et al.*, 1992; Qiu and Green, 1991). It is possible that a putative propagation signal (Figure 3c) may provide an immediate response to NGF, while long-term activation requires extensive migration of TrkA to the cell body. This long-term activation may be achieved by sequestering TrkA into vesicles, perhaps providing an environment where the NGF-TrkA complex is protected from degradation. At this stage the most convincing body of evidence points to a signalling vesicle carrying NGF and active TrkA to the cell body as in Figure 3B.

Discovering the mechanisms of retrograde transport for NGF and other growth factors is vital for understanding neuronal development. Knowledge of how these events occur will help in devising ways for promoting neuron survival after disease or injury.

1.13 THESIS OBJECTIVES

The main aim of this thesis was to characterise a downstream signalling molecule of the NGF-TrkA signalling pathway, using a fractionation protocol developed in our lab. We chose to

study ERK1 for several reasons: it is part of the best-characterised signalling pathway (the Ras/ERK pathway); it has been shown to be important for NGF-initiated differentiation in PC12 cells; and anti-ERK1 antibodies are available comercially, making it an easy protein to identify.

Several techniques were used to characterise NGF-stimulated ERK1 activity. Western blotting with anti-ERK antibodies was used to identify the distribution of ERK1 and 2 between cell fractions, using our fractionation protocol. In-gel kinase activity assays were performed with myelin basic protein as a substrate, to measure the activity of ERK and other serine/threonine kinases. ERK activity was also analysed with a non-radioactive MAPK assay kit purchased from New England Biolabs. Lastly, glycerol gradients were performed on some cell fractions to characterise the distribution and activity of ERK1 in these fractions.

CHAPTER 2 : MATERIALS AND METHODS

2.1 CHEMICALS AND SOLUTIONS

Almost all general chemicals were purchased from Sigma Chemical Company, St. Louis, MO. Non-radioactive MAPK reagents were purchased, including anti-phospho-ERK and anti-phospho-Elk1 antibodies, from New England Biolabs Inc. Beverly, MA. Anti-ERK antibodies were purchased from Upstate Biotechnology Inc. Lake Placid, NY, and Santa Cruz Biotechnology Inc., Santa Cruz, CA. Solutions were made using ddH_2O unless otherwise stated.

2.2 CELL CULTURE

PC12 cells were obtained by Dr. M. Grimes (Massey University, NZ) from Lloyd Greene (Columbia University, NY). The cells were grown in RPMI 1640 medium (Sigma) containing 10% horse serum (Gibco BRL), and 5% Foetal Calf Serum (Gibco BRL), with 5% CO_2 at 37°C. Fresh media was supplied three times a week, and replaced completely when needed. Cells were harvested and used, or passed to new plates (1 onto 4) within two weeks.

For optimum growth of PC12 cells the culture plates were coated with collagen. Rat tail collagen was used for this purpose, and was prepared as follows: four or five tails (stored at -75°C) were scrubbed with Virkon solution and rinsed in 70% ethanol. Each tail was skinned in turn and the tendons removed from the tip of the tail by snapping the last two or three vertebrae and pulling free the associated tendons, which were then cut from the bone. This procedure was repeated the entire length of the tail, making sure that associated muscle tissue was removed from the collected tendons. Approximately 2 g wet weight of tendon was collected and placed in 200 mL of 0.2% acetic acid. Two mL of chloroform was added to this mixture, and then rotated for two to seven days until a creamy viscous solution formed. This was centrifuged at 3 800g for 20 minutes at 4°C; the supernatant decanted and centrifuged

again at 9 100×g for 60 minutes at 4°C. The resulting supernatant was dialysed overnight against 3 L ddH_2O. The collagen solution was stored in a sterile bottle with 0.5 mL chloroform added as needed. For coating of plates, 2 to 3 mL was diluted in 100 mL 50% ethanol, then spread, 5 mL per plate, onto 20 plates. The ethanol solution was allowed to evaporate overnight in a tissue culture hood, leaving the collagen dried onto the plates. The collagen plates were stored at 4°C.

2.3 CELL FRACTIONATION PROTOCOL

Six plates of PC12 cells containing approximately 200 to 400×10^6 cells were harvested for each fractionation experiment. After the media was removed each plate was rinsed with 10 mL of warm PBS (phosphate buffered saline: 8 g NaCl, 0.2 g/L KCL, 1.44 g/L Na_2HPO_4, 0.24 g/L $KHPO_4$, to pH 7.2 to 7.4) and then the cells were harvested in 10mL PBS (warm). The cells were then centrifuged at 100×g for three minutes, and washed in ice cold PEE (PBS, 1mM EDTA, 1mM EGTA). All further washes were ice cold. The cells were resuspended in PGB (PBS + 1.0 g/L glucose + 1.0 g/L BSA) and evenly distributed into two tubes. They were then incubated for 1 hour with or without NGF (1 nm) at 4°C. The control and NGF samples were then each resuspended in 5 mL of PGB, followed by warming to 37°C for 10 minutes. The cells were then chilled rapidly in ice-water, washed in 5 mL each of PEE and then 2.5 mL each of bud buffer, which mimics the composition of the cytosol (BB = 38 mM aspartic acid, 38 mM gluconic acid, 38 mM glutamic acid, 20 mM MOPS, 10 mM potassium bicarbonate, 0.5 mM magnesium carbonate, 1 mM EDTA, 1 mM EGTA, adjusted to pH 7.4 with KOH, glutathione was freshly added from 100× stocks to a final concentration of 5 mM), before resuspending in 0.5 mL each of BB with protease inhibitors. The protease inhibitors were added from 100× stocks (17.9 mg/mL PMSF, 0.1 mg/mL o-phenathroline, in anhydrous ethanol) and 1000× stocks (10 µg/mL pepstatin, 10 µg/mL chymostatin, 10 µg/mL leupeptin, 10 µg/mL aprotinin, in DMSO). At this stage the cells were cracked by sending them through the ball homogeniser (see next section). After cracking the cell the volume of each sample was noted. A difference greater than 10% between samples was taken into account during calculations. The control and NGF samples were then split into '+ATP' and 'no ATP' samples; sodium orthovanadate (1 mM) was immediately added to the no ATP samples to inhibit phosphatase activity. An *in vitro* reaction was performed (+ATP samples) by adding

an ATP regenerating system from 100× stocks (1 mM ATP, 8 mM creatine phosphate, 5 mg/mL [240 units/mg] creatine kinase). The +ATP samples were then warmed for 15 minutes at 37°C, quenched in ice water, and sodium orthovanadate was added to inhibit phophatases. All the samples were then fractionated (see figure 4 for overview of cetrifugation protocol), with a first spin of 1000×g for 10 minutes. The pellet was solubilised by adding 0.5 mL PEE with 1% Igepal, including sodium orthovanadate and protease inhibitors. The pellet was allowed to sit on ice for 1 hour or longer, with occasional vortexing to aid break-up of the pellet. The supernatant of the 1 000×g centrifugation (S1) was spun at 8 000×g for 35 minutes, separating out the second pellet (P2). The supernatant (S2) was spun at 100 000×g for 60 minutes after layering over a 0.4 mL 10% sucrose pad (with 20 mM MOPS, 1 mM EGTA, sodium orthovanadate, and protease inhibitors). The supernatant was removed (S3). The P2 and P3 pellets were resuspended with an 18 gauge needle (P2), or 25 gauge needle (P3), in 200 µL of 10% sucrose with 20 mM MOPS and 1 mM EGTA, including sodium orthovanadate and protease inhibitors. During the entire fractionation procedure the utmost care was taken to keep the samples chilled at all stages, to minimise the action of phosphatases, kinases, and proteases.

2.4 CELL CRACKING

Cell cracking was performed using a stainless-steel ball homogeniser (see Figure 5), obtained from the European Molecular Biology Laboratory (Heidelberg, Germany). Tungsten carbide balls were obtained from Industrial Tectonics (Ann Arbor, MI). The ball homogeniser was assembled in a ddH_2O water bath. Air bubbles were removed from individual components prior to assembly, as air trapped in the instrument impedes the passage of cells. Any remaining air bubbles were removed by passing ddH_2O through the cracker with a syringe. The cell cracker was placed on ice and left to cool. Chilled (4°C) bud buffer was passed through (the homogeniser has a capacity of less than 1 mL) followed by 1mL of bud buffer containing protease inhibitors. The cells were cracked by passing once through the homogeniser. After cracking the homogeniser was disassembled, rinsed, and reassembled before the next sample was passed through.

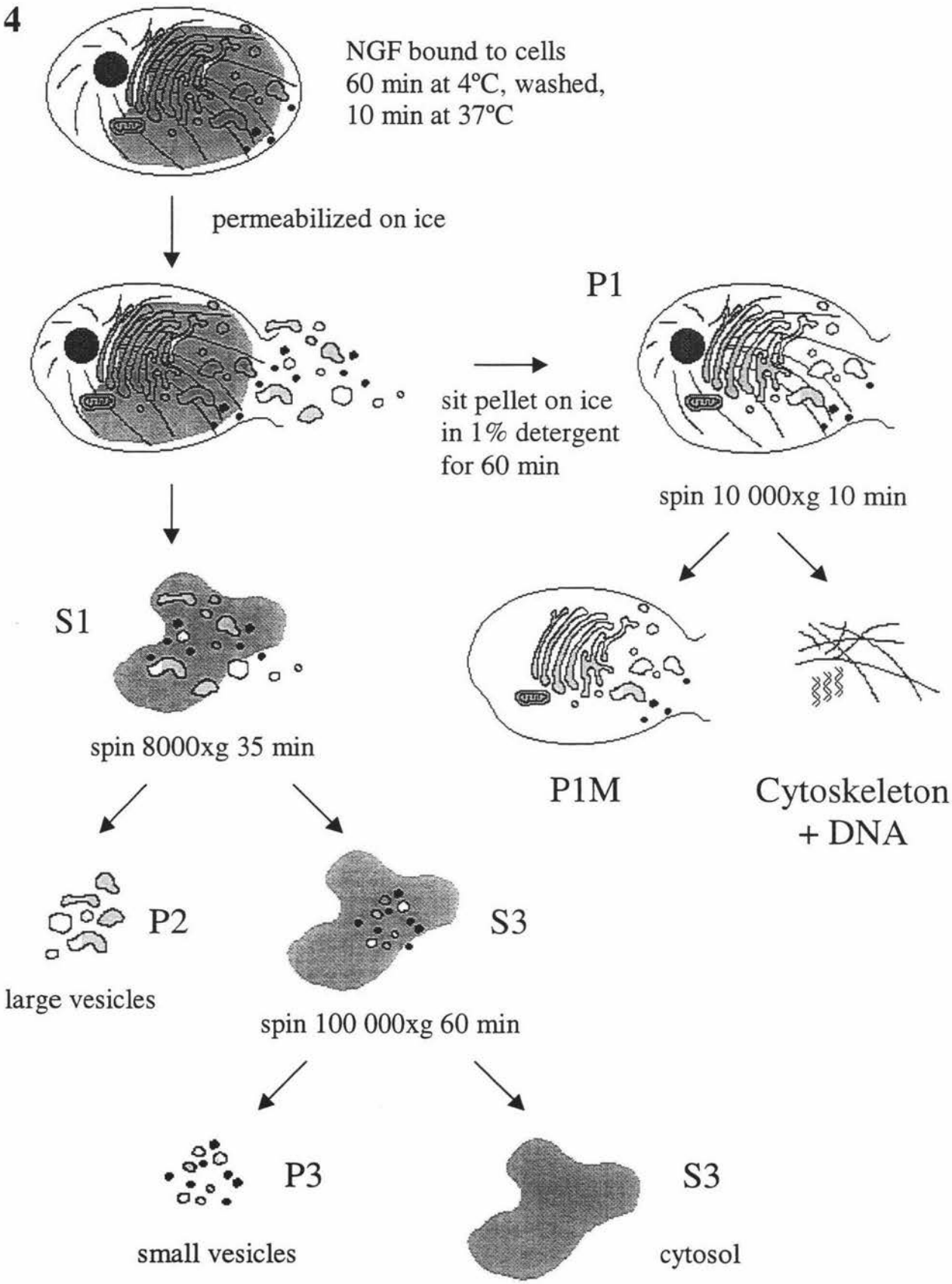


Figure 4: Overview of the cell fractionation protocol used for PC12 cells. P = pellet, S = supernatant, P1M = detergent solubilised membranes from pellet 1.

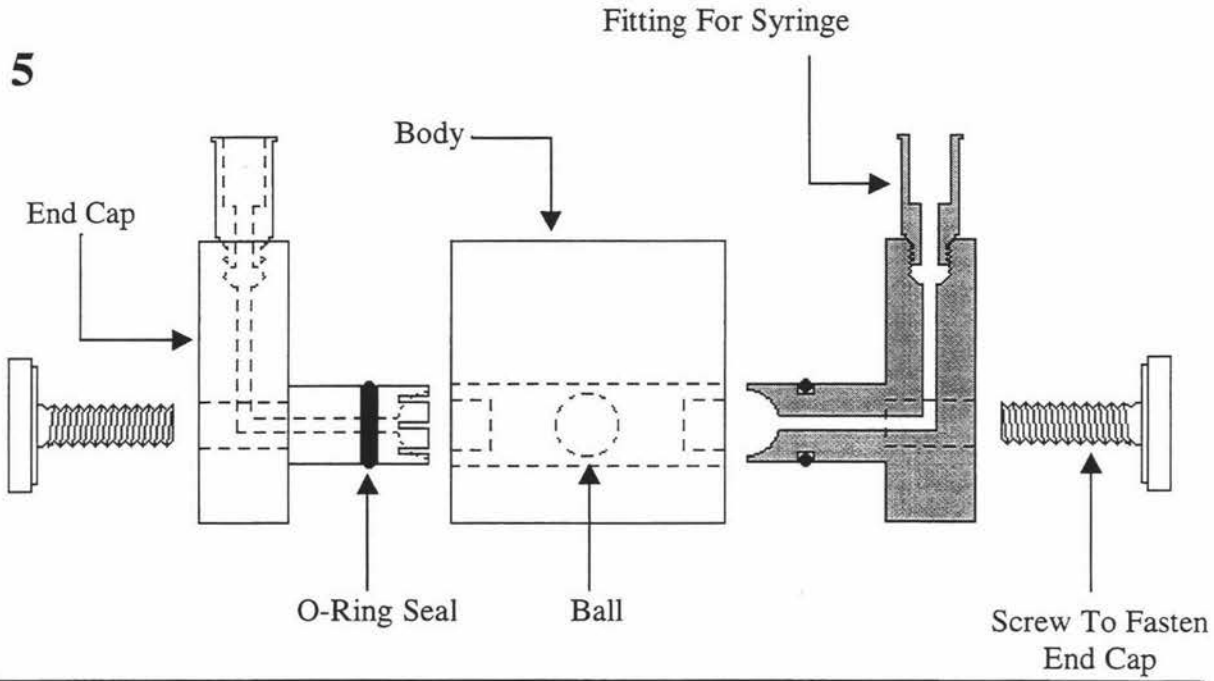


Figure 5: Diagrammatic representation of the stainless steel ball cracker. The ball and cylinder have a diameter of 8.0186 mm and 8.020 mm, respectively.

2.5 SDS-PAGE

SDS-PAGE was performed according to Laemelli (1970), with the following modifications. Stacking gels were made with 5.1% w/v (50:1) acrylamide: bisacrylamide, 0.125 M Tris HCl pH 6.95, 1 mM EDTA, 0.1% w/v SDS, 0.06% w/v APS, and 0.06% v/v TEMED. Resolving gels were made with 10-12.5% w/v (35:1 or 50:1) acrylamide: bisacrylamide, 0.375 M Tris HCl pH 8.95, 0.1% w/v SDS, 0.06% w/v APS, and 0.06% v/v TEMED. The electrophoresis buffer contained 40 mM Tris base, 0.2 M glycine, 1 mM EDTA, and 0.1% w/v SDS.

