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Purification of TrkA intracellular domain and the characterization of novel intracellular proteins

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Scott David Walker
Institute of Molecular Biosciences
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ABSTRACT

Nerve growth factor (NGF) binds to its receptor, TrkA, at the tips of nerve cell axons to inhibit apoptosis, causing survival and differentiation. Some factors within this process are largely unknown, such as the role of the p75 receptor and the molecular mechanisms that occur within the cell. NGF binding causes dimerization of TrkA, which activates the intracellular kinase domain. Autophosphorylation on tyrosine residues stimulates binding to the receptor of several intracellular proteins that mediate the NGF response. This receptor complex has been demonstrated to be retrogradely transported to the cell body. Retrograde transport is hypothesized to occur in small vesicles that have been isolated in our lab using a cell fractionation protocol using *in vitro* reactions with an ATP regenerating system. Discovering the initial molecular interactions that occur upon NGF binding could further our knowledge of NGF's inhibition of apoptosis, providing us with a possible tool for treatment of diseases that occur when the regulation of apoptosis no longer exists.

Novel proteins that were not previously identified were associated with TrkA in small vesicles after NGF activation. To isolate these proteins for further characterization, TrkA's intracellular domain (TrkAID) was expressed in *E. Coli*. This protein was found to be constitutively tyrosine-phosphorylated and therefore presumably active. In *E. Coli*, TrkAID protein was localized to the soluble fraction but smaller amounts were detected in the insoluble fraction. TrkAID was partially purified from the soluble fraction using a combination of salt disruption and denaturing techniques. The unpurified TrkAID was immunoprecipitated from the bacterial soluble fraction with an antibody to the C-terminus of TrkA, and some results suggest that immunoprecipitated TrkAID was able to stimulate ERK activation in untreated PC12 cells, but unfortunately this was not reproducible.

If the protein could be purified with a combination of techniques, then it would provide a useful tool for studying the initial events in NGF stimulation, that is, the recruitment of several intracellular proteins to the tyrosine-phosphorylated intracellular domain of TrkA.

LIST OF ABBREVIATIONS

1086	Antibody to the C-terminus of TrkA
Akt	serine/threonine protein kinase B
AP	Adapter protein
APAF	Apoptotic protease activating factor
APS	Ammonium persulphate
ATP	Adenosine Triphosphate
BB	Bud buffer
BL21 (DE3)	E. Coli expression system containing DE3 lysogen
BSA	Bovine serum albumin
CARD	Caspase recruitment domains
CM	Carboxymethyl
COS	Transformed African Green Monkey kidney cell line
CR	Conserved region
CRBP	<i>cis</i> -retinal binding protein
CRD	Cysteine rich domain
CuSO ₄	Copper sulphate
ddH ₂ O	Double-distilled water
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
ECV	Endosomal carrier vesicle
EDTA	Ethylenediamine tetraacetic acid
EGF	Epidermal growth factor
EGTA	Ethleneglycol-bis(β -aminoethyl ether) N,N,N',N'-tetraacetic acid
ERK	Extracellular regulated kinase
F	Phenylalanine
GDP	Guanosine diphosphate
GSK	Glycogen synthetase kinase
GTP	Guanosine triphosphate

Ha-Ras	Harvey Ras (mutant form of Ras)
HNGFR	High affinity NGF receptor
Hsc70	Uncoating ATPase
Hsp	Heat shock protein
IP	Immunoprecipitation
IPTG	isopropyl 1-thio-beta-D-galactopyranoside
K	Lysine
Kb	Kilobase
KDa	Kilo Dalton
KOH	Potassium hydroxide
KSR	Kinase suppressor of Ras
LB	Lennox Broth
LiCl	Lithium chloride
LNGFR	Low affinity NGF receptor
mA	milliamps
MAPK	Mitogen activated protein kinase
MAPKAP	MAPK activating protein (MEK)
MEK	MAPK or ERK kinase
Mg	Magnesium
MOPS	3-(N-Morpholino)propanesulphonic acid
MRNA	messenger ribonucleic acid
MVB	Multivesicular body
Na ₂ CO ₃	Sodium bicarbonate
NaCl	Sodium chloride
NaF	Sodium fluoride
NaOAc	Sodium acetate
NaOH	Sodium hydroxide
NGF	Nerve Growth Factor
NiCl	Nickel chloride
NIH 3T3	Contact inhibited NIH Swiss mouse embryo cell line
NSF	N-ethylmaleimide-sensitive fusion protein
PBS	Phosphate buffer saline
PC12	Adrenal Pheochromocytoma rat cell line

PEE	PBS with EDTA and EGTA
pET15b	Plasmid
pET15b-TrkAID	Plasmid containing the TrkAID construct
PGB	PBS with glucose and BSA
PH	Pleckstrin homology
PI-3K	Phosphatidylinositol-3' kinase
PLC- γ 1	Phospholipase C gamma 1
PMSF	Phenylmethylsulphonyl fluoride
Ptdins-3,4,5-P ₃	Phosphatidylinositol-3,4,5-trisphosphate
Ptdins-3,4-P ₂	Phosphatidylinositol-3,4-bisphosphate
Ptdins-3-P	Phosphatidylinositol-3-phosphate
Ras-GAP	Ras GTPase activating protein
RBD	Ras binding domain
RTA	Antibody to the extracellular domain of TrkA
SB	Sample buffer
SDS	Sodium dodecyl sulphate
SH	<i>src</i> homology
SHC	<i>src</i> homology containing protein
SNAP	SNARE associated protein
SNARE	SNAP receptors
SNT	suc-1-associated neurotrophic factor target
SOS	Son of sevenless
TAE	Tris-acetate plus EDTA
TBSI	Tris buffer saline plus IGEPAL
TE	Tris plus EDTA
TEMED	N,N,N',N' tetramethyl ethylene diamine
TGN	Trans golgi network
TNF	Tumour necrosis factor
TPA	12- <i>O</i> -tetradecanoylphorbol-13-acetate
TrkA	Tyrosine receptor kinase A
TrkAID	TrkA intracellular domain
UV	Ultraviolet

v/v	volume to volume
w/v	weight to volume
WD repeats	Tryptophan, aspartate repeats
Y	Tyrosine

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CHAPTER 1 INTRODUCTION

1.1: Overview of apoptosis

Apoptosis (regulated cell death) is an important mechanism in the development of neuronal cells. A cascade of intracellular reactions results in changes in cell structure, DNA and ultimately neuronal cell death (apoptosis). Cellular death is determined by the binding of the neurotrophin nerve growth factor (NGF) to its extracellular receptor resulting in one of two responses, proliferation or a mitogenic response *in vivo*. Which response occurs depends on the intracellular proteins that are activated.

NGF was first recognised as a neurotrophin by Levi-Montalcini and Angeletti (1963) and was shown to be important for the survival and differentiation of neurons in the peripheral and central nervous system (Barde, 1989). NGF also promoted the differentiation of the rat pheochromocytoma tumour cell line, PC12, into cells resembling sympathetic neurons (Greene and Tischler, 1976). For this reason the PC12 cell line provides a useful model to study NGF action.

The intracellular processes that occur during the inhibition of apoptosis can be split into three separate stages; the binding of NGF to its receptor on the neurite tip, transport of the NGF activated receptor from the neurite tip to the cell body, and the activation of the intracellular proteins that regulate NGF-induced inhibition of apoptosis.

Apoptosis must be tightly regulated to ensure brain function is not compromised. It has been theorised that if apoptosis is over active then the result is large amounts of cellular death resulting in the formation of certain types of dementia such as Alzheimer's disease. If apoptosis is inhibited then over-proliferation of cells is seen as in some forms of cancer. Understanding the mechanism of apoptosis may eventually lead to the ability to manipulate apoptosis as required.

1.2: Aim

The aim of the project was to characterize the initial events of NGF-induced survival by studying the intracellular proteins that bind the NGF-activated receptor and to identify any novel intracellular proteins that are activated by NGF. The following results show the existence of at least one 90 KDa intracellular protein that may not have been previously characterized or identified (Dr. M. L. Grimes, unpublished results). One other aim was to express and purify the intracellular domain of the NGF receptor, TrkA. These results could be used in conjunction with results from other members of the research group to characterize the whole process of apoptosis and to identify those steps at which regulation is crucial.

1.3: Identification of the NGF receptor

The first step in characterizing the inhibition of apoptosis by NGF was to identify the NGF receptor. Two types of NGF receptor were shown to exist in NGF-responsive primary neurons by Sutter *et al.* (1979). These receptors were also shown to exist on the membranes of NGF-responsive cell lines such as PC12 cells (Greene and Tischler, 1976, Bernd and Greene, 1984, Meakin and Shooter, 1991). One of the receptors, the low affinity NGF receptor (LNGFR), with a K_d value of 10^{-9} M, does not bind NGF very tightly and NGF dissociates from the receptor quite rapidly. The other receptor, the high affinity NGF receptor (HNGFR) has a K_d value of 10^{-11} M so NGF dissociates from this receptor in a slow manner.

In the paper by Meakin and Shooter (1991) binding of 125 I-NGF to the membranes of PC12 cells followed by chemical crosslinking showed the existence of these two NGF receptors on the cell membrane. The LNGFR resolved at 77 KDa on a SDS gel, and the HNGFR resolved at 135 KDa on the same SDS gel. They failed to immunoprecipitate any of the receptors with antibodies against *src*, *ras* or *raf-1*. This

suggested that these common intracellular signaling proteins were not in any kind of complex with either of the receptors. They also found phosphotyrosine residues on the HNGFR but not the LNGFR.

These results taken together showed that the NGF response is mediated through either one or both of the receptors. The identification of the phosphotyrosine residues on the HNGFR suggested that the HNGFR transduced the NGF signal through a kinase cascade discussed below. The next step was to characterize these receptors.

1.3.1: Identification of the HNGFR as TrkA

Kaplan *et al.* (1991a) identified the HNGFR as p140^{prototr_k}, a protein tyrosine kinase also called TrkA. TrkA is a member of the receptor tyrosine kinases that have a large extracellular ligand binding domain, a single transmembrane domain and an intracellular catalytic domain. Expression of *trk in vivo* is limited to the neural crest-derived portions of the nervous system (Martin-Zanca *et al.*, 1986).

Kaplan's results showed the activation of TrkA (tyrosine phosphorylation) in response to picomolar amounts of NGF. To test if NGF directly binding TrkA achieved activation of TrkA, ¹²⁵I-NGF was chemically crosslinked to cells and either NGF or TrkA was immunoprecipitated. In both of these immunoprecipitations, the presence of a 160 KDa protein was found. This co-precipitation was blocked by the addition of a peptide derived from TrkA. They concluded that NGF activates TrkA by directly binding it. The 160 KDa protein was believed to be TrkA and NGF. However Kaplan (1991a) did report that "p140^{prototr_k} alone does not have the binding characteristics of a high affinity receptor" suggesting a complex of the two receptors was required for the formation of a high affinity receptor for NGF. He also noted that NGF could activate TrkA in the absence of LNGFR, so TrkA might act independently of the second receptor. This begs the question, what is the role of the LNGFR?

Once the HNGFR was identified, the next step was to identify its effects *in vivo*. This was done in a study by Smeyne *et al.* (1994). They removed the *trk* proto-oncogene in mice embryonic stem cells. The resulting abnormalities showed that TrkA was the primary mediator of NGF action in the development of both the peripheral and central nervous system.

Hempstead *et al.* (1992) also demonstrated the importance of TrkA in mediating the NGF response. NGF-treated PC12 cells developed neurite extensions after two days following continual NGF exposure. When *trk* was overexpressed in PC12 cells NGF-mediated, neurite extension occurred in a matter of hours rather than days. This demonstrated that TrkA was important for NGF mediated neurite extension in PC12 cells and that NGF treatment of these cells resulted in an increased level of tyrosine phosphorylation of TrkA. This suggested a mechanism for the propagation of the NGF signal through the use of a signaling cascade system mediated by TrkA tyrosine kinase activity, which will be discussed in greater detail in section 1.4.

All these results had suggested a model for TrkA function. The function of LNGFR was less clear. It was also unclear whether TrkA had any effect on the activity of LNGFR. To look at these points the activity of the LNGFR needed to be determined.

1.3.2: The role of p75^{LNGFR} in NGF signaling.

The role of the LNGFR receptor (p75) had been debated for a number of years. It had been theorized that if the neurotrophins were present in picomolar concentrations, then the formation of high affinity binding sites would act to discriminate similar ligands. Since all the neurotrophins bound p75 it was possible that p75 assisted in the binding of the correct neurotrophin to the appropriate Trk receptor (Rodriguez-Tebar *et al.*, 1992). The important question was whether p75 acted as a presenter of NGF or whether p75 formed a complex with TrkA. Another possibility was that p75 acted as a separate receptor with either a separate signaling cascade system or some influence on the TrkA signaling cascade system.

p75 forming a complex with TrkA to form the high affinity binding site

There was conflicting evidence supporting the theory that p75 was in complex formation with TrkA. Low concentrations of NGF binding to p75 enhanced binding of NGF to TrkA and its autophosphorylation (Barker and Shooter, 1994; Verdi *et al.*, 1994) as well as activation of the immediate early gene *c-fos* (Barker and Shooter, 1994). Physical evidence for the formation of a complex between p75 and TrkA is scarce (Jing *et al.*, 1992).

One important event to note was that stimulation of PC12 cells with NGF resulted in the induction of p75 (Higgins *et al.*, 1989) and TrkA mRNA levels (Meakin *et al.*, 1992). When p75 and TrkA were co-expressed in the proper ratio, substantial numbers of high affinity binding sites were observed. Deletion mutants in p75 abolished high affinity binding sites (Hempstead *et al.*, 1990). This suggested that an intact p75 was required for the formation of the high affinity binding sites and that their formation was dependent on particular ratios of p75 to TrkA. The level of p75 mRNA was five to ten times greater than the level of TrkA mRNA in PC12 cells (Chao and Hempstead, 1994). These mRNA levels have been confirmed using ¹²⁵I labelled NGF to count the number of p75 and TrkA receptors (Meakin and Shooter, 1994). Studies by Ip *et al.*, (1993) and Ibanez *et al.*, (1992) excluded a role of p75 from neurotrophin action, but they did not analyse NGF binding under high affinity conditions, nor did they measure the mRNA levels of the two receptors.

The above results are evidence for the role of p75 as a partner for the high affinity-binding site for NGF. The high affinity-binding site could be assumed to be a multimeric complex of p75-TrkA proteins.

Does TrkA function dependently or independently of the LNGFR?

Below is depicted a diagram showing two possible models describing the co-operation of TrkA and p75.

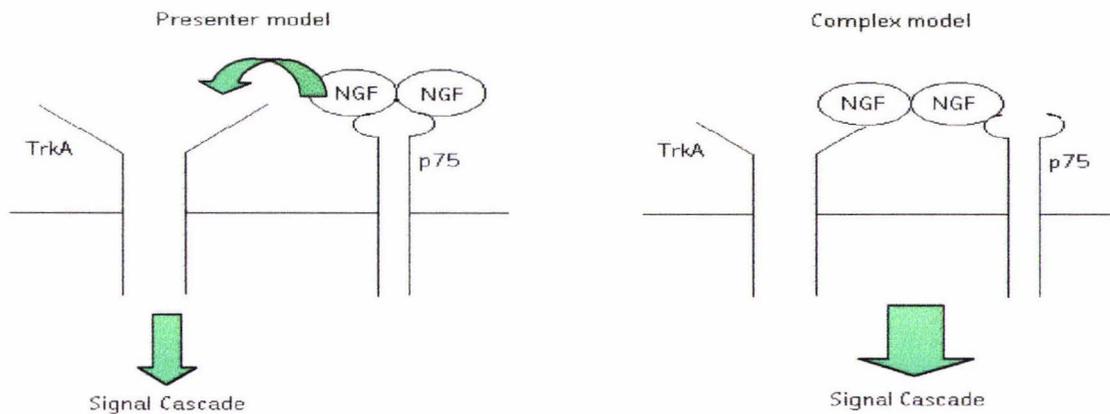


Figure 1
Function of p75

In the presenter model, p75 binds NGF with low affinity. P75 is co-localized on the plasma membrane with TrkA, therefore NGF is able to bind TrkA with higher affinity. Thus p75 acts as a presenter.

In the complex model, TrkA and p75 are in a complex to form the high affinity receptor for NGF. Once NGF is bound to the complex it stimulates a kinase cascade through TrkA

The function of the two receptors with different affinity for NGF was a matter of debate. Was the NGF binding site a combination of TrkA and different factors, such as the other NGF receptor? Was TrkA involved in both the low affinity and high affinity NGF binding sites or just one? How did TrkA function when NGF was bound? Evidence for the existence of the HNGFR being a complex between p75 and TrkA was put forward by Hempstead *et al.* (1992). Overexpression of *trk* resulted in an increase in both the high affinity and low affinity binding sites suggesting TrkA seemed to be involved in the formation of both these sites.

Ross *et al.* (1996) provided evidence for the existence of a complex of the two receptors. Using antibodies to p75^{LNGFR} and TrkA, followed by fluorescent secondary antibodies, they showed that p75^{LNGFR} co-patched with TrkA in both the absence and presence of NGF. p75^{LNGFR} was not found to co-patch with other receptor tyrosine kinases, so the association between the two receptors was quite specific. Expression of chimeric receptors identified the extracellular domain of TrkA as sufficient for this co-patching. A point mutation of the TrkA kinase domain did not affect the co-patching but a deletion of the intracellular domain reduced it. This paper indicated

that the p75^{LNGFR} and TrkA might form a complex to mediate the NGF response. Complex formation required the TrkA extracellular domain but the intracellular domain may also play a role.

This paper does not prove the existence of heterodimers of the two receptors; it only provides evidence for the co-localization of them. Therefore, this result could also be evidence for the presenting model. If the two receptors are in close proximity to each other then it is plausible that the p75^{LNGFR} acts as a recruiter of NGF for TrkA.

One study that disproved the complex theory and provided an answer to TrkA function was performed by Jing *et al.* (1992). Using cell lines expressing TrkA, p75^{LNGFR} or both together they found that NGF induced the tyrosine phosphorylation of TrkA in the presence and absence of p75^{LNGFR}. Crosslinking studies revealed the existence of TrkA and p75^{LNGFR} homodimers but no heterodimers were detected. Sutter *et al.* (1979) only mediated the NGF biological response through TrkA, which formed homodimers and exhibited a dissociation constant equivalent to that of the HNGFR reported previously. Co-expression of wild type and kinase deficient TrkA receptors in COS cells revealed that TrkA was capable of undergoing autophosphorylation on tyrosine residues in response to NGF. Furthermore, co-expression of the kinase deficient mutant and wild type TrkA inhibited the NGF response. These results suggested that the HNGFR consisted of TrkA alone. The NGF response appeared to be dependent on the formation of TrkA homodimers and this dimer formation was critical for the NGF-induced tyrosine phosphorylation of TrkA through its own kinase domain. The authors suggested that the p75^{LNGFR} functions as a “presenter” of NGF to TrkA by localizing NGF to regions of the membrane where TrkA receptors are clustered with p75^{LNGFR}.

Evidence for a functional dissociation between the two was also shown by Ibanez *et al.* (1992). They mutated critical lysine residues on NGF (K32, K34 and K95) required for NGF binding to p75^{LNGFR} and found that abolishing the binding of NGF to p75^{LNGFR} led to no inhibition of NGF biological activity.

Further evidence for the functional dissociation of the two receptors was provided by other groups (Cordon-Cardo *et al.*, 1991, Rovelli *et al.*, 1993). These studies used a

combination of experimental techniques. Cordon-Cardo *et al.* transfected NIH 3T3 cells with TrkA and found that a mitogenic response was induced upon treatment with NGF. This mitogenic response was abolished in cells not expressing TrkA. Rovelli *et al.* transfected PC12 cells with chimeric receptors consisting of the extracellular domain of tumour necrosis factor (TNF) receptor and the transmembrane domain and cytoplasmic domain of either TrkA or p75^{LN_GFR}. TNF treatment of these cells resulted in neurite extension in those cells transfected with the TNF-TrkA chimera. No response to TNF was observed in those cells transfected with the TNF-p75^{LN_GFR} chimera. This latter result supported the theory that TrkA is sufficient in mediating the NGF response.

All the above results have led to the following model of TrkA activation in NGF signalling. TrkA appears to be the primary receptor for NGF and the main mediator of the NGF response. Binding of NGF to TrkA results in the formation of TrkA homodimers. This results in the activation of the receptors kinase domain leading to cross-phosphorylation of tyrosine residues on the intracellular domain of the receptor providing a start for the signal to be transduced within the cell via a signaling cascade system. The protein pathways involved in this cascade will be discussed later in section 1.4. It remains unclear as to whether TrkA forms a complex with p75 or whether it acts independently to form the HNGFR. If it acts independently the question must be asked; what is the function of p75?

p75 stimulates a signaling cascade

Another possible role for p75 is that it initiates a separate signaling cascade system or modifies the TrkA signaling cascade. The suggestion of a signaling cascade function for p75 is due to its structural resemblance to the TNF receptor family. A number of protein kinases have been found to be associated with p75 (Ohmichi *et al.*, 1991) including the mitogen activated protein kinases (MAPK or extracellular regulated kinase, ERK) (Volonte *et al.*, 1993b). The manner in which p75 contributes to NGF signaling could either be through a direct interaction of NGF to p75 or indirectly via TrkA activation.

Canossa *et al.*, (1996) showed the association of a protein kinase approximately 120 KDa in size with the p75 receptor that was activated upon NGF treatment in PC12 cells. It was undetermined if the protein kinase was constitutively associated with the p75 receptor or whether the protein kinase was recruited upon NGF stimulation. This protein kinase was responsible for most of the kinase activity found associated with the receptor. In addition to this protein kinase they observed the association of two other protein kinases of approximately 44 KDa and 56 KDa. The authors suspected that the 44 KDa kinase may be ERK but this remains unclear. The identity of the 56 KDa kinase was also unknown. The rapid activation of the 120 KDa-protein kinase suggested that it lies upstream of ERK in the NGF signaling cascade. Since the p75 receptor was suggested to have some role in ceramide signaling (Dobrowsky *et al.*, 1994), Canossa *et al.* tested the protein kinase for activation with a ceramide homologue but found that the protein kinase was not a ceramide-activated protein kinase. The activation of this protein kinase was rapid when the NGF concentration was at low levels and was dependent on both TrkA autophosphorylation and NGF binding to p75. In the absence of NGF, or when the ratio of TrkA to p75 was decreased, only slow activation of this kinase was observed. The requirement for TrkA and TrkA activation in the activation of this protein kinase by NGF suggested a functional coupling of the two receptors, if not a physical coupling.

A review by Bredesen and Rabizadeh (1997) suggested TrkA-independent and TrkA-dependent effects for p75. Without ligand binding a pro-apoptotic signal is generated, once NGF binds TrkA the signal is blocked and other, anti-apoptotic signaling events take over. This pro-apoptotic signal may be stimulated by the p75 receptor and is believed to be TrkA independent. NGF binding to p75 resulted in ceramide formation (Dobrowsky *et al.*, 1994) and in some systems, ceramide has been shown to inhibit apoptosis (Ito and Horigome, 1995). This effect on p75 could require the interaction of the p75 transmembrane domain or extracellular domain with another molecule (Bredesen and Rabizadeh, 1997).

The TrkA-dependent effects of p75 include mutual signaling with TrkA and the role of p75 in the formation of high affinity binding sites with TrkA. Hempstead *et al.*, (1990,1991) demonstrated that co-expression of TrkA with p75 resulted in an increase

in the number of high affinity binding sites than TrkA alone. It was possible that the anti-apoptotic effect of low levels of NGF could require the presence of p75 without NGF stimulating a signal from p75. Also, as shown in the paper by Canossa *et al.*, (1996), the ratio of p75 to TrkA was critical to the cellular response indicating mutual effects on signalling by TrkA and p75. Finally, it was possible that p75 and TrkA exhibit mutual repression. The expression of one of the signalling cascades inhibited the anti-apoptotic effect of the other signaling pathway. A maximal signal was only generated by co-stimulation, which relieved the mutual repression (Bredesen and Rabizadeh, 1997). It could also be possible that p75 inhibits the anti-apoptotic effect of TrkA by binding directly to it. This would decrease the chance of TrkA dimerization thus decreasing the amount of anti-apoptotic signaling that would otherwise occur.

The p75 receptor may cause apoptosis when expressed in the absence of TrkA. It may also enhance binding and presentation to TrkA, promoting survival. To summarize the evidence presented here there is a suggestion of a role for p75 as a member of the high affinity-binding site (see Figure 2). TrkA can dimerise and initiate an anti-apoptotic signal (discussed above). Either p75 can signal independently of TrkA in a pro-apoptotic manner, or it can associate with TrkA to decrease the anti-apoptotic signal thus initiating a pro-apoptotic response. Upon NGF binding the pro-apoptotic signal is inhibited and the TrkA anti-apoptotic signal is stimulated.

The model shown in Figure 2 is a crude representation of what occurs within the cell, simplified for the convenience of explanation. With future study into the role of p75, a clearer model will be presented.

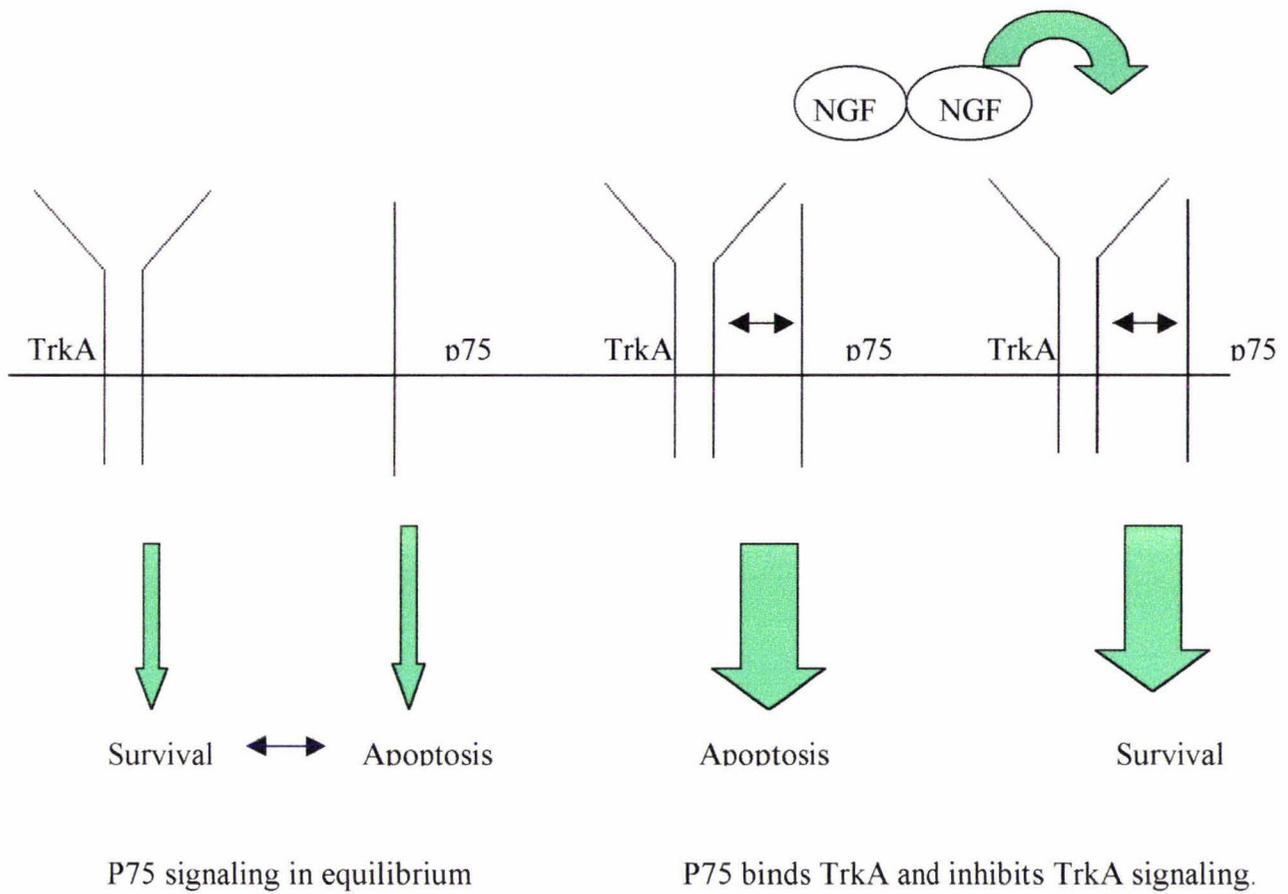


Figure 2
Role of p75 in signaling

P75 may be responsible for stimulating a signaling cascade that results in apoptosis. This cascade may be inhibited once TrkA, and thus survival, is stimulated by NGF binding. Therefore, the signaling cascades are in an equilibrium with each other. P75 may bind TrkA thus inhibiting the cascade that stimulates survival, resulting in apoptosis. Once NGF binds TrkA the survival signal is stimulated inhibiting apoptosis.

1.4: The TrkA Signaling Cascade System

Once the receptor for NGF had been discovered, the next issue to be determined was how the NGF signal was transduced from TrkA. The answer to this first came from the fact that TrkA underwent autophosphorylation on tyrosine residues in response to NGF (Kaplan *et al.*, 1991a). Hempstead *et al.*, (1992) showed the phosphorylation of several intracellular proteins in PC12 cells overexpressing TrkA, including the appearance of several tyrosine phosphorylated intracellular proteins in response to NGF (Maher *et al.*, 1988).

1.4.1: TrkA recruits intracellular proteins to its intracellular domain

The paper by Hempstead *et al.* (1992) identified two proteins that were tyrosine phosphorylated upon NGF stimulation, PLC- γ 1 and ERK-1. Vetter *et al.* (1991) linked PLC- γ 1 and TrkA activation by NGF. Immunoprecipitation of PLC- γ 1 from NGF-treated cells also co-precipitated a 140 KDa protein with kinase activity that they identified as TrkA by immunodepletion with antibodies against TrkA. These results taken together provided strong evidence for the propagation of the NGF signal in cells through TrkA tyrosine kinase activity.

Other intracellular proteins were activated in response to NGF. Phosphatidylinositol-3 kinase (PI-3K) was shown to interact with TrkA within minutes of NGF stimulation (Soltoff *et al.*, 1992). The activation of ERK-1 was also shown to occur after NGF treatment (Loeb *et al.*, 1992, Hempstead *et al.*, 1992). Rozakis-Adcock *et al.* (1992) showed that *src* homology containing proteins (SHC) are recruited to TrkA intracellular domain in response to NGF. An important step was identifying those proteins that associated with TrkA either directly or indirectly, and identifying the sites at which these proteins bind.

