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

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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	*cis*-retinal binding protein
CRD	Cysteine rich domain
$CuSO_4$	Copper sulphate
ddH_2O	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-galaetopyranoside
K	Lysine
Kb	Kilobsae
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_2CO_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-γ1	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	*src* homology
SHC	*src* 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-*O*-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 $p140^{prototrk}$, 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, ^{125}I-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 "$p140^{prototrk}$ 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 ^{125}I 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.

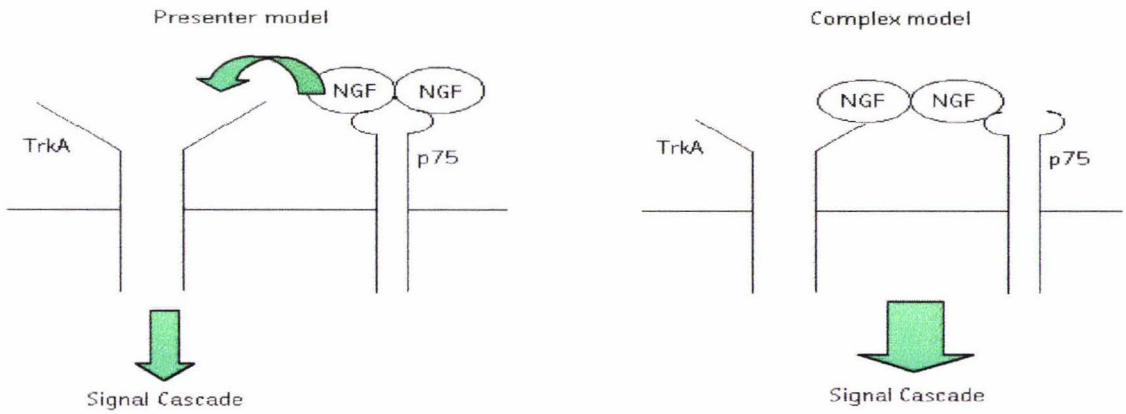

Figure 1
Function of p75

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 $p75^{LNGFR}$. 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-$p75^{LNGFR}$ 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.