2.6 WESTERN BLOTTING WITH ERK

Samples from fractionation experiments (10-20 µL) were dissolved in 7 M urea sample buffer (7 M urea, 125 mM Tris HCl pH 6.95, 1% w/v SDS, 0.1% w/v bromophenol blue, with 100 mM DTT added to sample buffer immediately before use), heated for 15 minutes at 55°C, and run on a SDS gel. Colour molecular weight markers (Sigma, # C3312, or Amersham # RPN 756), treated identically to the samples, were also loaded on the gel. After transfer of

proteins to nitrocellulose (Optitran™ supported nitrocellulose) using an electrophoresis tank (transfer buffer: 20% v/v methanol, 150 mM glycine, 20 mM Tris base), the blot was washed in ddH_2O, and stained with Ponceau S protein stain (0.2% Ponceau S in 3.0% TCA) for 10 minutes. The blot was then rinsed several times in ddH_2O to remove excess stain, and photocopied to locate position of molecular weight markers. To prevent non-specific binding the blot was blocked for 60 minutes in TBS (tris buffered saline: 10 mM Tris, 150 mM NaCl, to pH 7.7 with HCl) with 3% w/v non-fat milk powder. After removing blocking buffer, anti-ERK primary antibody was added, at 1:2500 dilution, in blocking buffer. Anti-ERK antibody was sourced from either UBI (# 06-182) or Santa Cruz Biotecnology (#sc-93). These antibodies are specific to ERK1 and ERK2, but react more strongly with ERK1. The blot was agitated in primary antibody solution, overnight at 4°C. It was then washed twice for 5 minutes each in ddH_2O, before adding the secondary antibody (horseradish peroxidase-conjugated anti-rabbit [Amersham], 1:5000 dilution), in blocking buffer for 1.5 hours. The blot was washed twice for 5 minutes each in ddH_2O, before a final wash in TBS with 0.05% Tween 20 for 5 minutes. An enhanced chemiluminescent reaction was then performed on the blot using an Amersham ECL™ western blotting detection kit.

2.7 ECL METHOD

ECL uses horseradish peroxidase (covalently coupled to the secondary antibody) and hydrogen peroxide to catalyse the oxidation of luminol, and consequently cause light emission. This reaction occurs in the presence of chemical enhancers (such as phenols), which increase light production and the time of light emission. The light produced by ECL peaks at 5 to 20 minutes and has a half-life of approximately 60 minutes.

The protocol followed was as recommended in the Amersham ECL Western blotting kit. Excess fluid was drained from the blot. Equal volumes of ECL detection reagents 1 and 2 were added to blot which was then incubated in the solution, with gentle agitation, for 1 minute. The excess reagent was drained from the blot, which was wrapped in a plastic wrap (SaranWrap) sandwich, protein side up, and exposed to x-ray film (Fuji medical x-ray film, HR-G 30). Initial exposures of 5 minutes were taken, with subsequent exposure times dictated by the first result.

2.8 MYELIN BASIC PROTEIN IN-GEL KINASE ASSAY

This assay is based on a protocol developed by Kameshita and Fujisawa, (1989). 0.25 mg/mL MBP (Myelin Basic Protein) was added to a SDS gel. The gel was run until the 30 kDa marker was close to running off. All non-essential acrylamide was trimmed from the gel, which was then gently agitated at room temperature in 2 x 250 mL (30 minutes each) of 20% isopropanol in 50mM Tris-HCl pH 8.0. This was followed 2 x 250 mL 30 minute washes, with agitation, in buffer A (50 mM Tris-HCl pH 8.0 with 5 mM β-mercaptoethanol). The gel was then denatured in 125 mL of 6 M guanidine-HCl in buffer A, for 60 minutes with agitation. Subsequent renaturation steps were without agitation. The gel was soaked at 4°C in pre-chilled buffer A, containing 0.04% (v/v) Tween 40, as follows: 2 x 250 mL for 30 minutes each, 1 x 1L overnight, 2 x 250 mL for 30 minutes each. The gel was then shaken in reaction buffer (40 mM MOPS pH 7.2, 15 mM $MgCl_2$, 1 mM $MnCl_2$, 300 μM Na_3VO_4, 0.1 mM EGTA, 2 mM DTT) for 30 minutes before removing the reaction buffer and adding 10 to 15 mL of the same buffer containing 25 μM ATP and 50 to 75 μCi/10 mL of γ^{32}P-ATP (Amersham Life Science). This was incubated for 60 minutes at room temperature with gentle agitation. Background radioactivity was removed by extensive washing with 5% TCA, 1% Pyrophosphate. When the background counts reached almost zero the gel was dried and exposed to x-ray film for 8 hours to 3 weeks.

2.9 IMMUNPRECIPITATION

For immunoprecipitation of ERK, 100 μL of sample was added to immunoprecipitation solution containing 3 μg anti-ERK antibody (Santa Cruz), 200 μL 5× deoxycholate buffer (5× = 5% BSA, 5% Igepal, 1.25% deoxycholate, 0.75 M NaCl, 5 mM sodium orthovanadate, 5 mM NaF, 5 mM EGTA, and 250 mM Tris.HCl pH 7.4), 100× and 1000× protease inhibitors, and 660 μL ddH_2O to make up a final volume of 1 mL. The immunoprecipitation solution was mixed with the sample and left overnight at 4°C. 20 μL 50% washed protein A beads (Pierce) were added and the sample rotated for 2.5 hours at 4°C. The protein A beads

were pelleted (1000×g, 3minutes) and the supernatant discarded. The pellet was washed twice in 500μL 1× immunoprecipitation buffer and once in 0.1× immunoprecipitation buffer. The final spin was 5000×g for 3 minutes. Urea sample buffer was added (with 100 mM DTT), vortexed, heated for 15 minutes at 55°C, and the sample spun at 10 000×g for 5 minutes to pellet the beads. The sample was loaded on the gel, and the pellet containing the protein A beads was discarded.

2.10 NON-RADIOACTIVE MAPK ASSAY

This method was modified from that recommended in the New England Biolabs MAPK assay kit (# 9800). 100 μL of each sample from a previous cell fractionation was used. To this 100 μL of lysis buffer with protease inhibitors was added, followed by 2 μg of anti-phospho-MAPK antibody (1:100 dilution.). The samples were vortexed, and allowed to sit overnight at 4°C. 20 μL of 50% protein-A sepharose beads were added, and then rotated for 2½ hours at 4°C. The samples were spun at 1000×g for 3 minutes to pellet the protein-A sepharose beads with attached antibody-protein complex. The supernatants were discarded and the sample pellets were washed twice with 500 μL each with lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM sodium orthovanadate, 1 μg/mL leupeptin, protease inhibitors) and then twice with 500 μL kinase buffer (25 mM Tris pH 7.5, 5 mM β-glycerolphosphate, 2 mM DTT, 0.1 mM sodium orthovanadate, 10 mM $MgCl_2$). Samples were kept on ice during handling. The pellets were re-suspended in 50 μL of kinase buffer containing 100 μM ATP and 1 μg Elk1 fusion protein (GST fused to Elk1 codons 307-428). The samples and reaction mixture were then incubated for 30 minutes at 30°C after which the reaction was ended by adding 25 μL 3× SDS sample buffer (1× SDS sample buffer: 62.5 mM Tris-HCl pH 6.8, 2% w/v SDS, 10% v/v glycerol, 50 mM DTT, 0.1% w/v bromphenol blue). The samples were boiled for 5 minutes, vortexed, and spun at 10 000×g for 10 minutes to pellet the protein-A sepharose beads, followed by sample loading on a SDS-PAGE gel. After protein transfer to nitrocellulose, the blot was washed with TBS for 5 minutes, and then blocked for 2 hours with blocking buffer (TBS, 0.1% Tween 20, 5% w/v non-fat milk powder). The blot was then incubated with anti-phospho-Elk1 antibody (1:1000 dilution), in antibody buffer containing TBS with 0.05% Tween 20 and 5% BSA, and agitated overnight at

4°C. Following this were three 5 minute washes in TBS with 0.1% Tween 20, after which secondary antibody (horseradish peroxidase conjugated anti-rabbit,1:2000 dilution in antibody buffer) was added for 60 minutes. The blot was washed three times for 5 minutes each in TBS with 0.1% Tween 20, followed by the ECL reaction and film exposure.

2.11 GLYCEROL GRADIENTS

Cell fractionation was as in section 2.3, except that 0.4 mL 5% glycerol was used in place of the 0.4 mL 10% sucrose pad (for the S2 spin), and 5% glycerol replaced the 10% sucrose use to dissolve the P2 and P3 pellets. After fractionation, the +ATP P3 fractions (100 µL) were immediately layered over glycerol gradients. Where indicated detergent was added; samples were incubated on ice for 60 minutes with 0.4% Igepal before layering over gradients. Glycerol gradients for each sample were made with starting solutions of 2.4 mL 5% and 2.2 mL 25% glycerol in BB with sodium orthovanadate and protease inhibitors, with a 0.4 mL 60% sucrose pad, using a Hoefer Scientific SG-5 gradient maker. Gradient samples were centrifuged at 200 000×g for 1 to 2 hours (note that halving the spin time did not change the sedimentation of the ERK particle). Approximately 200 µL gradient fractions were collected from the bottom of the tube. 750 µL ddH_2O and 100 µL TCA was added to each fraction and left overnight (4°C) to precipitate protein. Samples were centrifuged at 15 000×g for 15 minutes and supernatant discarded, followed by a wash in cold acetone. The samples were spun at 12 000×g. Acetone was removed and the precipitates were air dried at room temperature to remove all traces of acetone. 7 M urea sample buffer was added and samples were heated to 55°C for 15 minutes before loading on SDS gels.

2.12 STRIP AND REPROBE WITH SYNAPTOPHYSIN

Blots probed with anti-ERK antibodies were stripped using TBS pH 2.0, for 8 minutes. They were then rinsed three times in ddH_2O, followed by one rinse in TBS pH 8.0 for 10 minutes. The blots were then blocked with TBS pH 7.4, containing 0.05% Tween 20 and 5% non-fat milk powder, for 60 minutes. The blots were washed with anti-synaptophysin antibodies

(Boehringer Mannheim, # 902322), 1:500 dilution, in blocking buffer for 60 minutes, after which they were rinsed three times in TBS pH 7.4 with 0.05% Tween 20 for 5 minutes each. Secondary antibodies were added (horseradish peroxidase conjugated anti-mouse, 1:5000 dilution), for 60 minutes, followed by three washes in TBS pH 7.4 with 0.05% Tween 20, and one wash in TBS pH 7.4, before ECL using the Amersham western blotting detection kit for antibody detection.

2.13 NIH IMAGE

NIH Image is an image processing and analysis program for the Macintosh that can be downloaded at 'http://rsb.info.nih.gov/nih-image/download.html'. The figures of x-ray film in this thesis have been digitally scanned and processed to remove background by subtracting the image of a blank film. This removes lighting variations from the lightbox and camera that would otherwise interfere with the densitometry measurements. The only other alteration was to improve the image contrast, which makes the scan easier to see when printed but does not affect the densitometric analysis. The analysis works by measuring the greyscale of pixels in a defined area (a box encompassing the desired band) and creating a graph of the result. The area under the curve is computed and used as a measurement of density.

2.14 CALCULATIONS

Percentage of total ERK and percentage of total ERK activity data was calculated using raw values from NIH image scans adjusted for fraction volume. Percentage data was calculated for each set of fractions (i.e. the sum activity for P1M, P2, P3, and S3 NGF fractions equals 100%). P values were calculated using the students t-test.

CHAPTER 3 : RESULTS

3.1 ERK DISTRIBUTION IS UNEQUAL BETWEEN CELL FRACTIONS

As indicated in the introduction (section 1.10.6), previous work has shown ERK1 and 2 are found in the cytosol and the nucleus. ERK1 and 2 also accumulate on proximal and distal sides of axonal ligations in response to NGF, indicating they may be actively transported. We hypothesised ERK1 and 2 may be transported in association with signalling vesicles containing NGF and TrkA in axons and between the cell surface and the nucleus. We asked, where do ERK1 and 2 distribute in the fractionation scheme used to characterise vesicles? Although other groups have located ERK in the cytoplasm and cell nucleus, none have used our cell fractionation technique, which incorporates the use of a cell cracker. The cell cracker forces cells through a narrow gap that splits the plasma membrane but does not damage the contents of the cell. The nucleus and Golgi remain intact and attached to cracked cells ("cell ghosts"), while small organelles leak out (Grimes and Kelly, 1992a). Differential sedimentation separates large vesicles in the 8 000×g pellet (P2), and small vesicles in the 100 000×g pellet (P3) (Figure 4). The remainder of the cell membranes and organelles, minus the DNA and cytoskeleton, are found in the detergent solubilised cell ghost membranes (P1M), and the cytosol in the 100 000×g supernatant (S3, Figure 4).

We used this fractionation technique to determine the effects of NGF stimulation of PC12 cells on the location of ERK1 and 2. Before fractionation, NGF was allowed to bind to cells at 4°C for an hour, followed by 10 minutes of warming at 37°C. This allowed initiation of signal transduction and internalisation of NGF-TrkA receptor complexes. In addition, after mechanical permeabilisation, cells were subjected to an *in vitro* reaction using an ATP regenerating system. The reaction proceeded 15 minutes at 37°C in the presence of ATP, creatine phosphate, and creatine kinase. Previous work has shown that *in vitro* incubations with ATP promote vesicle budding reactions (Grimes and Kelly, 1992a). These procedures enabled us to compare events occurring just after TrkA activation with events occurring after

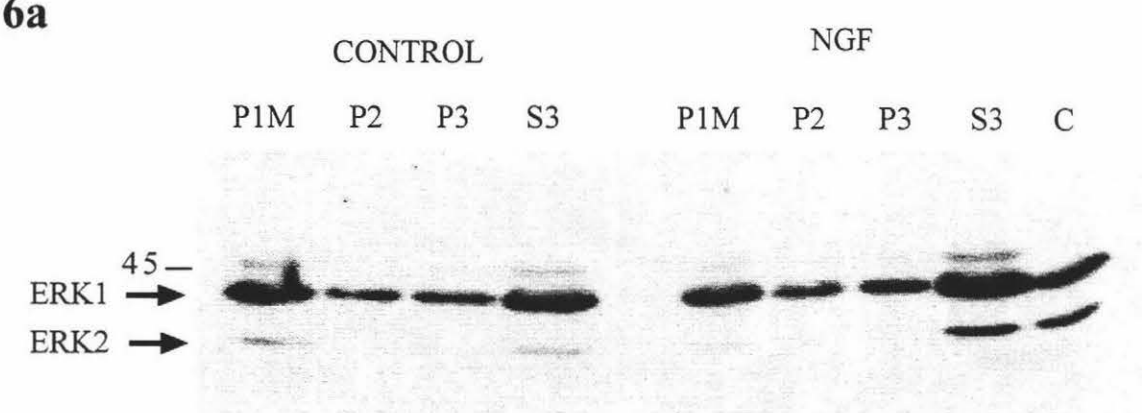
NGF-TrkA complexes were packaged into small vesicles. The focus of our attention was mainly on the 8 000×g and 100 000×g pellets, which have been shown to contain signalling vesicles associated with NGF-activated TrkA (Grimes *et al.*, 1997; Grimes *et al.*, 1996). It is important to note that not all the organelles that emerge from cracked cells have been characterised, and not all the vesicles in these fractions contain NGF-activated TrkA.

SDS-PAGE and western blotting were used to identify ERK distribution in cell fractions. Proteins in cell fractions were separated on SDS gels, then transferred to nitrocellulose, and probed with anti-ERK antibody. Secondary antibodies were subsequently bound to the anti-ERK antibodies which contained a horseradish peroxidase enzyme that catalyses a chemi-luminescent reaction when substrate is added, resulting in a band on x-ray film.