Intracellular proteins interact with TrkA on specific phosphotyrosine residues

Obermeier et al., (1993a) showed NGF activation of TrkA caused the association and activation of PLC- γ 1, ras GTPase activating protein (ras-GAP), and the non-catalytic subunit of PI-3K (p85). They constructed chimeric receptors consisting of the EGF extracellular domain and the TrkA transmembrane and intracellular domains. This chimeric receptor showed the association with TrkA and tyrosine phosphorylation of PLC- γ 1, rasGAP and PI-3K. A deletion of the cytoplasmic tail, involving the removal of the terminal 15 amino acids including Y-785 inhibited activation and association of the intracellular proteins with TrkA. A single point mutation (Y785F) resulted in the loss of PLC- γ 1 activation and association with TrkA, but only a small reduction in activation of PI-3K and rasGAP was seen. These results indicated that the cytoplasmic tail of TrkA is critical for protein recruitment and activation. It also showed that PLC- γ 1 binds TrkA at Y785.

In a subsequent study (Obermeier *et al.*, 1993b) the binding and activation of two other signaling proteins, PI-3K and SHC, was investigated. Rozakis-Adcock *et al.* (1992) had previously shown that SHC links TrkA to the *ras* pathway through other intracellular proteins. Obermeier *et al.* (1993b) expressed a number of peptides that contained each of the tyrosine residues found on the intracellular domain of TrkA. They discovered that the peptide containing Y-490 inhibited the TrkA-dependent activation of SHC and inhibited the association of SHC with TrkA. The peptide containing Y-751 blocked the TrkA dependent activation of p85 as well as the association of p85 with TrkA. This paper identified the binding sites of p85 (Y-751) and SHC (Y-490).

SHC had been shown to contain a *src* homology domain by Pelicci *et al.* (1992). Other intracellular signaling proteins that had been shown to associate with NGF-activated TrkA also contain an SH2 domain. It is through these SH2 domains that proteins are able to specifically bind NGF-activated TrkA on the receptors phosphotyrosine residues (reviewed in Cantley *et al.*, 1991).

Different signaling cascades are activated by TrkA to mediate the NGF response

Once the binding sites of these intracellular proteins had been identified, the next step was to determine the roles of these proteins. Stephens et al. (1994) did this by mutating the binding sites of PLC- γ 1 (Y-785) and SHC (Y-490) on TrkA. They expressed these mutant receptors in cells that do not express any endogenous TrkA. Mutations in either of these binding sites had no effect on NGF-mediated neurite extension and ERK-1 activation. Mutations in both binding sites resulted in the inhibition of NGF-mediated neurite extension and ERK-1 activation. Although neurite extension and ERK-1 activation did not occur upon NGF treatment of the receptors containing the double mutation, phosphorylation of an intracellular protein called src 1-associated neurotrophic factor target (SNT) still occurred. These results provided evidence for the existence of two independent signaling pathways. There is some redundancy in NGF signaling; if one pathway is inhibited then the other pathway can still mediate NGF-induced neurite extension. The protein SNT seems to be involved in a separate, ERK-1 independent, signaling pathway.

The roles of the different domains of TrkA in NGF signaling were determined in a study by Peng *et al.* (1995). They deleted a conserved region of the juxtamembrane domain. NGF treatment of cells expressing these mutant receptors did not promote neurite extension but did enhance survival. Cells expressing the mutant receptors were still receptive to NGF-stimulated survival and were capable of autophosphorylation as well as activation of the proteins involved in the *ras*-dependent pathway. SNT was not activated upon treatment with NGF. These results indicated that *ras*-dependent pathways mediate NGF-stimulated survival. The results also suggested that *ras*-independent pathways mediate neurite extension induced by NGF. One of the *ras*-independent pathways involves the activation of the protein SNT. This result also shows that TrkA can stimulate signaling pathways through methods other than kinase activity. This does not exclude the theory that both *ras*-dependent and independent pathways mediate neurite extension. There is evidence for the requirement of *ras* for NGF-stimulated neurite extension (Thomas *et al.*, 1992, Hagag *et al.*, 1986).

The binding of NGF to its receptor stimulates signaling cascades within the cell that mediate the response. These signaling pathways occur in the cell body with the protein targets being located in the nucleus. For these targets to be activated the signal needs to be transported from the plasma membrane through signaling vesicles (Grimes *et al.* 1996, 1997). These signaling vesicles are produced by endocytosis.

1.5: Endocytosis

The method of endocytosis discussed in this section is clathrin-mediated, also known as receptor-mediated endocytosis. Clathrin-independent endocytosis can also occur but will not be dealt with in this discussion. Endocytosis involves budding of a region of the plasma membrane containing NGF-bound TrkA to form a clathrin-coated vesicle. This vesicle containing activated TrkA as well as several intracellular proteins fuses and buds off from various intracellular organelles to arrive at its destination within the cell body. These organelles include early and late endosomes, lysosomes, endoplasmic reticulum and golgi. Endocytosis occurs for receptor recycling, protein degradation as well as transport of the NGF signal.

Coated vesicles are composed of clathrin triskelia, clathrin light and heavy chains arranged into a three-armed (triskelion) molecule. Triskelion molecules arrange themselves into “cages” to coat the vesicle. Assembly of the clathrin coat is dependent on the recruitment of AP2 proteins. AP2 recruitment to the section of plasma membrane that is to be budded off is believed to occur through its high affinity for the cytoplasmic domains of various proteins that associate with the plasma membrane. This AP2 lattice then provides a site where the clathrin triskelia can form a coat (Robinson, 1994; De Camilli and Takei, 1996). Formation of clathrin coats is ATP and GTP-dependent. Subsequent bending of the coated patch of membrane to form a dome shaped vesicle then a vesicle with a long narrow neck can occur in ATP-depleted preparations (Schmid, 1993).

The formation of a free vesicle from this budded state requires the activity of a protein called dynamin. Dynamin forms a ring at the neck of invaginated clathrin pits and is able to stimulate GTP hydrolysis. Hydrolysis of GTP could twist the ring conformation of dynamin to sever the neck (reviewed in DeCamilli and Takei, 1996). Dynamin contains a pleckstrin homology (PH) domain and proline rich C-terminus. This proline rich sequence can bind SH3 domains of various intracellular proteins (Gout *et al.*, 1993). The neuronal protein amphiphysin is co-localized with dynamin nerve terminals and interacts with AP2 (Wang *et al.*, 1995; David *et al.*, 1996). Therefore amphiphysin may play a role in recruiting dynamin at clathrin coats. Dynamin could also interact directly with AP2 or proteins that contain SH3 domain such as Grb2, PI3K or PLC γ -1.

After fission, the vesicles need to shed their clathrin coats. This occurs via an ATP-dependent reaction that involves the heat shock protein Hsc70 (uncoating ATPase) and Auxilin (Ungewickell *et al.*, 1995). Auxilin binds to the clathrin coat recruiting Hsc70 to it. This disrupts the clathrin lattice and releases the coat (Ungewickell *et al.*, 1995).

The requirement for ATP in vesicle budding and fusion can be utilized for our purposes (see Figure 3). *In vivo*, vesicles fuse with the target compartment in an ATP-dependent manner. In cells that have been permeabilised, the vesicles will leak out of the cell instead of fusing with their target compartment. The addition of an *in vitro* reaction with an ATP regenerating system (ATP, creatine kinase and creatine phosphate) will increase the number of vesicles formed and these vesicles will be unable to fuse with intracellular organelles. This would provide an opportunity for selection of a large population of signaling vesicles in permeabilised PC12 cells.

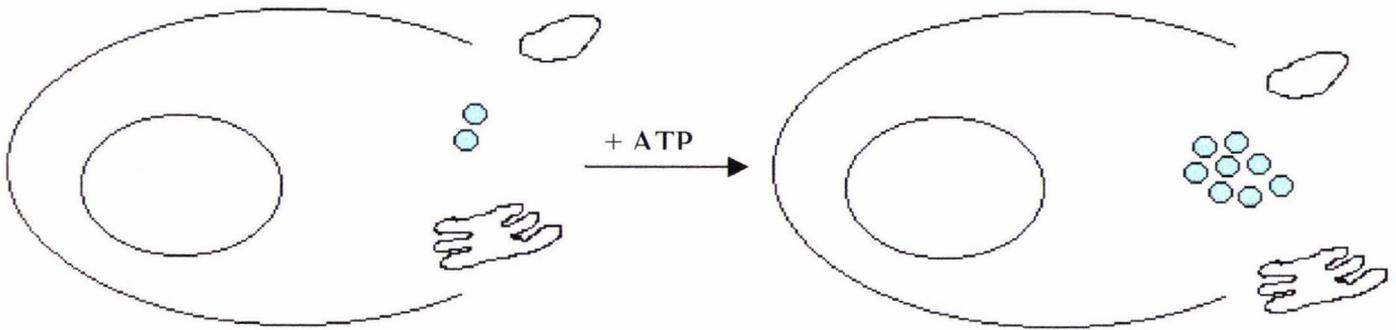


Figure 3

In vitro reaction with ATP

After mechanical permeabilisation, cells are exposed to an *in vitro* reaction with ATP (ATP, creatine kinase and creatine phosphate) for 15 minutes at 37°C. This allows the formation of small vesicles, which leak out of the cell rather than fusing with their target organelles. This enables us to select for a larger population of small vesicles than would be obtained in the absence of an *in vitro* reaction with ATP.

Clathrin-coated vesicles can fuse with early endosomes but not with late endosomes. This specificity is due to the presence of SNAP proteins, target SNAP receptors (t-SNARE) and NSF on the surface of some intracellular compartments. The vesicle has on its surface vesicle SNAP receptors (v-SNAREs) which bind the SNAP proteins on the surface of the compartment. NSF then stimulates fusion of the vesicle to the compartment (Rothman and Warren, 1994; Diaz *et al.*, 1989). After fusion with the early endosomes, proteins that are destined for the late endosome are separated from those that are recycled to the plasma membrane. Those proteins destined for the late endosome are packaged into small spherical vesicles called multivesicular bodies (MVBs) or endosomal carrier vesicles (ECVs) (reviewed in Gruenberg and Maxfield, 1995). Sorting and recycling of proteins occurs in regions of the early endosome that differ in their structure (Gruenberg and Maxfield, 1995).

Proteins that have been implicated in membrane transport are small GTPases of the Rab family. Early and late endosomes each contain a unique set of Rab proteins. Rab

5 mediates early endosome fusion (Gorvel *et al.*, 1991). Rab 4 has been implicated in the recycling process from early endosomes back to the plasma membrane (Van der Sluijs *et al.*, 1992). Rab 9 is required for transport from the late endosome to the trans-golgi network (TGN) and lysosome biogenesis (Riederer *et al.*, 1994). The exact role of Rab proteins is unclear, but it is believed that GTP hydrolysis aids vesicle fusion.

Evidence for the internalization of activated TrkA can be found in studies by Grimes *et al.*, (1996) and Grimes *et al.*, (1997). Through confocal microscopy they determined that TrkA was localized in intracellular organelles near the plasma membrane. Clathrin co-localized with TrkA indicating that the intracellular organelles were clathrin-coated vesicles from endocytosis. They found NGF present in both large and small vesicles along with activated TrkA. This was good evidence signaling endosomes containing activated TrkA could retrogradely transport the NGF from the neurite tip to the cell body.

Once a vesicle containing NGF-bound TrkA and several intracellular proteins also bound to TrkA, such as Ras and Raf (discussed in section 1.6.1) is generated then the vesicle can be transported to the nucleus, where target proteins such as MEK and ERK are located, to stimulate signal transduction.

1.6: Signal Transduction

1.6.1: The MAPK/ERK pathway

The signaling pathway leading to the activation of MAPK, or ERK, is the best-characterized pathway and is summarized above (Figure 4). Ras binds and activates Raf. Raf then activates MAPK or ERK kinase (MEK), which is responsible for the activation of ERK. Activation of ERK by NGF has been seen in previous studies (Loeb *et al.*, 1992, Hempstead *et al.*, 1992). The ERK pathway has been shown

to be important for neuronal differentiation in PC12 cells (Pang *et al.*, 1995). When the activity of MEK was inhibited the NGF-dependent formation of neurites was consequently inhibited suggesting the ERK pathway is responsible for mediating NGF-stimulated neurite extension. ERK activates a number of essential proteins within the cell in response to NGF signaling and so the pathways that stimulate ERK have been closely studied.

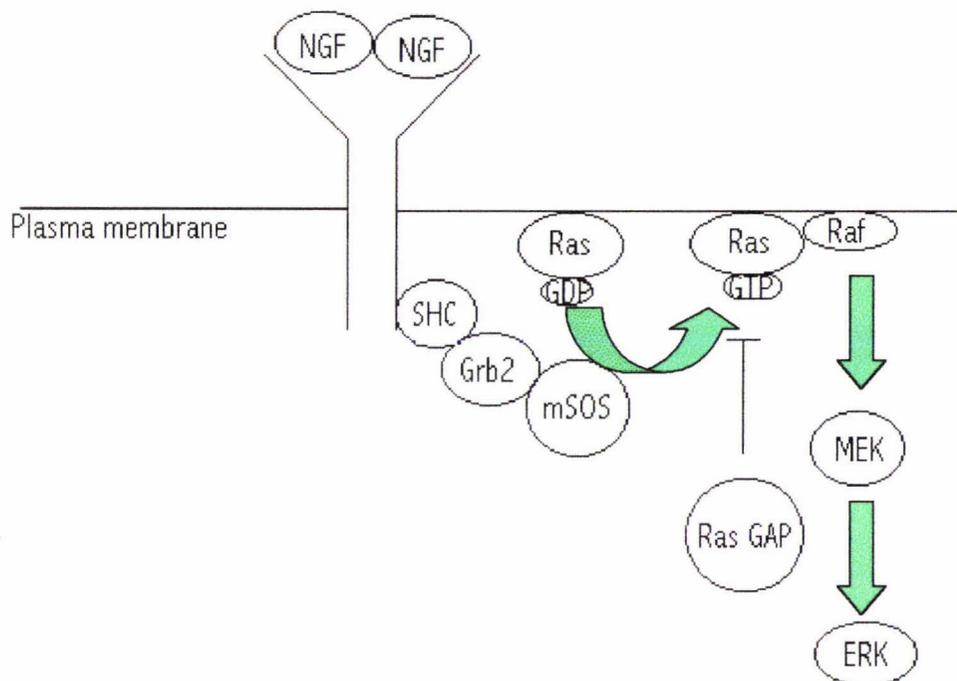


Figure 4
ERK Pathway

Ras activation of the ERK pathway

The protein Ras was believed to have played a pivotal role in the signal transduction of many cell types (Barbacid, 1987). Activated Ras (Ras bound to GTP) was able to induce the differentiation of PC12 cells to sympathetic neuron-like cells (Bar-Sagi and Feramisco, 1985; Noda *et al.*, 1985). Ras has some GTPase activity so over time, the

complex becomes Ras bound to GDP, the inactive form of Ras. The substitution of GDP for GTP is catalyzed by a guanidine nucleotide exchange factor that has sequence homology to the *Drosophila* protein, son of sevenless (mSOS). Ras GTPase activating protein (Ras-GAP) is responsible for stimulating the hydrolysis of GTP to GDP thus deactivating Ras. Ras-GAP has been shown to be a substrate of TrkA by Cantley *et al.*, (1991) and it accumulates in PC12 cells upon NGF stimulation (Qui and Green, 1991; Muroya *et al.*, 1992; Nakafu *et al.*, 1992). This demonstrates the complexity of NGF signalling since these results indicate NGF binding to TrkA activates Ras-GAP. Nakafu *et al.*, (1992) demonstrated that Ras-GTP accumulated in cells and that this accumulation could be inhibited by the addition of a tyrosine kinase inhibitor, so Ras must have some role in NGF signaling.

Once Ras had been shown to participate in NGF signaling, Thomas *et al.* (1992) determined its role. They expressed a dominant inhibitory Ras mutant in PC12 cells. After exposure to NGF, cells expressing the mutant Ras were deficient in the activation of ERK-1 and 2. Expression of a constitutively active form of Ras in PC12 cells was sufficient to activate ERK-1 and 2. They looked at other possible roles for Ras. Ras played no part in activating the kinase activity of TrkA, nor did Ras affect the tyrosine phosphorylation of PLC γ -1. This report separated distinct signaling pathways activated in response to NGF. These are a Ras-dependent pathway (ERK activation), and a Ras-independent pathway (PLC γ -1 activation, SNT activation).

The next step was identifying the mechanism by which Ras is activated. Ras is active when it is complexed to GTP and inactive when it is complexed to GDP, therefore the activation of Ras must require the activity of the nucleotide exchange factor (mSOS). In *C. elegans* a small protein, Sem-5, is believed to be responsible for the formation of a protein complex that results in the activation of Ras (Clark *et al.*, 1992; Pawson, 1992). A mammalian homologue, Grb2, has been cloned in screens for SH2 containing proteins (Matuoka *et al.*, 1992). Grb2 contains two SH3 domains in addition to the SH2 domain.

The protein SHC binds the intracellular domain of TrkA (Rozakis-Adcock *et al.*, 1992). Grb2 has been shown to stimulate the formation of a multiprotein complex. Once SHC binds to the intracellular domain of TrkA it is phosphorylated on specific

tyrosine residues. Grb2 binds SHC through its SH2 domain. Egan *et al.* (1993) showed that the Ras guanidine nucleotide exchange protein binds the SH3 domain of Grb2 through its proline rich domain, localizing mSOS to the plasma membrane for guanidine nucleotide exchange on Ras.

Ras binds and activates Raf-1

The function of Ras in the signaling pathway was clarified once it was discovered Ras is complexed with the serine/threonine kinase Raf-1 (Avruch *et al.*, 1994; Vojitek *et al.*, 1993). It is thought Ras localizes Raf-1 to the plasma membrane. Raf-1 activation is dependent upon Ras' C-terminal farnesylation (Lerner *et al.*, 1995; Okada *et al.*, 1996). Inhibition of Ras farnesylation leads to the formation of Ras-Raf complexes in the cytosol (Lerner *et al.*, 1995) indicating the addition of long chain fatty acids to the C-terminus of Ras is required for Ras localization to the plasma membrane consequently localizing Raf to the plasma membrane. Raf-1 activation in the absence of Ras can be achieved if Raf-1 is targeted to the plasma membrane using a membrane-targeting signal (Leevers *et al.*, 1994).

The site of Ras binding on Raf was subsequently identified and termed the Ras binding domain (RBD). It was shown to be contained within residues 51-131 (Vojitek *et al.*, 1993; Nassar *et al.*, 1995; Gorman *et al.*, 1996; Herrmann *et al.* 1995; Hermann *et al.*, 1996). Ras binds Raf through this domain with high affinity (Herrmann *et al.*, 1995; Herrmann *et al.* 1996).

A second Ras binding domain containing the cysteine rich domain (CRD) was found to also contain a zinc finger (Mott *et al.*, 1996). This cysteine rich domain spans residues 139-184. It is unclear whether Ras binding to CRD is GTP-dependent or whether Ras needs to be modified with a long chain fatty acid to bind. Roy *et al.* (1997) showed the RBD was sufficient for the recruitment of Raf-1 to the plasma membrane and the Zinc finger played no part in this recruitment. They also demonstrated that the presence of the Zinc finger was required for membrane localization of Raf-1. By using a constitutively active form of Raf-1, they found Raf-1 activity was only fully achieved when Ras was co-expressed even though Raf-1 was constitutively associated with the plasma membrane. This sensitivity to Ras was abolished by a mutation in the Zinc

finger region of Raf-1, suggesting two roles for Ras in Raf-1 activation; binding of Ras to the RBD for localization to the plasma membrane and Ras binding to the CRD for Raf-1 activation in a manner that requires the Zinc finger.

The co-association of Ras and Rap-1A, a protein with an identical effector domain for Raf-1 binding, interferes with the Ras-dependent activation of Raf-1 (Hu *et al.*, 1997) because of its ability to bind Raf-1. Although Rap-1A has high sequence homology with Ras as well as an identical effector domain it was not able to activate Raf-1. Critical residues in the Ras effector domain were replaced with residues in the same position on Rap-1A. This mutant form of Ras (Ha-Ras) was found to bind Raf-1 with high affinity, as was Rap-1A, through their RBD and CRD respectively. Association of Ha-Ras and Rap-1A with Raf-1 prevented its activation. This proved Ras binds to Raf-1 on two domains. One domain for localization to the plasma membrane (RBD) and a second Ras interaction site that is required for Raf-1 activation.

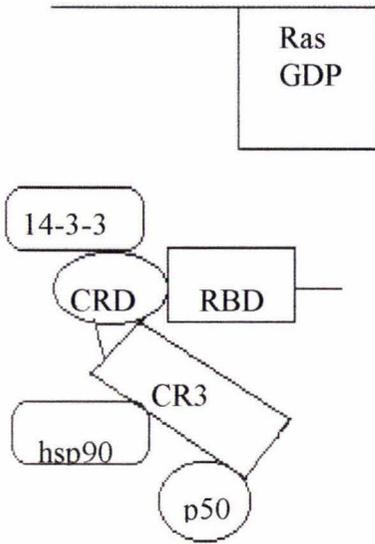
The activation of Raf-1 is more complicated than the association of Ras with Raf-1. The physiological substrate of Raf-1, MEK, is also believed to bind Raf-1 (Van Aelst *et al.*, 1993; Catling *et al.*, 1995) as are members of the protein family 14-3-3 (Morrison, 1994). The 14-3-3 interaction was particularly confusing because it has been shown to be constitutively associated with Raf-1 regardless of the activation state of Raf-1, and has also been shown to suppress Raf-1 activity (Irie *et al.*, 1994; Fantl *et al.*, 1994; Freed *et al.*, 1994; Michaud *et al.*, 1995). 14-3-3 is a specific phosphoserine binding protein and Raf-1 has two such phosphorylation sites, S-259 and S-621. Mutations in the CRD of Raf-1 abolish 14-3-3 binding suggesting that 14-3-3 interacts with Raf-1 in the CRD (Michaud *et al.*, 1995). Other proteins that have been shown to bind Raf-1 include the heat shock proteins hsp90 and hsp50 (Wartmann *et al.*, 1994; Stepanova *et al.*, 1996). Kinase suppressor of Ras (KSR) also binds to Raf-1 at the plasma membrane in a Ras-dependent manner (Therrien *et al.*, 1996).

A model of Raf-1 activation was proposed (Morrison and Cutler, 1997) (Figure 5). There are three conserved regions of Raf-1. Conserved region 1 (CR1) contains the RBD and CRD. CR2 (residues 254-269) is rich in threonine and serine residues. CR3 is the kinase domain of Raf-1. Ras binds to the RBD of Raf-1 causing plasma membrane localization. This allows the binding of Ras to the second binding site

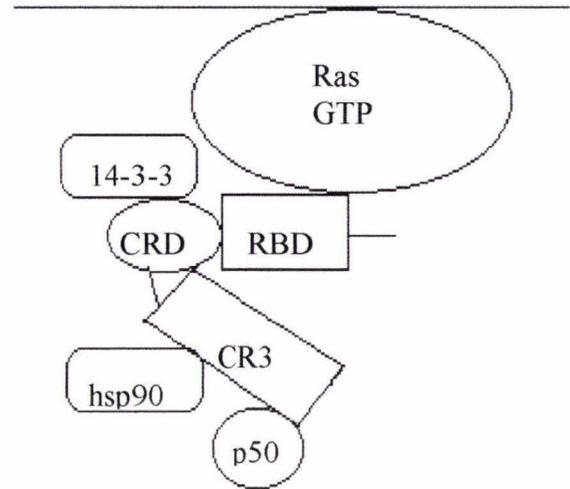
(CRD). This second binding cannot occur without Ras binding first to the RBD (Drugan *et al.*, 1996). The CRD may also be responsible for bringing together other protein members of the protein complex (Michaud *et al.*, 1995). The presence of 14-3-3 may maintain Raf-1 in a conformation that prevents Ras binding to CRD. This would reduce the chance of cytosolic active Ras-Raf-1 complexes, which could harm the cell. This was proposed because the mutations that allow Ras to bind CRD in the absence of RBD binding occur in the residues responsible for 14-3-3 binding (Michaud *et al.*, 1995). 14-3-3 may play a secondary role in stabilizing the active conformation of Raf-1. This interaction would prevent the inactivation of Raf-1 by phosphatase treatment since it could interact with phosphoserine 621 (Muslin *et al.*, 1996; Dent *et al.*, 1995). 14-3-3 molecules are able to dimerise to form heterodimers. This would suggest a role for 14-3-3 as a protein that is responsible for the formation of higher order protein complexes (Xiao *et al.*, 1995; Liu *et al.*, 1995). Hsp50 and hsp90 act as stabilizing proteins whereas the binding of MEK is believed to place it in close proximity to Raf-1 for activation. The possibility of phosphorylation as a mediator of Raf-1 activity has been noted (Morrison *et al.*, 1993). In this model it has been suggested that phosphorylation of serine, and possibly other residues would then provide specific binding sites for regulatory proteins such as 14-3-3 binding phosphoserine residues. The regulation of Raf-1 is a complex mechanism and is an important step in the survival of the cell in response to NGF.

The importance of Raf-1 for cell signaling was shown in a study by Lenormand *et al.* (1996). They showed Raf-1 was responsible for the regulation of S6 kinase activity. They expressed an oestradiol-regulated form of Raf-1 in Chinese hamster lung fibroblast cells and blocked the activation of ERK with phosphatase treatment. In these conditions, the activation of Raf-1 was able to mediate S6 kinase activation. In addition to this, Raf-1 was found to activate MEK, the kinase responsible for the activation of ERK (Catling *et al.*, 1995).

A.



B.



C.

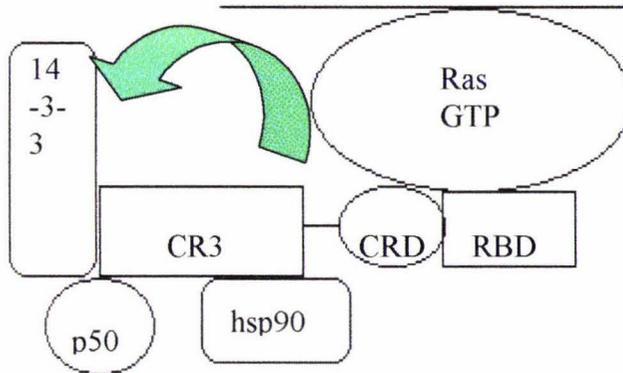


Figure 5
Ras binding and activating Raf

14-3-3 is believed to play an essential role in Raf activation. It is bound to Raf in a way that prevents the cysteine rich domain from binding Ras-GTP. Once Raf binds Ras through the Ras binding domain, 14-3-3 is displaced and binds to the cysteine rich-3 region stabilizing the Ras-Raf complex. Other proteins are believed to participate in Raf activation.

The importance of MEK in the ERK pathway

As mentioned previously, MEK is believed to bind Raf-1 and is consequently activated by Raf-1. MEK is a tyrosine/threonine kinase that is responsible for activating ERK (Kosako *et al.*, 1992; Crews *et al.* 1992; Robbins *et al.*, 1993). ERK is active once its threonine and tyrosine residues have been phosphorylated. MEK is unable to recognize denatured ERK (Seger *et al.*, 1992). This suggests that the three dimensional structure of ERK is important for MEK mediated activation. Several isoforms of MEK and ERK exist so it was hypothesized that there must be some structural recognition to ensure that the correct MAPK family member is activated by the appropriate MEK. Robinson *et al.* (1996) produced several ERK mutants and their activation by the corresponding MEKs was studied. Mutations were introduced in the phosphorylation loop of ERK, either at the phosphorylation sites within the loop, or at residues between these sites. Mutations were also introduced to shorten the loop. They found that the length of the loop does not affect MEK specificity and that the only major determinant for specificity was the kinase backbone.

It has also been proposed ERK can bind MEK on a docking site contained at the N-terminus of MEK (Bardwell and Thorner, 1996). Binding of ERK to MEK at this site would provide another level of specificity. The authors also suggest that the MEK-ERK complex would be too tight to allow the transfer of phosphate from ATP to ERK. Therefore, the activation of ERK would need to be a two step process. The first step would be the binding of the correct ERK and MEK determined by the kinase domain and N-terminal specificity, and the second step would require a dissociation of the two proteins, which would remain in close proximity for ATP to bind and phosphotransfer to occur.

MEK activates ERK in the cytoplasm (Lenormand *et al.*, 1993; Zheng *et al.*, 1994b) so the question asked was; how does MEK become localized to the cytoplasm? This question was looked at by Fukuda *et al.*, (1996). Using deletion mutants, they discovered that MEK contains a nuclear export signal within its N-terminal domain. The model proposed for the MEK-dependent activation of ERK was that MEK is translocated to the cytoplasm through its nuclear export signal where it binds ERK and activates it.

It is possible that other proteins have a role in regulating the activation of MEK. It is widely accepted that Raf is responsible for the activation of MEK. Zheng *et al.* (1994) reported that they immunodepleted Raf from PC12 cells and observed an NGF-dependent activation of MEK. One group has identified a protein kinase that plays a role in MEK activation. Pang *et al.* (1995b) have characterized this novel kinase in PC12 cells and found that its activity was stimulated three-fold in response to NGF. It is approximately 50 KDa in size with an isoelectric point of 7.3. The kinase activity was specific for ATP and requires Mg^{2+} as a cofactor. The enzyme was not activated when a dominant inhibitory mutant form of Ras was expressed in PC12 cells suggesting that this kinase is downstream of Ras. Site directed mutation of the residues on MEK that are required for activation by Raf-1 showed a decreased, but no inhibition, of MEK phosphorylation suggesting this kinase may share some common residue targets for phosphorylation of MEK as well as targeting novel residues.