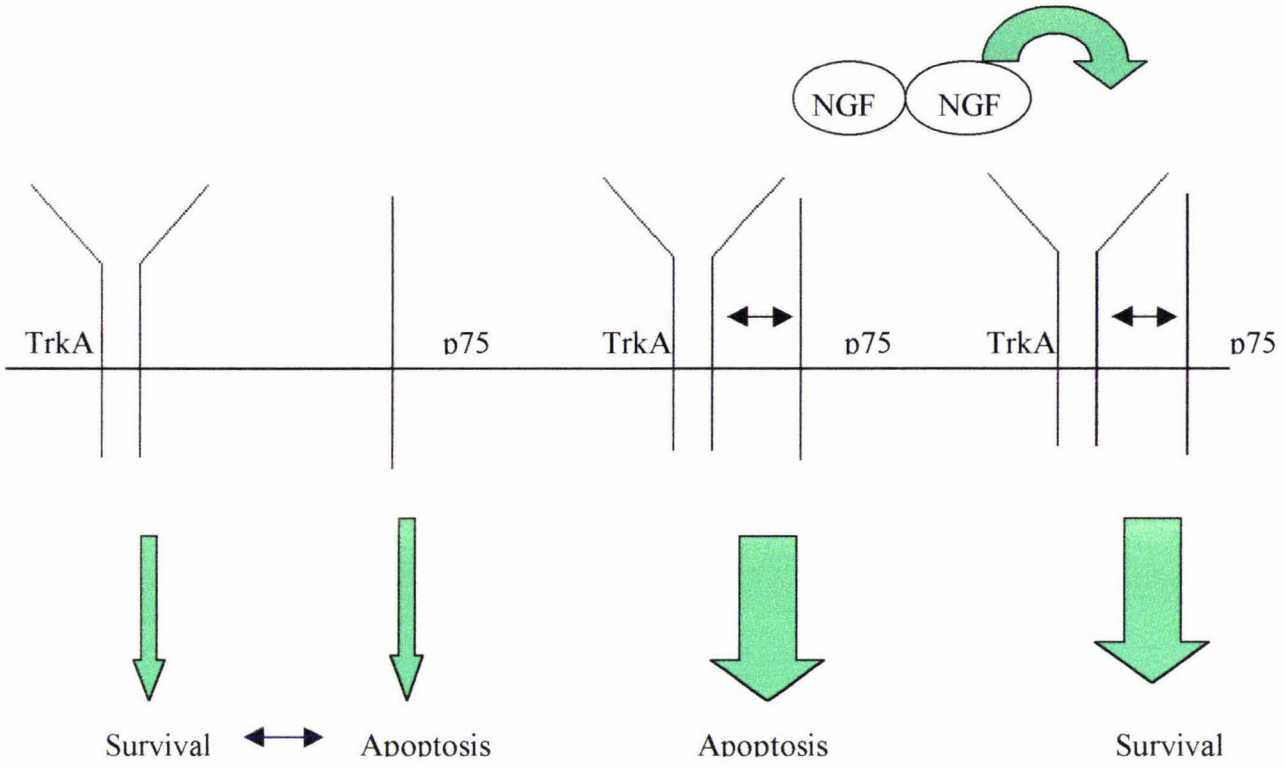

Figure 2
Role of p75 in signaling

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-γ1 and ERK-1. Vetter *et al.* (1991) linked PLC-γ1 and TrkA activation by NGF. Immunoprecipitation of PLC-γ1 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-γ1, 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-γ1, 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-γ1 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-γ1 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-γ1 (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 suc 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 PLCγ-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.

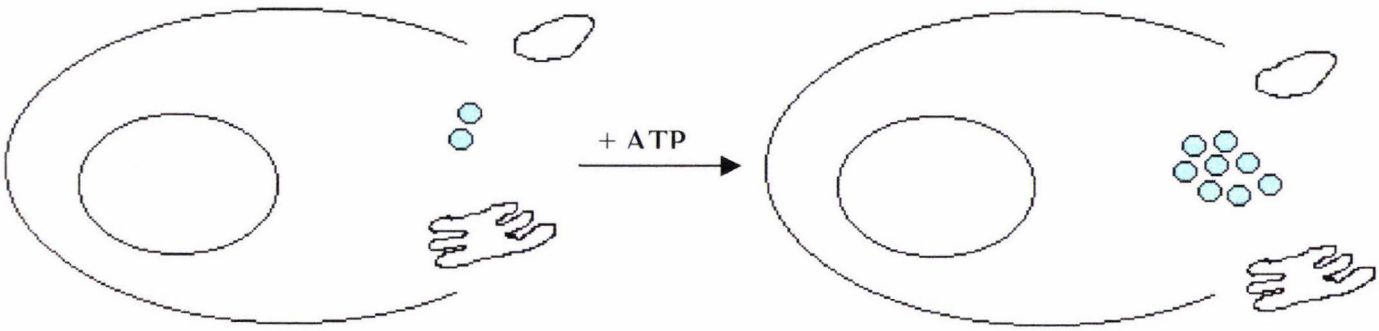

Figure 3
In vitro reaction with ATP

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.

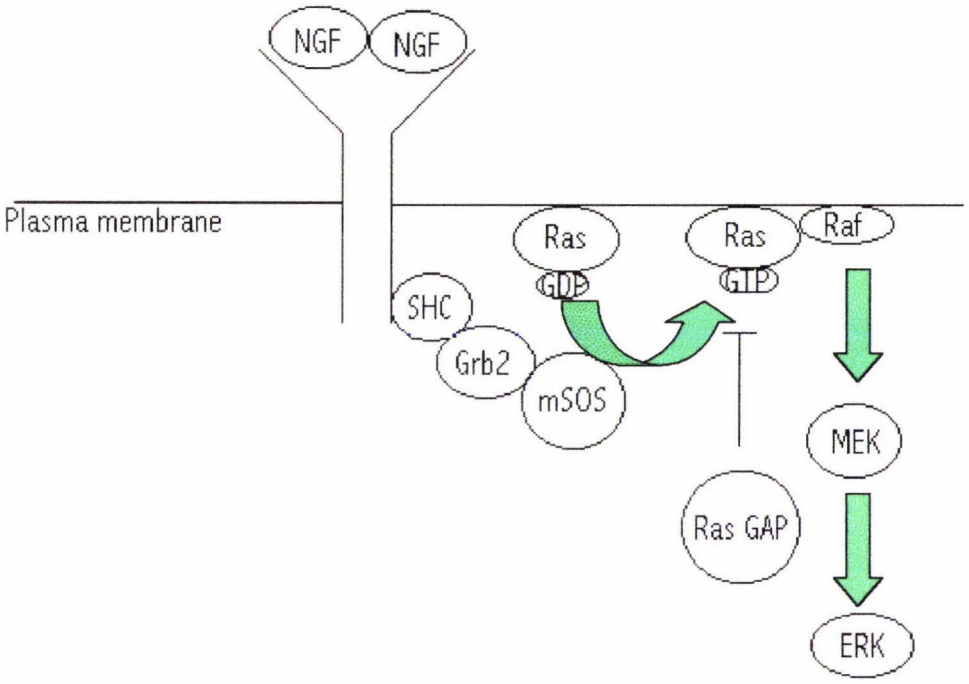

Figure 4
ERK Pathway

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 PLCγ-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 (PLCγ-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).