Figure 6 shows results from an experiment using anti-ERK antibodies that are specific for rat ERK1 and ERK2. A positive control provided with the antibodies is shown in the far-right lane. ERK1 (44kDa) is the higher band running just under the 45kDa marker, and ERK2 is the lower band. In both the control and NGF fractions ERK1 was found in all the fractions, mostly in the cell ghost (P1M) and cytosol (S3), but also at significant levels in the 8 000×g (P2) and 100 000×g (P3) pellets. ERK2 was detected in much smaller quantities in all the fractions compared to ERK1, and did not show up in the 8 000×g and 100 000×g pellets with this film exposure, but an overnight exposure did detect ERK2 in these pellets (Figure 6b). The experiment shown was an early one, but with refining of the fractionation technique highly reproducible results were obtained for ERK distribution. These results are discussed below.

Figure 7a shows ERK1 distribution calculated as a percentage of the total in all cell fractions. The amount of ERK1 in the samples was measured by densitometry using NIH image (see methods). Results were normalised to total ERK1 in combined P1M, P2, P3 and S3 fractions (i.e. the whole cell), for each condition (for example, one condition is 'NGF', another is 'control +ATP'). In all cases 70% or more of total ERK1 is found in the cytosol (S3) fraction. 18 to 26% was found in the cell ghost (P1M), and less than 5% was found in the 8 000×g (P2) and 100 000×g (P3) pellets combined. There was a statistically significant increase in ERK1 levels in the NGF-treated cell ghosts when compared to their controls; a 4.4% increase, ($p <$

6a



6b

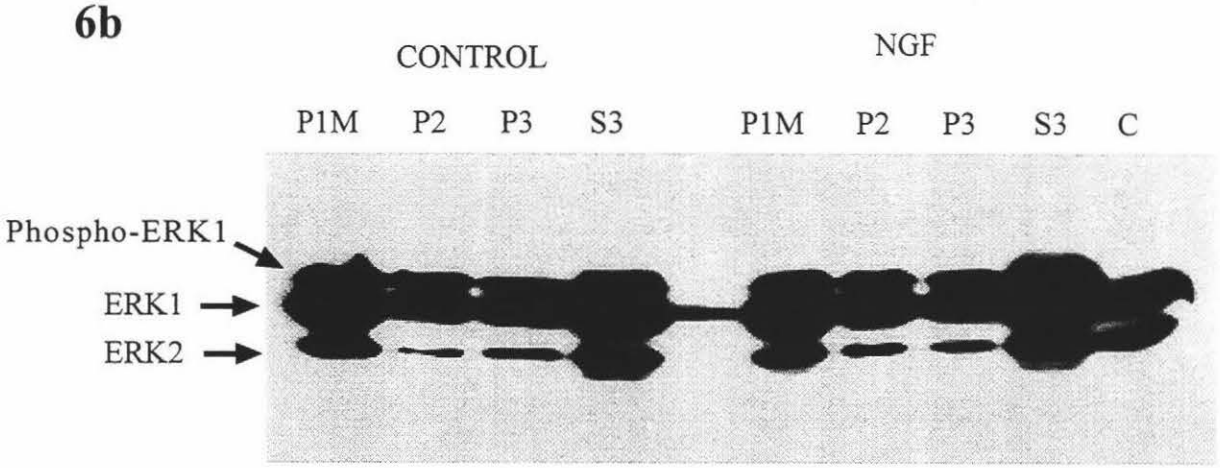
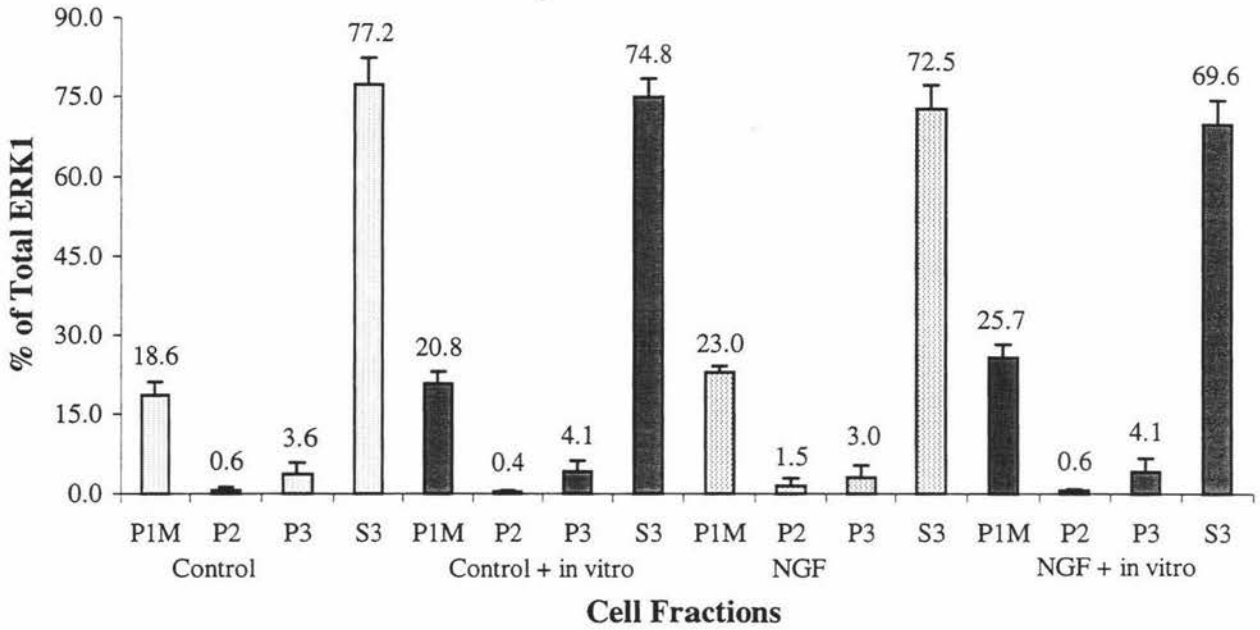


Figure 6: Identification of ERK1 and 2 in PC12 cell fractions. PC12 cells incubated with or without NGF (1 nM) at 4°C were washed, warmed 10 minutes (37°C, chilled 4°C), permeabilized and fractionated. Samples were run on a SDS gel, western blotted and probed with anti-ERK (UBI). The far-right lane shows a positive control provided with the antibodies (NIH 3T3 cell lysate). The position of the 45 kDa molecular weight marker is shown on the far left. 6a was a 5 second exposure, and 6b a 30 second exposure, showing hyperphosphorylated ERK1, and traces of ERK2 in the P2 and P3 fractions.

7a ERK1 AVERAGE DISTRIBUTION FROM WESTERN BLOTS



7b ERK2 AVERAGE DISTIBUTION FROM WESTERN BLOTS

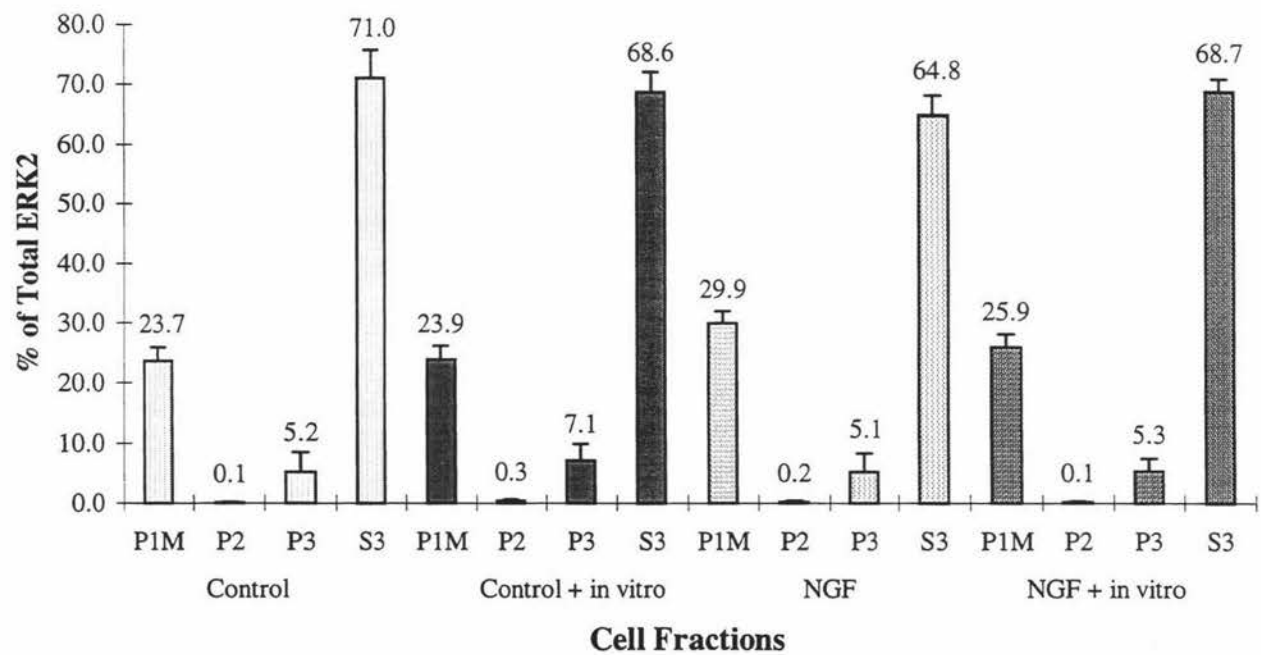


Figure 7: Averaged values for ERK distribution between cell fractions. The density of bands for ERK1 (7a) and ERK2 (7b) from western blots (such as Figures 6 and 8) were measured using NIH image, quantified, averaged, and plotted as a percentage of the total in all fractions (P1M + P2 + P3 + S3). Averages were calculated from three experiments (from samples without *in vitro* reactions) or four experiments (from samples with *in vitro* reaction) for ERK1, and from three experiments for ERK2. Figures above bars represent average values.

0.05), without *in vitro* reactions and a 4.9% increase with *in vitro* reactions. Those increases were reflected in slight decreases in their corresponding cytosolic fractions. ERK2 distribution, (Figure 7b), was similar to that of ERK1, except there were slightly higher levels of ERK2 in the cell ghosts and lower levels in the cytosolic fractions compared with ERK1. There was also a slightly higher percentage of ERK2 in the 100 000×g (P3) pellets compared with ERK1. There was significant movement of ERK2 into the NGF-treated cell ghost compared to the control, an increase of 6.2% ($p > 0.02$), but no other significant change in ERK2 distribution was noted.

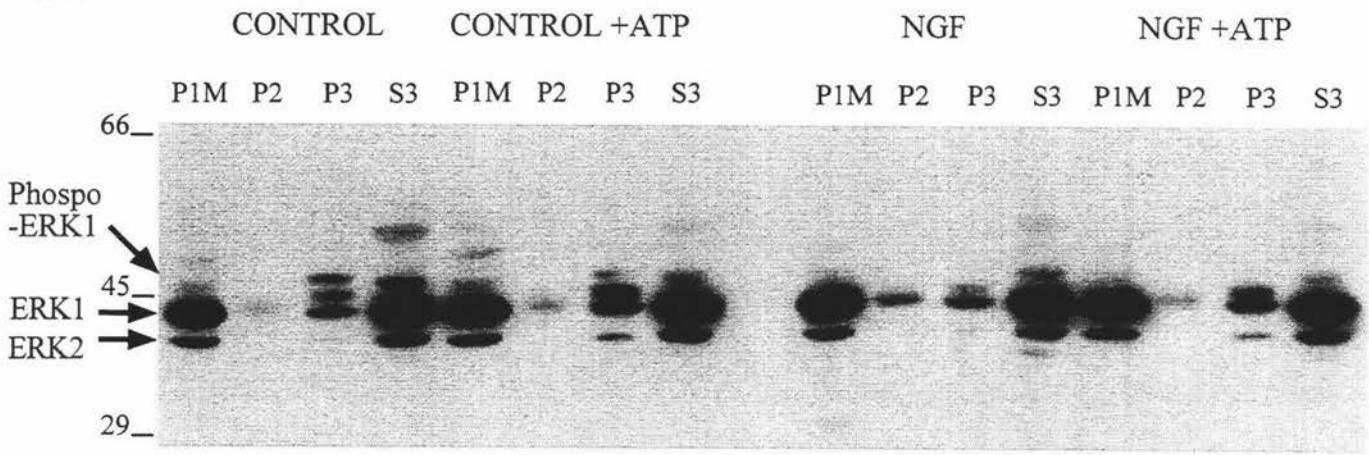
On closer examination, many of the western blots for ERK1 and 2 showed the presence of higher bands running just above ERK1 (Figure 6b). There is evidence that these higher bands correlate with phosphorylated, active ERK1, which is of slightly higher molecular weight than unphosphorylated ERK1 (Virdee and Tolkovsky, 1995; Peng *et al.*, 1996; Zhao *et al.*, 1996). Figures 8a and 8b show two experiments that included *in vitro* reactions with ATP, where higher migrating bands above ERK1 can clearly be seen. Importantly, while hyperphosphorylated ERK1 was found in some of the cell ghost and cytosolic fractions, these bands appear to be concentrated in the 100 000×g (P3) pellets. The experiment in Figure 8b shows a small amount of hyperphosphorylated ERK1 was also detected in the 8 000×g (P2) pellets.

We were interested to see if okadaic acid (a serine/threonine phosphatase inhibitor used by some researchers to prolong ERK activity) would enhance the ERK signal, or change its subcellular distribution. No major differences in distribution of ERK or phosphorylated species were observed using okadaic acid (Figure 8b). Due to the expense of okadaic acid, combined with the number of steps during the fractionation process that would require it, okadaic acid was omitted from subsequent experiments.

These results suggest that stimulation of the cells with NGF and subsequent *in vitro* reactions caused movement of ERK1 and 2 out of the cytosol (S3), and into vesicle or nuclear compartments (P1M). The level of ERKs in the P2 and P3 fractions remained constant, at least to the level of sensitivity of the technique used, suggesting there is no major redistribution of ERK1 and 2 in the 8 000×g (P2) and 100 000×g (P3) pellets. It is possible

that the increase of ERK1 and 2 in the cell ghost (P1M) is due to movement into the nucleus. Moreover, hyperphosphorylated ERK1 was detected, which appeared to be concentrated in 100 000×g (P3) pellets.

8a



8b

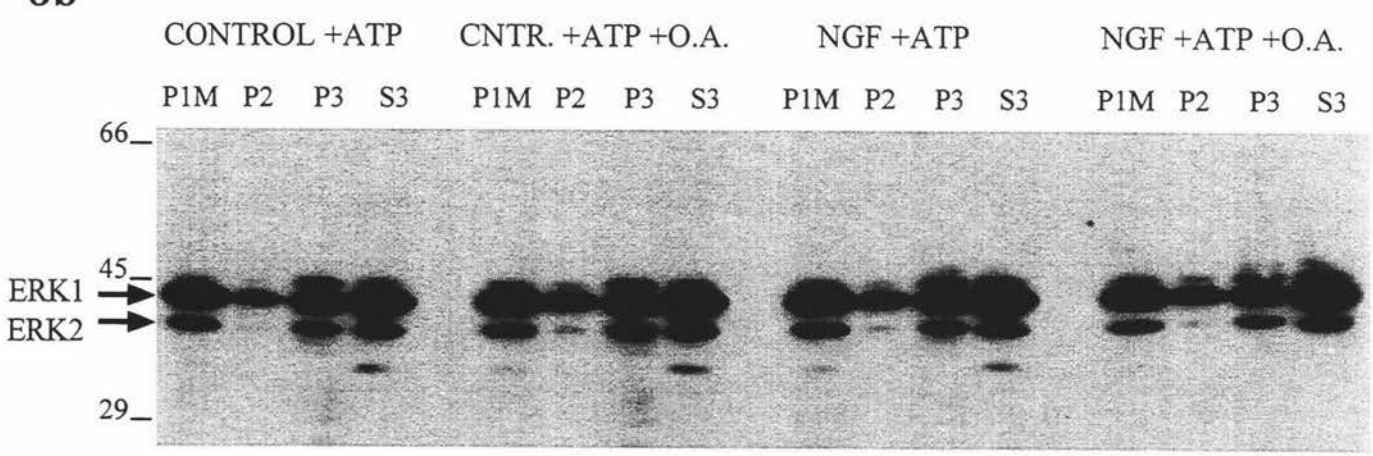


Figure 8: Identification of ERK1 and 2 in PC12 cell fractions with *in vitro* reactions. (8a) Samples were treated in the same way as in Figure 6, except after permeabilization some samples were subjected to an *in vitro* reaction with an ATP regenerating system (1 mM ATP, 8 mM creatine phosphate, 5 µg/mL creatine kinase), for 15 minutes at 37°C, before fractionation. (8b) *In vitro* reactions were performed without (control) and with (control + O.A.) 10 nM okadaic acid and processed as in (8a).