ERK

The importance of ERK activation can be seen by the different responses that occur as a result of ERK's duration of activation. After NGF stimulation the activity of ERK remains active for hours, yet EGF stimulation results in a short lived ERK activity, one that can be measured in minutes (Heasley and Johnson, 1992; Traverse *et al.*, 1992; Nguyen *et al.*, 1993). The result is that NGF stimulates a differential response while EGF stimulates a proliferative one. The only other difference in ERK activation between the two responses is that ERK activation is achieved through a complex formation of SHC, Grb2 and mSOS in response to NGF. However in response to EGF, ERK is activated through the formation of a Grb2, mSOS complex (Obermeier *et al.*, 1994; Stephens *et al.*, 1994). The EGF receptor is more rapidly down regulated than TrkA through internalization and phosphorylation (Countaway *et al.*, 1992) suggesting it is the number of active cell surface receptors that determines the time period that ERK is active. Why the difference in activation times? Transient activation leads to a different cellular response than a sustained activation. During sustained ERK activation, ERK is translocated to the nucleus (Chen *et al.*, 1992; Traverse *et al.*, 1992; Nguyen *et al.*, 1993). The nuclear translocation of ERK would mean that certain transcription factors are targets for ERK activity. A sustained ERK activity could lead

to critical changes in gene expression. This could stimulate the expression of those genes that are required for cell survival.

Various isoforms of ERK exist. They are all serine/threonine kinases and require phosphorylation of their tyrosine and threonine residues for maximal activation. The localization of ERK was expected to be in the cytosol because ERK is a target for the EGF receptor (Northwood *et al.*, 1991). Alvarez *et al.*, (1991) showed that ERK was also localized to the nucleus. Thus, the translocation of ERK to the nucleus provides a physical link between NGF binding to its receptor and signaling events within the nucleus.

ERK targets includes kinases and phosphatases. Raf and MEK are phosphorylated by ERK *in vitro* (Anderson *et al.*, 1991; Lee *et al.*, 1992; Matsuda *et al.*, 1993) suggesting ERK is capable of self-regulation. ERK has also been shown to phosphorylate S6 kinase. Two different types of S6 kinase are phosphorylated in response to NGF and EGF treatment (Mutoh *et al.* 1988). This showed that the prolonged activation of ERK seen with NGF stimulation activated a different S6 kinase than EGF signaling. This could account for the fact that NGF results in differentiation while EGF stimulates proliferation.

Other factors could be involved in ERK activation

Other proteins have been shown to regulate the activation of the ERK signaling cascade. Cells treated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) show activation of ERK (Ray *et al.*, 1987; Hoshi *et al.*, 1988; Rossomando *et al.*, 1989) suggesting an involvement of PKC in this pathway. A direct demonstration of this effect was shown by Ueda *et al.*, (1996) when they found that TPA mediated activation of ERK was not blocked when a dominant negative form of Ras was expressed. They demonstrated that PKC δ could activate ERK but Raf was required. This event appeared to be Ras-independent but TPA modulates effectors of Ras so any result for Ras could not be taken into account. Evidence has also been provided for the requirement of other signaling cascades in ERK activation (Grammer and Blenis, 1997). These results demonstrate that the activation of ERK is not just a clear linear

pathway. Its activation could be a complex system of cross talk between different pathways activated by NGF.

To summarize the activation of ERK through NGF stimulation: the protein Shc binds to a phosphotyrosine residue on the intracellular domain of TrkA. This phosphorylates tyrosine residues on Shc providing a binding site for Grb2. Grb2 localizes mSOS to the plasma membrane. mSOS exchanges GDP for GTP on Ras thus activating it. Plasma membrane-bound Ras binds the RBD on Raf-1. This achieves two things; it localizes Raf-1 to the plasma membrane and it displaces 14-3-3, which is bound to phosphoserine residues on Raf-1. This displacement exposes a CRD on Raf-1 to which Ras is able to bind resulting in Raf-1 activation. Raf-1 then activates MEK, which has been localized to the cytoplasm through its nuclear export signal. MEK in turn phosphorylates, and activates, ERK. ERK then continues the signal by phosphorylating other signaling proteins and transcription factors. These targets of ERK trigger events that are responsible for the survival of the cell.

1.6.2: The PI3K pathway

As mentioned in section 1.3.3, other intracellular proteins are recruited to the intracellular domain of TrkA in response to NGF binding. These proteins trigger other signaling cascades that also have importance in cellular survival. The binding of phosphatidylinositol-3 kinase (PI-3K) to the intracellular domain triggers one such pathway. Ohmichi *et al.* (1992) showed tyrosine phosphorylation of PI3K in anti-phosphotyrosine immunoprecipitates but not in anti-TrkA immunoprecipitates. This suggested PI3K is tyrosine phosphorylated in response to NGF but it is not associated directly with TrkA. They proposed that there is an SH2 containing protein that links PI-3K to TrkA intracellular domain. In contrast some studies have provided evidence for PI-3K binding to the TrkA intracellular domain through its p85 regulatory subunit (Soltoff *et al.*, 1992; Obermeier *et al.*, 1993a; Obermeier *et al.*, 1993b). It remains unclear as to whether PI-3K directly or indirectly interacts with the intracellular domain of TrkA.

The action of PI-3K is in the formation of phosphoinositides, which act as secondary messenger molecules. PI-3K can phosphorylate various forms of phosphoinositides to form phosphatidylinositol-3-phosphate (Ptdins-3-P), phosphatidylinositol-3,4-bisphosphate (Ptdins-3,4-P₂) and phosphatidylinositol-3,4,5-trisphosphate (Ptdins-3,4,5-P₃). The formation of Ptdins-3-P can occur without NGF stimulation but the formation of Ptdins-3,4,5-P₃ occurs only in response to NGF. The formation of Ptdins-3,4-P₂ and Ptdins-3,4,5-P₃ is regulated through a complex series of kinases and phosphatases. This is important because certain SH2 and Pleckstrin Homology (PH) domains can interact with Ptdins-3,4,5-P₃ (Carpenter and Cantley, 1996). In addition, the serine/threonine protein kinase B (Akt) is activated by Ptdins-3,4-P₂ (Franke *et al.*, 1997; Klippel *et al.*, 1997).

PI3K activates Akt

The activity of Akt is regulated by growth factors that regulate the activity of PI-3K (Franke *et al.*, 1995; Burgering and Coffey, 1995). These studies found receptor mutants deficient in activating PI-3K failed to activate Akt and the expression of dominant inhibitory alleles of PI-3K also prevented Akt activation. Finally, they found constitutively active PI-3K increased Akt activity independent of growth factors and the PI-3K inhibitor wortmannin inhibited Akt activity. Franke *et al.* (1995) also showed Akt activation was partially dependent on Ras. This had been noted by Klippel *et al.* (1996) who demonstrated that activated mutants of Ras might stimulate Akt by activating PI-3K. However, Akt is not involved in the activation of ERK (Franke *et al.*, 1995). Another pathway, independent of PI-3K, that plays a role in Akt activation could involve p38/HOG (Alessi *et al.*, 1996).

Akt molecules have the ability to form homodimers and interact with other intracellular proteins through its PH domain (Datta *et al.*, 1995). Treatment of Akt with serine and threonine phosphatases has shown the importance of phosphorylation on these residues for Akt activity. The critical phosphorylation sites have been shown to be threonine 308 and serine 473 (Alessi *et al.*, 1996). p38/HOG activates MAPKAP2 (MAPK activating protein also known as MEK) under conditions of cellular stress when PI-3K is not activated (Alessi *et al.*, 1996). Therefore, it may be possible that this pathway is responsible for the phosphorylation on S-473 *in vivo*.

It is still unclear how PI-3K activates Akt. Klippel *et al.*, (1997) used vesicles containing Ptdins-3-P, Ptdins-3,4-P₂ or Ptdins-3,4,5-P₃ to observe whether the products of PI-3K are able to activate Akt *in vivo*. They found that only those vesicles that contained Ptdins-3,4-P₂ were able to activate Akt. This activation was dependent on an intact PH domain, which has the ability to bind phosphoinositides. This result was also shown by Franke *et al.* (1997) who found that binding of Ptdins-3,4-P₂ stimulated the dimerization of Akt. Stephens *et al.* (1998) showed binding of Ptdins-3,4,5-P₃ occurs on the PH domain of Akt. This caused translocation of Akt to the plasma membrane and allowed upstream kinases to phosphorylate threonine 308. Four isoforms of this kinase were isolated from sheep brain cytosol. From these it was discovered the kinase contains an N-terminal catalytic domain and a C-terminal PH domain. Expression of this kinase augmented receptor activation of Akt. In another study, chromatographic separation of cytosol revealed a kinase activity that was able to phosphorylate Akt (Stokoe *et al.*, 1998). This phosphorylation, exclusively on threonine 308, could only occur in the presence of Ptdins-3,4,5-P₃.

Several downstream targets of Akt have recently been identified. Targets include glycogen synthetase kinase-3 (GSK3) which is not involved in cellular survival. This suggests that Akt has other roles within the cell besides cellular survival. p70 S6 kinase is downstream of Akt (Burgering and Coffey, 1995). Most importantly Akt has been implicated in having an effect on members of the Bcl-2 family.

1.6.3: The Bcl-2 family and Caspases.

The cell machinery that has been shown to be responsible for apoptosis involves caspases and Bcl-2 family members. Caspases are specific proteases that can be defined into two major groups; those responsible for the terminal phase of apoptosis (executors), and those that activate the executors (initiators). The Bcl-2 family members are groups of proteins that can either be pro-apoptotic (Bax, Bad, and Bak) or anti-apoptotic (Bcl-2, Bcl-X_L, A1, and Bag-1). In general the Bcl-2 family members regulate the activation of caspases through interactions with other intracellular proteins

and/or through the control of ion fluxes across intracellular membranes. Most of these proteins have been studied for their roles in apoptosis by looking at the simpler model of apoptosis in *C. elegans*.

The Bcl-2 family

The pathways involving the Bcl-2 family members and caspases are responsible for regulating the final event of apoptosis. They in turn are regulated by those signaling pathways we have discussed so far – those activated by proteins that directly associate with the intracellular domain of TrkA. One such link is the phosphorylation of Bad by Akt (Datta *et al.*, 1997; del Peso *et al.*, 1997). Scheid and Duronio (1998) also demonstrated this result as well as providing evidence for the phosphorylation of Bad through a PI-3K independent pathway, probably through MEK.

Phosphorylation of Bad, a protein that normally dimerises with Bcl-2 and Bcl-X_L through interaction of the BH3 domain of Bad (Zha *et al.*, 1997; Otilie *et al.*, 1997; Kelekar *et al.*, 1997), results in inhibition of apoptosis. Phosphorylation of Bad occurs on two serine residues and this allows Bad to bind a member of the 14-3-3 family of proteins (Zha *et al.*, 1996). This association frees Bcl-X_L for binding to Bax preventing the pro-apoptotic effect of Bax. Mutation of these phosphorylation sites on Bad result in loss of cell survival by cytokines (Zha *et al.*, 1996).

The roles of the Bcl-2 family members remain unclear. Bcl-2 has been overexpressed in many cell types and has been shown to inhibit the apoptotic response induced by a variety of factors (Reed, 1994). Endogenous and exogenous Bcl-2 are anchored via their hydrophobic C-terminal domains to the outer membranes of mitochondria, nuclei, and the endoplasmic reticulum with most of the protein being exposed to the cytosol (Nguyen *et al.*, 1993; Chen-Levy and Cleary, 1990; Hockenberry *et al.*, 1990). Membrane localization of Bcl-2 is required for maximal activation (Hockenberry *et al.*, 1993; Tanaka *et al.*, 1993). Bcl-2, which has been implicated as an anti-oxidant (Hockenberry *et al.*, 1993; Kane *et al.*, 1993), preserves the potential of mitochondrial membranes (Hennet *et al.*, 1993), and blocks the release of calcium from the intracellular stores (Baffy *et al.*, 1993). Its most characterized function is as an inhibitor of caspases. Bcl-2 is able to bind other members of the family that have a

pro-apoptotic role, such as Bax. Otter *et al.*, (1998) showed the degree of protection from apoptosis does not correlate to the number of Bcl-2-Bax heterodimers. It was regulated by the amount of Bcl-2 that is free of Bax. Their study determined the Bcl-2-Bax heterodimer is a pro-apoptotic complex and Bcl-2 acts as an enzyme, either as a homodimer or as multimer.

Bcl-X_L has been shown to have structural homology to a bacterial pore forming protein (Muchmore *et al.*, 1996) which has been shown to form ion channels in synthetic bilipid bilayers (Minn *et al.*, 1997). By analogy, it is possible Bcl-2 also has the ability to form ion channels. These ion channels could regulate homeostasis of organelles during the apoptotic process.

During apoptosis, the mitochondrial matrix swells and hyper-polarization of the mitochondrial inner membrane occurs resulting in the loss of electron transport and oxidative phosphorylation. Consequently, disruption of the outer membrane occurs resulting in the release of cytochrome c. These events can be prevented by the expression of Bcl-X_L (Vander Heiden *et al.*, 1997). This is good evidence for the suggestion Bcl-X_L forms an ion channel in the mitochondrial membrane.

The Bcl-X_L ion channel was studied by Lam *et al.* (1998). They expressed full length Bcl-X_L in a lipid bilayer reconstitution system and found Bcl-X_L formed a sodium-conducting channel, which was inhibited by luminal calcium.

The release of cytochrome c from mitochondria during apoptosis is an essential event because it has been implicated in regulating the activation of caspases. In particular it is known to activate caspase-3 (Liu *et al.*, 1996). The release of cytochrome c has been proposed to occur by two possible methods. Firstly, the mitochondrial disruption of the outer membrane could lead to the formation of pores through which cytochrome c could leak. This has been suggested because during apoptosis the permeability transition of mitochondria increases (Petit *et al.*, 1996). The other possibility is that Bax and Bad form specific channels. This is supported by the fact Bax induces cytochrome c release from mitochondria when expressed in yeast (Manon *et al.*, 1997).

Caspase Activation

The next area of study was the activation of caspases by cytochrome c. Most of the knowledge about caspase action has been gained by studying the nematode *C. elegans*. In *C. elegans* three genes have been identified that control apoptosis. Two genes, *ced-3* and *ced-4*, are required for apoptosis to occur (Yuan and Horvitz, 1990). A third gene, *ced-9*, negatively regulates the functions of *ced-3* and *ced-4*. It is proposed to lie upstream of the other two genes (Hengartner *et al.*, 1992). This apoptotic process is conserved in mammals but with more complexity. Bcl-2 is the homologue of *ced-9* (Hengartner and Horvitz, 1994), a series of cysteine proteases are homologues of *ced-3* (Yuan *et al.*, 1993; Fernandes-Alnemeri *et al.*, 1994; Alnemeri *et al.*, 1996) and Apaf-1 (apoptotic protease activating factor) is homologous to *ced-4* (Zou *et al.*, 1997).

Apaf-1 has the ability to activate caspase-3. Cytochrome c had also been previously found to play a role in caspase-3 activation (Liu *et al.*, 1996). This activation by cytochrome c could be inhibited by the expression of Bcl-2 (Yang *et al.*, 1997). Apaf-1 has a CED-4 like domain that is flanked on one side by a CED-3 like domain (this also has sequence homology to caspases-2, -9, -11) and caspase recruitment domains (CARD) (Irmeler *et al.* 1997). On the other side there are 12 WD repeats (Zou *et al.*, 1997).

In *C. elegans* CED-3, CED-4 and CED-9 are in a complex with each other (Chinnaiyan *et al.*, 1997; Wu *et al.*, 1997; Spector *et al.*, 1997). In mammals, Apaf-1 can bind caspase-9 (previously known as Apaf-3, Li *et al.*, 1997) in the presence of cytochrome c and ATP. This protein complex is able to activate caspase-3 (Liu *et al.*, 1996; Vaux 1997). This interaction is mediated through the CARD domains. Pan *et al.* (1998) showed Bcl-X_L was bound to Apaf-1 and caspase-9 in a ternary complex, which inhibits the apoptotic response. They also found expression of dominant negative caspase-9 inhibited apoptosis suggesting caspase-9 is a downstream target of Apaf-1 and its activation is critical for apoptosis.

A model is presented where cytochrome c binds to the WD repeats of Apaf-1 inducing a conformational change. This change in conformation allows Apaf-1 to bind caspase-9 in an ATP dependent manner (Reed, 1997). Caspase-9 then cleaves

caspase-3 leading to its activation. Caspase-3 activates further downstream caspases inducing apoptosis. Bcl-2/Bcl-X_L is able to inhibit this process by binding the Apaf-1-caspase-9 ternary complex. This prevents the activation of caspase-3, protecting the cell from apoptosis.

Other methods of caspase-3 activation occur within the cell. Ceramide has been linked to caspase-3 activation (Yoshimura *et al.* 1998) suggesting a method whereby the p75 receptor, which has been linked to ceramide formation (Dobrowsky *et al.*, 1994), can induce apoptosis via caspase-3 activation. Calcium has been found to increase the activity of caspase-3 (Juin *et al.*, 1998). This was consistent with their discovery that the addition of calcium to a cell free system triggered nuclear apoptosis (DNA fragmentation).

Apoptosis needs to be tightly regulated to ensure the cell functions in an appropriate manner. This requires a certain amount of cross talk between different pathways as has been discussed in previous sections. NGF-activated TrkA activates several signaling pathways at the plasma membrane. Other signaling pathways are activated by intracellular organelles containing active TrkA. These signals activate downstream pathways responsible for the physical effects of apoptosis such as mitochondrial swelling and DNA fragmentation.

Many intracellular pathways activate or inhibit apoptosis. To be able to manipulate apoptosis we need to understand its control at the molecular level. The purpose of the projects described in this thesis is to examine the initial molecular interactions that occur upon NGF binding. That is, the recruitment of several intracellular proteins to the intracellular domain of activated TrkA. Several of these proteins have already been identified (discussed in section 1.3.3). The intracellular domain of TrkA contains many tyrosine residues that are phosphorylated upon NGF binding. These residues could provide binding sites for additional intracellular proteins that have not yet been identified as TrkA binding proteins. A set of such proteins had been seen previously (Dr. M. L. Grimes, unpublished results). My aim was to isolate and characterize these proteins. To achieve this I attempted to express and purify the intracellular domain of TrkA in bacteria. The protein was found to be expressed in a tyrosine-phosphorylated form suggesting that it was constitutively active. This provides us with an opportunity

to identify proteins that are activated and those that bind to activated TrkA by adding the protein to cytosol from PC12 cells that have not been treated with NGF. This will further clarify the molecular regulation of apoptosis and bring us closer to being able to manipulate the decision to undergo apoptosis.

CHAPTER 2 : MATERIALS AND METHODS

2.1: Chemicals and Reagents

All chemicals and reagents were purchased from Sigma chemical company, St. Louis. MO unless otherwise stated. All buffers were made to volume with ddH₂O unless otherwise stated.

2.2: Preparation of Collagen

Pre-frozen rat-tails were scrubbed with vircon and sterilized in 70 % ethanol. The tails were skinned 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 we then cut from the bone. Approximately 2 g of tendons was collected and placed in 200 ml sterile 0.2 % acetic acid. 2 ml of chloroform were added and this suspension was gently rotated for 2-7 days at 4°C until a creamy viscous solution formed. The solution was centrifuged at 3 800xg for 20 minutes at 4°C. The supernatant was removed, centrifuged at 9 100xg for 60 minutes at 4°C and the supernatant dialysed overnight against ddH₂O. The collagen was stored in a sterile bottle at 4°C. For coating 20 plates, 2-3 ml was diluted in 100 ml 50 % ethanol, and 5 ml was spread over each plate. The ethanol solution was allowed to dry overnight in a tissue culture hood, leaving the collagen dried onto the plates. The collagen plates were stored at 4°C

2.3: Tissue Culture

Falcon plates coated with rat tail collagen were used to grow the PC12 cells obtained by Dr. Grimes from Dr. Lloyd Greene, (Columbia University, New York). Cells were grown in RPM1-1640 media [+ 2.2 g Sodium Carbonate + 10 % horse serum (Gibco BRL) + 5 % foetal calf serum (Gibco BRL)] at 37°C in 5 % Carbon Dioxide mixed with air in an incubator. Fresh media was added to the cells three times a week with the media being replaced completely when required. The cells were grown to confluence and passed onto plates at a ratio of 1:4.

2.4: Passage of Cells

The media was removed and the plates washed with 10 ml PBS (Phosphate buffer saline: 8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na₂HPO₄, 0.2 g/L KHPO₄ pH 7.4). The PBS was removed and the cells were harvested with 10 ml of ice cold PEE (PBS + 1 mM EDTA + 1 mM EGTA). The cells were centrifuged at 100xg at 4°C for 3 minutes, washed with ice cold PEE, centrifuged again at 100xg for 3 minutes at 4°C and resuspended in 4 ml of media and 4 ml of PBS by passage through a small-hole pasteur pipette to break apart clumps of cells. 1 ml of this suspension was added to each falcon plate containing 24 ml of media.

2.5: Harvesting, Permeabilisation and Fractionation of Cells

The media was removed and the plates washed with 10 ml PBS. The PBS was removed and the cells were harvested with 10 ml of ice cold PEE, centrifuged at 100xg at 4°C for 3 minutes and washed with ice cold PEE as above. Cells were resuspended

in 10 ml ice cold PGB (PBS + 1 g/L glucose + 1 g/L BSA) by passing the cell suspension through a small hole pasteur pipette and the suspension was distributed equally into 2 falcon 15 ml tubes, with 5 ml of cells in each. One tube (+NGF) was treated with 1 nM NGF (diluted in PBS) and the other tube had no further additions (raw). The tubes were rotated for 1 hour at 4°C. After incubation the cells were centrifuged at 100xg for 3 minutes at 4°C, resuspended in 5 ml PGB and warmed at 37°C for 10 minutes to allow the internalization of the NGF-bound receptor. To prevent extended internalization within the cell the suspension was quenched in ice water for 15 minutes. Cells were washed with 5 ml cold PEE, then washed in 2.5 ml cold BB (bud buffer: 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 pH 7.4 with KOH; glutathione was added fresh from 100X stocks to a final concentration of 5 mM). The cells were resuspended in 0.5 ml BB with a syringe and 18G needle. 0.2 mM Sodium Orthovanadate (a tyrosine phosphatase inhibitor) was added to both tubes before the cells were passed through a stainless steel ball homogeniser (European Molecular Biology Laboratory, Heidelberg, Germany) to permeabilise the cells. (Tungsten carbide balls were purchased from Industrial tectonics Ann Arbor, MI).

After mechanical permeabilisation, the cells were spun at 1000xg for 10 minutes at 4°C. The pellet was resuspended in 0.45 ml PEE with 1 % detergent (IGEPAL) containing protease inhibitors added from 100X stocks (17.9 mg/ml PMSF, 0.1 mg/ml o-phenanthroline in anhydrous ethanol) and 1 000X stocks (10 µg/ml pepstatin, 10 µg/ml chymostatin, 10 µg/ml leupeptin, 10 µg/ml aprotinin in DMSO) and 0.2 mM sodium orthovanadate. This suspension was incubated on ice for one hour then centrifuged at 10 000xg for 10 minutes at 4°C. This separated the cytoskeleton (pellet) from the plasma membrane (P1M - supernatant). The supernatant of the 1 000xg centrifugation (S1) was spun at 8 000xg for 35 minutes at 4°C. The resulting pellet (P2 - large intracellular organelles) was resuspended in 160 µL immunoprecipitation buffer (IP; 0.25 % deoxycholate, 0.15 M NaCl, 1 mM NaF, 1 mM EGTA and 50 mM Tris.HCl pH 7.4) with 2 % IGEPAL also containing protease inhibitors and 1 mM sodium orthovanadate. The supernatant was layered over a sucrose pad (10 % sucrose

with 20 mM MOPS pH 7.4) containing phosphatase inhibitors and sodium orthovanadate as above and centrifuged 100 000xg for 1 hour at 4°C. The pellet (P3) contained small intracellular organelles and the supernatant is the cytosolic fraction (S3). P3 was resuspended in 160 µL IP buffer containing 1 % IGEPAL, phosphatase inhibitors and sodium orthovanadate. The fractions were either immunoprecipitated or stored at -70°C. Great care was taken to ensure the samples remained cold at all times throughout the fractionation procedure. This fractionation procedure is depicted in Figure 6 (Chapter 3).

2.6: In Vitro reaction with ATP

In some cases, the permeabilised cells were subjected to an *in vitro* reaction with an ATP regenerating system before fractionation of the organelles that emerged from the permeabilised cells. 5 mg/ml (240 units/mg) creatine kinase, 8 mM creatine phosphate and 1 mM ATP was taken from 100X stocks stored at -70°C and added to cracked PC12 cells that were either NGF-treated or untreated. The cells were incubated at 37°C for 15 minutes. Cells were quenched in ice water for 5 minutes after the *in vitro* reaction.

2.7: Immunoprecipitation

100 µl of each fraction was immunoprecipitated with approximately 10 µg of antibody in the presence of IP (defined above) buffer + 1 % BSA, 0.2 mM sodium orthovanadate and protease inhibitors. The contents were mixed and incubated at 4°C overnight.

200 µl of 50 % Protein A beads was added to 800 µl of 1X IP buffer. These 10 % Protein A beads were washed twice in 1X IP buffer. 100 µl of washed 10 % protein A

beads was added to the immunoprecipitation and mixed on a rotating device at room temperature for 3-4 hours. The samples were then centrifuged at 1 000xg for 3 minutes and the beads were washed twice in 1X IP buffer, once in 0.1X IP buffer and centrifuged at 5 000xg for 5 minutes. 4X SB (sample buffer: 0.5 M Tris pH 6.95, 0.45 M DTT, 4 % w/v SDS, 4 mM EDTA, 40 % sucrose w/v and 0.4 % bromophenol blue) was added, and the samples heated for 15 minutes at 60°C, centrifuged at 10 000xg for 10 minutes and the supernatant loaded onto a SDS gel.

2.8: Protein Concentration Determinations

Protein concentration was estimated by the Lowry method (Waterberg and Matthews). 1 ml of diluted or undiluted sample was added to 1 ml of reaction mix, consisting of a 100:1:1 ratio of solution A (2 % w/v Na₂CO₃ in 0.1 M NaOH): solution B (1 % w/v CuSO₄.5H₂O in ddH₂O): solution C (2 % w/v Sodium potassium tartrate in ddH₂O) respectively. This mixture was allowed to stand at room temperature for 10 minutes after which 0.1 ml of Folin-Ciocalteu reagent was added and this was allowed to stand for a further 30 minutes. The absorbance was read at 550 nm on a Hitachi U-1100 spectrophotometer. If the protein concentration was 500 µg/ml or more then the absorbance was read at 750 nm.

2.9: SDS Gels

SDS gels were prepared as described by Laemmli (1970) with some modifications. The stacking gel was made with 5 % w/v (50:1) acrylamide: bisacrylamide, 0.125 M Tris.HCl pH 6.95, 1 mM EDTA, 0.1 % w/v SDS, 0.06 % w/v APS and 0.06 % v/v TEMED. The resolving gel was made with 8-15 % w/v (50:1 or 100:1) acrylamide: bisacrylamide, 0.375 M Tris.HCl pH 8.95, 0.1 % w/v SDS, 0.06 % w/v APS and 0.06 % w/v TEMED. The volumes of ddH₂O and acrylamide were altered to make the

different acrylamide percentage and the volumes of acrylamide and bisacrylamide were altered to make the different crosslinking ratios. The gels were cast between two glass plates with rubber tubing providing the seal. A comb was used to form the wells in the stacking gel. Once the gel was set the tubing and comb was removed. The lanes were filled with stacking buffer (stacking gel without the APS or TEMED) for sample loading. Samples were loaded in the presence of 4X SB. Phosphorplus™ biotinylated protein markers (New England Biolabs), Sigma colour markers (high range) or Sigma SDS 7 markers were used as size markers. The electrophoresis buffer consisted of 40 mM Tris, 0.2 M glycine, 1 mM EDTA and 0.1 % w/v SDS.

2.10: Coomassie Staining

Gels were incubated in coomassie brilliant blue R-250 for 30 minutes at room temperature. The gel was destained in acetic acid, methanol and water for 10 minutes. Fresh destain solution was added and the gel was incubated in this for 1 hour at room temperature, then overnight at room temperature in fresh destain.

2.11: Electrophoretic transfer of Proteins to Nitrocellulose

SDS gels were covered with nitrocellulose (Optitran™ supported nitrocellulose) and sandwiched between four pieces of Whatman blotting paper. This sandwich was placed between two sponges and placed into a blotting apparatus. The tank was filled with transfer buffer (9.69 g Tris, 45.04 g glycine, 0.8 L methanol and ddH₂O was used to bring the volume to 4 L). Proteins were transferred from the gel onto the nitrocellulose at 100 mA for 16 hours. The time of blotting and current was decreased or increased if necessary, keeping the number of amp.hours constant at 1.6.

2.12: Immuno blotting

The nitrocellulose blot was washed in ddH₂O for 10 minutes, stained in 0.2 % Ponceau S in 3 % TCA for 15 minutes, washed in ddH₂O for 10 minutes and a Xerox copy of the stained blot was made. For blocking, the blot was incubated in 5 % BSA in TBSI (20 mM Tris pH 8.0, 150 mM NaCl and 0.5 % IGEPAL) for one hour at room temperature. The blot was then incubated in primary antibody for one hour at room temperature, or overnight at 4°C, washed for 15 minutes three times in TBSI and incubated in secondary antibody, either anti-rabbit or anti-mouse conjugated to horseradish peroxidase (see below), for one hour at room temperature. The blot was washed three times in TBSI for 15 minutes before being exposed to a chemiluminescent reaction.

2.13: Chemiluminescence Reaction

Enhanced chemiluminescence (ECL) uses horseradish peroxidase covalently linked to the secondary antibody (anti-mouse or anti-rabbit) and hydrogen peroxide to oxidise luminol and consequently cause light emission. The reaction occurs in the presence of chemical enhancers (*eg* phenols), which increase light production and the time of light emission. Light produced by the ECL reaction peaks at 5-20 minutes and has a half-life of approximately 60 minutes.

The procedure was that recommended in the Amersham ECL kit. Equal volumes of solution 1 and solution 2 were added to cover the nitrocellulose blot and the reaction was allowed to continue for 1 minute. The excess solution was removed from the blot and it was placed onto a piece of cardboard wrapped with SaranWrap (Dupont) before being covered with more SaranWrap. The blot was exposed to Fuji medical x-ray film, HR-G 30, for 5 minutes initially and was exposed to another film for longer periods of time to increase the signal exposure.

2.14: Stripping the Blot

To strip off the antibodies for the purpose of reprobing, the blot was incubated in TBS pH 2.0 (20 mM Tris pH 2.0, 150 mM NaCl) for 10 minutes, then washed in ddH₂O twice for 10 minutes and TBS pH 8.0 for 10 minutes. The blot was blocked in 5 % BSA in TBSI for 30 minutes at room temperature, probed with primary antibody for one hour at room temperature, and treated as described above.

