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The identification and characterization of a nerve growth factor-activated Fos kinase from PC12 cells

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Case Western Reserve University, 1994
THE IDENTIFICATION AND CHARACTERIZATION OF A NERVE GROWTH FACTOR-ACTIVATED FOS KINASE FROM PC12 CELLS

by

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Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

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GRADUATE STUDIES

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*We also certify that written approval has been obtained for any proprietary material contained therein.
THE IDENTIFICATION AND CHARACTERIZATION OF A NERVE GROWTH FACTOR-ACTIVATED FOS KINASE FROM PC12 CELLS

Abstract

by

LORI KELL TAYLOR

Nerve growth factor (NGF) is the prototypic member of the neurotrophin family, which are highly related trophic factors required for the development and maintenance of populations of central and peripheral neurons. NGF elicits its biological actions through binding to the Trk receptor, initiating a signaling cascade propagated through the cytosol primarily by regulated protein phosphorylation resulting from serial activation of protein kinases. Protein kinases mediate the transduction of this signal into alterations in gene transcription through the phosphorylation of transcription factors, leading to the synthesis of new protein products responsible for the eventual biological response.

NGF stimulation of PC12 cells induces the differentiation of these cells into a neuronal phenotype. An immediate effect of NGF action is the transcription and synthesis of c-Fos, a bifunctional transcriptional regulator and phosphoprotein. c-Fos acts as a transcriptional activator, but can also repress transcription from some promoters, including its own. Phosphorylation of c-Fos is responsible for its ability to exhibit transrepressive activity, and potential phosphorylation sites at the C-terminus of this protein are required for this function. In vivo, c-Fos becomes phosphorylated at these sites by a growth factor-activated protein kinase.
I have identified a NGF-stimulated, depolarization-insensitive Fos kinase in PC12 cells using a peptide substrate corresponding to a sequence within the C-terminus of c-Fos. This enzyme is a novel protein kinase and phosphorylates authentic c-Fos at its C-terminus, likely at Ser^{362}, a site required for transrepression. Growth factor responsive Fos kinase activity has been detected in fibroblasts, lymphocytes, and epidermal cells, indicating that this enzyme is widely expressed. NGF stimulation of Fos kinase requires that the enzyme be phosphorylated at serine/threonine residues. Fos kinase has been purified to near homogeneity from PC12 cells and is a 37kDa protein with a pI of 5.95. Its activation requires p21^{ras} action, however, an activator of Fos kinase has not yet been detected. The protein kinase identified and characterized here is likely an important participant in NGF signaling cascades and may regulate the transrepressive action and transcriptional inactivation of c-Fos.
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# TABLE OF CONTENTS

**Chapter 1 - Introduction**  1

I. Role of Neurotrophins in Development and Maintenance of the Nervous System  2
II. Neurotrophin Receptors  10
III. Signal Transduction Mechanisms of NGF  15
IV. Transcriptional Regulation in Response to Growth Factors  28

Figures  36
Tables  40
References  43

**Chapter 2 - Identification of a Nerve Growth Factor and Epidermal Growth Factor-Regulated Protein Kinase which Phosphorylates the Proto-Oncogene Product c-Fos**  62

Introduction  63
Materials and Methods  65
Results  67
Discussion  70
Figures  74
References  79

**Chapter 3 - Isolation and Characterization of a Nerve Growth Factor-Regulated Fos Kinase in PC12 Cells**  83

Introduction  84
Materials and Methods  87
Results  95
Discussion  103
Figures  110
Tables  128
References  131

**Appendix - Chapter 3 - Inhibition of Fos Kinase *in vitro* by Protein Kinase Inhibitors**  136

Figures  138
References  140
Chapter 4 - Identification of a Growth Factor Stimulated Fos Kinase in NIH3T3 Cells and A431 Cells

Introduction 142
Materials and Methods 145
Results 148
Discussion 151
Figures 155
References 165

Appendix - Chapter 4 - Identification of Fos Kinase in Jurkat T-Lymphocytes

Introduction 169
Materials and Methods 171
Results 173
Discussion 175
Figures 178
References 184

Chapter 5 - Mechanisms which Regulate Nerve Growth Factor Stimulation of Fos Kinase Activation in PC12 Cells

Introduction 188
Materials and Methods 192
Results 196
Discussion 200
Figures 206
References 216

Chapter 6 - Discussion

Figures 233
References 234

Chapter 7 - Bibliography

237
LIST OF FIGURES

Chapter 1

Figure 1 - NGF signal transduction mechanisms 36
Figure 2 - Regulation of p21ras 38