3.2 ERK ACTIVITY CAN BE MEASURED BY IN GEL KINASE ASSAYS

A possible trigger for movement of ERKs from one cell fraction to another is their activation by NGF stimulation. The purpose of the next experiment was to ask which cell fractions contained active ERKs, and to see if this correlated with the movement of total ERK seen using anti-ERK antibodies. Although anti-ERK antibodies show the distribution of ERKs between cell fractions, they do not reliably distinguish between active and inactive ERKs.

To determine ERK activity in cell fractions another method was used. Myelin Basic Protein (MBP) in-gel kinase assays have been used by other groups to show ERK activity in whole cell extracts (Seger *et al.*, 1991; Rosen *et al.*, 1994; Schwenger *et al.*, 1996). This assay measures the kinase activity of serine/threonine kinases by using MBP, incorporated into the gel, as a substrate for transfer of phosphate from γ-^{32}P-ATP. Bands corresponding to ^{32}P-phosphorylated MBP show up on x-ray film and indicate an active kinase at that position in the gel. The advantage of this method is that only active kinases will label the MBP.

Figure 9 (right), shows an MBP kinase gel run with samples from experiments similar to those above. A number of serine/threonine kinases show up in MBP kinase gels, including two bands that run at 44 and 42 kDa. Although it was likely that these corresponded to ERK1 and ERK2, it was necessary for us to positively identify these bands. Anti-ERK antibodies were used to immunoprecipitate ERK out of the cell fractions, and these samples were run on the same gel (Figure 9, left). Active ERK bands in the immunoprecipitated control +ATP and NGF +ATP P1M fractions were in the same position as the bands of the samples on the right, of approximately 43-44 kDa.

The ERK1 bands vary slightly in size between different fractions. As indicated above, we hypothesise the slower migrating bands represent phosphorylated or hyper-phosphorylated, ERK1. These bands are found in the P2 and P3 fractions of all the samples, again suggesting, as with the western blots, that the 8 000×g and 100 000×g pellets contain a pool of active

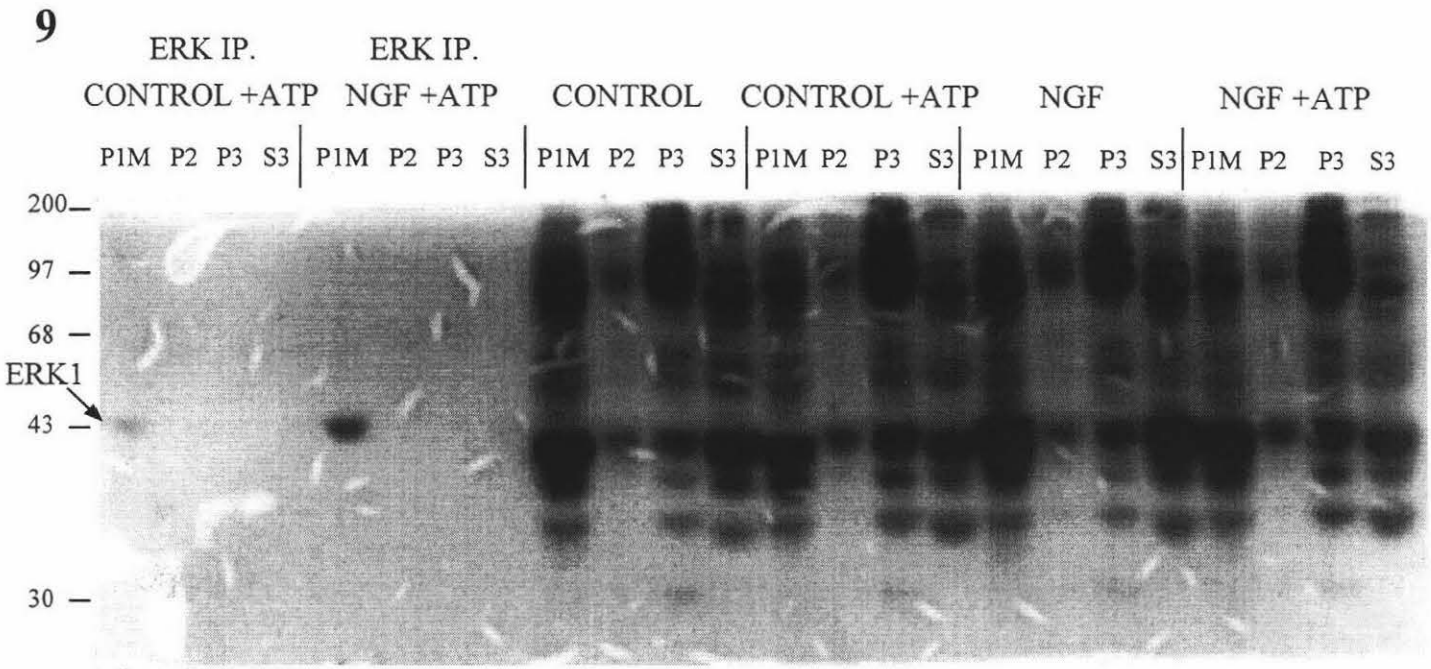


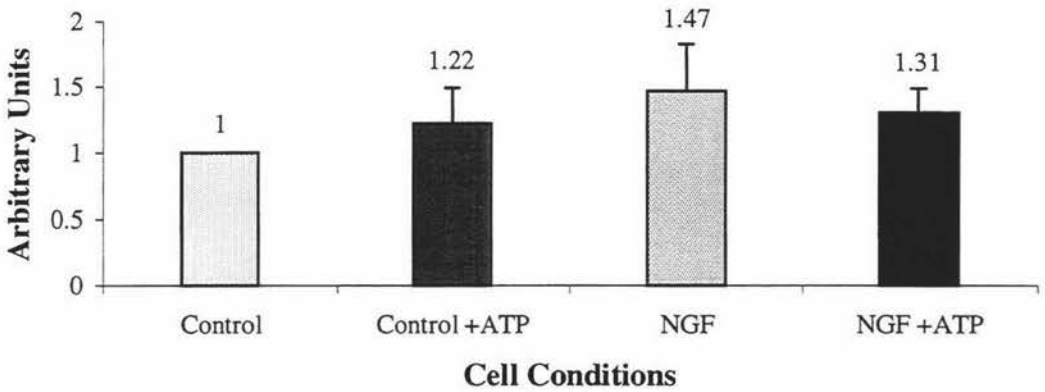
Figure 9: In-gel kinase activity assay on samples treated as in Figure 8a (control, control +ATP, NGF, NGF +ATP). In addition, ERK1 was immunoprecipitated in post *in vitro* reaction samples (ERK IP., left). MBP (0.25 mg/mL) was incorporated in the gel (see methods). After electrophoresis, the gel was denatured and renatured in a series of washes, 50-75 μCi/10 mL of γ^{32}P-ATP in reaction buffer was added for 1 hour. Background radioactivity was removed by extensive washing in 5% TCA, 1% pyrophosphate. The gel was dried and exposed to x-ray film for 20 days.

ERK1. The NGF-treated samples also displayed higher bands in the cell ghost and cytosolic fraction, although after the *in vitro* reaction activity decreased, especially in the cytosolic fraction. The P2 and P3 ERK1 bands in the NGF samples migrate to an equivalent position as the vesicle fractions for the other samples. Note that the immunoprecipitated samples show much weaker signals, indicating that immunoprecipitation did not bind all the ERK present in the samples. Also, ERK2, which does not bind strongly to this antibody, was not immunoprecipitated, nor were any bands immunoprecipitated from the other fractions. Although it is possible that there are multiple kinases that run at the same molecular weight, we favour the interpretation that ERK immunoprecipitation is inefficient and that P1M signals for ERK1 were preferentially detected due to greater activity in that fraction. It is also possible that in other fractions ERK binds to proteins that prevent antibody interaction. In the following analysis we assume that the active kinases at 44 and 42 kDa are ERK1 and ERK2 respectively.

Total ERK activity between conditions, determined by densitometry of in-gel kinase autoradiograms, was compared from several experiments to determine the effect of NGF stimulation and *in vitro* reactions on ERK activity. Total ERK activity increased with NGF-treatment for all conditions. The most significant increase in ERK1 activity (Figure 10a) was after NGF-treatment (1.47 times the total ERK1 activity of the control, $p< 0.05$). NGF treatment with the *in vitro* reaction caused 1.3 times as much activity as the control, and there was 1.2 times as much activity after the *in vitro* reaction alone. ERK2 activity also increased with NGF treatment (Figure 10b), most dramatically after *in vitro* reactions, with 1.5 times as much activity, as the total control ERK2 activity (although this is not statistically significant due to variability). Total ERK2 activity was only 0.50(control) to 0.80(NGF+ATP) times the ERK1 control total activity.

We analysed a number of these experiments in attempt to understand the dynamics of ERK activation and redistribution. Of particular interest is the comparison of kinase activity with the distribution of ERK1 and 2, or in other words, the specific activity of ERK1 and 2 between different conditions and different fractions. With early indications of increased activity in the 8 000×g and 100 000×g pellets, we expected specific activity to be high in these fractions. These results are discussed in the sections below.

10a **ERK1 TOTAL ACTIVITY USING MBP IN-GEL KINASE ASSAYS**



10b **ERK2 TOTAL ACTIVITY USING MBP IN-GEL KINASE ASSAYS**

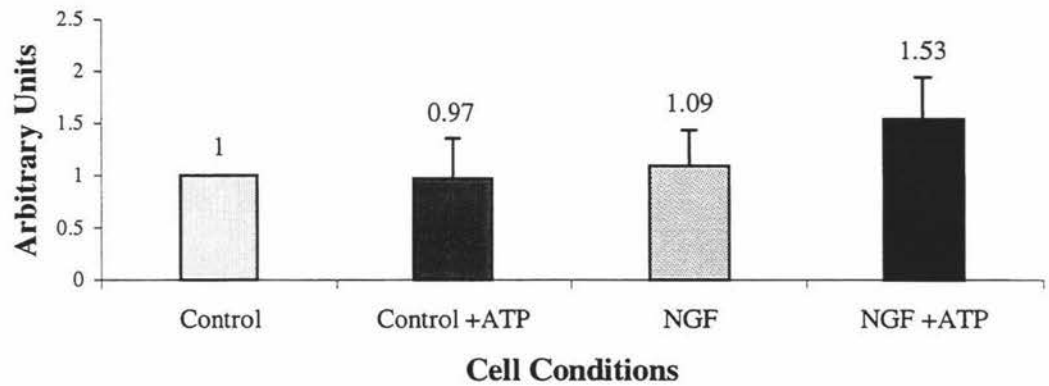


Figure 10: The effect of *in vitro* reactions and NGF treatment of PC12 cells on ERK activity. Total activity for each condition was calculated from the sum of the raw data from P1M, P2, P3, and S3 fractions. Conditions were normalised to the control (control = 1) for both ERK1 (10a) and ERK2 (10b). Total activity was calculated from three experiments for 10a and for 10b.

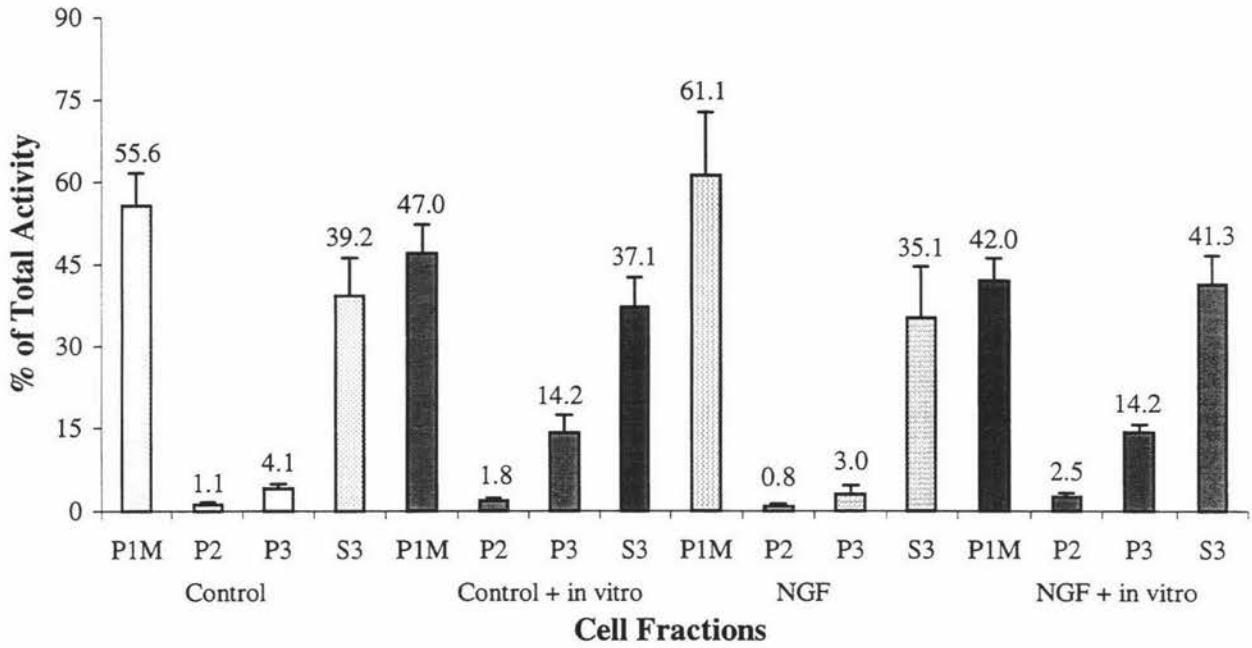
3.3 ERK ACTIVITY DOES NOT MATCH ERK DISTRIBUTION

The results for a number of activity assays were analysed by densitometry, in the same way as for the western blots of ERK1 and 2 distribution. Figure 11a shows the average ERK1 activity from three experiments. If Figure 11a is compared with Figure 7a it is immediately obvious that the percentage distribution of ERK1 between P1M and S3 fractions does not equate with percentage activity. If ERK1 were equally active in all fractions we would expect approximately 70% of activity in the S3 fractions. Figure 11a shows less than 45% of total activity was found in the S3 fractions. Most activity was found in the P1M fractions, suggesting ERK1 associated with membranes (or in the nucleus) was more active than ERK1 present in the cytosol.

Figure 11a shows a decrease in ERK1 activity in cell ghosts after *in vitro* reactions. This was most notable between NGF and NGF +ATP P1M fractions, with a 19% decrease ($p<0.05$) in activity after the *in vitro* reaction; there was no such decrease in ERK1 distribution. In fact, in terms of distribution, there was slightly more ERK1 in the P1M after NGF and *in vitro* reactions than with just NGF stimulation, although differences were not statistically significant (23% of total ERK1 in NGF P1M versus 26% in NGF +ATP P1M).