2.15: Transformation of E.Coli

2 µl of the TrkAID plasmid (pET15b (Novagen) containing the TrkAID construct, a gift from Alan Bates, UCSF) was added to 100 µl of competent BL21 (DE3) E.Coli transformed with pLysS (a gift from Michelle Sullivan, Massey University). This was incubated on ice for 30 minutes. 25 µl was spread onto pre-warmed LB agar plates (10 g LB + 7.5 g agar made up to 500 ml with ddH₂O. 20 ml of this was added to plates and the agar was allowed to dry near a flame at room temperature, then stored at 4°C) containing either one or both of 50 µg Ampicillin, 34 µg chloramphenicol. The plates were incubated at 37°C overnight.

2.16: Plasmid preparation

A 5 ml culture of BL21 (DE3) bacteria transformed with the TrkAID construct were grown overnight at 37°C. This culture was spun at 5K rpm for 10 minutes and the pellet was resuspended in 1 µl RNaseA and 200 µl of 50 mM glucose, 25 mM Tris pH 8.0, 10 mM EDTA and was incubated on ice for 5 minutes. 0.4 ml of 0.2 M NaOH + 1 % SDS was added and the tube mixed by inversion and incubated on ice for

5 minutes before 0.3 ml of 7.5 M ammonium acetate pH 4.8 was added. This was mixed by inversion, incubated on ice for 15 minutes and centrifuged 13K rpm for 15 minutes. An equal volume of phenol/chloroform equilibrated with 0.1 M Tris.Cl pH 8.0, was added to the supernatant. After mixing the sample was centrifuged 13K rpm for 15 minutes and the top layer was DNA precipitated with 0.6 volume of isopropanol and one tenth volume of 3 M sodium acetate followed by centrifugation at 13K rpm for 10 minutes. The pellet was washed in 0.4 ml of 20 % ethanol, centrifuged at 13K rpm for 5 minutes, and dried in the speed-vac for 15 minutes. The pellet was then resuspended in 20 µl TE. This was stored at either -20°C for short-term storage or -70°C for long-term storage. The plasmid was loaded on a 0.6 % agarose gel for visualization.

2.17: DNA sequencing

DNA was sequenced at the DNA sequencing unit at Massey University by Lorraine Barry.

2.18: Restriction Digests

Plasmid was digested with one or both of BamHI (Gibco BRL, # 15201-023) and XhoI (Gibco BRL, # 15231-012). The plasmid was incubated in restriction cocktail (restriction enzyme or enzymes, 10X NE buffer and ddH₂O) for 1-2 hours at 37°C. The digest was run on a 0.6 % agarose gel in 6X DNA loading dye.

2.19: DNA gels

0.6 % agarose in ddH₂O was heated until the agarose was dissolved. The gel was cast in a DNA gel apparatus with a comb to make the lanes. 1 µl of 6X DNA loading dye was added to 1 or 2 µl of sample and the volume was made up to 6 µl with ddH₂O before being loaded onto a gel along with a 1Kb DNA ladder (Gibco BRL, FNG 701). The gel was run with TAE buffer (0.04 M Tris-Acetate +1 mM EDTA). After electrophoresis the gel was stained in ethidium bromide in ddH₂O for 30 minutes, destained in ddH₂O for 10 minutes and visualized under UV.

2.20: Making Competent Cells

A 100 ml culture of BL21 (DE3), with or without pLysS, was grown to an optical density of 0.5 (measured by reading the absorbance at 600 nm). The cells were chilled on ice for 2 hours, centrifuged at 2 500xg for 20 minutes at 4°C and resuspended in 100 ml of ice cold solution A (100 mM CaCl₂·2H₂O, 70 mM MgCl₂·6H₂O, 40 mM NaOAc pH5.2, and made up to 200 ml volume with ddH₂O, pH to 5.5 and filter sterilized). The cells were centrifuged at 4 800 rpm for 10 minutes at 4°C, resuspended in 10 ml ice cold solution A, pelleted at 4 800 rpm for 10 minutes at 4°C and resuspended in 2 ml ice cold solution A for storage at -70°C in 15 % glycerol.

2.21: Expression of TrkAID

A colony of BL21 (DE3) pLysS transformed with the TrkAID construct and grown on a LB plate in the presence of ampicillin and chloramphenicol at 37°C, was used to inoculate 5 ml of LB broth + 50 µg ampicillin +34 µg chloramphenicol, which was

grown at 37°C overnight. This culture was used to inoculate 1L of LB broth + 50 µg ampicillin + 34 µg chloramphenicol and bacteria were allowed to grow to an optical density of 0.4-0.6 (measured at 600 nm). At this stage 50 ml was taken as a before induction (bi) sample, 0.4 mM of IPTG was added to the culture and the culture was grown for a period of time either taking samples every hour or taking the sample once a period of time had elapsed. Cells were harvested by centrifugation at 4 800 rpm for 10 minutes, frozen in 40 ml of 20 mM phosphate buffer pH 6.8 and thawed the next day. To break up the DNA, the cells were sonicated on ice for 3 minutes in 30-second bursts with 15-second intervals or passed through a syringe and 18G needle for smaller volumes. The suspension was centrifuged at 4 800 rpm for 10 minutes and the supernatant was loaded directly onto a SDS gel with 4X SB.

The pellets were resuspended in 40 ml of 20 mM phosphate buffer pH 6.8 + 0.01 % IGEPAL. This was incubated for 20 minutes at 30°C. They were sonicated for four minutes in 30-second bursts with 15-second intervals and loaded onto a SDS gel with 4X SB.

2.22: Nickel Column Purification

A 1 ml-affinity column (Biorad) was washed with 5 column volumes of ddH₂O, 5 column volumes of wash buffer (20 mM phosphate buffer pH 6.8, 100 mM, 10 mM or no imidazole), charged with 0.5 ml of 0.1 M NiCl or 0.5 ml of 0.1 M CuSO₄ and equilibrated with 5 column volumes of wash buffer. Sample was loaded onto the column either in the presence or in absence of imidazole. The column was washed with 5 column volumes of wash buffer and the protein eluted with 5 column volumes of elution buffer (20 mM phosphate buffer, 250 mM or 100 mM imidazole) before being washed again with final wash buffer (20 mM phosphate buffer, 500 mM imidazole). All samples collected during the sample loading, wash, elution and final wash stages were stored at -20°C. The nickel was stripped off the column with wash buffer + 0.05 M EDTA and the column washed with 5 column volumes of ddH₂O

followed by 5 column volumes of 20 % ethanol. The column was stored in 20 % ethanol at room temperature.

2.23: Dialysis

Dialysis tubing was prepared by boiling the tubing for 10 minutes in 2 % w/v sodium bicarbonate + 1 mM EDTA pH 8.0, washing thoroughly in ddH₂O and boiling for a further 10 minutes in 1 mM EDTA pH 8.0 before being rinsed again in ddH₂O. The tubing was autoclaved and stored in ddH₂O.

Samples were secured in dialysis tubing and dialyzed against the appropriate buffer for one hour at 4°C. The buffer was replaced and samples dialyzed for a further one hour at 4°C before the buffer was once more renewed and the samples were left to dialyze overnight at 4°C.

2.24: Inclusion Body Purification

The affinity column (used in Nickel column purification) was washed with 5 column volumes of ddH₂O, 5 column volumes of wash buffer (20 mM phosphate buffer, 6 M guanidine hydrochloride), charged with 0.5 ml of 0.1 M NiCl and equilibrated with 5 column volumes of wash buffer. The sample was denatured in 6 M guanidine hydrochloride and loaded onto the column. The column was washed with 5 column volumes of wash buffer, washed with a gradient of 8 – 0 M urea (in 20 mM phosphate buffer) for 80 minutes with an econo-system (Biorad) at a speed of 1 ml/minute and washed again with 3 column volumes of wash buffer without 6 M guanidine hydrochloride. Protein was eluted with 5 column volumes of elution buffer (20 mM phosphate buffer, 250 mM imidazole) and the column washed again with 5 column volumes of final wash buffer (20 mM phosphate buffer, 500 mM imidazole). The

samples were dialyzed to remove the urea and guanidine hydrochloride and stored at -20°C. Nickel was stripped off the column with 5 column volumes of 20 mM phosphate buffer + 0.05 M EDTA. The column was washed and stored as described previously.

2.25: Inclusion Body Purification in the Presence of Lithium Chloride

A 1 ml-affinity column was washed with 5 column volumes of ddH₂O followed by 5 column volumes of wash buffer (20 mM Tris pH 8.0, 1 M LiCl, + 6 M guanidine hydrochloride). The column was charged with 0.5 ml 0.1 M NiCl and equilibrated with 5 column volumes of wash buffer. Sample was denatured with the addition of 6 M guanidine hydrochloride in the presence of 1 M LiCl and loaded onto the column, which was washed with 5 column volumes of wash buffer, a gradient wash of 8 – 0 M urea (in 20 mM Tris pH 8.0 + 1 M LiCl) and finally with 3 column volumes of wash buffer without the guanidine hydrochloride. Protein was eluted with 5 column volumes of elution buffer (20 mM Tris pH 8.0, 1 M LiCl, and 250 mM imidazole) and the column was washed again with final wash buffer (20 mM Tris pH 8.0, 1 M LiCl + 500 mM imidazole). Nickel was stripped off the column with 20 mM Tris pH 8.0 + 0.05 M EDTA. The column was washed and stored as described previously.

2.26: Ion Exchange

A 1-ml ion exchange column (a generous gift from Dr. Gill Norris) was washed with 5 column volumes of ddH₂O and equilibrated overnight with 10 mM acetic acid pH 5.5 for CM ion exchange or 10 mM Tris pH 8.0 for mono Q ion exchange. The sample was dialyzed against the appropriate buffer before being loaded onto the column. The column was washed with 20 ml equilibration buffer, the proteins eluted with a gradient wash of 0-1 M NaCl in equilibration buffer and the column was washed again with

20 ml equilibration buffer + 1.5 M NaCl. The samples were dialyzed to remove the salt and stored at -20°C. The column was washed with 20 ml ddH₂O followed by 10 ml of 20 % ethanol. The columns were stored in 20 % ethanol at 4°C.

2.27: Ammonium Sulphate Precipitation

Ammonium sulphate was added to soluble sample collected from bacteria expressing TrkAID to 10 % saturation. The solution was stirred for 30 minutes and centrifuged at 3 300 xg for 30 minutes. The pellet was resuspended in 20 mM MOPS (3-[N-Morpholino] propane-sulphonic acid) pH 7.2 and a sample taken from the supernatant. More ammonium sulphate was added to the remainder of the supernatant to 20 % saturation. The process was repeated until 100 % saturation was reached in 10 % intervals.

2.28: *In vitro* activation of ERK with TrkAID

Soluble material was collected from bacteria transformed with pET15b-TrkAID as described in section 2.21. TrkAID was immunoprecipitated from this with 2 µl of 1086 as described in section 2.7. PC12 cells were harvested and permeabilised as described in section 2.5. Half the cells were exposed to an *in vitro* reaction with ATP (described in section 2.6) before fractionation. Both sets of cells were centrifuged 100 000Xg for 1 hour at 4°C to pellet the membranes and vesicles. Supernatant (cytosol) was added to the immunoprecipitated TrkAID and was exposed to an *in vitro* experiment with ATP for 15 minutes as described in section 2.6. Some cytosol was added to immunoprecipitated TrkAID but was not exposed to an *in vitro* reaction. S3 collected from NGF-treated PC12 cells was also added to immunoprecipitated TrkAID and treated in the same manner.

2.29: Data evaluation and recording

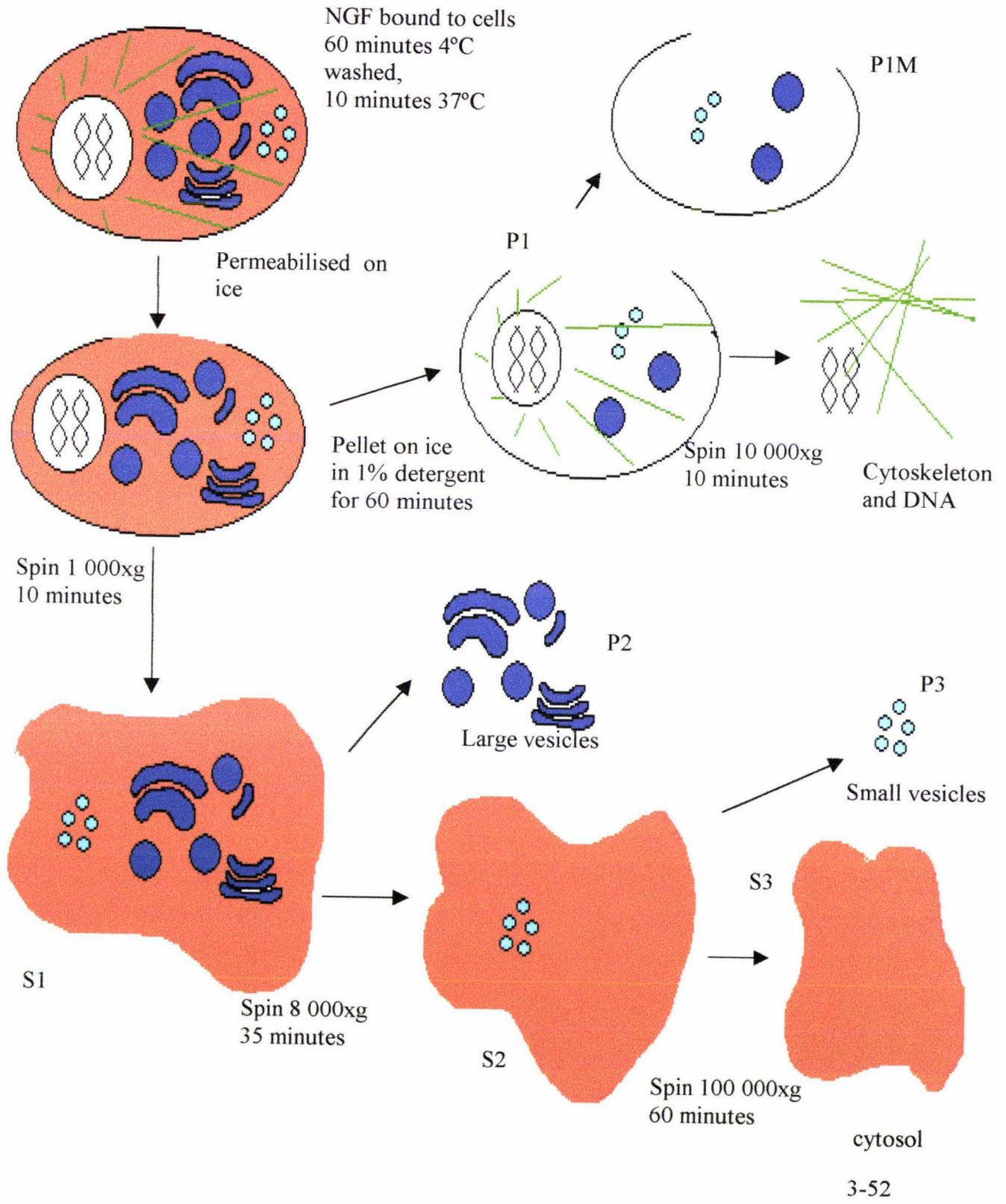
Exposed films were recorded on the Gel Documentation system. The white, black and gamma factors were reduced or increased as required to visualize all the bands on the film. A blank film was recorded with same settings and used as a blank to remove the background from the lightbox. The films were analyzed with Scion image (a PC version of NIH image) downloaded from <http://www.scioncorp.com>. The background was removed using scion and the result imported to Microsoft® PowerPoint where text was added to the picture. The resulting figures were then imported to Microsoft® Word. All DNA sequence analysis was done with the GCG program. The growth curve (Figure 13) was done with Kaleidagraph and Table 1 and Figure 23 were done with Microsoft® Excel

CHAPTER 3 : IDENTIFICATION OF NOVEL INTRACELLULAR PROTEINS ASSOCIATED WITH TRKA

As was discussed in section 1.3.3, previous studies have shown the association of intracellular proteins such as PLC- γ 1 (Vetter *et al.*, 1991; Obermeier *et al.*, 1993a) and SHC (Obermeier *et al.*, 1993b) with TrkA. It is possible that other, as yet uncharacterized intracellular proteins interact with TrkA upon NGF treatment, particularly in intracellular organelles. In previous experiments, my supervisor Dr. M. L. Grimes had detected a triplet of 90 KDa bands that appear to be associated with TrkA in intracellular organelles in the presence of NGF (unpublished results). The aim of these following experiments was to reproduce these results and isolate these proteins for characterization.

TrkA is incorporated into signaling vesicles (Grimes *et al.*, 1997; Grimes *et al.*, 1998) and any proteins that associate with TrkA will be incorporated into these signaling vesicles. I discussed in section 1.5 how the formation of vesicles is ATP dependent and how an *in vitro* reaction with ATP could select for a larger population of small intracellular organelles including signaling vesicles. For studying intracellular proteins that associate with TrkA in signaling vesicles, we needed to select for a large population of these signaling vesicles. Consequently, several of the following experiments included an ATP *in vitro* reaction.

Figure 6



3.1: A 90 KDa protein is activated by TrkA

PC12 cells, untreated or treated with NGF, were mechanically permeabilised by passage through a ball homogenizer. The cells treated with NGF were split into two fractions. One fraction was exposed to an *in vitro* reaction with ATP (as described in section 2.6) and the other fraction was kept on ice. The cells were fractionated as described in section 2.5 (depicted in Figure 6) to separate different cellular compartments. A low speed spin (1 000Xg) separated the cell ghost from the cell contents. The cell ghost membranes, including the plasma membrane (P1M), were solubilised in detergent. Organelles that emerged from the permeabilised cells were centrifuged at 8 000Xg to pellet the large intracellular organelles (P2) and the supernatant was subjected to a high-speed spin (100 000Xg) to pellet the small organelles (P3) and isolate the remaining supernatant containing soluble proteins (cytosol, S3). All fractions, except the S3 fractions, were immunoprecipitated with an antibody to the extracellular domain of TrkA (RTA), resolved on a SDS gel, western blotted and probed with an antibody to phosphotyrosine (4G10). The results are shown in Figure 7.

Tyrosine-phosphorylated TrkA (140 KDa) can only be detected in those fractions that were treated with NGF and exposed to an *in vitro* reaction (lanes 13-16). Most of the activated TrkA was found in the plasma membrane (P1M) fraction with smaller amounts in the large organelle (P2) and small organelle (P3) fractions. No other proteins were detected except for the antibody band at approximately 55 KDa. The cytosol fraction in lane 16 contains many tyrosine-phosphorylated proteins but the other S3 fractions do not. This experiment was repeated several times and the result was the same in each case.

To observe tyrosine-phosphorylated proteins, including TrkA, the experiment needed to be performed in the presence of a tyrosine phosphatase inhibitor (sodium orthovanadate). The experiment described above was repeated except the cells were not exposed to an *in vitro* reaction with ATP and the fractionation was done in the presence of sodium orthovanadate. The results are shown in Figure 8.

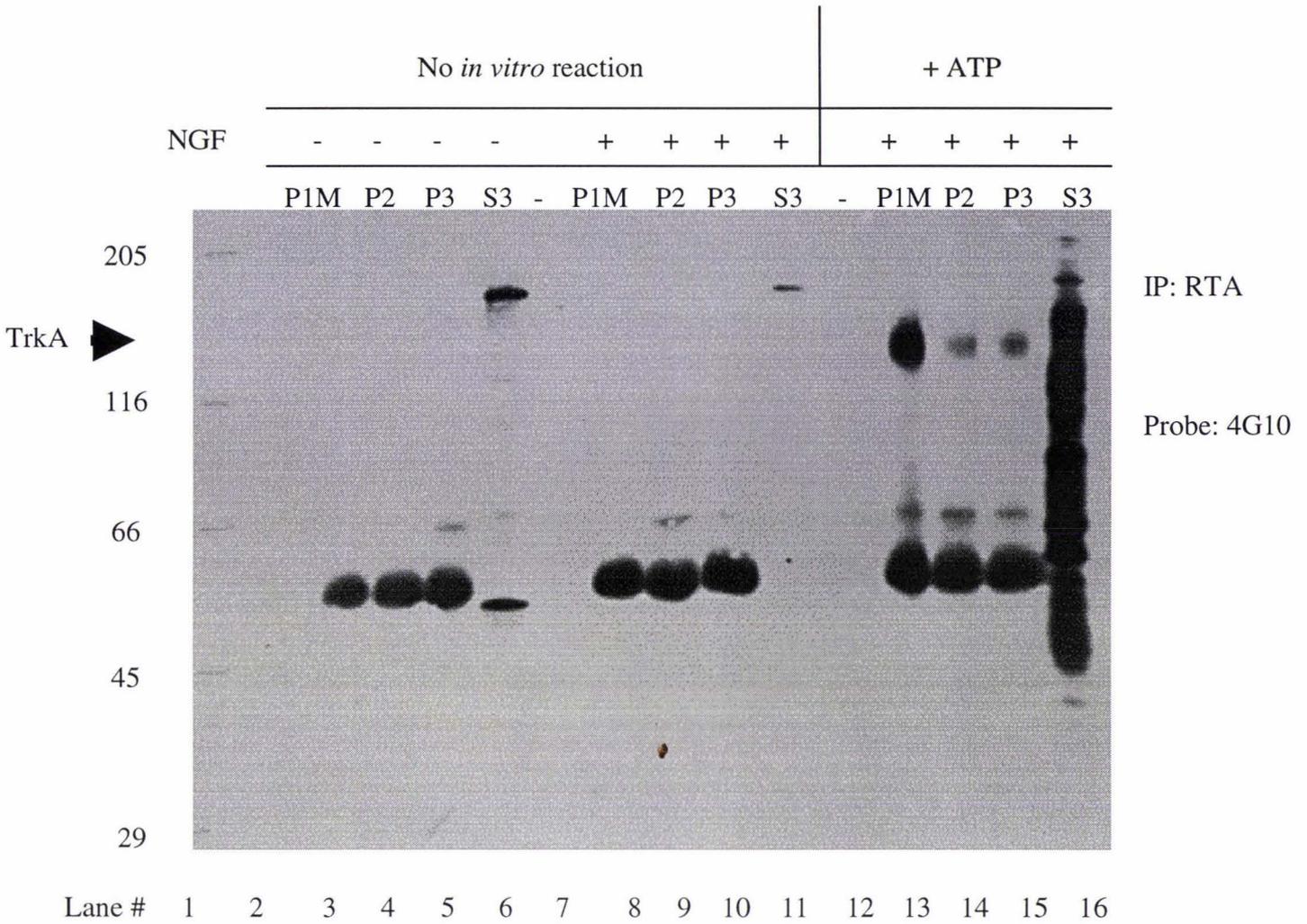


Figure 7
 PC12 fractions exposed to an *in vitro* reaction in the absence of sodium orthovanadate.

PC12 cells untreated or treated with NGF were mechanically permeabilised by passage through a ball homogeniser. After permeabilisation half of the cells treated with NGF were exposed to an *in vitro* reaction with ATP (as described in section 2.6), in the absence of sodium orthovanadate. The cells were centrifuged at 1 000Xg for 10 minutes. The pellet was resuspended in detergent and centrifuged at 10000Xg for 10 minutes to separate the plasma membrane (P1M) from the cytoskeleton (pellet). The 1000Xg supernatant was centrifuged at 8000Xg for 35 minutes to pellet the large organelles (P2). The 8000Xg supernatant was centrifuged at 100 000Xg to pellet the small organelles (P3). The 100 000Xg supernatant was the cell cytosol (S3). The P1M, P2 and P3 fractions were immunoprecipitated with an antibody to the extracellular domain of TrkA (RTA) and resolved on a SDS gel along with Sigma molecular weight markers. 20 μ l of the S3 fraction was loaded directly on the SDS gel as a positive control for the blot. The proteins were transferred onto a nitrocellulose membrane and the blot probed with a 1:1000 dilution of an antibody to phosphotyrosine (4G10). The blot was incubated in a 1:10 000 dilution of anti-mouse antibody covalently linked to horseradish peroxidase. A signal was generated by a chemiluminescence reaction.

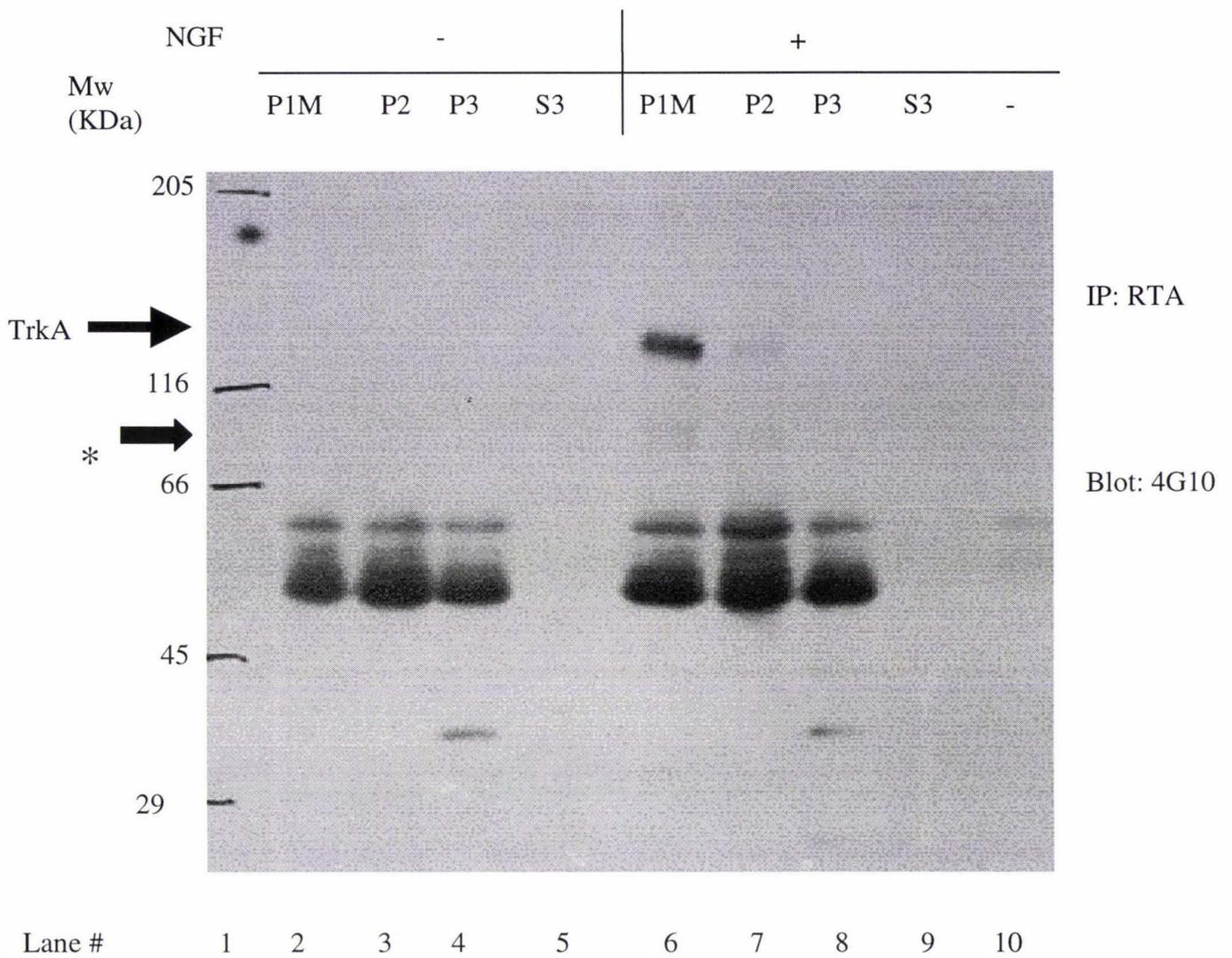


Figure 8

The appearance of a tyrosine-phosphorylated 90 KDa proteins in PC12 fractions exposed to sodium orthovanadate.

PC12 cells either untreated or NGF-treated were permeabilised and fractionated as described in Figure 7, except they were fractionated in the presence of sodium orthovanadate and an *in vitro* reaction was performed. The fractions were immunoprecipitated with RTA, run on a SDS gel and probed with 4G10 as in Figure 7. Note the faint tyrosine phosphorylated band that appears in lane 6 (*).

Tyrosine-phosphorylated TrkA can be seen only in those fractions that had been treated with NGF. It was found mainly in the plasma membrane (P1M) fraction (lane 6) and a small amount was detected in the large organelle (P2) fraction (lane 7). A faint band approximately 90 KDa in size was found associated with TrkA in the P1M fraction (lane 6).

As mentioned previously, TrkA is incorporated into signaling vesicles. Figure 8 shows the association of the 90 KDa protein with TrkA. We hypothesized that the 90 KDa proteins would therefore be incorporated into signaling vesicles along with TrkA. To better visualize this we needed to repeat the above experiment but expose the cells to an *in vitro* reaction with ATP.

3.2: The 90 KDa Protein is Associated with TrkA in signaling vesicles

PC12 cells, untreated or treated with NGF were permeabilised by passage through a ball homogeniser, exposed to an *in vitro* ATP reaction and fractionated in the presence of sodium orthovanadate. All fractions, except the cytosol (S3), were immunoprecipitated with RTA, and all the samples were treated as described for Figure 7. The results of two such experiments can be seen in Figures 9A and 9B.

In Figure 9A a large amount of tyrosine-phosphorylated TrkA was detected in the P2 fraction (lane 4) with a smaller amount in the P1M fraction (lane 3) in the absence of NGF. When cells were exposed to NGF more activated TrkA was observed in the P1M fraction (lane 6) with a comparable amount detected in the P2 fraction (lane 7). No activated TrkA was detected in the P3 fraction, either in the presence or in absence of NGF (lanes 5 and 9).

The 90 KDa proteins were seen to be associated with TrkA in the P2 fraction both in the presence and absence of NGF (lanes 4 and 8), but were only seen in the P1M fraction in the presence of NGF (lane 7).