Chapter 2

Figure 1 - Time course of NGF-activation of Fos kinase 74
Figure 2 - Ligand specificity of Fos kinase 75
Figure 3 - Phosphorylation of c-Fos 76
Figure 4 - Cation exchange chromatography of Fos kinase 77
Figure 5 - Sequence analysis of the phosphorylated Fos peptide 78

Chapter 3

Figure 1 - Mono Q profile of Fos kinase 111
Figure 2 - Mono Q profiles with other peptide substrates 113
Figure 3 - Profile of Fos kinase from phenyl Sepharose chromatography 114
Figure 4 - Mono S profile of Fos kinase 115
Figure 5 - Mono P profile of Fos kinase 116
Figure 6 - Mono Q chromatography of purified Fos kinase 117
Figure 7 - Gel filtration chromatography 118
Figure 8 - Purification of Fos kinase 120
Figure 9 - Substrate specificity of Fos kinase 121
Figure 10 - Required elements in the Fos kinase recognition sequence 122
Figure 11 - Physical properties of Fos kinase 123
Figure 12 - The dose response relationship between NGF and Fos kinase 125
Figure 13 - Subcellular fractionation of Fos kinase 126
Figure 14 - Immunoprecipitation of c-Fos 127

Chapter 3 - Appendix

Figure 1 - Staurosporine inhibition of Fos kinase activity 138
Figure 2 - K252a inhibition of Fos kinase activity 139
Chapter 4

Figure 1 - Time course of Fos kinase activation 155
Figure 2 - Ligand activation of Fos kinase 157
Figure 3 - Chromatographic behavior of Fos kinase from NIH3T3 cells 159
Figure 4 - Chromatographic characterization of Fos kinase from A431 cells 161
Figure 5 - Phosphorylation of c-Fos by Fos kinase from NIH3T3 and A431 cells 164

Chapter 4 - Appendix

Figure 1 - Chromatographic behavior of Fos kinase and other serine/threonine kinases 178
Figure 2 - Fos kinase phosphorylated authentic c-Fos 183

Chapter 5

Figure 1 - Phosphatase effects on Fos kinase activity 206
Figure 2 - Okadaic acid stimulation of Fos kinase activity 208
Figure 3 - Ca²⁺ independence of Fos kinase activation 209
Figure 4 - Effect of protein kinase C down regulation on Fos kinase activity 210
Figure 5 - Rapamycin resistance of Fos kinase activation 211
Figure 6 - Ras dependence of Fos kinase activity 212
Figure 7 - MEK1, ERK2, and p90RSK as Fos kinase substrates and activators 213
Figure 8 - Putative model for the NGF signal transduction cascade 215

Chapter 6

Figure 1 - Transcriptional regulation from the c-fos promoter 233
LIST OF TABLES

Chapter 1

Table 1 - Neurotrophin family 40
Table 2 - Summary of immediate early genes 41
Table 3 - Mechanisms of transcription factor regulation by phosphorylation 42

Chapter 3

Table 1 - Purification of Fos kinase 128
Table 2 - Kinetic properties of Fos kinase 130
CHAPTER 1

Introduction
I. Role of Neurotrophins in Development and Maintenance of the Nervous System.

A neuron and its target have a symbiotic relationship, in which the target tissue requires innervation to receive signals from the nervous system, and a neuron requires its target for survival. The trophic dependence of neurons was first demonstrated in the seminal studies of Hamburger and Levi-Montalcini where target extirpation induced hypoplasia of the innervating ganglia (Hamburger and Levi-Montalcini, 1949). The addition of large target fields leads to increased neuronal survival, rescuing some neurons from naturally occurring cell death, a phenomenon resulting in the elimination of differentiated neurons due to limited trophic support (Barde, 1989). These observations have provided the basis of the neurotrophin hypothesis, whereby trophic support is present at the target in the form of a diffusible factor. The trophic factor concentration is limited, and neurons not receiving appropriate support are eliminated by naturally occurring cell death. The identification of other neurotrophins related to NGF, their sites of synthesis, and neuronal targets has highlighted the general use of this mechanism for development and maintenance of the nervous system.