While *in vitro* reactions caused a decrease in cell ghost ERK1 activity, there was a concomitant increase in the 100 000×g pellets (Figure 11a). Activity in the pellets increased from 3% of total activity (NGF), to 14.2% of total activity (NGF +ATP). This increase in activity was highly significant ($p<0.001$). There was also a slight increase in activity in the 8 000×g pellets, from 0.8% (NGF) to 2.5% (NGF +ATP, $p<0.05$). The control *in vitro* reaction also caused a significant increase ($p<0.01$) in ERK1 activity from 4.1% (control) to 14.2% (control +ATP). In contrast to increased activity, the *in vitro* reaction caused no significant change in ERK1 distribution in the 100 000×g pellets, as values for distribution varied from 3.0 to 4.1% across all samples (Figure 7a). Therefore, the increase in activity in the *in vitro* stimulated P3 fractions was not due to a large redistribution of ERK1.

11a ERK1 AVERAGE ACTIVITY FROM MBP KINASE GELS



11b ERK2 AVERAGE ACTIVITY FROM MBP KINASE GELS

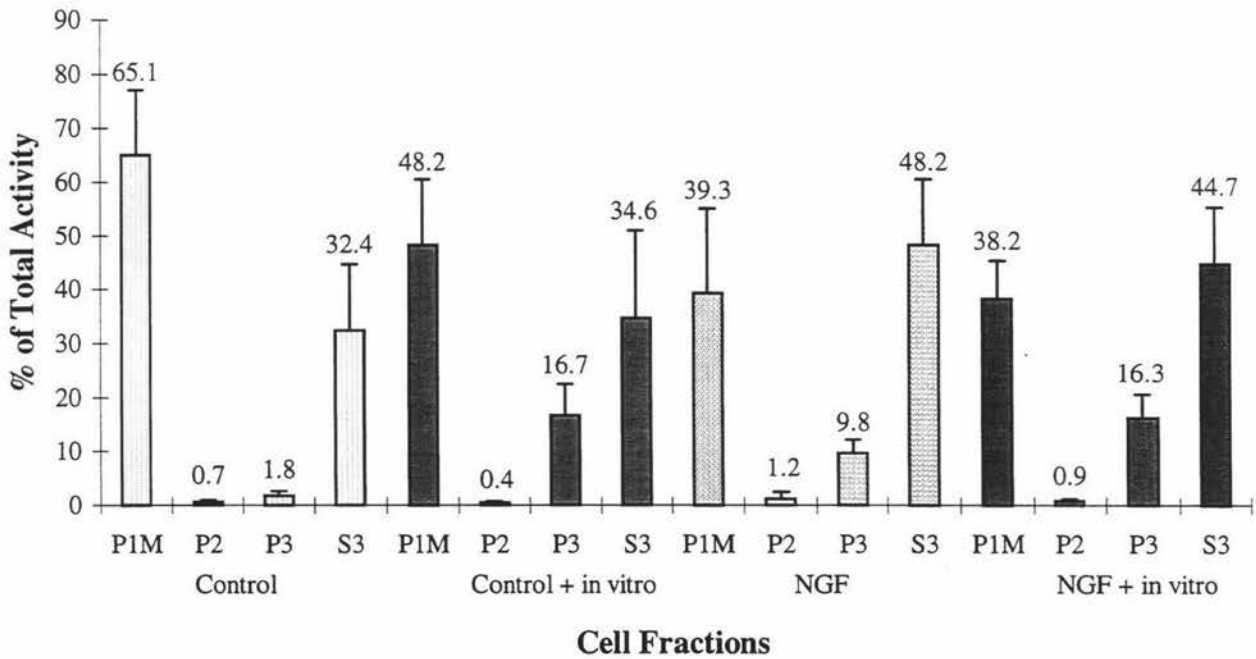


Figure 11: Averaged values for ERK activity between cell fractions. Results for ERK activity were analysed and plotted as a percentage of total activity in the same way as for ERK distribution (Figure 7). (a) Results for ERK1 activity were calculated from three (control, NGF), five (control +ATP), or six (NGF +ATP) experiments. (b) Results for ERK2 activity were calculated from three (control), four (NGF), or five (control +ATP, NGF +ATP) experiments.

Comparison of ERK2 activity (Figure 7b) with distribution (Figure 11b) with shows both similarities and differences to ERK1. The NGF and NGF +ATP cell ghost activities for ERK2 were actually lower than the corresponding cytosolic activities for ERK1, although the error bars are larger. However, the western blot profile for ERK2 was very similar to that for ERK1 (Figures 6, 7, 8), so the actual specific activity for the NGF and NGF +ATP cell ghost fractions was still higher than for the cytosolic fractions. ERK2 also showed strong 100 000×g pellet activity and specific activity for samples subjected to *in vitro* reactions. The major difference from ERK1 was the increase in activity in this pellet from 1.8% to 9.8% after NGF stimulation ($p<0.01$), although after *in vitro* reactions this difference was masked (16.7% for control +ATP P3, 16.3% for NGF +ATP; Figure 11b).

Figure 12 shows the specific activity of ERK1. This graph is a representation of activity divided by distribution, from individual experiments where both in-gel kinase assays and western blots were performed. While specific activity has been inferred from the above results showing relative distribution of activity and protein, measuring it in each experiment was difficult because of low western blot signals, in P2 fractions in particular, so the results in these graphs are not statistically significant. However, they are useful in that they confirmed the overall trends predicted in the above analyses. Specific activity was several times higher in the cell ghosts compared to cytosolic fractions. Also, in the cell ghosts ERK1 specific activity decreased slightly with *in vitro* and NGF reactions. Conversely, in the 100 000×g pellets, specific activity for ERK1 was higher after *in vitro* stimulation, at least for NGF treated samples (note that it was difficult to measure in the control sample because of little protein present). ERK2 specific activity could not be calculated due to low ERK2 measurements from western blots. Even so, these preliminary results are intriguing and suggest that the distribution of active ERK in the cell is dynamic.

Because both the distribution and activity of ERK1 bordered on the limits of detection (therefore with relatively large errors), small changes (half a percent increase in distribution or activity) could halve or double apparent specific activity. This problem could be solved in future experiments by increasing the sample loading on gels, and thus the signal strength, of P2 and P3 samples.

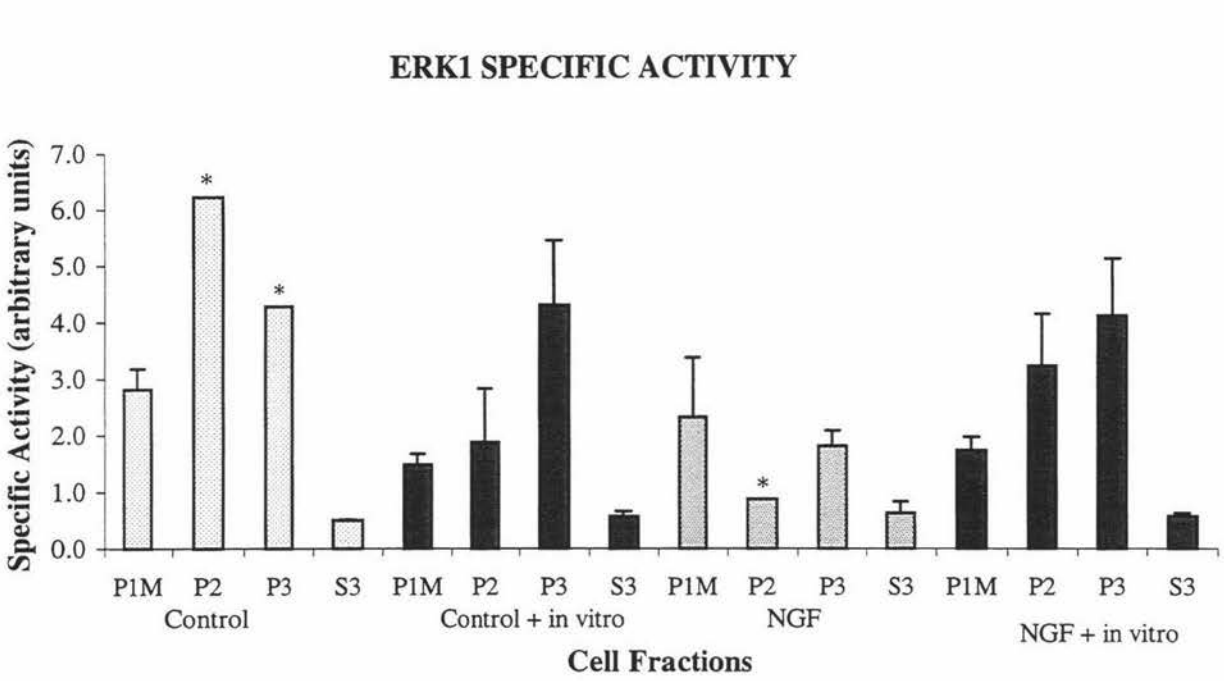


Figure 12: ERK1 specific activity. Specific activity was calculated by dividing raw results for ERK1 activity from MBP in gel kinase assays with ERK distribution from western blots of the same experiments. This comparison gives an indication of how much active ERK1 compared to inactive ERK1 is found in each fraction. Results were calculated from two to four experiments, except data marked with asterisks which denote one experiment.

3.4 ERK ACTIVITY IS ASSOCIATED WITH P2 AND P3 FRACTIONS

The data above suggest that ERK kinase activity may be associated with membrane fractions. *In vitro* reactions, which increase the production of small vesicles, caused increased activity of ERK1 and 2 in the 100 000×g pellet, the fraction which contains small vesicles, even though there is no increase in total ERK detected by western blotting in these fractions. In response to NGF stimulation, ERK2 also increased in activity in the 100 000×g pellet. We do not know why ERK2 but not ERK1 increased in activity in this fraction, as both kinases are stimulated by the same NGF-activated cascade. In fact, although averaged results showed no significant increase in ERK1 activity in the 100 000×g pellet with NGF stimulation, in some experiments marked increases were seen. Figure 13 shows an example of strong stimulation of ERK1 activty in NGF-treated cells after an *in vitro* reaction (NGF +ATP, P3); there was four times as much activity as in the corresponding untreated control (control +ATP P3), and

at least twice as much activity as in the other P3 fractions. There was also 11% more ERK1 activity in the NGF-treated P3 fraction compared to the control P3 fraction. ERK2 was more than twice as active in the NGF +ATP P3 fraction than in the control +ATP P3 fraction. Importantly, these increases were not due to increased proteins in those lanes, because higher molecular weight kinases showed a range of differences of only 1.5 to 8.2% between conditions.

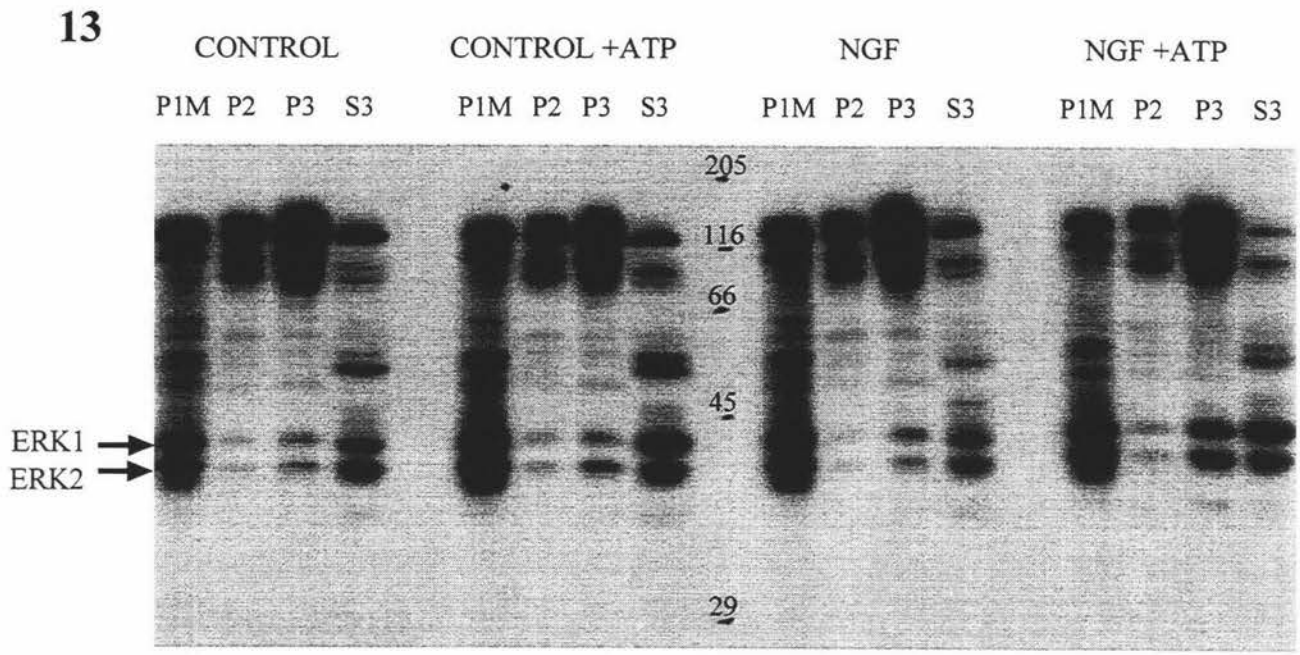


Figure 13: An example of an experiment where ERK1 and 2 activity increases in the NGF treated cells P3 fraction after the *in vitro* reaction. Samples were treated as the non-immunoprecipitated samples in Figure 9. Molecular weight markers are shown in the middle of the gel.

To investigate further the activity of ERK1 and 2 in the pellet fractions we employed a different method to measure ERK activation, a non-radioactive *in vitro* MAPK assay. This assay, which has recently become commercially available, involves precipitating active ERK from cell fractions with an anti-phospho-ERK antibody that only recognises tyrosine phosphorylated MAPK. An *in vitro* reaction is then initiated by adding ATP and the ERK substrate Elk1, a transcription factor, to the precipitated ERK. ERK1 and 2 are the only known activators of Elk1 (Gille *et al.*, 1995). Active ERK phosphorylates Elk1, which is then

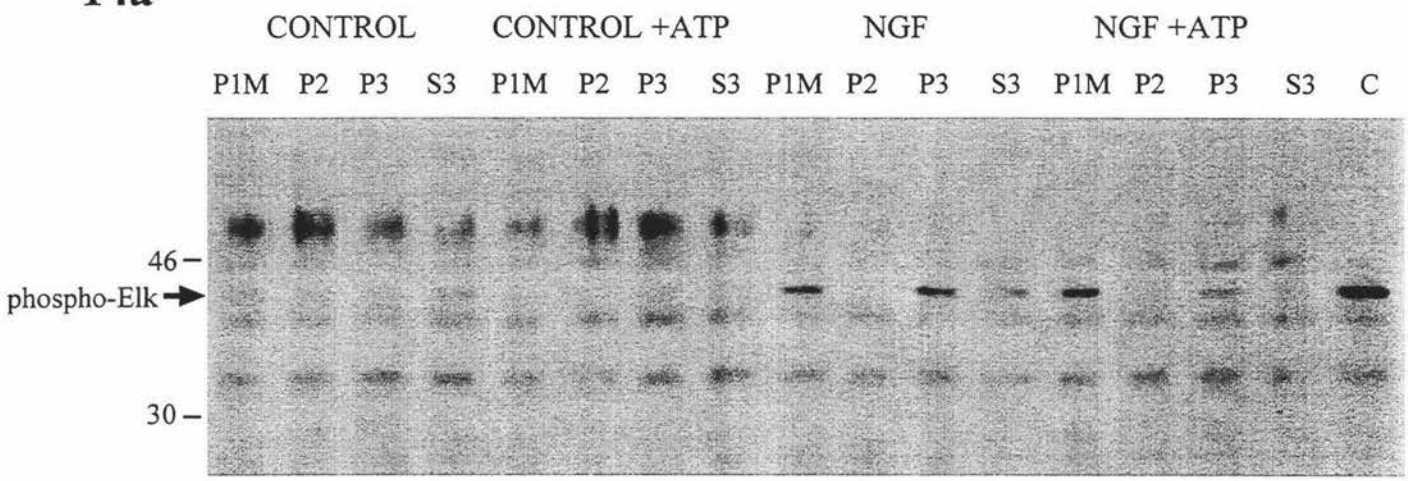
detected by western blotting with anti-phospho-Elk1. Because the ERK antibody used binds to both ERK1 and ERK2, and Elk1 is a substrate for both kinases, the individual activities for each kinase cannot be determined.