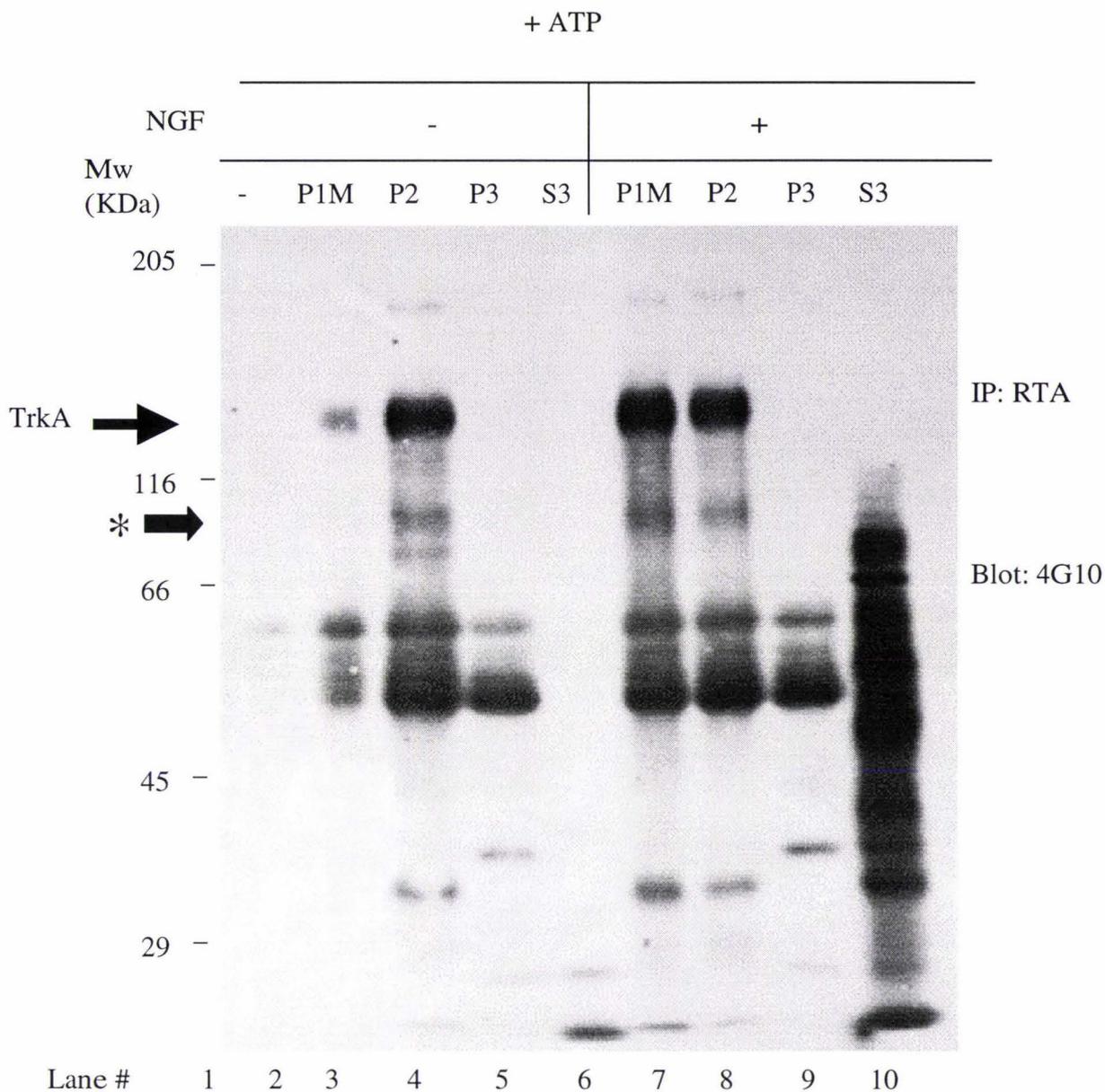


Figure 9A

The appearance of tyrosine-phosphorylated 90 KDa proteins in fractions exposed to an *in vitro* reaction in the presence of sodium orthovanadate.

NGF-treated or untreated PC12 cells were permeabilised, exposed to an *in vitro* reaction (described in section 2.6) and fractionated (as described for Figure 7) in the presence of sodium orthovanadate. Samples were treated as previously described for Figures 7 and 8. Note the presence of a tyrosine-phosphorylated band at approximately 90 KDa (*).

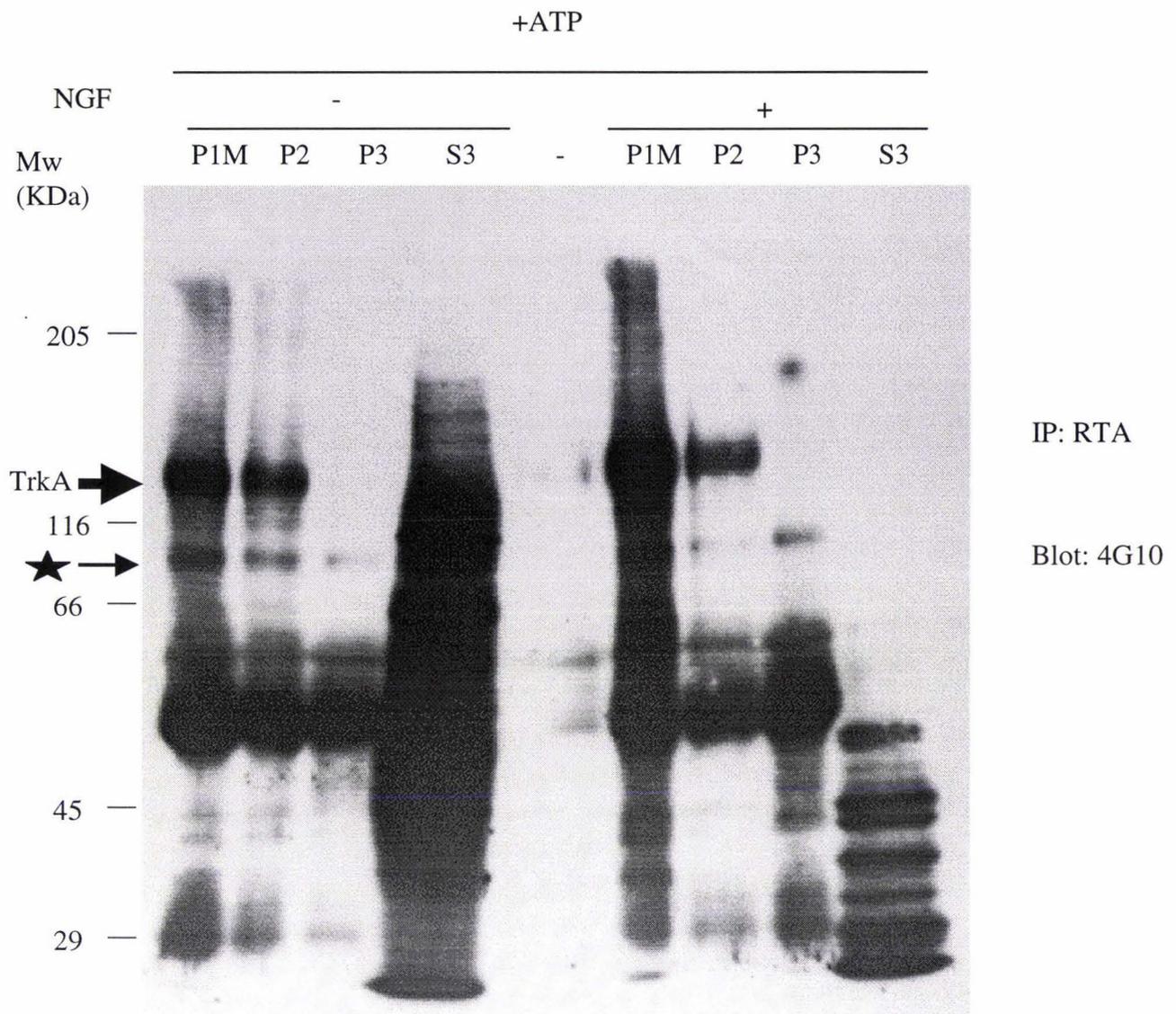


Figure 9B

The appearance of tyrosine-phosphorylated 90 KDa proteins in fractions exposed to an *in vitro* reaction in the presence of sodium orthovanadate.

PC12 cells either untreated or treated with NGF were treated as in Figure 9A. Note the presence of a tyrosine-phosphorylated band at approximately 90 KDa (★).

Tyrosine-phosphorylated TrkA was detected in the plasma membrane and large vesicle fractions in both the presence and absence of NGF in Figure 9B. No activated TrkA was observed in the P3 fractions. The important point to note in these results is the appearance of a band at 90 KDa associated with TrkA in all the fractions where TrkA was detected. The band was also detected in the P3 fractions. When the cells were stimulated with NGF, the 90 KDa band decreases in the P2 fraction and increases in the P3 fraction in comparison to those fractions that were not treated with NGF.

3.3: Discussion

The 90 KDa protein seen in Figures 8 and 9 is a possible candidate as a novel protein. It was detected in fractions that also contained tyrosine-phosphorylated TrkA (Figures 8 and 9) and was immunoprecipitated by anti-TrkA indicating that it is associated with TrkA. A small amount of this protein was seen in the absence of activated TrkA in the small vesicle fraction upon NGF stimulation (Figure 9B). In Figure 9B this 90 KDa protein appears to move from the P2 fraction to the P3 fraction, *i.e.* from large organelles to small organelles, upon NGF treatment. This indicates the protein is localized to small vesicles with TrkA to mediate the NGF signal, consistent with the model of the activated TrkA and associated proteins being transported from the plasma membrane to the cell body in signaling vesicles (Grimes *et al*, 1997). The absence of the tyrosine-phosphorylated 90 KDa proteins in Figure 7 is consistent with the fact that the tyrosine phosphatases are active due to the absence of an inhibitor. The tyrosine phosphatase inhibitor also causes artifactual buildup of tyrosine-phosphorylated TrkA, as seen in Figure 9A (lanes 3-5) and Figure 9B (-NGF + ATP).

Activated TrkA was not detected in the P3 fractions (Figures 8 and 9). The reason for this is unclear but I suspect that the blot was not covered with sufficient antibody or reagent and so a signal was not generated. The reason I believe this is the S3 fraction treated with NGF and exposed to an *in vitro* reaction should contain high molecular weight, tyrosine-phosphorylated proteins as well as low molecular weight proteins (compare to S3 fraction not treated with NGF in Figure 9B and S3 fraction (lane 10) in

Figure 9A). It was detected in the P3 fraction when the experiment was performed in the absence of sodium orthovanadate (Figure 7). *In vitro* reactions in the presence of orthovanadate may promote aggregation, causing more signaling proteins to form complexes with one another and thus sediment into the 8 000xg P2 fraction. The amount of activated TrkA seen in lane 3 (Figure 9A) is less than expected. I believe this to be due to sample loss since the antibody band is also reduced. The presence of activated TrkA in lane 6 (Figure 8) is expected because NGF treatment results in TrkA activation. A small amount is also observed in the P2 fraction demonstrating the movement of TrkA from the plasma membrane to large vesicles during the 10 minute warming stage after NGF treatment (receptor internalization).

These experiments have shown the existence of at least one, possibly novel, 90 KDa protein found to be associated with TrkA. Dr. M. L. Grimes had seen a triplet of 90 KDa proteins in his unpublished results but I was unable to show this clearly. The 90 KDa protein, or proteins, could associate with TrkA by interaction with the intracellular domain of TrkA since they are tyrosine-phosphorylated. Bacterial expression of the intracellular domain of TrkA would facilitate isolation of any novel intracellular proteins that associate with TrkA including the 90 KDa proteins. This would then enable us to better characterize these proteins and further our understanding of the initial events of NGF signaling.

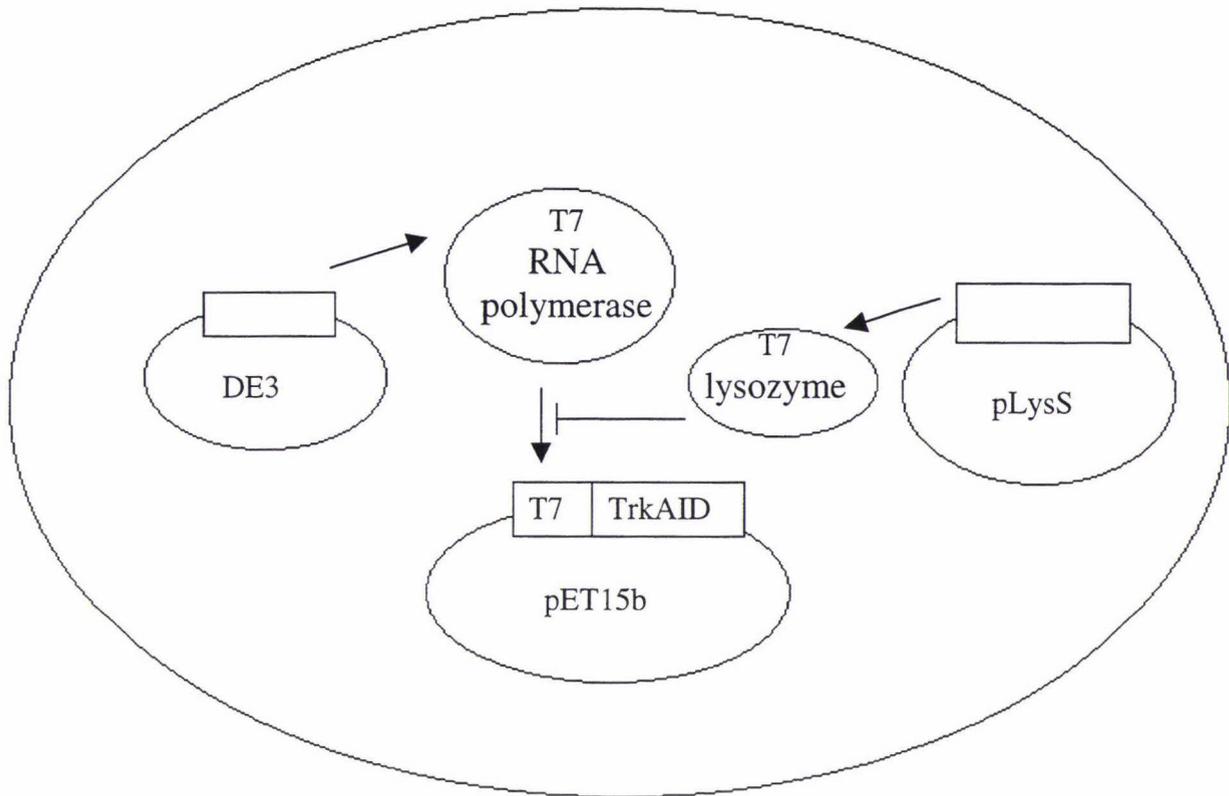


Figure 11
Expression System

DE3 lysogen expresses the T7 polymerase, which controls protein expression through the T7 promoter. pLysS expresses small amounts of T7 lysozyme, which degrades the T7 polymerase being expressed at a basal rate. This allows a population of bacteria containing the TrkAID construct to become established. This effect is overcome once expression is induced with IPTG and greater amounts of T7 polymerase are expressed.

The BL21 (DE3) *E.Coli* expression system, depicted in Figure 11, is an extensively used bacterial system for protein expression. These bacteria have an established DE3 lysogenic plasmid, which is responsible for the production of the T7 polymerase required for protein expression. The production of T7 polymerase is under control of the *lac* operon, so expression is dependent on the presence of IPTG.

Some proteins expressed in *E.Coli* are toxic. This means that if the protein is expressed constitutively then the bacteria would die and a sufficient population could not be established. In the BL21 system, even in the absence of IPTG, the *lac* operon

model has a basal rate of expression, producing small amounts of T7 RNA polymerase, sufficient to express small amounts of the protein.

To overcome this problem of toxicity the protein was expressed in the presence of pLysS. pLysS contains a gene that expresses small quantities of T7 lysozyme, which degrades the T7 polymerase being expressed at a basal rate. Consequently, no protein is expressed before induction with IPTG. Once IPTG is added to the culture, the production of the T7 RNA polymerase is stimulated, resulting in excessive levels of T7 RNA polymerase. The small amount of T7 lysozyme is insufficient to degrade all the T7 RNA polymerase produced thus allowing expression of the protein.

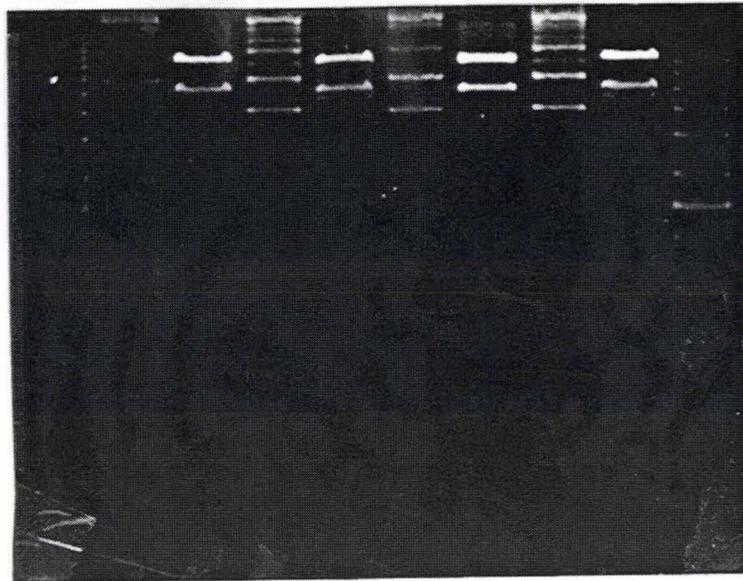
Bacteria were lysed after a period of time to release the expressed protein, which was believed to be soluble. Freeze/thawing the culture breaks open the outer membrane and the T7 lysozyme is able to degrade the peptidoglycan layer, therefore freeze/thawing in the presence of pLysS is sufficient to lyse the bacteria in this system. After centrifugation, the expressed protein was believed to be localized to the supernatant.

4.2: TrkAID Construct

It was important to ensure the TrkAID construct was inserted in the correct orientation, the construct contained the expected amino acid sequence and to verify the cloning sites due to contradictions in the accompanying information. To verify the cloning sites we performed a restriction digest of the plasmid. BL21 (DE3) *E.Coli* were transformed with the plasmid as described in section 2.18 and plasmid DNA was isolated from two colony types as described in section 2.16. There was some confusion as to whether TrkAID had been cloned into the NdeI and BamHI sites or NdeI and XhoI sites. If the construct had been cloned into the BamHI site then the XhoI site would no longer be present but if the XhoI site was used then the BamHI site would still be present. So the plasmid was digested with BamHI (B), XhoI (X) or both enzymes together (dd). The samples were run on an agarose gel along with a 1Kb

DNA ladder and plasmid DNA not cut with restriction enzymes (U). The digest is shown in Figure 12.

Ladder U B X dd U B X dd Ladder



Lane # 1 2 3 4 5 6 7 8 9 10

Figure 12
Restriction Digest

TrkAID plasmid was uncut (U) or cut with BamHI (B), XhoI (X) or both together (dd). The samples were run on a 1 % agarose gel and the bands visualized with ethidium bromide under UV light. The XhoI digest (lanes 4 and 8) are the same as the uncut TrkAID plasmid (lanes 2 and 6). The BamHI digest however is different, indicating BamHI has cut the plasmid.

XhoI did not cut the plasmid so the site must no longer be present demonstrating TrkAID was cloned into the NdeI and BamHI sites.

There was also some confusion as to the start point of the construct since there was an indication that it began within the transmembrane domain. This would not be beneficial for purification because the expressed protein would be insoluble and therefore more difficult to collect and purify. If this were the case then the methods utilized for purification would need to be revised. To check this and the orientation of the construct, Lorraine Barry in the Massey University sequencing unit sequenced the plasmid using a T7 primer (kind gift from Dr. J. S. Lott). The sequencing data was paired with the theoretical sequence of pET15b containing TrkAID on the GCG using the Bestfit program. The data is shown in Figure 13.

The Bestfit program showed the TrkAID construct cloned into pET15b was TrkAID (91 % similarity and 85 % identity). The sequencing data also confirmed that the construct was cloned in the correct orientation and was in the correct reading frame. The appearance of "N" bases within the TrkAID sequence is a consequence of the sequencing unit being unable to positively identify these residues. As the sequencing extends further along the DNA the amount of errors occurring and thus the appearance of "N" residues increases. Translation of the TrkAID sequence using the Translate program on the GCG identified the starting residue as Asparagine₄₄₃ and the final residue as Glycine₇₉₉. This means that the construct begins within the intracellular domain, consequently the standard methods of collection and purification could be used. Use of the Proteinsort program on the GCG for the TrkAID sequence calculated the molecular mass of the expressed protein to be 42.3 KDa and the isoelectric point to be 7.59.

4.3: TrkAID expression in E.Coli

Whenever the bacteria were grown on LB plates in the presence of ampicillin and chloramphenicol two bacterial morphology types were seen, large colonies and small colonies. The pET15b manual states that the growth of bacteria may be impaired in those bacteria that are expressing high levels of toxic protein (Novagen). To observe if there was any difference in TrkAID expression levels, cultures of transformed bacteria were grown for both the large and small colonies. A single colony was used to inoculate LB broth containing ampicillin and chloramphenicol (large-L and small-S). A negative control of BL21 (DE3) transformed with pLysS but not TrkAID-pET15b was grown in parallel (P). Protein expression was induced with the addition of IPTG. The cultures were grown for a further three hours with samples being removed before induction and each hour after induction. A typical growth curve is shown below in Figure 14.

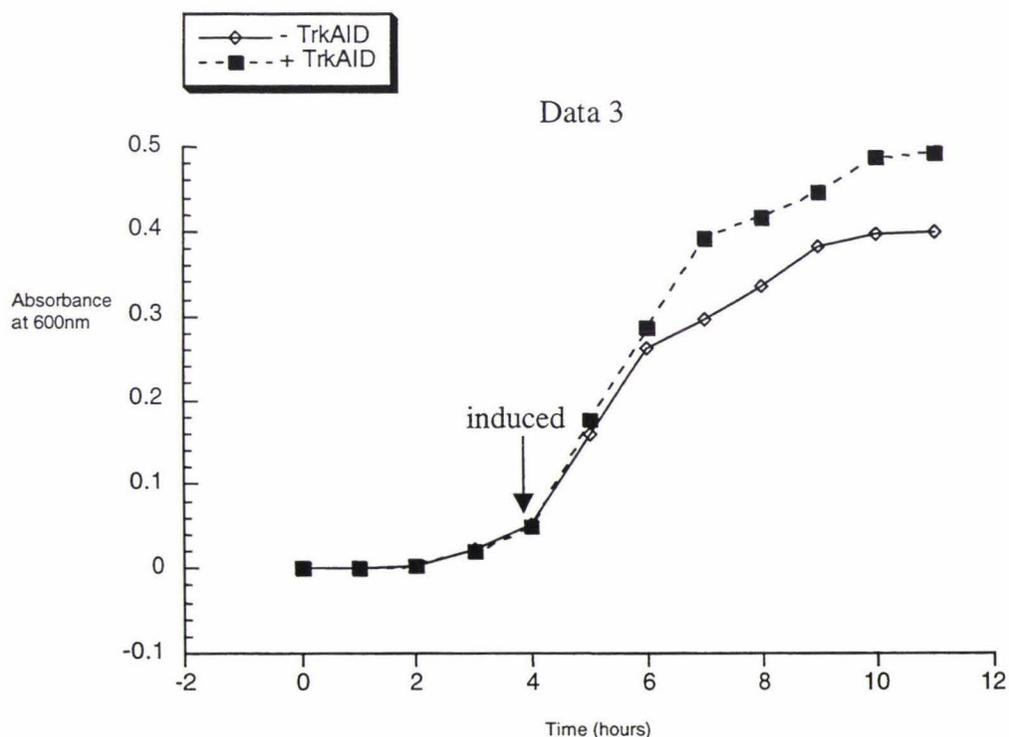


Figure 14

Growth Curve

Two colonies of bacteria, one expressing TrkAID (+ TrkAID) and the other not expressing TrkAID (- TrkAID), were grown until the optical density (measured at 600nm) was approximately 0.4 (4 hours growth). At this stage they were induced with 0.4mM IPTG and grown for a further 8 hours. Samples were taken every hour and the optical density measured for each sample and plotted against time of growth.

The bacteria were lysed by freeze/thawing and the bacterial DNA was sheared by passage through a syringe and 18G needle. Soluble material containing TrkAID was removed from the insoluble material by centrifugation, resolved on a SDS gel and stained with coomassie blue as described in section 2.10 (Figure 15).

No obvious band corresponding to TrkAID could be seen in either of the samples taken from transformed cultures. To determine if TrkAID was being expressed the experiment was repeated and the SDS gel western blotted and probed with an antibody to the C-terminal tail of TrkA (1086) as described in sections 2.11-2.13. The results are shown in Figure 16.

TrkAID was expressed in both the small colonies and large colonies. There was no significant difference in TrkAID expression levels between the two morphology types except a difference was noted at two hours growth (L2 and S2). No TrkAID was seen in bacteria not transformed with TrkAID-pET15b (P1-3) or in bacteria not induced with IPTG (bi).

There appeared to be an increase in the levels of TrkAID expressed from two to three hours. To obtain the maximal amount of protein expression a time course experiment was performed to identify the time point at which maximal TrkAID expression was achieved (Figure 17). Bacteria were transformed and grown as described for the previous experiment except one flask (S) was grown for two hours after induction to obtain material for purification trials. Another flask (E) was grown for 7 hours with samples being removed before induction and each hour after induction. A negative control flask was grown as in the last experiment (P). The samples were treated as before and equal protein concentrations were loaded onto the SDS gel. The gel was western blotted and probed with 1086 as before. The results are shown in Figure 17.

No significant increase in TrkAID levels was observed after two hours of growth proceeding induction.

The conclusion drawn from Figures 16 and 17 was that TrkAID was expressed in *E. Coli* and that maximal levels of expression were achieved two hours after induction. For future experiments, the sample was collected two hours after induction.

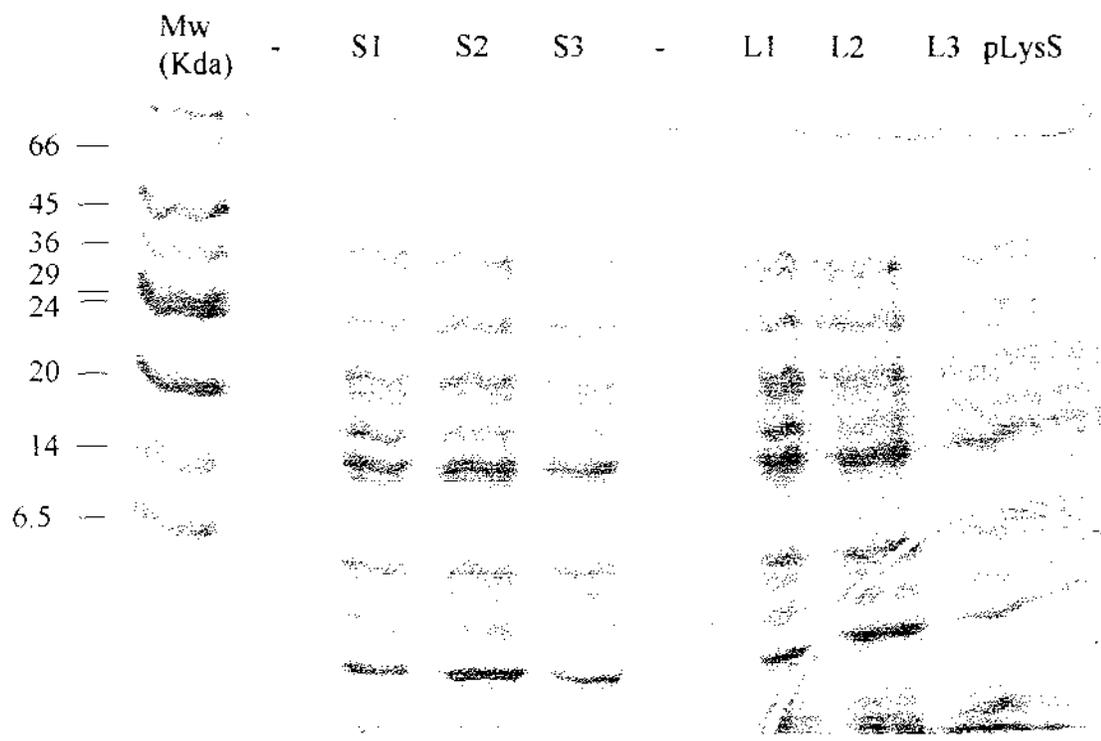


Figure 15
Coomassie Stain of TrkAID Expression

Three Small (S1-3) and Large (L1-3) colonies of bacteria expressing TrkAID were collected and used to grow 6 separate cultures of bacteria. A colony not expressing TrkAID (pLysS) was also used to inoculate another culture of bacteria. These bacteria were induced with IPTG and allowed to grow for a further three hours. The bacteria collected were centrifuged, resuspended in 20 mM phosphate buffer and frozen overnight. The samples were thawed the next day and passed through a syringe and 18G needle. The samples were centrifuged and the soluble material resolved on a SDS gel and stained with coomassie.

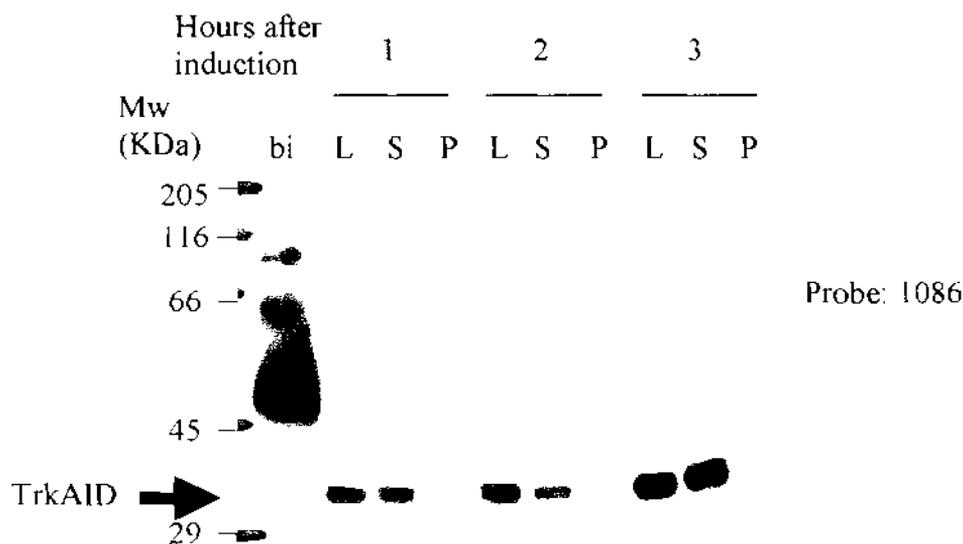


Figure 16
Comparison of TrkAID expression levels over time in different bacterial colonies.