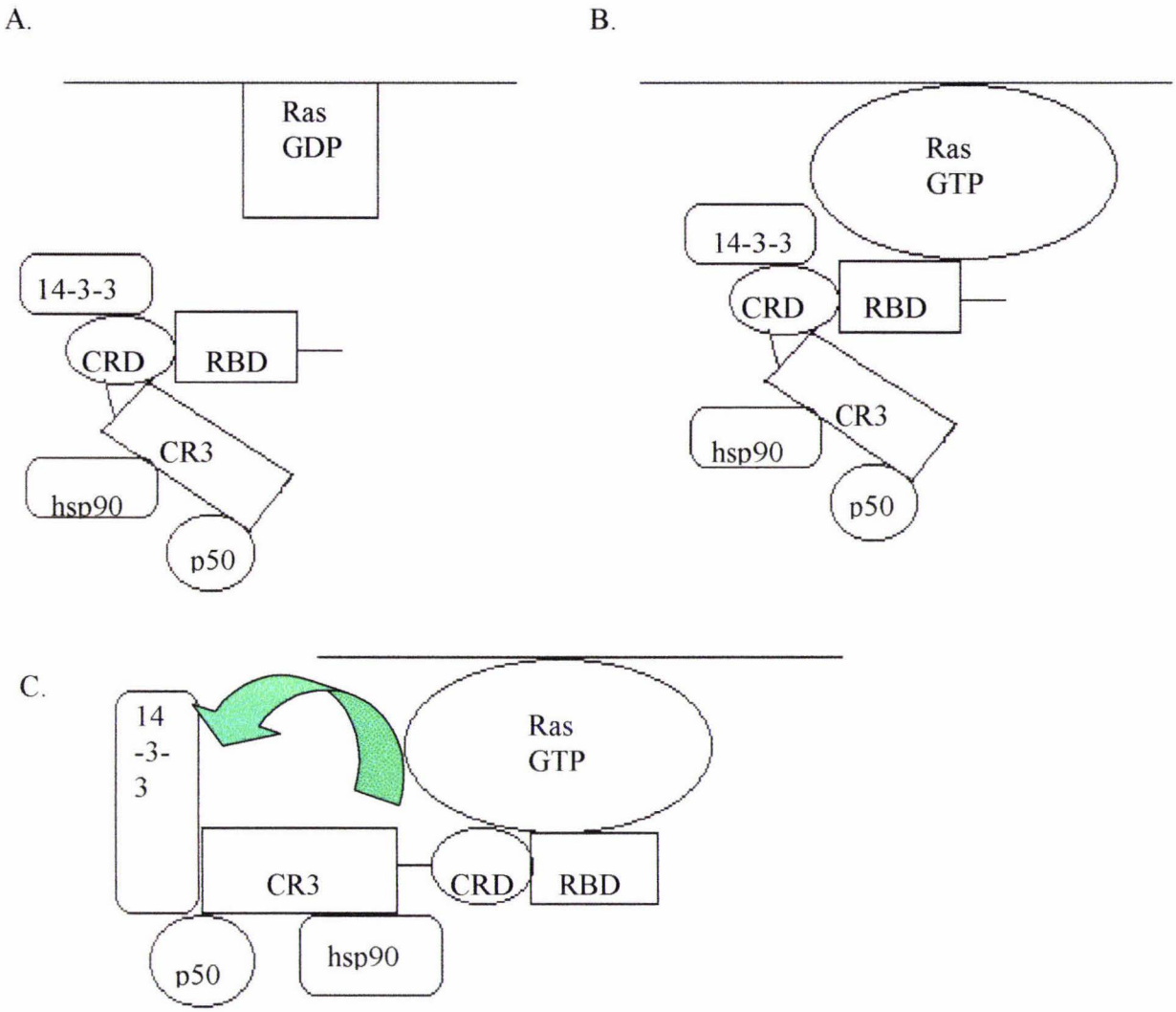

Figure 5
Ras binding and activating Raf

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 PKCδ 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 phosphotidylinositol-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-P_2) and phosphatidylinositol-3,4,5-trisphosphate (Ptdins-3,4,5-P_3). The formation of Ptdins-3-P can occur without NGF stimulation but the formation of Ptdins-3,4,5-P_3 occurs only in response to NGF. The formation of Ptdins-3,4-P_2 and Ptdins-3,4,5-P_3 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-P_3 (Carpenter and Cantley, 1996). In addition, the serine/threonine protein kinase B (Akt) is activated by Ptdins-3,4-P_2 (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 Coffer, 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-P_2 or Ptdins-3,4,5-P_3 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-P_2 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-P_2 stimulated the dimerization of Akt. Stephens *et al.* (1998) showed binding of Ptdins-3,4,5-P_3 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-P_3.

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 Coffer, 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; Ottilie *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 biolipid 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) (Irmler *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.