In the first experiment, phospho-Elk1 is identified in the control lane of Figure 14a (C, right), and is distinguished by its molecular weight and strong signal over background bands. The first experiment (Figure 14a) showed strong activation of ERK in the NGF-treated P3 fraction, which persisted after the *in vitro* reaction (NGF +ATP P3). Figure 14b is a longer exposure of the same experiment, in which this can be seen more clearly. The second experiment, in Figure 14d and e, shows strong ERK activity in some of the control samples, but there is still 1.8 times more activity in the NGF P2 than in the control P2; 2 times as much activity in the NGF P3 than the control P3; and 2.4 times as much activity in the NGF +ATP P3 than the control +ATP P3.

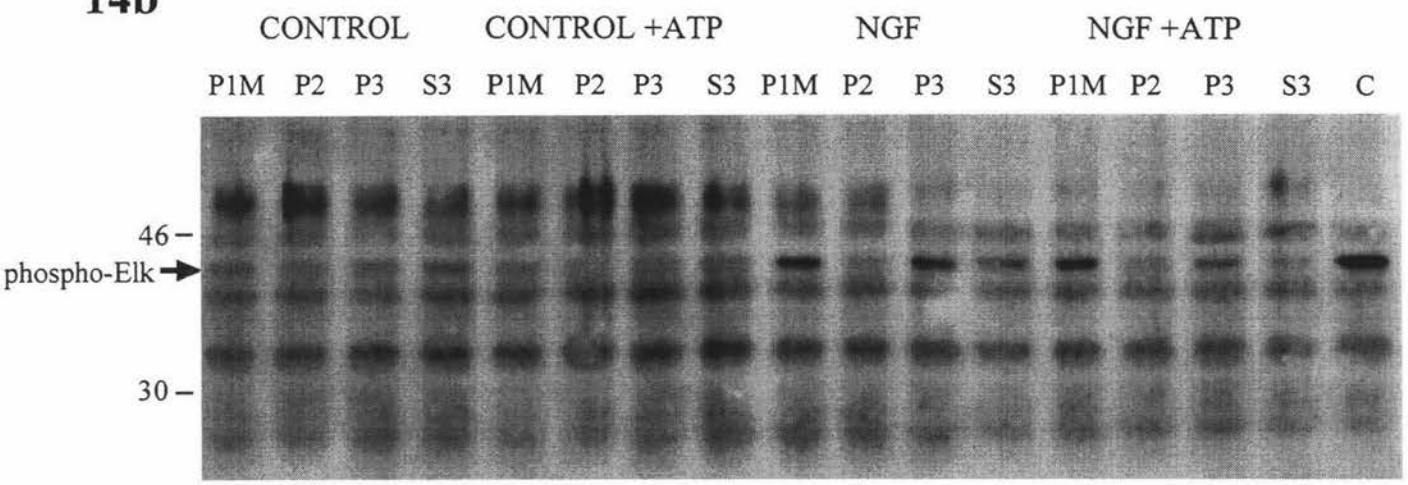
These results are similar to those for ERK2 from the MBP assay; they show that NGF stimulation caused increased ERK activity in the 100 000×g pellet. In the Elk1 assay it cannot be determined whether this increase was due to ERK1 activity or ERK2 activity or both. Although there was strong stimulation of ERK1 and 2 activity in the P2 +NGF lane of Figure 14e, activity was found in the P3 +NGF lane of Figure 14a and b. This variability may be due to experimental error or to the dynamics of these kinases. However, after *in vitro* reactions in both experiments, there was strong ERK activity in the 100 000×g pellet, but little activity in the corresponding 8 000×g pellet. This suggests that active ERK is making a transition to small organelle fractions during the course of the *in vitro* reaction. The active ERK in small organelle fractions could come from larger organelles originating in the cell ghost and 8 000×g pellet, or alternatively could be due to recruitment from the cytosolic fraction. There is also evidence for this transition in some western blots. Figure 8a shows that ERK1 was present in the 8 000×g pellet after NGF stimulation, but not present in this fraction after the *in vitro* reaction.

Total ERK activity (Figure 14c, f), as measured by this assay, increased in NGF treated cells by 1.25 to 1.6 times (calculated from the stronger exposures, (Figure 14b, e) over the control

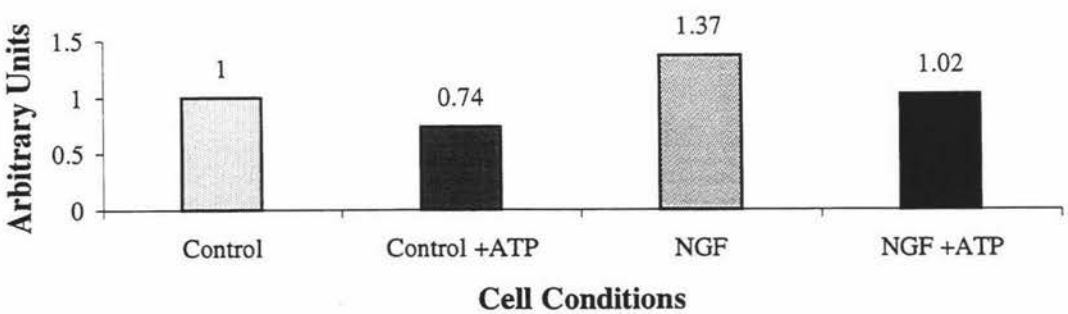
14a



14b



14c



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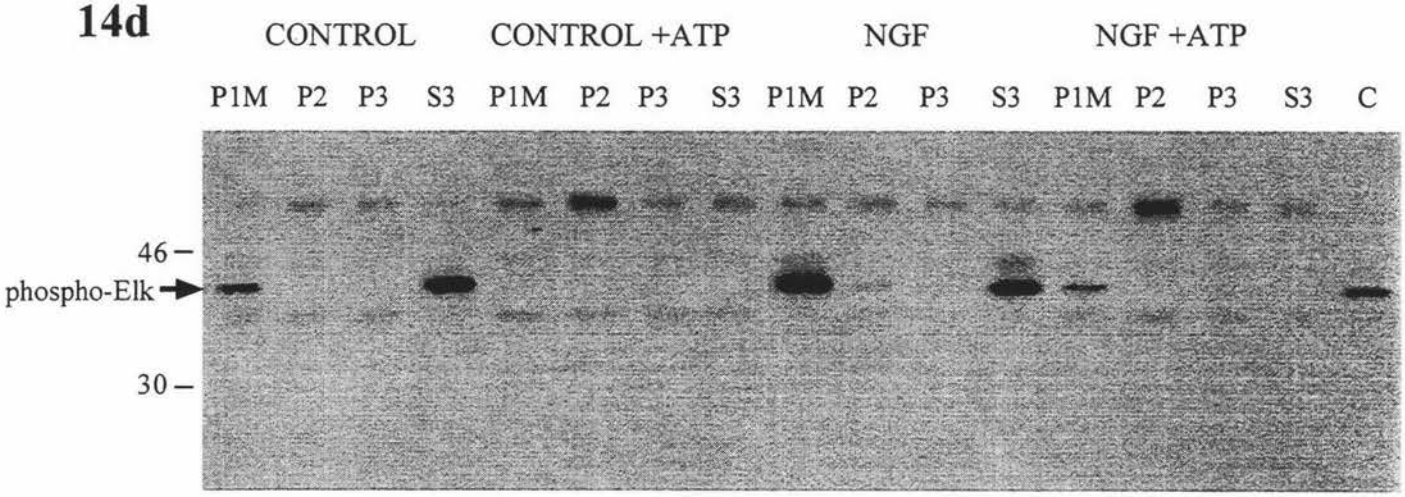
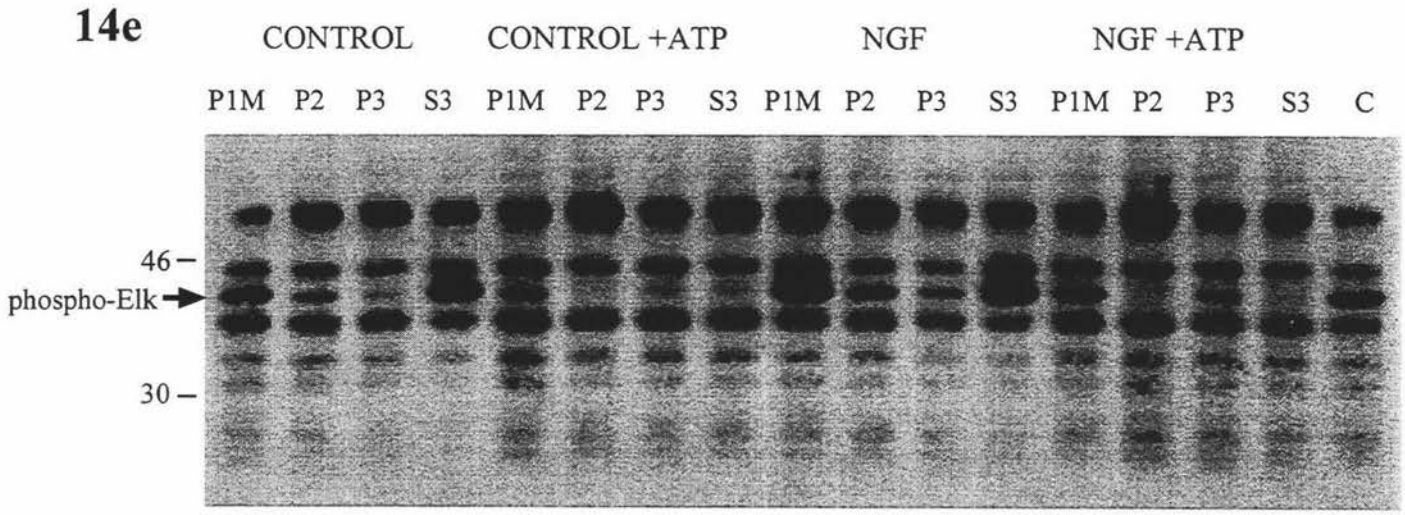




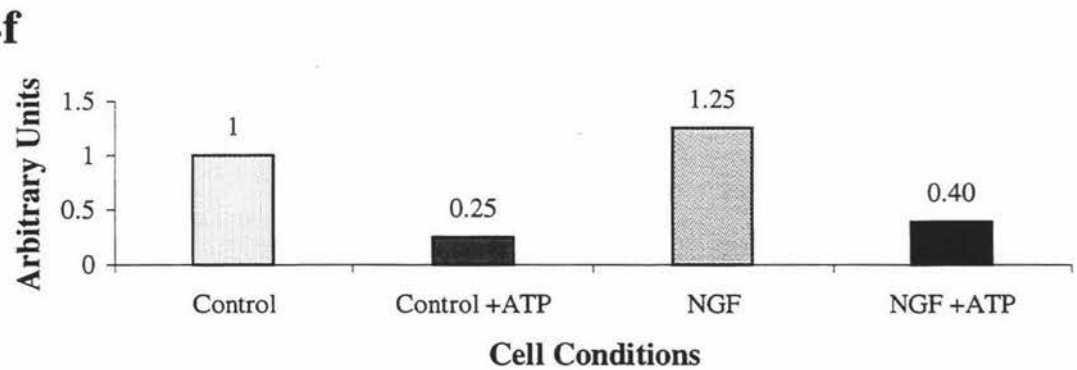


Figure 14: ERK activity is associated with NGF and NGF +ATP P2 and P3 fractions. Samples were treated as in Figure 8a. After fractionation 100 µL of each sample was immunoprecipitated with anti-phosphoERK antibodies. PhosphoERK was then incubated with the ERK substrate Elk1, before running the samples on SDS-PAGE. ERK activity was detected by blotting with anti-phosphoElk1 antibodies. (a) and (c) are two individual experiments. (b) and (d) are longer exposures of the blots in (a) and (c), respectively. On all blots the far right lane (C) is the control, which was active ERK2 provided with the kit. (c) and (f) are graphs of the total activity measured for each condition from the blots in (b) and (d) respectively. Data was adjusted for sample volumes before calculating total activity, and results shown are relative to the controls.

activities in the two experiments performed with this kit. This increase in ERK activity after cells are stimulated with NGF is consistent with the results from MBP in-gel kinase assays (Figure 10a and b). Note that the considerably lower total activities for samples that underwent the *in vitro* reaction are probably due to phosphatase action on the kinases.

The anti-phospho-ERK antibodies used for the non-radioactive MAPK assays can also be used to probe western blots directly, which is another method for detecting phospho-ERK. Figure 15 shows a western blot of samples from the same cell fractionation as in Figure 14a. The results clearly do not match; in NGF treated cells, the cytosolic fraction contains the majority of phosphorylated ERK, and less was detected in the 100 000×g pellet. In contrast, the activity assay detected strong ERK activity in the +NGF 100 000×g pellet and only weak activity in the corresponding cytosolic fraction. Neither did the western blot (Figure 15) reflect the moderate ERK activity in the NGF-treated cells after *in vitro* reactions (Figure 14a, NGF +ATP P1M, P3). This discrepancy between western blots and the activity assay indicates that tyrosine phosphorylation is not necessarily an indicator of ERK activity.

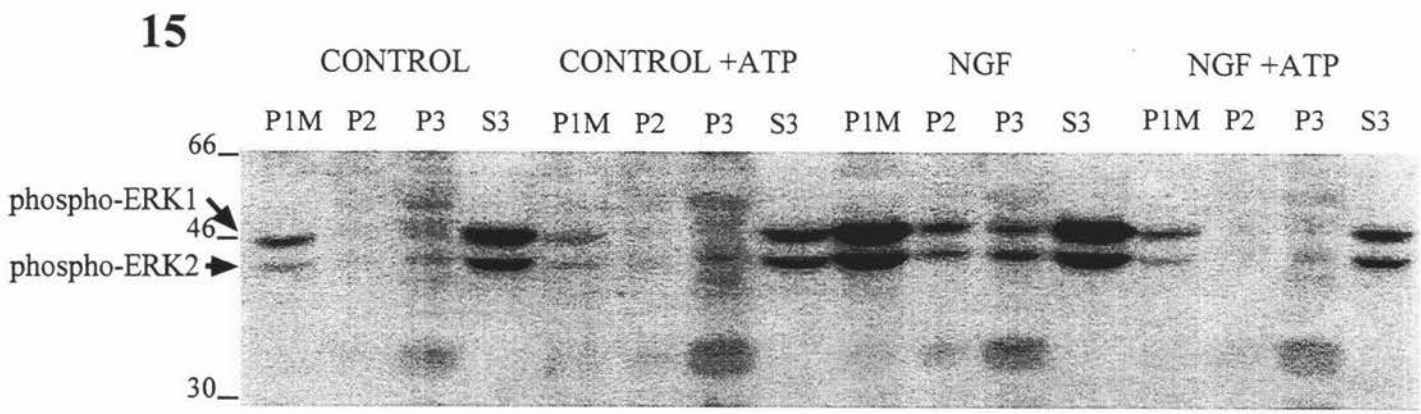


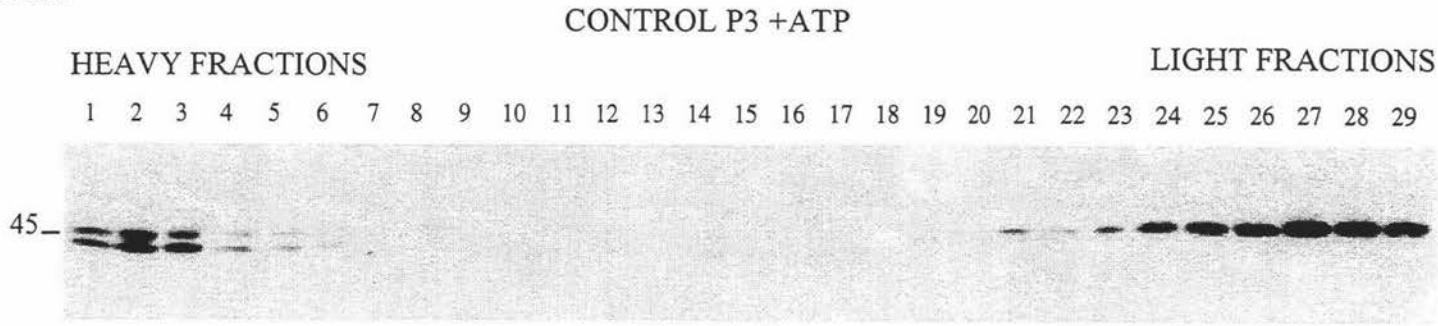
Figure 15: Results from western blotting using anti-phospho-ERK antibodies do not match results using the non-radioactive MAPK assay. Figure 15 shows a western blot using samples from the same fractionation experiment shown in Figure 14a and b. Cell fractions were western blotted directly without immunoprecipitation as in Figure 8a, except that the primary antibody was anti-phospho-ERK (1:1000 dilution), and the secondary antibody was anti-rabbit (1:2000 dilution).