BL21 (DE3) E.Coli transformed with pLysS alone (P) or pLysS and pET-15b-TrkAID (large-L and small-S colonies) were grown for three hours after induction with a sample being removed before induction (bi) and each hour after induction with IPTG. The bacteria were lysed by freeze – thawing and the DNA was sheared by passing the resuspended bacteria through a 18G needle. The bacteria were fractionated into soluble and insoluble fractions by centrifugation at 4 800 rpm for 10 minutes. The supernatant was loaded onto a 15 % acrylamide SDS gel. The proteins were transferred and the blot probed with an antibody raised to peptides corresponding to the C-terminal tail of TrkA (1086). This was followed by a reaction with an anti-rabbit antibody covalently linked to horseradish peroxidase as described previously. A signal was generated via a chemiluminescent reaction.



Figure 17
Extended time course for TrkAID expression levels.

Three cultures of BL21 (DE3) E.Coli transformed with either pLysS alone (P) or with pET-15b-TrkAID (E and S) were induced as in Figure 16. One culture of bacteria was harvested two hours after induction (S) for purification trials. The other flasks (P and E) were grown for 7 hours after induction. Samples were taken before induction (bi) or each hour for seven hours after induction with IPTG (E1-7, P1-7 and S1-2). Samples were prepared as described for Figure 16 and equal protein concentrations of the soluble material was loaded onto a 15 % acrylamide SDS gel, western blotted and probed with 1086 as in Figure 16.

4.4: TrkAID is Found in the Soluble and Insoluble Fractions

The levels of expression were not as high as predicted by Alan Bates, *i.e.* the band could not be visualized by coomassie staining. In section 4.1, I have discussed the inclusion of pLysS in the expression system to increase the levels of TrkAID expression. I wanted to determine if removing pLysS, thus removing the T7 lysozyme, from the expression system would increase TrkAID expression levels.

Bacteria transformed with TrkAID-pET15b, pLysS or both together were grown in three flasks. Protein expression was induced with IPTG and the bacteria grown for a further two hours with samples being taken before induction and each hour after induction. The samples were collected as described for Figure 15 and treated as described for Figure 16. The results are shown in Figure 18A. No visible difference in TrkAID expression levels was noted when pLysS was present or absent.

It was thought TrkAID might be partially localized to the membrane fractions (pellet) since the levels of expression were lower than expected (Figure 15). In addition, the sequencing data in Figure 13 shows that TrkAID contains a proline residue after the histidine tag. Proline residues are either required in turn structure or their introduction will possibly break secondary structure elements (Genetics Computer Group, 1991). If this were true, TrkAID could be in some insoluble conformation that would result in it being found in the bacterial pellet. The pellets were resuspended as described in section 2.21 and equal protein concentrations were resolved on a SDS gel. TrkAID was detected in the pellet. (Figure 18B).

Since TrkA was localized to both the soluble and insoluble fractions, we wanted to determine which fraction contained the most TrkAID. The fraction that contains the most TrkAID would be the one used for purification. Equal protein concentrations of the pellet and supernatant fractions were resolved on a SDS gel, western blotted and probed with 1086, (Figure 19). It was clear from the results that the supernatant contains the bulk of the expressed TrkAID.

Figure 18A

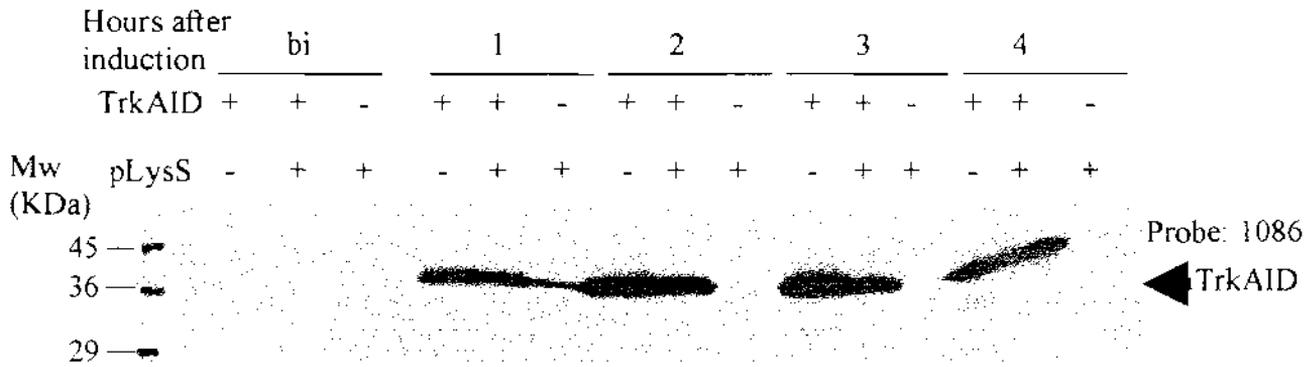


Figure 18B

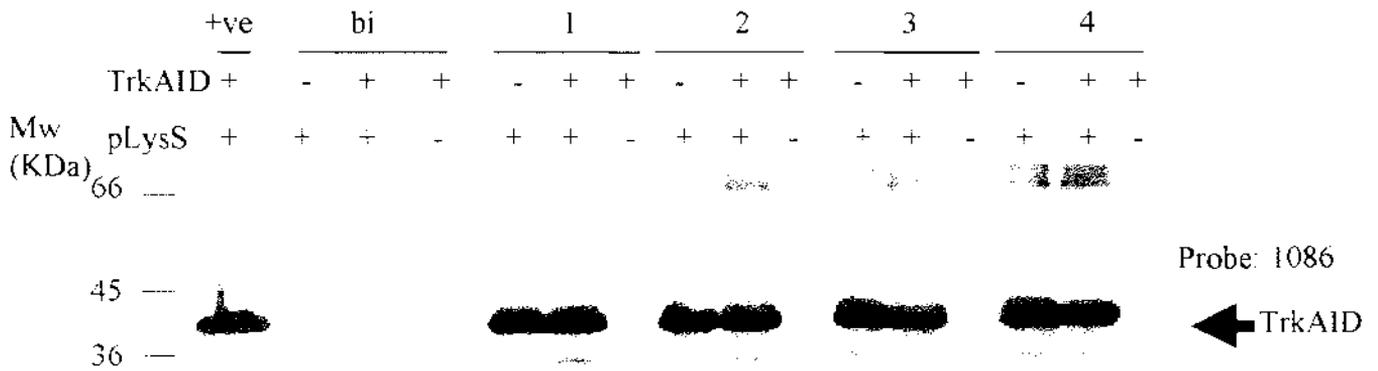


Figure 18

Effect of pLysS on TrkAID expression levels and TrkAID localization to the soluble (A) and insoluble (B) fractions.

BL21 (DE3) E.Coli transformed with or without pLysS, pET15b-TrkAID or both were grown for four hours after induction with IPTG. Samples were taken before induction (bi) and every hour after induction. The samples were treated as in Figure 16 and equal protein concentrations of the material loaded onto a 15 % acrylamide SDS gel and western blotted. Blots were probed with 1086 as in Figure 16.

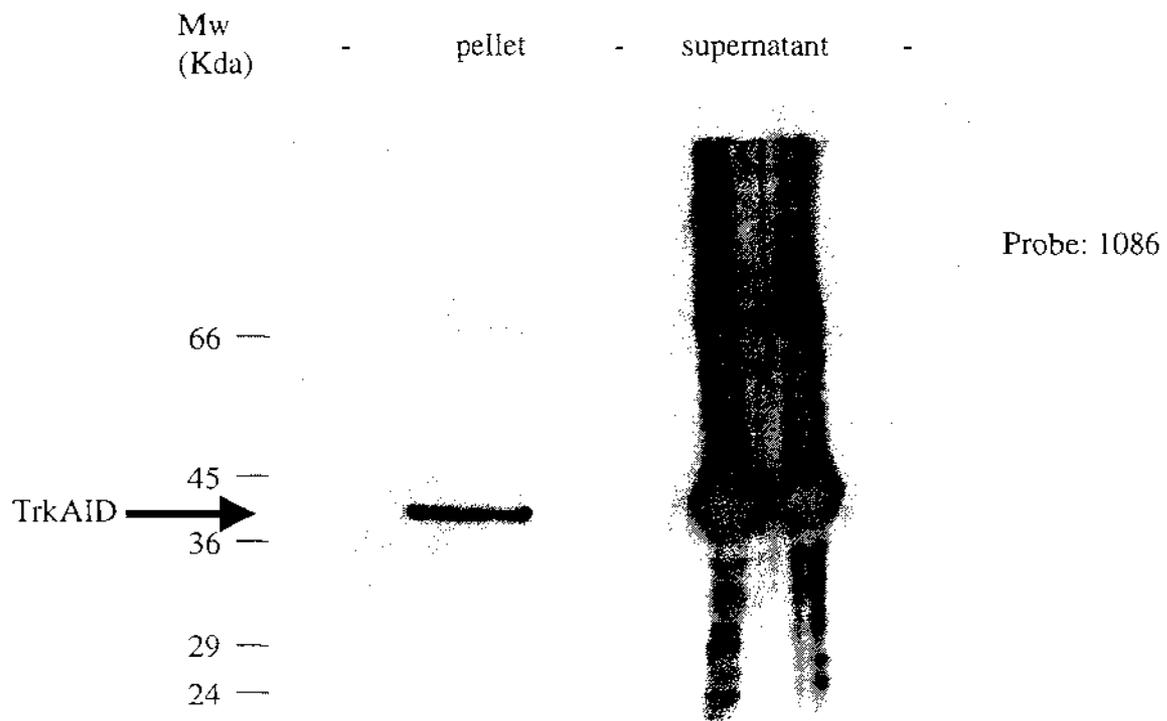


Figure 19
 Comparison of TrkAID levels in pellet and supernatant fractions.

Equal protein concentrations of soluble and insoluble material collected from bacteria expressing TrkAID as described previously, was loaded onto a SDS gel, western blotted and probed with 1086 as described for previous experiments.

4.5: TrkAID is expressed in an active state

The intention was to use purified TrkAID for binding studies and raising antibodies. For intracellular proteins to bind TrkA, TrkA tyrosine residues need to be phosphorylated, so it would be useful to determine if TrkAID is expressed in an active state, *ie* tyrosine phosphorylated. To achieve this the blots from Figures 18A and 18B were stripped and reprobed with 4G10 as described in sections 2.13 and 2.14. The results are shown in Figure 20. Both blots showed that TrkAID was expressed in an active state and that the presence or absence of pLysS made no difference to the levels of tyrosine phosphorylation.

4.6: TrkAID can be immunoprecipitated by 1086 in an active state

There are several tyrosine residues phosphorylated in response to NGF (described in section 1.3). Identifying which tyrosine residues are phosphorylated on TrkAID would determine which proteins associate with TrkAID *in vitro*. If certain tyrosine residues are not phosphorylated then we may not be able to identify novel intracellular proteins that may associate with TrkA in response to NGF. TrkAID was immunoprecipitated from the soluble material with 1086. Samples were resolved on a SDS gel, western blotted and probed with 1086 to ensure TrkAID was immunoprecipitated. The blot was stripped and reprobed with 4G10 to ensure that TrkAID was immunoprecipitated in an active state and then reprobed again with antibodies raised to specific phosphotyrosine residues (Y490 and Y674/675). All blots are shown in Figure 21.

Figure 20A

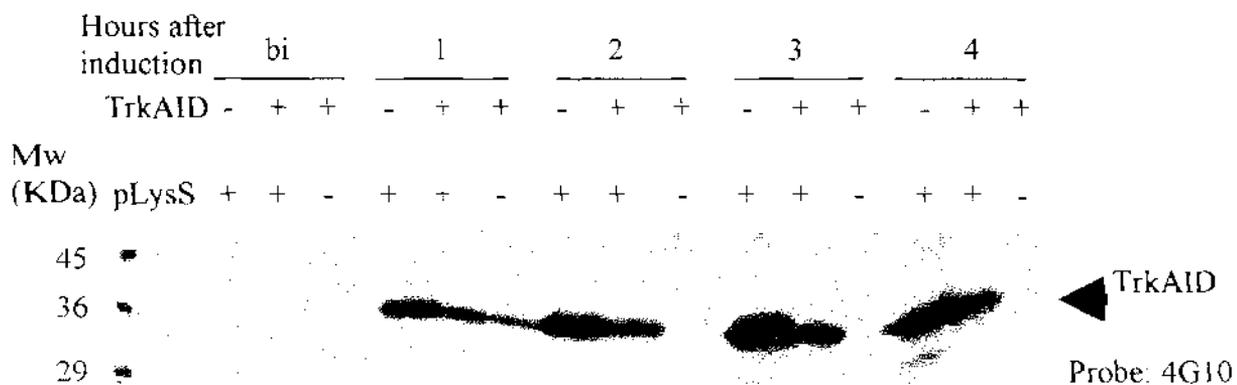


Figure 20B

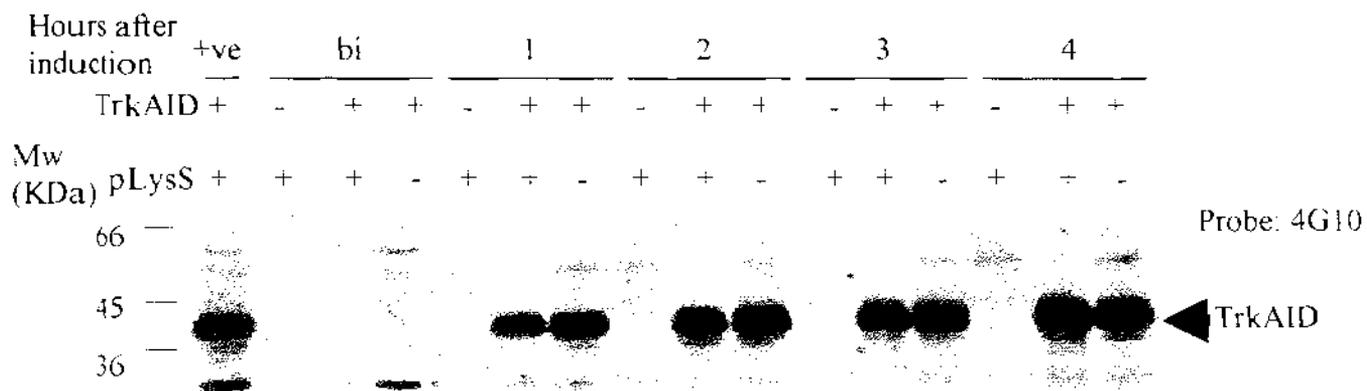


Figure 20

TrkAID is expressed in an active state in both the soluble and insoluble fractions.

The blots from Figure 18 were stripped in acid wash for 10 minutes and reprobed with anti-phosphotyrosine (4G10) as described in section 2.14.

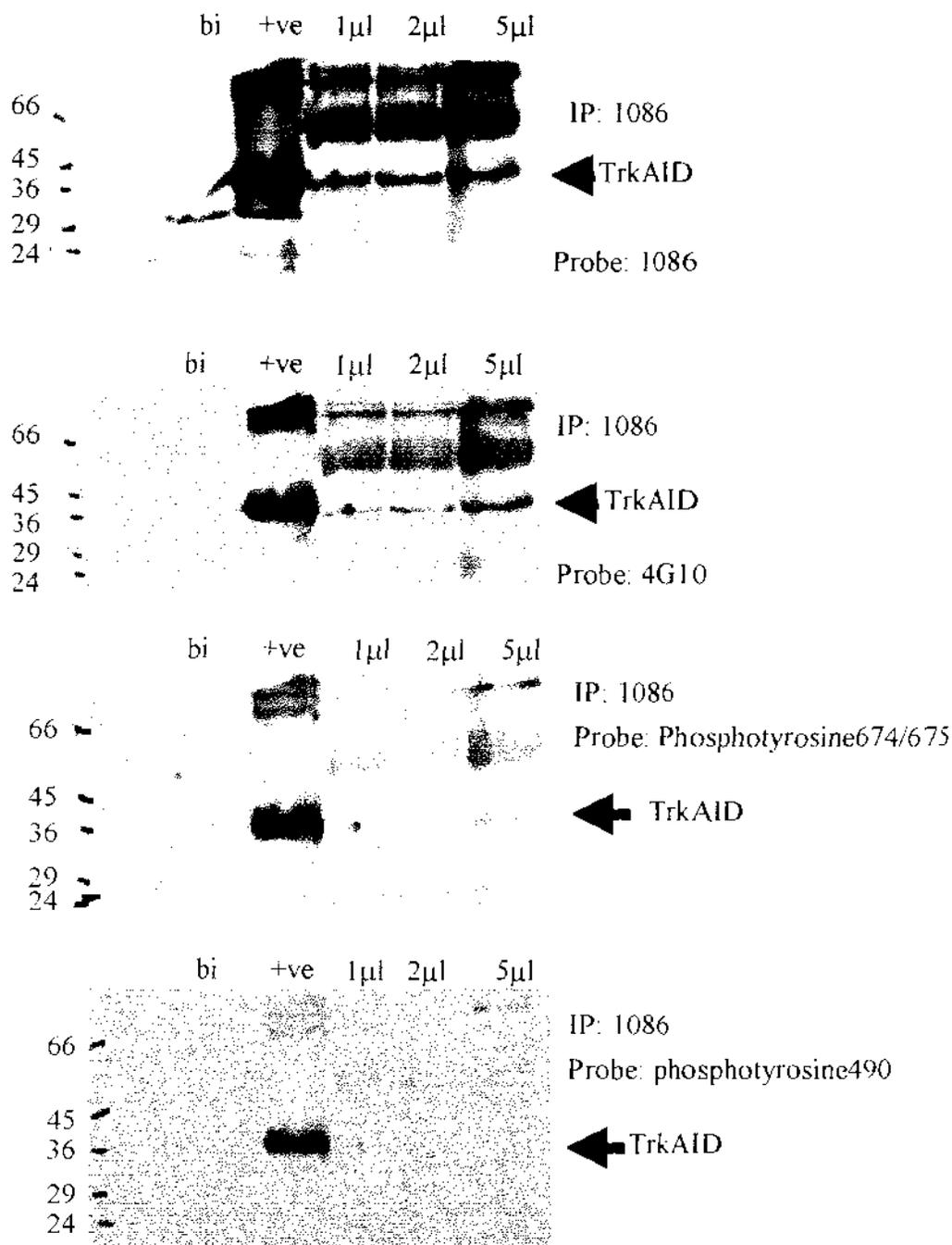


Figure 21
TrkAID can be immunoprecipitated with 1086.

BL21 (DE3) E.Coli were transformed with pLysS, TrkAID-pET15b or both. The bacteria were treated as for Figure 16. The soluble material was immunoprecipitated with 1, 2, or 5μl of 1086 and resolved on a 15% acrylamide SDS gel along with samples taken before induction (bi) and before immunoprecipitation (+ve). A significant increase in the levels of TrkAID was observed between 1 and 2μl but not between 2 and 5μl. The levels of the contaminating proteins also increase with increasing amounts of antibody. The gel was western blotted and probed with 1086. The blot was stripped and reprobed with anti-phosphotyrosine (4G10) as in Figure 20. The blot was stripped again and reprobed with anti-TrkA phosphotyrosine 674/675 and anti-TrkA phosphotyrosine 490.

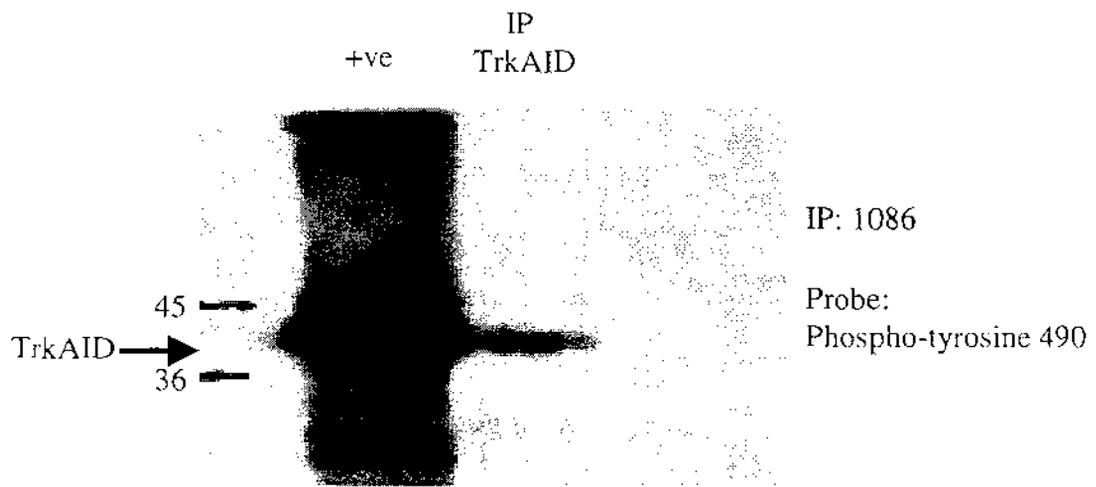


Figure 22
TrkAID is phosphorylated on Y490.

TrkAID was immunoprecipitated with 1086 from soluble material as described for Figure 21, resolved on a SDS gel, western blotted and probed with phosphotyrosine 490 as described for previous figures.

The 1086 probe shows that TrkAID was immunoprecipitated. Other bands on the blot are assumed to be from the antibody. The probe with 4G10 shows TrkAID was immunoprecipitated in tyrosine phosphorylated state. The levels of tyrosine phosphorylated TrkAID is significantly higher with 5 μ l of antibody. The probe with the phosphotyrosine-490 TrkA and phosphotyrosine-674/675 antibodies show that TrkAID appears to be phosphorylated on tyrosine 674/675 but not tyrosine 490. However, TrkAID in the lysate (positive control) reacted with the two antibodies so it is probable that TrkAID is phosphorylated on tyrosine 490 and tyrosine 674/675.

To confirm this, the experiment was repeated with the blot being probed first with phosphotyrosine 490 antibodies (Figure 22). In this case, a stronger band was observed in the immunoprecipitated samples, confirming that tyrosine 490 is phosphorylated in bacterial lysates.

4.7: Discussion

TrkAID was expressed in bacteria but the levels of expression were very low (Figure 15). The reason for this is unclear but the Novagen pET15b manual does note that when a protein is toxic to bacteria then they may not express the protein at high levels and those that do express the protein at high levels die. The low levels of expression meant that we were not able to visualize expression by staining the gel with coomassie. Instead, we had to probe with an antibody raised to the C-terminal tail of TrkA (1086) since this method is more sensitive for detecting protein.

The appearance of small bacterial colonies when the transformants were grown (section 4.3, Figures 15 and 16) indicated that some bacteria may be expressing higher levels of TrkAID but this resulted in a decrease in their ability to grow because of the energy required to express the protein and survive. The difference seen in the expression levels between the small and large colonies (Figure 16) is not significant because equal amounts of protein were not loaded on the gel. It would have been beneficial to repeat the experiment but load equal protein on the gel. I believe the small colonies to be at earlier stages of growth.

The result in Figure 16 also showed TrkAID expression increasing at three hours after induction. This result was due to the unequal protein loading in the lanes as confirmed when equal protein was loaded on the lanes (Figure 17). It showed maximal TrkAID expression was reached by two hours after induction.

An investigation into the localization of TrkAID in bacteria revealed it could be found in both the soluble and insoluble fractions (Figure 18). For this experiment the culture of bacteria were allowed to grow for a longer period of time after induction to observe whether pLysS has an effect on TrkAID expression at later stages of growth. The reason for the localization of TrkAID to the soluble and insoluble fractions is unclear, but the sequencing data (Figure 13) shows the presence of a proline residue after the histidine tag. This could be a candidate for a site of α -helix formation, which would result in the protein misfolding. This protein could be in some insoluble conformation or have some hydrophobic residues exposed for interaction with membranes. It is strange that TrkAID is found in both the soluble and insoluble fractions. However, if TrkAID misfolds to form soluble aggregates then it would have a partial distribution in the soluble fraction. Interestingly, a comparison of the distribution of TrkAID revealed it was found mostly in the soluble fraction (Figure 19) providing support for the formation of soluble aggregates. The appearance of phosphate groups on TrkAID (Figure 20) suggests that the protein be properly folded with constitutively active kinase activity. The phosphate groups could cause extra interactions that could affect TrkAID distribution in bacteria. These negatively charged phosphate groups might interact with the positively charged histidine groups causing TrkAID to undergo some conformational change. However, the expression of tyrosine phosphorylated TrkAID is useful since the tyrosine residues need to be phosphorylated if intracellular proteins are to interact with the protein for binding studies (discussed in section 1.3.3).

Immunoprecipitation with 1086 was successful with 2 μ l of antibody immunoprecipitating higher levels of TrkAID than 1 μ l but equal levels to 5 μ l (Figure 21). The efficiency with which 1086 immunoprecipitated TrkAID was quite low when compared to the levels of TrkAID in the lysate (positive control). The reason for this is unclear. The results also show that TrkAID was tyrosine-phosphorylated.

Knowledge of which tyrosine residues are phosphorylated will facilitate analysis of which intracellular proteins interact with TrkAID since the intracellular proteins all bind different phosphotyrosine residues (discussed in section 1.3.3). The gel was probed with antibodies raised to phosphotyrosine-490 and phosphotyrosine-674/675 on TrkA (Figures 21 and 22). The bands in these two blots were very faint, which is probably an artifact of the repeated stripping of the blot for each reprobe. This was confirmed by the relatively strong signal in the lysate for the two blots, which was also decreasing with each reprobe (the blots were probed with the different antibodies in the order represented in Figure 21). The signals seen for the lysates could also be a result of insufficient stripping, if the antibody was not totally stripped off the blot then a signal would be generated once the ECL reagents were added again to the blot. Another confirmation comes from a gel with immunoprecipitated TrkAID that was probed with anti-TrkA phosphotyrosine-490 first (Figure 22). In this blot, the signal was more intense confirming that TrkAID did react with this antibody. Therefore, TrkAID must be phosphorylated on tyrosine-490.

The next step was to purify TrkAID to obtain enough material for further studies and to raise antibodies to it. Purified TrkAID could be added to untreated PC12 cytosol to identify those proteins that interact with TrkA upon NGF treatment.

CHAPTER 5 : PURIFICATION OF TRKAID

5.1: Nickel Column Purification of TrkAID

The N-terminal 6-histidine tag was utilized to purify TrkAID expressed in bacteria. This histidine tag can bind to a positively charged ion such as nickel with high affinity, which is mediated by free pairs of electrons in secondary amine groups of histidine coordinating with the positively charged nickel. Once bound to nickel, the interaction can be disrupted by the addition of imidazole, which competes with the histidine groups for nickel binding. Therefore, imidazole can be used for eluting the protein from the nickel column.

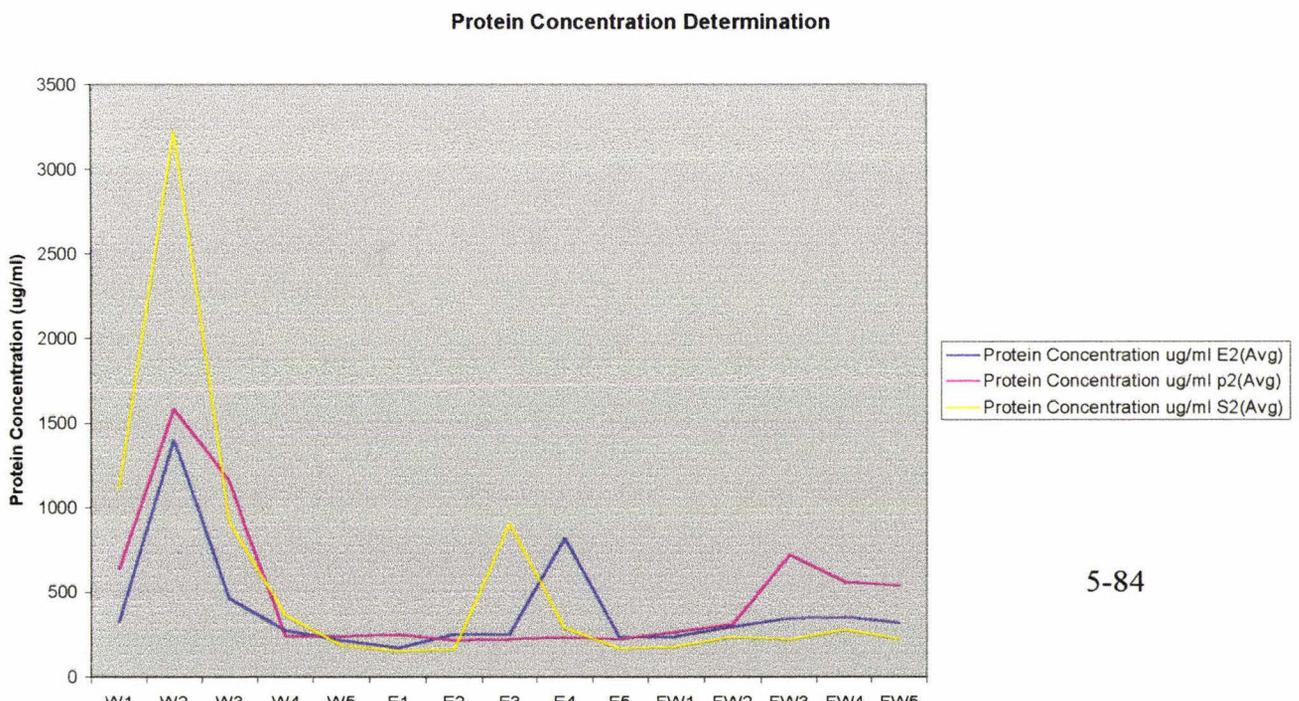
Soluble material obtained from bacteria transformed with pLysS and TrkAID-pET15b (E2 and S2) and bacteria transformed with pLysS alone (-2) were taken two hours after induction. Samples were passed through a nickel-charged affinity column and the column was washed with a low concentration of imidazole to remove non-specific proteins that do not bind to the column. The protein was eluted with a higher concentration of imidazole and the column was washed with a high concentration of imidazole to remove any proteins off the column that may interact with the column with a very high affinity. The column was stripped and recharged with nickel for each sample loading. Fractions were collected throughout this procedure and their protein concentration was measured using the Lowry method (section 2.8). The protein concentrations are shown in Figure 23. Those fractions that contained protein were resolved on a SDS gel, western blotted and probed with 1086 (Figure 24).

Fraction	Protein Concentration $\mu\text{g/ml}$		
	E2(Avg)	p2(Avg)	S2(Avg)
W1	325	638.75	1116.25
W2	1397.5	1587.5	3225
W3	465	1156.25	920
W4	272.5	247.5	365
W5	220	242.5	190
E1	175	252.5	151.5
E2	252.5	217.5	165
E3	252.5	227.5	910
E4	823.75	237.5	287.5
E5	240	225	167.5
FW1	242.5	267.5	180
FW2	295	312.5	237.5
FW3	345	725	222.5
FW4	352.5	565	277.5
FW5	320	539	225

Table 1
Protein Concentration of Fractions Collected During TrkAID Purification on a Nickel Column

Bacteria transformed with pLysS and TrkAID (E2 and S2) or pLysS alone (p2) were induced with 0.4mM IPTG. Samples were collected two hours after induction and were passed through a nickel column as described in materials and methods. Fractions collected during the procedure were assayed for protein concentration by the Lowry method. Triplicate results were averaged and plotted in Figure 23.

Figure 23



TrkAID was detected in the initial wash fractions for both samples of bacteria transformed with TrkAID-pET15b (E2 and S2), indicating TrkAID was not binding to the column with high affinity (Figure 24). A higher protein concentration was observed for a few of the eluted fractions from the sample taken from bacteria that had been transformed with TrkAID-pET15b (Figure 24). However, in the blot no TrkAID was detected in these fractions.

This experiment was repeated three times and the result was the same each time. The appearance of TrkAID in the washes could also be an indication that the method is unsuitable. A positive control was needed to ensure that the column was working and the method was correct.

The positive control used was the *cis*-retinal binding protein (CRBP) purified by Dr. Treena Blythe. CRBP was expressed and purified using identical methods for TrkAID. The result shown in Figure 25 is a coomassie stain of a gel of fractions collected from a nickel column. CRBP was bound to and eluted from the column, proving that the column and method of elution were working.

Why then was TrkAID not purified? It is possible that TrkAID binds strongly to the column and is not eluted. If this were the case then TrkAID would not be eluted in any of the washes but once the nickel is stripped off the column with EDTA then TrkAID would also be eluted. Another sample was passed through the nickel charged affinity column as described for Figure 20. Fractions were collected when the nickel was stripped off the column, dialyzed to remove the nickel and EDTA and resolved on a SDS gel along with fractions collected during the wash and elution steps. The gel was western blotted with anti-TrkAID (1086). The stripped fractions did not contain TrkAID (data not shown). We therefore concluded that TrkAID was binding with very low affinity to the nickel column, causing it to be eluted during the washing procedure.

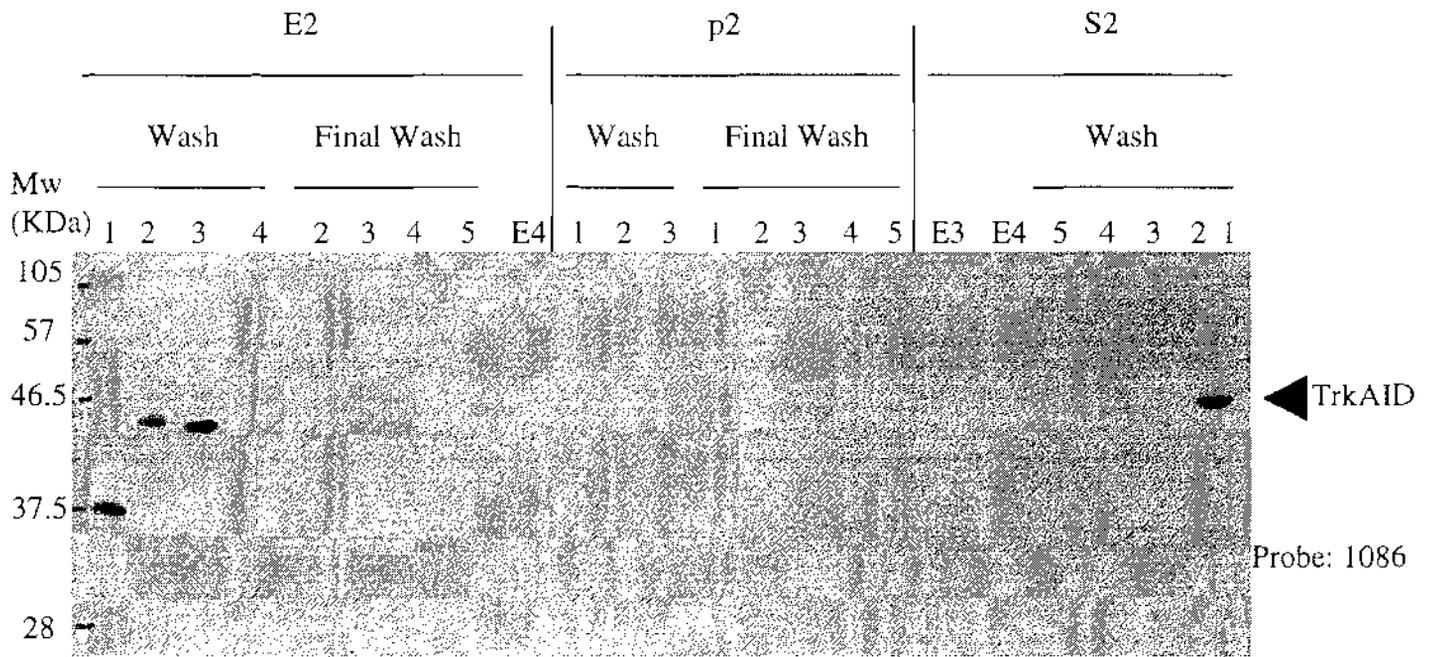


Figure 24
 Trial for nickel column purification of TrkAID.

BL21 (DE3) bacteria transformed with pLysS (p2) or with pLysS and pET15b-TrkAID (E2 and S2) were harvested two hours after induction with IPTG. The bacteria were treated the same way as for Figure 16 and the soluble fraction was run through a nickel charged affinity column. The column was washed with 100mM imidazole and the protein eluted with 250mM imidazole. The column was washed again with 500mM imidazole. Five 1ml fractions were collected at each step (W1-5, E1-5 and FW1-5) and the protein concentration of each fraction was measured. Those fractions found to contain protein were resolved on a 15% acrylamide SDS gel and western blotted. The blot was probed with 1086 as in Figure 16.

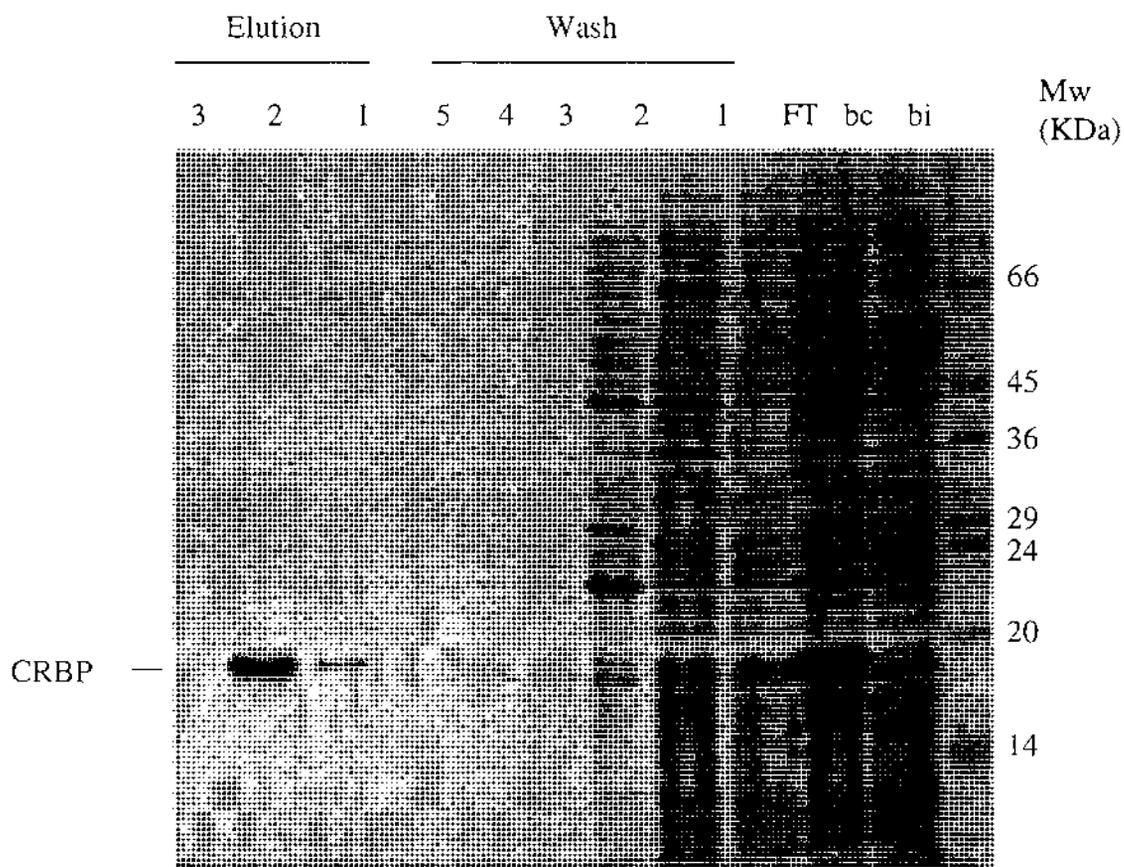


Figure 25

Purification of *cis*-retinal binding protein (CRBP) on a nickel-charged affinity column.

BL21 (DE3) E.Coli transformed with pLysS and pET-15b-CRBP were grown for 3 hours after induction with IPTG. Samples were taken before induction (bi), before the sample was passed through the column (bc) and collected three hours after induction. The bacteria were treated as described in Figure 16 and the soluble material was passed through a nickel charged affinity column as described for Figure 23. The fractions collected (flow through – FT, wash and elution) were run on a 15 % acrylamide SDS gel and stained with coomassie blue for 30 minutes.

5.2: Maximizing TrkAID Binding to the Nickel Column

The affinity of TrkAID for the column may be increased by charging the column with a positively charged ion that has higher affinity for histidine groups such as copper. Soluble material collected from TrkAID-expressing bacteria was passed through a column charged with copper. The procedure was the same as before except the protein was eluted with 300 mM imidazole. Fractions collected during the procedure were resolved on a SDS gel, western blotted and probed with 1086 (Figure 26).

While TrkAID was still found in the initial wash fractions, using copper instead of nickel to charge the affinity column did increase the amount of TrkAID that was retained and eluted with imidazole. However, other proteins were also eluted, some of which were seen in the blot (Figure 26) and more clearly in the Ponceau stain of the blot (data not shown). A small amount of TrkAID was seen within the final wash fractions indicating the increase in TrkAID affinity for the column.

We concluded that this method would not provide us with any advantage for TrkAID purification. Charging the column with copper increased the binding of TrkAID to the column but it also increased the binding of other proteins to the column. These proteins bound the column with very similar affinity to TrkAID.

It is possible that TrkAID's affinity for the nickel column is so low that low concentrations of imidazole elute it. This was tested using elution with an imidazole concentration gradient. TrkAID was expressed in bacteria as for past expression trials and the sample treated as before (section 5.2). The nickel column was washed in the absence of imidazole and the protein was eluted with a low gradient elution (0-100 mM imidazole). The column was washed again with 500 mM imidazole. The result is shown in Figure 27.

TrkAID was eluted in small amounts at approximately 50 mM imidazole and some came off in the wash. The absence of imidazole in the wash and the low concentration of imidazole in the elution buffer appeared to enhance TrkAID's binding and elution, but even in these conditions, the amount of TrkAID eluted was small.

Although the low imidazole concentration gradient wash only retained a small amount of TrkAID on the column, this appears to provide more hope for purification. If we could increase TrkAID specific binding to the column then we could purify the protein in reasonable quantities.

TrkAID has been found to be expressed in an active state (Figures 20 and 22). The phosphate-histidine interactions discussed in section 4.7 may be preventing histidine binding to the nickel. If this were true then the interaction of TrkAID with the nickel might be strengthened by the addition of salt to the sample, this would disrupt phosphate-histidine interactions.

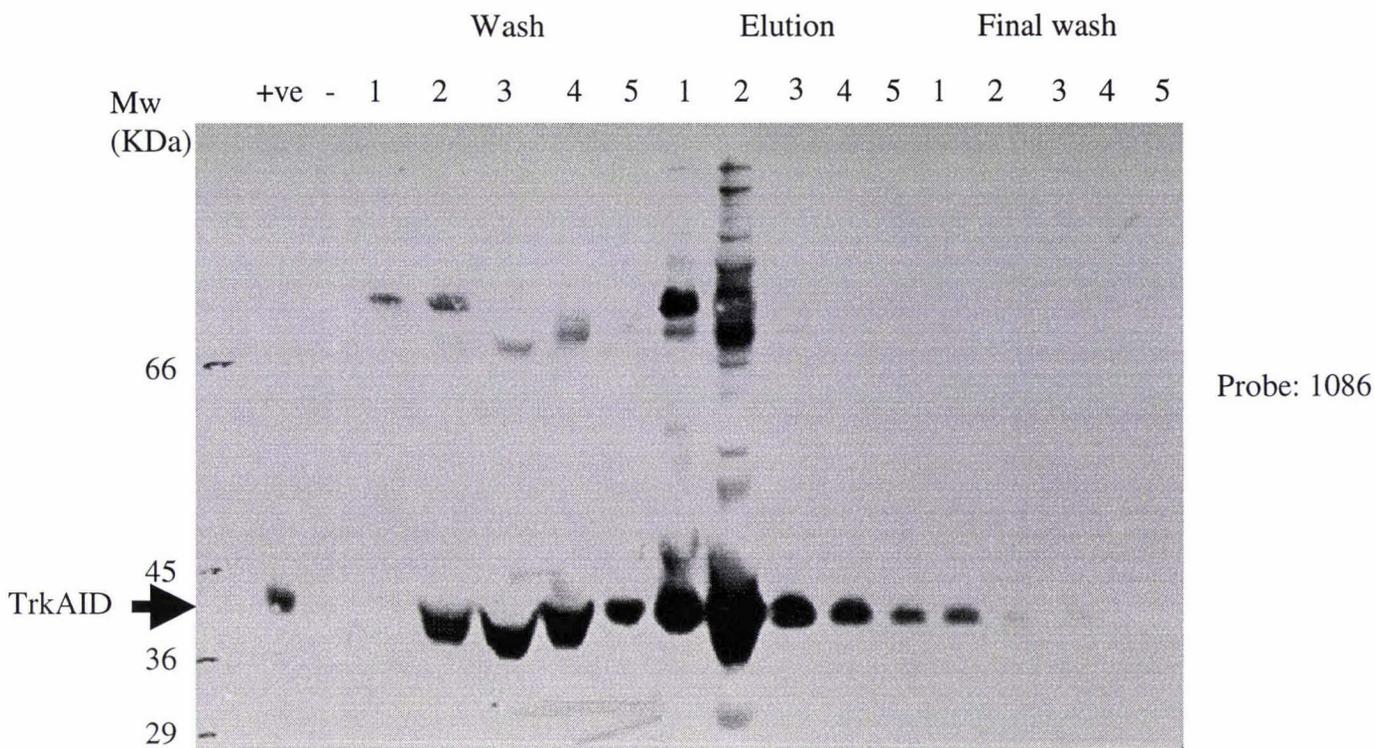


Figure 26
Purification of TrkAID on a copper-charged affinity column.

BL21 (DE3) E.Coli transformed with pLysS and pET15b-TrkAID were grown for two hours after induction with IPTG. Soluble material was passed through a copper - charged affinity column. The column was washed with 20 mM imidazole and the protein eluted with 300 mM imidazole, then a final wash was performed with 500 mM imidazole. The collected fractions (Wash 1-5, Elution 1-5 and Final Wash 1-5) and sample taken before passage through the column (+ve) were resolved on a 15% acrylamide SDS gel, western blotted and probed with anti-TrkA intracellular domain (1086) as described previously.

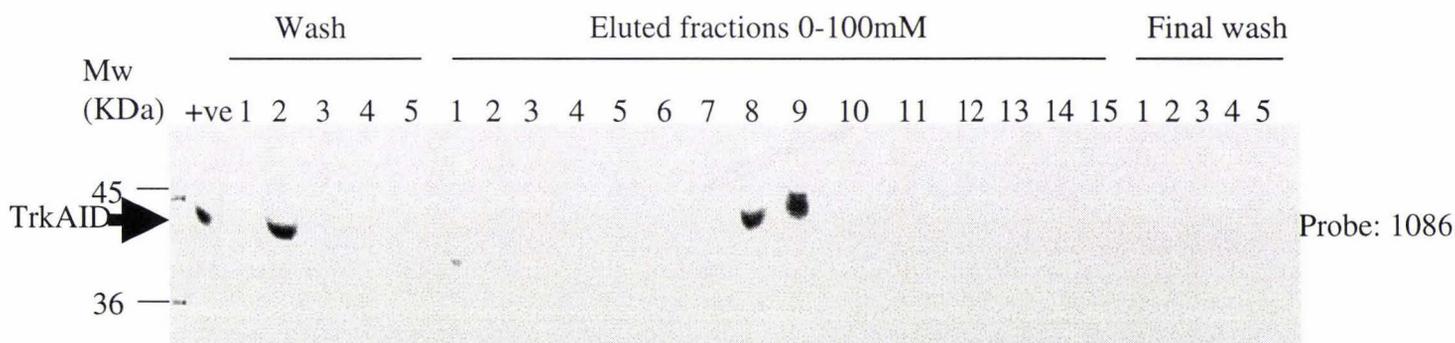


Figure 27
Elution of TrkAID from nickel column with 0-100 mM imidazole.

BL21 (DE3) E.Coli transformed with pLysS and pET15b-TrkAID were grown for two hours after induction. Soluble material was passed through a nickel - charged affinity column. The column was washed with buffer containing no imidazole and the protein was eluted with a gradient of 0-100mM imidazole. The column was washed finally with 500mM imidazole. The collected fractions (Wash 1-5, Eluted fractions 1-5 and Final Wash 1-5) and sample taken before passage through the column (+ve) were treated as above.

5.3: Disruption of Phosphate-Histidine Interactions within TrkAID

Lithium is a small ion that can be added in high concentrations to disrupt the salt bridges without denaturing protein. Soluble bacterial lysate containing TrkAID was collected as described for previous experiments. The sample was passed through a nickel column as for Figure 27 with and without lithium chloride present from the binding to the elution stages. Fractions collected throughout the procedure were dialyzed to remove the salt, resolved on a SDS gel and treated as for previous experiments (Figure 28).

In the presence of lithium chloride, a large amount was still seen in the flow through fraction, *ie* a large amount had not bound to the column and TrkAID was found in the initial wash fractions. No TrkAID was seen in the eluted fractions but a small amount of TrkAID was detected in the final wash fraction indicating that lithium chloride had increased the affinity of TrkAID for the column slightly over controls without lithium chloride (Figure 28).

5.4: Purification of TrkAID Using Denaturation and Renaturation

The above data suggests that TrkAID does not interact with the nickel column with high affinity (Figures 24 and 27), which may be due to conformation or the presence of phosphate groups that are masking the histidine tag. There is a method normally used for purification of soluble proteins that misfold and aggregate to yield inactive proteins during expression that may be useful for purification of TrkAID (Shi *et al.*, 1997). The method involves denaturing the protein, binding it to a column, renaturing the protein on a column then eluting the renatured protein.

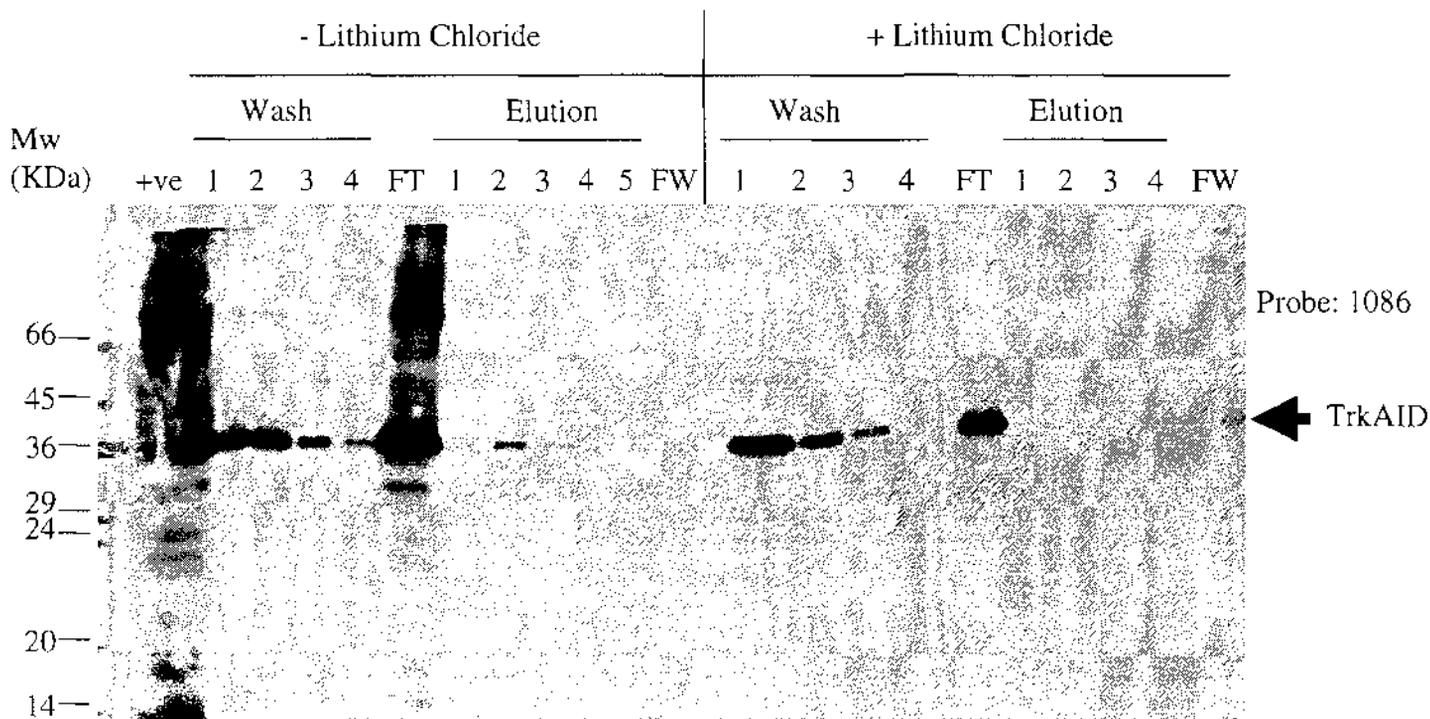


Figure 28

Purification of TrkAID by disrupting ionic interactions with lithium chloride.

BL21 (DE3) E.Coli transformed with pLysS and pET15b-TrkAID were grown for two hours after induction with IPTG. The cells were treated as in Figure 16 and the soluble material was passed through a nickel - charged affinity column in the presence or absence of lithium chloride. The sample that did not bind to the column (flow through, FT) was collected as well as the wash, elution and final wash (FW) fractions for both conditions. The fractions and a sample taken before the cells were passed through the column (+ve) were resolved on a 15% acrylamide SDS gel and treated as in Figure 16.

Shi *et al* (1997) suggested that a strong denaturant (guanidine hydrochloride) be used for denaturing the protein and a milder denaturant (urea) be used for renaturing the protein on the column to maximize the recovery of the purified protein. Soluble lysate was passed through a nickel column under denaturing conditions, renatured on the column and eluted as described in section 2.24. Fractions were collected throughout the procedure and those fractions containing salt were dialyzed. All the collected fractions were resolved on a SDS gel, western blotted and probed with 1086, (Figure 29).

TrkAID was detected in the wash fractions taken after TrkAID was renatured on the column (Wash a). Importantly, the blot also shows that TrkAID was eluted from the column. A coomassie stain of an identical SDS gel with the same samples showed that the amount of TrkAID eluted off the column was very small but the band could be visualized for the first time (data not shown).

Since TrkAID was also found in the insoluble fraction (Figure 18B), this method may be useful in purifying TrkAID from this fraction. Samples collected from the insoluble fraction were passed through a nickel column and treated as described for Figure 29. The results are shown in Figures 30 and 31. No TrkAID was detected in the wash or eluted fractions. TrkAID was detected in the initial wash fractions but not in the renaturation fractions confirming TrkAID from the insoluble fraction was not interacting with the nickel column even under denaturing conditions.

The fact TrkAID from the soluble fraction was binding to, eluting off the column in small quantities (Figure 29) suggested the interaction between TrkAID, and the nickel column was still very weak. This pointed to other factors playing a role in disrupting TrkAID binding. We had observed in Figure 28 that lithium chloride increased TrkAID binding to the nickel column. Therefore, a combination of the two methods of purification may increase the amount of TrkAID that can be purified.

Soluble lysate was passed through a nickel column using the method described in section 2.25 (inclusion body purification in the presence of lithium chloride). The collected fractions were western blotted as above Figure 32.

A large amount of TrkAID was still seen in the flow through and initial wash fractions under these conditions, so TrkAID was still not fully interacting with the nickel column. However, a larger amount was detected in the eluted fractions than had been seen previously. This was the most successful cation affinity column protocol, suggesting that disrupting charge-charge interactions with high salt was a useful strategy when used in conjunction with denaturation and renaturation. However, much TrkAID passed straight through the column and quantities purified were still not satisfactory.

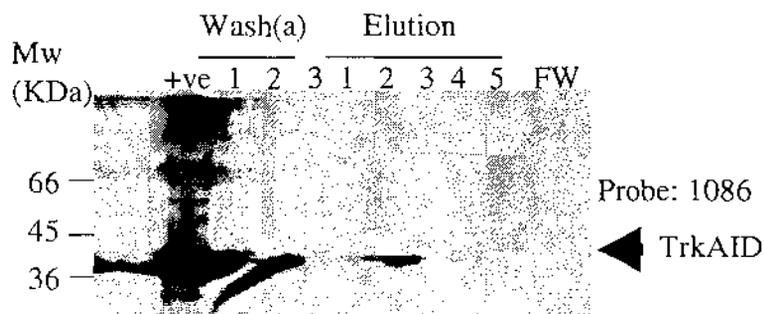


Figure 29

Purification of denatured TrkAID from the soluble fraction on a nickel-charged affinity column and the elution of the renatured protein.

BL21 (DE3) transformed with pLysS and pET15b-TrkAID were grown for two hours after induction with IPTG. Soluble material was passed through a nickel - charged affinity column in the presence of guanidine hydrochloride. The column was washed with no imidazole and guanidine hydrochloride. The protein was renatured on the column with a gradient wash 8 M-0 M urea and washed again with no imidazole and no guanidine hydrochloride. The protein was eluted with 250 mM imidazole and washed finally with 500 mM imidazole. The wash fractions collected after renaturation of the protein on the column [Wash (a)] the eluted fractions (1-5), and the final wash (FW) fraction were resolved on a 15% acrylamide SDS gel, western blotted and probed with 1086 as in Figure 16.

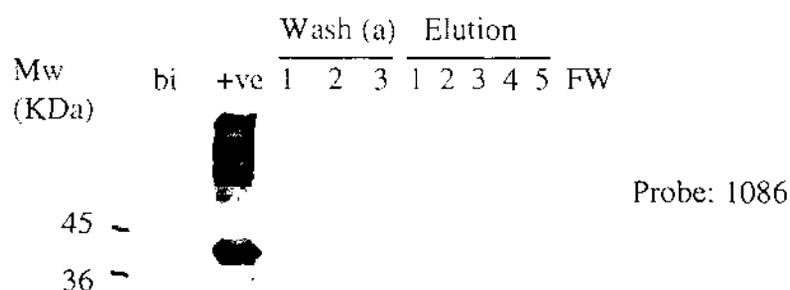


Figure 30

Purification of denatured TrkAID from the insoluble fraction on a nickel-charged affinity column and the elution of the renatured protein.

BL21 (DE3) E.Coli transformed with pLysS and pET15b-TrkAID were grown for two hours after induction with IPTG. The cells were treated as for Figure 16 and the insoluble material was passed through a nickel charged affinity column under denaturing conditions and treated as described in Figure 28.

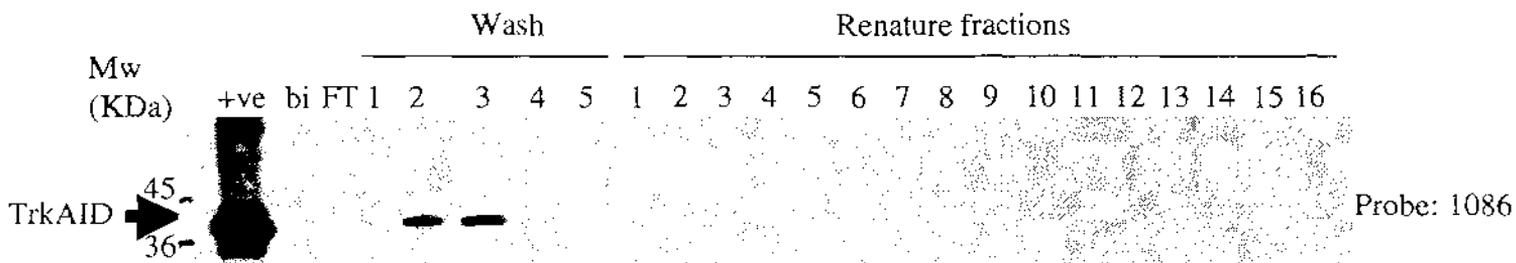


Figure 31

Analysis of the initial washes and washes during renaturation for purification of denatured TrkAID from the insoluble fraction.

BL21 (DE3) E.Coli transformed with pLysS and pET15b-TrkAID were grown for two hours after induction with IPTG. Fractions collected during the initial washes (before renaturation) and as the protein was being renatured on the column (1-16) (in Figure 29) were dialysed against 20 mM phosphate buffer pH 8.0. These fractions and samples collected before induction (bi) and before passage through the column (+ve) were loaded onto a 15 % acrylamide SDS gel, which was treated as described for Figure 28.