Overall, both the MBP and non-radioactive ERK activity assays indicated that *in vitro* reactions and NGF stimulation caused increased activity in 8 000×g and 100 000×g pellets. The variability in these results (i.e. the amount of activity detected in the P2 and P3 fractions) will be further dealt with in the discussion. However, because ERK was found in the 100 000×g pellet in an active state, and this fraction contains vesicles with tyrosine phosphorylated TrkA that may initiate signal transduction (Grimes *et al.*, 1997), we chose to further investigate the properties of ERK in this fraction. It is possible that TrkA activates ERK directly via a complex with signalling intermediates in these vesicle fractions. The fact that ERK1 was reported to be bound to TrkA (Loeb *et al.*, 1992) supports this hypothesis. Another possibility is that ERK1 and 2 are associated with other proteins in a "signalling particle". Although it has not been shown in mammalian cells, kinases of the MAPK pathway are bound together in a scaffold in yeast cells (Choi *et al.*, 1994; Printen and Sprague, 1994), which allows for efficient activation of the kinase cascade and keeps similar MAPK pathways separate from each other. A similar system as in yeast may be used in mammals. If ERK is bound in a protein scaffold similar to that found in yeast, we would expect it to be in a relatively heavy particle whose sedimentation properties may be similar to transport vesicles or synaptic vesicles. To investigate this, we submitted the 100 000×g pellets containing active ERK1 to glycerol velocity gradient sedimentation.

3.5 ERK IS FOUND IN HEAVY AND LIGHT P3 GRADIENT FRACTIONS

The 100 000×g pellet, from cells fractionated after *in vitro* reactions, contained active ERK1 and signalling vesicles. Glycerol gradients have been used to resolve signalling vesicles, synaptic vesicles, and soluble protein from one another (Grimes and Kelly, 1992a; Grimes and Kelly, 1992b). Two separate experiments were performed (Figures 16, 17). ERK1 was present in both the heaviest and the lightest fractions of the glycerol gradient for both the control and NGF-treated P3 cell fractions after *in vitro* reactions (Figure 16 and Figure 17a and c). Soluble protein is expected in the lighter, less dense fractions at the top of the gradient, as small molecules do not migrate very far. ERK1 was found in these fractions (fractions 22-29) and also in the heavy fractions (1-6) at the bottom of the gradient. ERK1

16a



16b

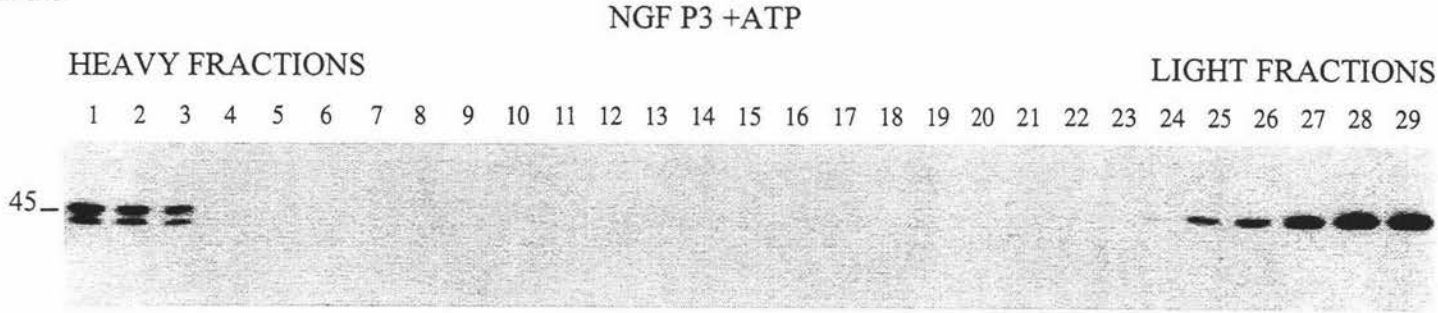


Figure 16: ERK is present in light and heavy fractions of glycerol velocity gradients. After *in vitro* reactions and fractionation as in Figure 8a, P3 samples were layered over a glycerol gradient (5-25% glycerol over a 60% sucrose pad) and spun at 200 000×g for 2 hours. Headings indicate the particular sample submitted to glycerol gradient sedimentation. Gradient fractions were collected from the bottom of the tube. Protein was precipitated from each fraction using TCA. Precipitated protein was redissolved in urea sample buffer, submitted to SDS PAGE and western blotted with anti-ERK antibodies (UBI). In this experiment, ERK1 and phospho-ERK1 were detected. The heavy fractions contained both, whereas the lighter fractions did not contain phospho-ERK1. Although not visible on the figures, on the original film lighter bands could be traced directly from the heavy fractions to the light fractions.

found in the heavy fractions may be bound in a complex or be attached to vesicle membranes, as this is the location at which TrkA-containing vesicles migrate (Grimes *et al.*, 1997). To distinguish these possibilities, the 100 000×g pellet was split, and one half was dissolved in mild detergent prior to glycerol gradients (Figure 17b, d,). The concentration of detergent used (0.4% Igepal) would be expected to dissolve vesicle membranes. Membrane disruption had no effect on the distribution of ERK. To confirm membranes were disrupted as predicted, the ERK blots were re-probed with anti-synaptophysin antibodies, shown in Figure 18. Synaptophysin, a 38 kDa protein associated with constitutive secretory vesicles (fractions 1-6) and synaptic vesicles (fractions 7-11), moved from these fractions to the lightest fractions when the samples were pre-treated with detergent prior to gradients. These results indicate

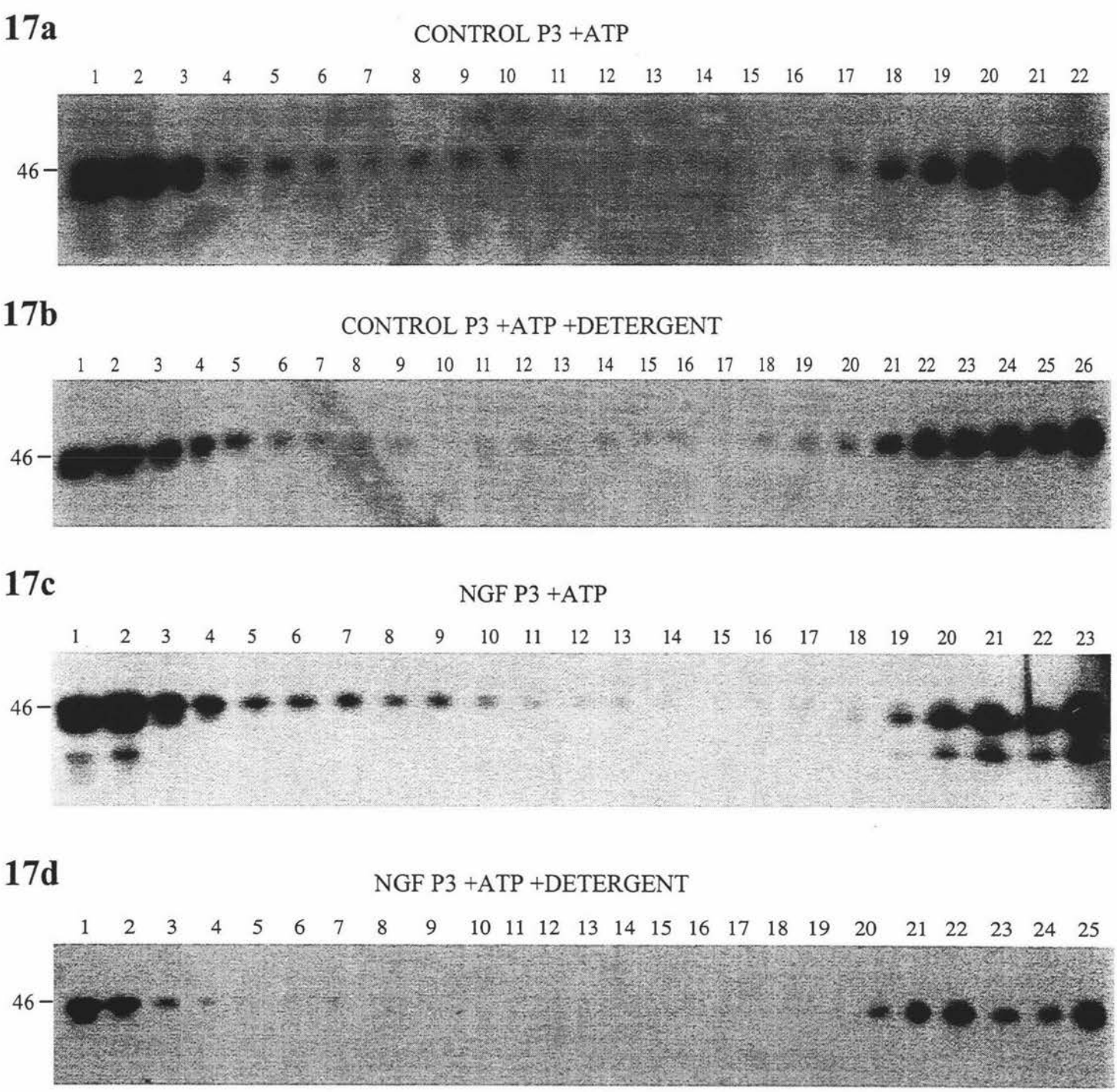
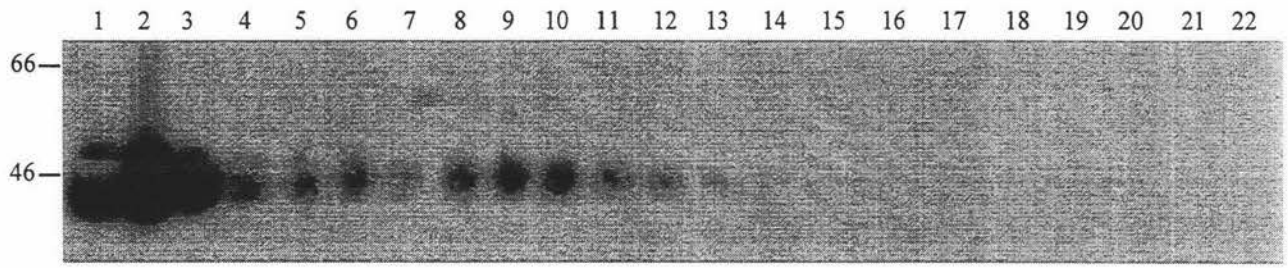


Figure 17: Detergent solubilisation did not affect ERK sedimentation in glycerol velocity gradients. Samples in a and c were treated as in Figure 14 except the glycerol gradient was spun at 200 000×g for 60 minutes and the blot was probed with Santa Cruz anti-ERK antibodies. Samples in b and d were treated as above except they were dissolved in 0.4% Igepal before loading on the glycerol gradients. ERK1 was the major species detected in this experiment, and the phosphorylated form was distributed mostly at the bottom of the gradient as in Figure 16. ERK2 was weakly detected, and was present in both soluble and particulate fractions.

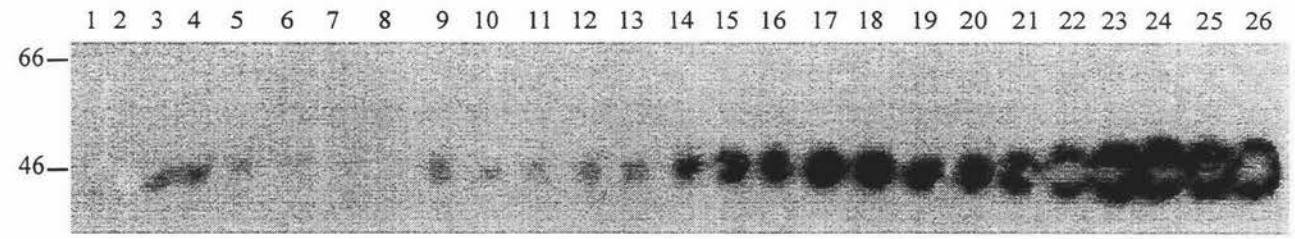
18a

CONTROL P3 +ATP



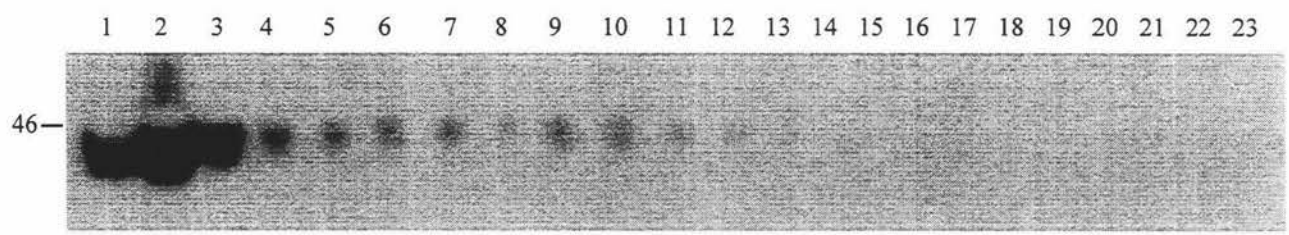
18b

CONTROL P3 +ATP +DETERGENT



18c

NGF P3 +ATP



18d

NGF P3 +ATP +DETERGENT

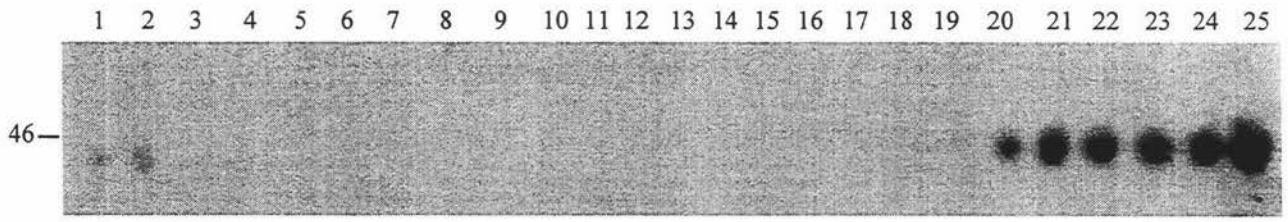


Figure 18: Blots shown in Figure 17 were stripped and reprobed using anti-synaptophysin antibodies. Detergent solubilisation caused synaptophysin to shift from heavy to light fractions.

that membrane-containing vesicles were disrupted by detergent treatment. Since detergent did not affect ERK1 migration, this data suggests that it is not bound to vesicle membranes, and suggests that ERK1 present in the heavy fractions may be part of a multi-protein complex.