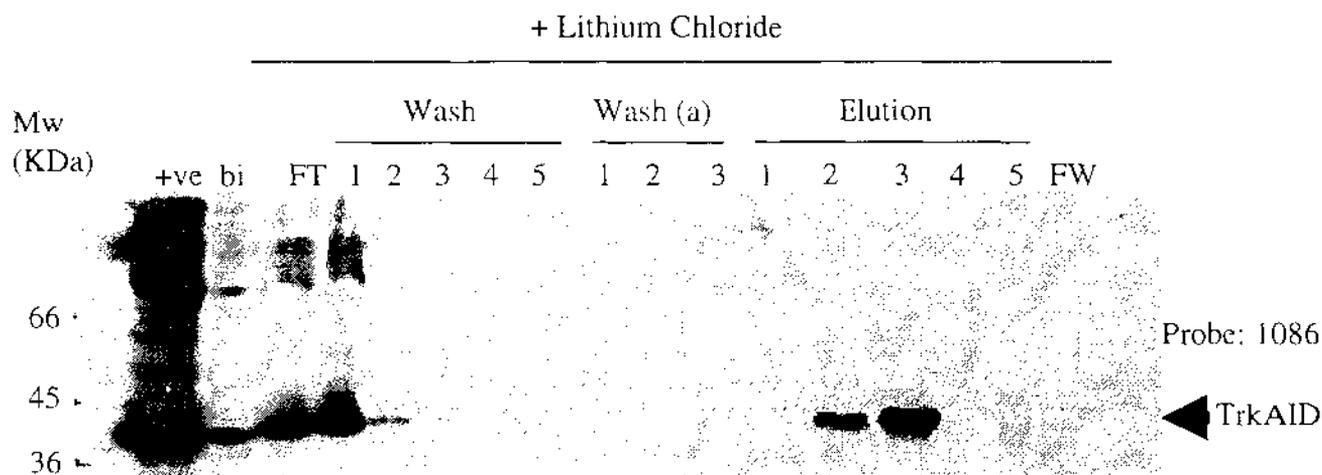


Figure 32

Combination of lithium chloride and denaturation methods for purification of TrkAID from the soluble fraction.

BL21 (DE3) E.Coli transformed with pLysS and pET15b-TrkAID were grown for two hours after induction with IPTG. The cells were treated as in Figure 16 and the soluble material was passed through a nickel charged affinity column in denaturing conditions as in Figure 28 except in the presence of 1 M lithium chloride. Fractions collected during the initial washes (before renaturation) (Wash 1-5), washes after renaturation [wash (a) 1-5], elutions (E1-5) and the final wash (FW) were dialysed against 20 mM phosphate buffer pH 8.0. These were resolved on a SDS gel along with samples taken before induction (bi), before passage through the column (+ve) and while the sample was loaded on the column (FT). The proteins were transferred and the blot was probed with 1086 as in Figure 16.

5.5: Other Methods of Purification

More standard methods of purification, such as ammonium sulphate precipitation and ion exchange, were attempted to purify TrkAID.

For sequential ammonium sulphate precipitation, 10 % ammonium sulphate was added to the soluble fraction containing TrkAID and allowed to mix at room temperature. The precipitate was pelleted by centrifugation and a further 10 % ammonium sulphate was added. The procedure was repeated until 100 % saturation was reached. Samples were taken from the supernatants and pellets at each stage and resolved on a SDS gel, western blotted and probed with 1086 (Figure 33). An identical gel was run and stained in coomassie blue (data not shown).

The bulk of TrkAID was precipitated with 50 % saturation, but unfortunately most of the protein in the sample was also precipitated at this stage (Figure 33). This method of purification was not helpful for TrkAID purification since it was unable to resolve TrkAID from the other proteins.

We explored ion exchange chromatography as a method for purification. TrkAID has a theoretical isoelectric point of 7.59. At pH 5.5 TrkAID was unable to bind a negatively charged column (CM) and at pH 8.0 TrkAID was able to bind a positively charged column (monoQ) (Figures 34 and 35). Supernatant from induced cultures was dialysed against MOPS pH 5.5 and the sample passed through a CM column (kind gift from Dr. Gill. Norris). Proteins were eluted with 1 M sodium chloride and fractions were resolved on a SDS gel and western blotted as described above. An identical gel was run and stained with coomassie blue. These results are shown in Figure 34.

TrkAID was not detected in the eluted fractions. The coomassie gel shows that other proteins bound the CM column and were eluted with 1M sodium chloride. Therefore, using the CM column may be used in purification of TrkAID to remove other proteins.

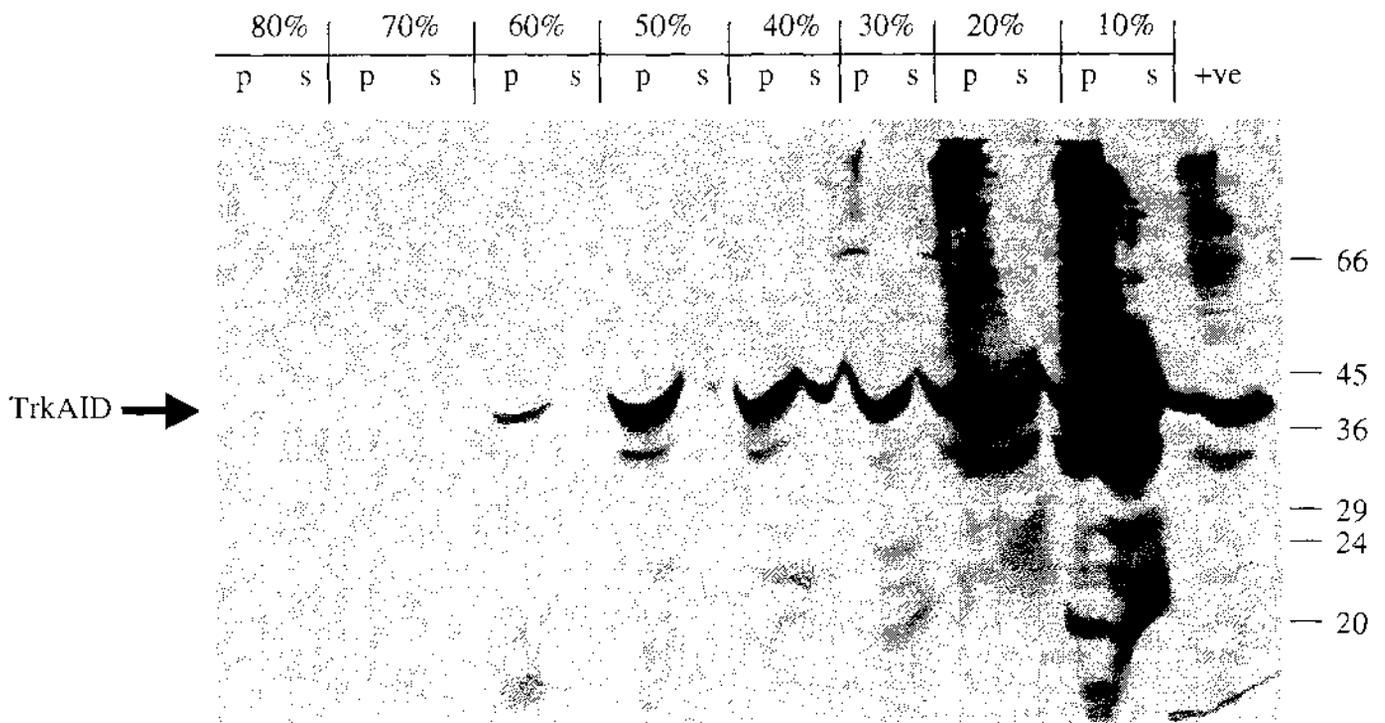


Figure 33
Ammonium sulphate precipitation of protein from the soluble fraction.

Soluble material was collected from bacteria transformed with pET15b-TrkAID as described previously. Ammonium sulphate was added to the sample at 10% saturation and the sample was centrifuged at 3 300xg for 30 minutes. The pellet was resuspended in 20 mM MOPS pH 7.2 and a sample taken from the supernatant. The process was repeated in 10 % intervals until 100 % saturation had been reached. The pellet samples (p) were dialysed against 20 mM MOPS pH 7.2 and were loaded on a SDS gel along with the supernatant samples (s) and a sample taken before precipitation (+ve). The proteins were transferred and probed with 1086 as for Figure 16.

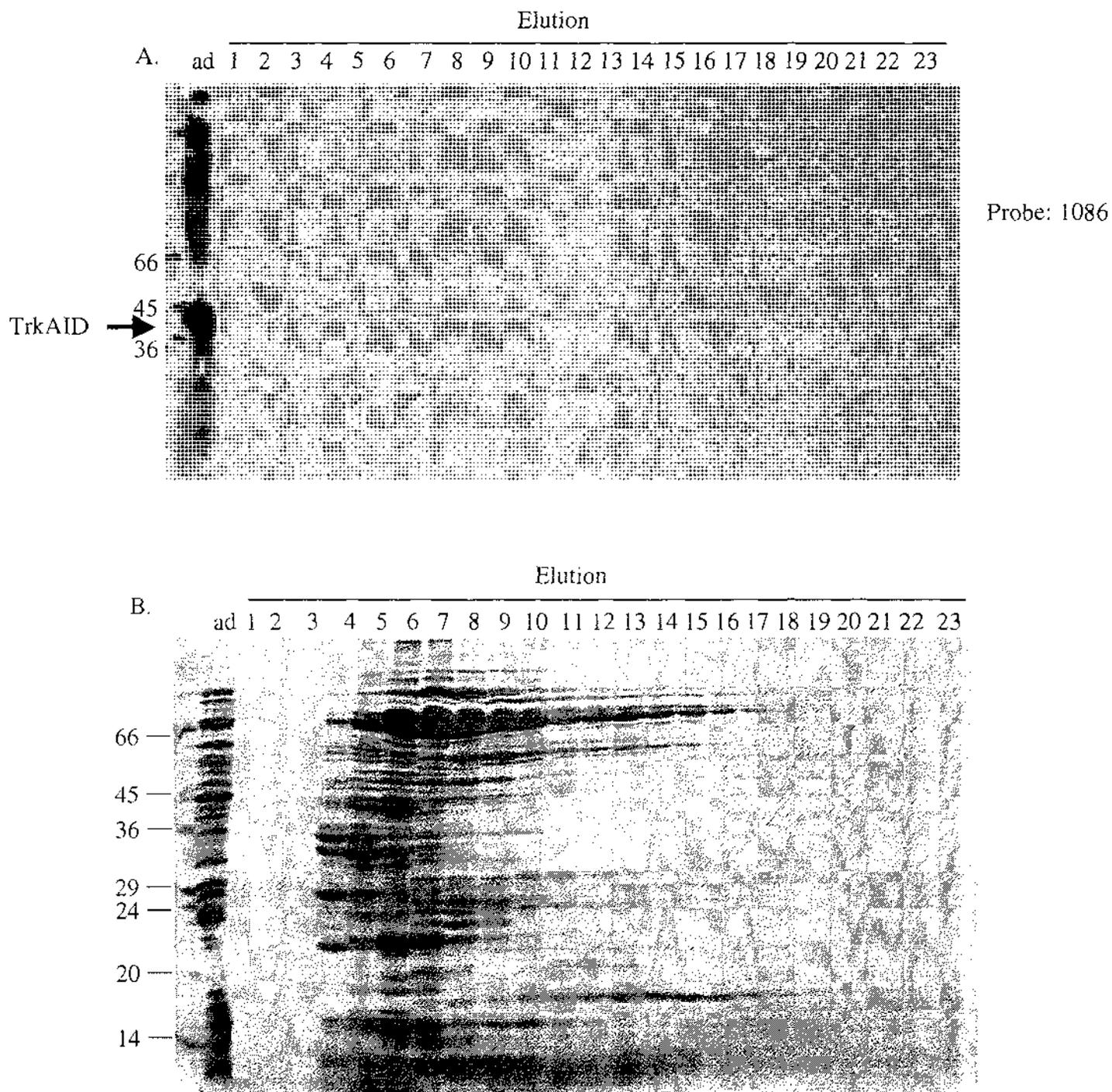


Figure 34
Purification of TrkAID from the soluble fraction using CM ion exchange chromatography.

Soluble material collected from bacteria transformed with pET15b-TrkAID was dialysed against 10 mM acetic acid pH 5.5 and passed through a CM ion exchange column. The column was washed with 10 mM acetic acid pH 5.5 and the protein eluted with 10mM acetic acid pH 5.5 + 1 M NaCl. Fractions collected during the elution and a sample taken after dialysis before passage through the column (ad) were resolved on two SDS gels. One gel was stained with coomassie blue (B) (as described in section 2.10) and the other gel was western blotted and probed with 1086 (A) as described previously.

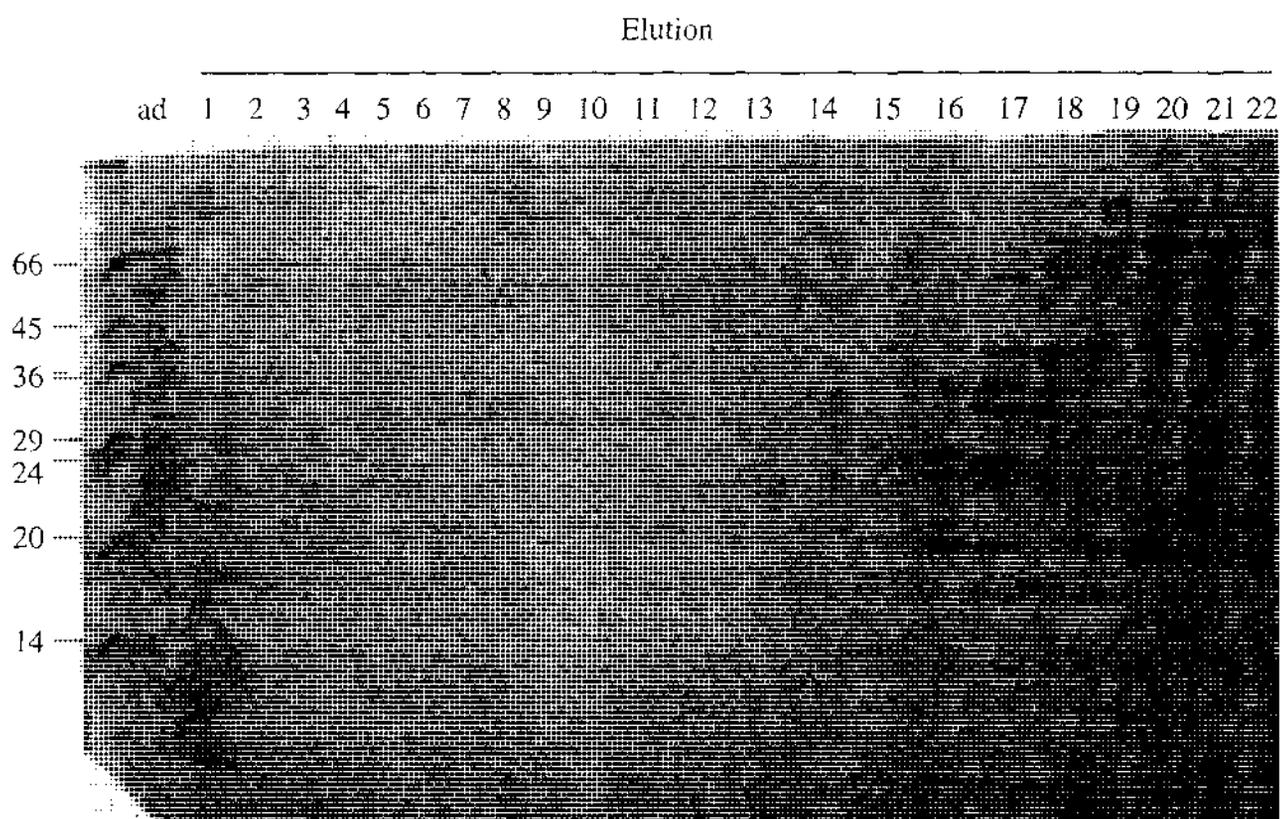
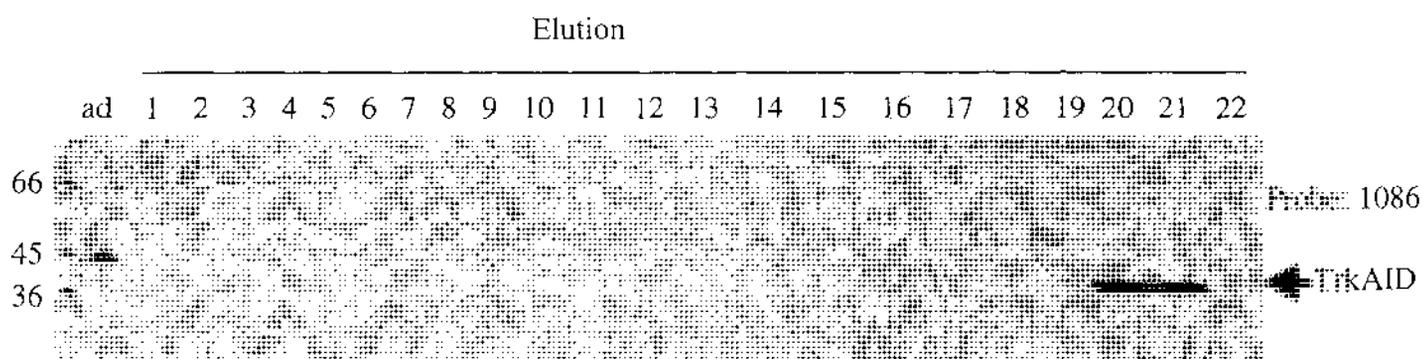


Figure 35
Purification of TrkAID from the soluble fraction using a monoQ ion exchange column.

Soluble material collected from bacteria transformed with pET15b-TrkAID was dialysed against 10 mM Tris pH 8.0 and passed through a monoQ ion exchange column. The column was washed with 10 mM Tris pH 8.0 and the protein eluted with 10 mM Tris pH 8.0 + 1 M NaCl. Fractions collected during elution and a sample collected after dialysis before passage through the column (ad) were resolved on two SDS gels and treated as described for Figure 33. (A) is a western blot probed with 1086 and (B) is a coomassie stained gel of the same samples.

Soluble bacterial lysate was dialyzed against Tris pH 8.0 and passed through a monoQ column at pH 8.0. Fractions were collected and run on gels as described above (Figure 35).

TrkAID was found in the eluted fractions and none was found to flow straight through the column. A comparison of this with the coomassie stain shows that the eluted fractions contain quite a few other proteins also. Therefore, this method may be useful in a purification step since TrkAID does interact with a monoQ column.

5.6: TrkAID can activate ERK *in vitro*

Since TrkAID was tyrosine phosphorylated (Figures 20 and 22), it may be enzymatically active as a tyrosine kinase and it may be able to stimulate the activation of ERK in untreated PC12 cytosol. TrkAID was immunoprecipitated from soluble bacterial lysates with 1086 and added to untreated PC12 cytosol (see section 1.6). Immunoprecipitated TrkAID was also added to cytosol that had been exposed to an *in vitro* reaction with ATP (as a control to observe whether ERK was activated by the ATP *in vitro* reaction) and cytosol from NGF-treated cells (as a control for ERK activation by NGF treatment). The samples were exposed to an *in vitro* reaction at 37°C for 15 minutes. A control was performed where the samples were not exposed to an *in vitro* reaction after immunoprecipitated TrkAID was added to them. The samples were resolved on a SDS gel, western blotted and probed with anti-PhosphoERK and anti-ERK (Figure 36).

Two bands approximately 42 and 44 KDa (corresponding to phospho-ERK-1 and phospho-ERK-2) were detected in untreated cytosol and NGF-treated cytosol that were exposed to an *in vitro* reaction in the presence of immunoprecipitated TrkAID (lanes 2 and 9). They were also observed in NGF-treated cytosol containing immunoprecipitated TrkAID that was not exposed to an *in vitro* reaction (lane 1). No bands were detected in the other lanes. ERK-1 and -2 were detected in unequal amounts in all lanes where sample was loaded, except in lane 7. Only weak signals

were detected in lanes 4, 6 and 11 and the ERK-2 signal was much weaker than ERK-1, which is probably a result of ERK-1 specificity by the antibody.

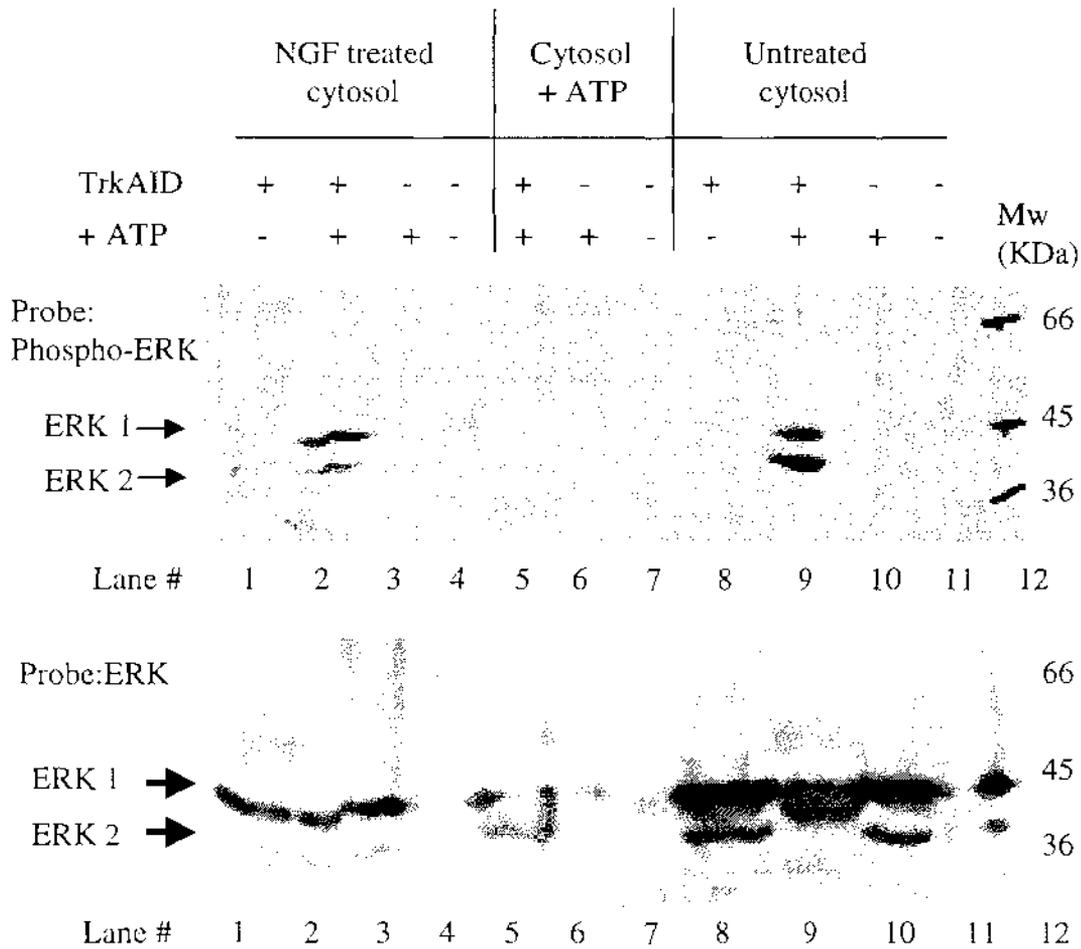


Figure 36

TrkAID is able to phosphorylate ERK in untreated PC12 cytosol in the presence of ATP.

TrkAID was immunoprecipitated with 1086 from the soluble bacterial lysates and was added to untreated PC12 cytosol, NGF-treated cytosol and PC12 cytosol exposed to an ATP *in vitro* reaction before fractionation (cytosol + ATP). Some of these samples were exposed to an *in vitro* reaction with ATP as depicted in the figure (+ ATP row). These samples and untreated cytosol, NGF-treated cytosol and ATP treated cytosol without an *in vitro* reaction with ATP were loaded on a 15 % acrylamide SDS gel. Untreated cytosol, NGF-treated cytosol and ATP treated cytosol exposed to an *in vitro* reaction in the absence of immunoprecipitated TrkAID were also loaded onto the gel. The gel was western blotted and probed with an antibody to phospho-ERK (A). The blot was stripped by acid wash and reprobed with anti-ERK (B). Signals were generated by chemiluminescence.

This data is the best of three experiments and the other two unfortunately did not give the same results. Therefore, it is difficult to draw any firm conclusions about the ability of the cytoplasmic domain of TrkA to activate downstream kinases *in vitro*.

5.7: Discussion

TrkAID could not be purified by the standard method of nickel purification (Figure 24) since it was not being retained on the column. Elution of the protein with a low concentration gradient of imidazole resulted in elution at approximately 50 mM (Figure 27). The flow through fractions contained high amounts of TrkAID, as did the initial washes (Figures 22-30). A different protein with a histidine tag (CRBP) could be purified on the same column using the same method (Figure 25). Therefore, the histidine tag on TrkAID must be somehow masked. Using copper instead of nickel caused more protein to bind non-specifically to the column but did not aid in TrkAID purification (Figure 26).

In the Novagen manual it notes that if one or more of the histidine residues are cleaved or masked then the efficiency for binding to nickel decreases in a logarithmic fashion. One reason could be the possible disruption of the secondary structure by the proline residue (Figure 13). Phosphate residues might interact with the histidine tag, causing some conformational change of TrkAID. That this is the case is suggested by the addition of lithium chloride to denature samples increased affinity of the protein for the column (Figure 32). This method worked for the soluble material (Figure 32), but was not attempted for the insoluble fraction since TrkAID was not partially purified using the denaturing method from this fraction (Figure 29). This combination of guanidinium denaturation and lithium treatment was successful in increasing the amount of TrkAID that was eluted from the column, but was not sufficient to cause most of the TrkAID to bind to the column, since TrkAID was detected in the flow through and initial wash fractions. The amount purified in Figure 32 was unfortunately very small.

For conventional protein purification trials, the soluble material was used because most of the TrkAID was localized to the soluble fraction (Figure 19). Salt precipitation was ineffective for TrkAID purification since the point at which TrkAID was mostly precipitated was also the point at which many other proteins were precipitated. However, ion exchange did show some promise. At pH 5.5 TrkAID did not bind to the CM column, although other proteins did (Figure 33), suggesting the possibility that TrkAID is neutral at this pH. TrkAID did bind to the monoQ column at pH 8.0, along with some other proteins (Figure 34) suggesting that at this pH TrkAID is negatively charged. The fact that ion exchange removes some proteins (CM) and can bind TrkAID (monoQ) suggests a combination of these two methods would be useful for TrkAID purification.

The aim of these experiments was to purify TrkAID for use in identifying proteins that bind to the cytoplasmic domain of TrkA. The ability of immunoprecipitated TrkAID to activate downstream kinases, such as ERK-1 and -2 in untreated PC12 cytosol was tested in Figure 36. In the one experiment shown, phospho-ERK was only detected in cytosol that was added to immunoprecipitated TrkAID and exposed to an *in vitro* reaction. The absence of phospho-ERK in the cytosol + ATP (lane 5) could possibly be explained by the reduced amount of ERK present in that sample. This experiment was repeated twice (data not shown) and in each case the result was different which makes it impossible to draw any conclusions at this initial stage. It may be beneficial to ensure equal concentrations of protein are loaded onto the lanes in future experiments so that the proportion of ERK to activated ERK can be determined for the samples. This would determine whether TrkAID is truly stimulating the activation of ERK. The fact that TrkAID stimulates the activation of ERK in one experiment suggests TrkAID might have the capability to activate downstream kinases and thus would provide a useful tool to study the molecular mechanisms that occur when NGF binds to a neuronal cell via TrkA.

CHAPTER 6 : CONCLUSION

6.1: Conclusion

Tyrosine-phosphorylated TrkAID was expressed at low levels in bacteria and localized to the soluble and insoluble fractions but was found mostly in the soluble fraction. Its purification was hindered by possible conformational changes because of phosphate interactions with the histidine tag and a possible disruption of its secondary structure by a proline residue. These factors could be partially overcome with different methods of purification but not adequately for purification of any quantity of the protein. The use of ion exchange may aid the purification process during the later stages.

If TrkAID were to be purified it would be a useful tool to study the initial stages of intracellular proteins interacting with TrkA upon NGF stimulation. The possible existence of novel proteins that associate with TrkA in small vesicles may be explored using purified TrkAID.

6.2: Future Work

It is possible that other methods of purification such as hydrophobic interaction or size chromatography, could be utilized for TrkAID purification. However, it appears likely that a combination of purification techniques is required since there are a number of factors having an effect on purification. The difficulty in expression could be an artifact of the pET15b system. With time, the TrkA intracellular domain could be cloned here at Massey as well as making clones with mutations such as removal of the juxtamembrane region or point mutations at different tyrosine residues. I hope that

cloning these constructs into a different plasmid may increase expression and make purification a simple one step process.

Once purified, the protein could be injected into rabbits for antibody production. These antibodies could be used for binding studies. Addition of purified TrkAID to untreated PC12 cells would allow intracellular proteins to associate with it since it is already tyrosine phosphorylated. Immunoprecipitating or purifying this protein complex from the permeabilised cells and running the complex on a SDS gel would allow us to identify the proteins that associate with tyrosine phosphorylated TrkAID. This would also allow us to identify any novel proteins that interact with tyrosine phosphorylated TrkA.

These novel proteins could be studied by protein sequencing and searching for similarities with other known proteins. This would provide general guidelines as to the function of the proteins.

Constructs containing mutations within the TrkAID would be useful in these binding studies. Deletion of certain regions of the TrkAID could locate binding sites for novel proteins, and mutations at specific tyrosine residues could identify protein-binding sites. Expression of these mutated receptors in PC12 cells that do not express any native TrkA would provide clues as to the function of these proteins, whether the cells extend neurites with certain mutations or whether the cell survives. Once the binding sites for any novel proteins are identified we could block the binding of these proteins to TrkA and observe which proteins are no longer activated. This would further unravel the signaling cascade systems that are activated upon NGF stimulation as well as further our understanding of any cross-talk that occurs amongst the many cascades. The information obtained from these studies can be used in conjunction with data from other members of the lab in studying more downstream events such as the effect on ERK activation, caspase activation or vesicle transport.

These studies would bring us closer to understanding the molecular mechanisms of apoptosis bringing us closer to being able to manipulate the process as required for purposes such as the treatment of some forms of cancer.

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