3.6 EVIDENCE FOR PHOSPHORYLATED ERK

The gradient experiments appear to show two (Figure 16) or three (Figure 17) distinct ERK bands. Based on their molecular weights (see Figures 6 and 8), we hypothesise that the highest band is phospho-ERK1, the middle band is unphosphorylated ERK1, and the lower band, which was only weakly detected, if at all, is ERK2. There are several reasons for suggesting this. Firstly there is evidence from published literature that described a mobility shift due to ERK being phosphorylated (Virdee and Tolkovsky, 1995; Peng *et al.*, 1996; Zhao *et al.*, 1996).

Secondly, the anti-ERK antibodies used in these experiments, especially from UBI (Figure 16), react more strongly with ERK1 than ERK2. Unphosphorylated ERK1 (Figure 16a) was found in fractions 1-6 and accounts for the entire signal in fractions 20-29. This is consistent with previous western blots that detect ERK1 and phosphorylated ERK1 as the major species in the 100 000×g pellet (Figure 6 and 8), although ERK2 activity was detected (Figure 9 and 13). In most western blots ERK2 only shows up in the 100 000×g after a long exposure. Comparison of ERK1 to ERK2 ratios in the P3 +ATP fractions in Figure 8 indicate that there is a 5 to 30 times greater signal for ERK1 than ERK2, and in fact, a stronger signal for ERK2 than ERK1 was never observed on western blots.

As mentioned in section 3.1, phosphorylated ERK1 was detected on western blots in the 100 000×g pellet. In glycerol gradient experiments, the highest molecular weight band in this pellet, phosphorylated ERK1, increased in amount relative to unphophorylated ERK1 with NGF stimulation (Figure 16b). This is consistent with NGF causing an increase in ERK1 activation. Importantly, this band was only present in the heavy particles that migrate to the bottom of the glycerol gradient (fractions 1-6). The western blots in Figure 17 were probed

with different ERK antibodies, and the resolution is not as sharp, but again there was evidence that the higher molecular weight band of phospho-ERK1 was present exclusively in heavy particles (fractions 1 to 6), whereas unphosphorylated ERK1 was present in these and in the soluble pool (fractions 20 to 23). Comparison of fractions 1 to 3 in Figure 17a and 17c shows the ratio of the higher band to the lower band was greater in the NGF-treated sample than in the control.

These results suggest that ERK1, activated by NGF stimulation, becomes hyperphosphorylated and associated with a protein complex. This complex was detected in the 100 000×g pellet and migrates to the bottom of glycerol velocity gradients.

CHAPTER 4 : DISCUSSION

4.1 DISCUSSION AND CONCLUSIONS

Studies have shown that TrkA, MEK and ERK are retrogradely transported down NGF stimulated axons (Bhattacharyya *et al.*, 1997; Johanson *et al.*, 1995). NGF-activated TrkA retrograde transport may be facilitated by transport vesicles, which can be partially isolated following the cell fractionation technique of Grimes *et al.* (1997). It was the aim of our research to investigate further the distribution of ERKs 1 and 2 and their activities, using the cell fractionation scheme used to characterise NGF-activated TrkA containing vesicles. Since ERKs are activated by NGF, and have been found to associate with TrkA, we hypothesised ERKs may also be associated with these vesicles. Unexpectedly, we found ERK was not associated with membranes in small vesicle fractions, but was found in detergent-insoluble particles.

To investigate ERK distribution and activity, we were able to separate cell components into five fractions. The 'cell ghost' or membrane fraction (P1M) resulted from the pellet of the low speed centrifugation (1 000×g, 10 minutes) of the cracked cells. This was solubilised in detergent and centrifuged again, at 10 000×g for 10 minutes, to remove cytoskeleton and DNA. Note that we did not measure ERK content in the cytoskeletal pellet, although it would be interesting to do so in the future as its activity and distribution in this pellet may vary significantly after NGF treatment. Supernatant from the low speed centrifugation was spun at 8 000×g (35 minutes) to pellet large organelles (P2). Supernatant from the 8 000×g spin was centrifuged at 100 000×g (60 minutes) to pellet small organelles (P3). The remaining supernatant was termed the cytosol (S3) fraction. Using electron microscopy the 8 000×g pellet has been reported to contain mitochondria, dense bodies, ribosomes, and uncoated vesicles with a mean diameter of 180 ± 71 nm (Grimes *et al.*, 1996). The 100 000×g pellet contains several types of smaller vesicles (the largest with a mean diameter of 94 ± 24 nm or less), a few mitochondira, and dense bodies. It is important to note that the 8 000×g and

100 000×g pellets have been characterised in terms of the identified organelles, however, they also contain a large amount of dense matter that has not been identified. Before fractionation, some of the cells were exposed to an *in vitro* reaction, this promotes vesicle budding that enhances the recovery of small vesicles (Grimes, M. and Kelly, R.B. 1992a). Using two conditions (+NGF, NGF +ATP), we were able to analyse the effect of NGF on ERK activity and distribution soon after NGF binding and activation of TrkA (+NGF), and compare this with events occurring after 15 minutes of vesicle transport and budding reactions (NGF +ATP).

Comparison of different cell fractions, by western blotting with anti-ERK antibodies and by MBP in-gel kinase assays, demonstrated that ERK distribution did not match ERK activity. 65 to 77% of all ERK was found in the cytosol (S3), and only 18 to 30% in the cell ghost (P1M). In contrast, the majority of the active ERK was found in the cell ghost, in all except two conditions, which showed equal activities in the cell ghost and cytosol (ERK2, NGF, NGF+ATP; Figure 7b). ERK activity decreased in the cytosol fractions with *in vitro* and NGF-treatment, while at the same time increasing in the cell ghost. Previous research suggests ERK is bound to MEK in the cytosol and once activated is released from MEK and translocates to the nucleus (Lenormand *et al.*, 1993; Fukuda *et al.*, 1997). As the cell ghost (P1M) contains the contents of the nucleus minus the DNA, the increase in ERK activity in this fraction may in part be due to nuclear translocation.

Data for the 8 000×g pellet (P2) indicated a small and sometimes undetectable presence of ERK. This may indicate that ERK mainly associates with small vesicles after their formation (and therefore is not associated with components of the P2 fraction), perhaps by binding directly to activated TrkA (Loeb *et al.*, 1992). There was however a slight increase in total ERK in the P2 NGF fraction. This may be an indication that ERK is associating with TrkA or a protein scaffold at the time of, or before, vesicle budding from endosomes.

The P3 fractions showed high specific ERK activity for both the control and NGF *in vitro* reactions. This would suggest ERK activity is stimulated by the *in vitro* reaction alone, and does not require NGF stimulation. However, ERK2 in the P3 fraction showed strong stimulation by NGF compared to the control ($P > 0.01$), and although averaged results for

ERK1 activity did not show NGF activation, two experiments (one is shown in Figure 12) had 4 times and 2.4 times more active ERK1 in the NGF treated P3 fractions after *in vitro* reactions compared to controls.

Why this inconsistency? Results from the non-radioactive MAPK assays may give some clue. Using these assays ERK activation occurred strongly in the 8 000×g or 100 000×g pellets, but not both together. Activation of ERK in the 8 000×g pellet only occurred with NGF stimulation without the *in vitro* reaction. This suggests timing is critical for determining the location of active ERK. The fractionation protocol used for these experiments was designed to follow the extracellular binding and internalisation of NGF to TrkA, not to monitor ERK activity. To consistently localise active ERK to the 100 000×g pellet with NGF stimulation the timing of the NGF warming reaction (10 min at 37°C) may have to be investigated by time-course experiments where various times of warming are tested. Alternatively the experiment could be redesigned. By using two conditions, one just after NGF internalisation (10 min at 37°C), and the other after vesicle budding (15 min +ATP at 37°C), we are trying to take a snapshot of two time points during NGF activated signal transduction. In reality, reactions going on in the cell remain dynamic, and although the speeds of these reactions are minimised by performing experiments between 0-4°C, some changes may occur. Cell fraction experiments with NGF and *in vitro* reactions can take 8 to 12 hours and at each stage can be inadvertently warmed during handling. Therefore timing of each sample preparation stage in the experiment can vary and this may affect the result. To minimise time delays and preparation stages (and thus minimise signal transduction), the fractionation procedure could be modified by re-suspending washed cells directly into PBS containing NGF (ice cold). These cells would be immediately warmed for perhaps 5 to 25 minutes, followed by cell cracking and fractionation. The timing of warming would be adjusted for the desired snapshot of the signalling pathway. The advantage of this method would be a reduced experiment time and less *in vitro* manipulation of the cells.

It is not obvious why there are discrepancies between MBP kinase assays and non-radioactive MAPK assays. The MBP kinase assay shows general activity in the control samples that may simply be intrinsic to the method, although there was a significant increase in ERK1 activity after NGF-treatment of cells ($P < 0.05$). It is possible that denaturing protein kinases in SDS and subsequently renaturing them in guanidine-HCl causes artificial activation, especially as

ERKs are capable of auto-activation (Seger *et al.*, 1991). In support of this there is generally little or no ERK activity in the control lanes of the non-radioactive MAPK assays for both experiments, in contrast to data from MBP assays. Total ERK activity as measured by densitometry did increase by a similar amount, after NGF stimulation of cells and *in vitro* reactions, in both types of assay. However, low activity is artificially increased compared to high activity in long exposure western blots (compare NGF and control P1M fractions in Figure 14a versus 14b). Interestingly, the immunoprecipitated P1M samples in Figure 9 do show a strong contrast in control and +NGF ERK1 activity, suggesting immunoprecipitating all samples would give better results. Unfortunately, the cost of antibodies makes this prohibitive. The non-radioactive MAPK assay uses phospho-ERK antibodies, but these antibodies, raised against tyrosine phosphorylated ERKs, may not detect all ERK activity. ERK phosphorylated only on threonine may be more active than ERK phosphorylated only on tyrosine residues. Full ERK activity requires both tyrosine and threonine phosphorylation activation (Anderson *et al.*, 1990; Boulton and Cobb, 1991). Fully active ERK kinases would be detected using the non-radioactive MAPK assay, unless hyper-phosphorylated ERK does not immunoprecipitate well. However, the one western blot performed with the phospho-ERK antibodies showed very different results from the non-radioactive MAPK assay performed using the same samples. This suggests western blotting with anti tyrosine-phosphorylated ERK could be detecting partially active ERK. A number of antibodies have recently been developed that are specific to both tyrosine and threonine-phosphorylated ERK, which may resolve this problem.

However, despite some inconsistencies, the results clearly showed that ERK1 activity could be localised to the P3 fraction. This prompted closer investigation of the P3 fraction by glycerol velocity gradient sedimentation, which separates particles according to their size. Using this method we found ERK present in both the heaviest and lightest fractions of both control +ATP and NGF +ATP samples. The presence of ERK1 in the heaviest fractions suggests it must be bound to something like a large vesicle or a protein complex. To determine if it was bound to a vesicle we dissolved the P3 fractions in detergent to disrupt membranes. If ERK1 was attached to the membrane we would have expected ERK1 present in the heaviest fractions to shift to the lightest fractions. However, detergent did not change the location of ERK1. To verify that detergent dissolved the vesicle membranes the blots were reprobed with synaptophysin. Membrane disruption was confirmed as synaptophysin

shifted from heavy fractions in samples without detergent to light fractions in samples with added detergent.

As well as demonstrating the presence of ERK in a heavy complex, the presence of higher molecular weight bands in the ERK blots of the gradient fractions revealed differences in the distribution of phosphorylated ERK isoforms. Western blots showed two species of ERK1 in the P3 fraction. Gradient fraction experiments showed the higher band, phosphorylated ERK1, to be distributed exclusively in the particles, whereas the lower band, unphosphorylated ERK1, was in both the particulate and soluble fractions. These data are exciting because there is a larger proportion of phosphorylated ERK1 present in the particles after NGF treatment (compare Figures 15a and b). This suggests there is a higher ratio of active ERK1 to normal ERK1 in the 100 000×g pellet after NGF stimulation. Of concern in these experiments is the lack of positive controls. Future gradients will include a small sample of P1M or S3 fractions to compare relative positions of ERK1 and 2. No controls are available for phospho-ERK1 or 2.

If ERK1 is activated with NGF stimulation and recruited into a heavy particle containing other proteins, this would provide an alternative explanation for retrograde transport of the NGF-activated signal from the nerve axon to the cell body. This heavy particle may consist of multiple proteins, such as TrkA, MEK, and ERK1, with perhaps a scaffold protein analogous to STE5. Such a particle would explain observed retrograde transport of TrkA, MEK, and ERK1. It has recently been shown that ERK2 homodimerizes and binds to MAP kinase phosphatase 3, a phosphatase specific to ERK. These results also suggest ERK1 homodimerizes (Khokhlatchev *et al.*, 1998) and binds MAP kinase phosphatase 3 (Camps *et al.*, 1998) which may indicate the formation of a multi-protein complex.

In summary, we found that the serine/threonine kinases ERK1 and 2 were largely located in the cytosolic fraction of PC12 cells in an inactive state, where as the majority of kinase activity was found in the P1M, P2, and P3 fractions. Investigation of the P3 fraction using glycerol velocity gradients identified ERK1 that sedimented into dense fractions was not affected by membrane disruption, suggesting it may be part of a protein complex. Stimulation of the cells with ATP or NGF caused increased ERK activity either in the P2 or P3 fractions,

indicating that ERK may associate with these fractions at different time points, or that the ERK particle varies in size or density.

4.2 FUTURE WORK

Elements of this work are exciting but not yet ready for publication. The immediate aim of further work would be to investigate the ERK1 particle. One experiment would be to subject the P3 fraction to high salt buffer before adding to the glycerol gradient, to ask if the particle is disrupted. Native gels could be run to test if ERK1 and 2 homodimerize, as has been recently reported. Other proteins that might bind to ERK could be tentatively identified by re-probing gradient fraction blots with antibodies, where available, to see if they co-localise. Candidate proteins would be MEK, which has been shown to bind to ERK in the cytosol, MAP kinase phosphatase 3, and other proteins upstream in the MAPK pathway such as Ras and Raf. Whether or not TrkA and ERK co-localise in these gradient fractions in the presence and absence of detergent should also be investigated. If they are bound in the same complex then they may co-immunoprecipitate, as has been reported by one group of investigators (Loeb *et al.*, 1992). However, the fact that detergent did not affect the ERK particles, but solubilised synaptophysin-containing membranes, makes this possibility unlikely, since TrkA is in a vesicle. This work could also be investigated by immunofluoresence. Fixed PC12 cells could be probed with anti-TrkA and anti-ERK antibodies, or antibodies for any other candidate proteins, by using separate fluorescent secondary antibodies. Separate secondary antibodies that emit at different wavelengths would be used to differentiate anti-TrkA and anti-ERK primary antibodies. Co-localisation would be detected by photomicroscopy. Fluorescent co-localisation studies would provide an exciting avenue of research, as they have already been performed successfully in our lab.

Although electron microscopy of the P3 fraction does not show obvious cytoskeletal elements (Grimes *et al.*, 1996) it would be useful to assay the P3 and P2 fractions for cytoskeletal elements such as actin or intermediate elements, as ERK associates with the cytoskeleton and phosphorylates MAPs.

A long-term goal would be to test the ability of signalling vesicles containing activated TrkA to perpetuate signal transduction *in vivo*. NGF-activated P3 fractions or vesicles pooled from gradient fractions, could be added to naïve cytosol or permeabilized cells to test for activation of the MAPK signalling pathway. Increased ERK activation in the cell extracts or activation of downstream transcription factors, such as c-fos, will indicate whether signalling vesicles initiate activation of downstream events. As a variation, different cell fractions could be combined individually with cell extracts to determine if the complex containing ERK is capable of perpetuating signal transduction. If these experiments are successful, and activated TrkA-containing vesicles are capable of signal transduction as assessed by activation of downstream events *in vitro*, then activated vesicles could be micro-injected *in vivo* to cells in the early stage of apoptosis to determine if they can reverse the processes of cell death. This would suggest that signalling vesicles containing activated TrkA are capable of conveying to the cell body, by retrograde transport, the NGF-activated signal.

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