Nerve Growth Factor

Nerve growth promoting activity was identified by Hamburger and Levi-Montalcini in some mouse sarcomas (Levi-Montalcini and Hamburger, 1951). It was also later discovered serendipitously to be present in large amounts in
both snake venom and mouse submaxillary glands (Cohen, 1959; Cohen and Levi-Montalcini, 1956). This nerve growth promoting activity was the result of the actions of a single, diffusible protein, termed nerve growth factor (NGF). This protein is a basic 118 amino acid polypeptide, which exists as a dimer in solution. The identification of an abundant source of this protein has lead to extensive characterization of its actions (Yankner and Shooter, 1982) and determination of its three-dimensional structure (McDonald, et al., 1991). NGF acts by binding to its specific receptor at the nerve terminal and is retrogradely transported through the axon to the cell body (Levi-Montalcini, 1987). However, trophic support can also be supplied by a presynaptic source, by non neuronal cells that are not targets, or through an autocrine mechanism (Korsching, 1993). More recently, it has been appreciated that NGF is a member of a family of neurotrophic factors termed the neurotrophins (Raffioni, et al., 1993). These proteins have significant sequence homology, signal through similar receptors, and act upon overlapping but distinct populations of neurons.

*Action of NGF on Sympathetic Neurons*

Neurons of the sympathetic nervous system are primary targets of NGF action. Early studies by Levi-Montalcini and colleagues documented the hypertrophy of these ganglia upon the provision of exogenous NGF (Levi-Montalcini, 1987), and subsequently demonstrated that treatment of neonatal mice with anti-NGF antibodies resulted in the loss of the majority of these cells (Levi-Montalcini and Booker, 1960). Autoimmunization of mature animals does not induce complete immunosympathectomy, but causes a 30-
40% loss of neurons in the superior cervical ganglia (Gorin and Johnson, 1979), demonstrating the continued dependence upon NGF for maintenance of sympathetic neurons.

*Action of NGF on Sensory Neurons*

The NGF dependence of sensory neurons is not as dramatic as for sympathetic populations, however, some sensory neurons are responsive to this factor. Administration of NGF to the embryo decreases naturally occurring cell death in the dorsal root ganglia (DRG; Hamburger, et al., 1981). NGF is important for the survival of subpopulations of neurons in the DRG as offspring of mothers autoimmunized against NGF show a reduction in the number of these neurons (Johnson, et al., 1986). Other neurotrophin family members are responsible for the trophic support of other neuronal populations in the DRG (see below; Barde, 1989). Administration of anti-NGF antibodies through autoimmunization of the mature animal shows that NGF is not required for the continued survival of DRG neurons (Johnson, et al., 1986), in contrast to sympathetic populations. However, NGF is required for biochemical maintenance of DRG neurons, as demonstrated by decreased neurotransmitter synthesis in the presence of anti-NGF antibodies.

NGF also appears to function in the regeneration of peripheral axons. Neuronal death in the DRG following sciatic nerve transection can be reduced by the application of exogenous NGF (Johnson, E.M., et al., 1986). Moreover, sciatic nerve transection induces expression of NGF in non-neuronal cells surrounding and distal to the cut (Heumann, et al., 1987).
Upon regeneration of sensory neuron axons, levels of NGF mRNA decrease in the surrounding cells.

**Actions of NGF in the Central Nervous System**

Historically, NGF was not believed to be present in the CNS, nor to have effects on these neurons. This view was based on the observation that, unlike their peripheral counterparts, central noradrenergic neurons were unresponsive to NGF and antibody treatments were without effect (Hefti, et al., 1989). However, the recognition that the cholinergic neurons of the basal forebrain were NGF responsive indicated that NGF could act as a trophic factor in the CNS (Schwab, et al., 1979). Moreover, intraventricular injections of NGF reduce the loss of basal cholinergic neurons resulting from the interruption of their tract to the hippocampus through the lesion of the fimbria fornix (Hefti, 1986). In addition, NGF treatment of the lesioned animals improves their response in a maze learning exercise compared to lesioned, untreated animals (Will and Hefti, 1985).

The expression of NGF correlates with its potential function as a trophic factor for basal forebrain cholinergic neurons. The level of NGF mRNA is high in both the hippocampal and cortical regions that receive projections from the basal forebrain cholinergic neurons, however, the NGF protein level here is low (Large, et al., 1986). Conversely, NGF mRNA expression in the basal forebrain is low but the protein level is high. These data suggest that the hippocampal and cortical targets of the basal forebrain cholinergic neurons produce NGF, which is retrogradely transported by these neurons to the cell bodies in the basal forebrain. It should be noted that
degeneration of the basal forebrain cholinergic neurons is a consistent pathological feature of Alzheimer's disease, and it has been hypothesized that NGF plays a role in this disease (Hefti, et al., 1989). However, no change is observed in cortical levels of NGF in Alzheimer's patients compared to controls (Goedert, et al., 1986).

Other Neurotrophins

The existence of other neurotrophins was hypothesized due to the trophic action of NGF upon only limited neuronal populations. However, no fortuitous source of other neurotrophins was available, making the characterization of additional trophic factors exceedingly difficult. The advent of microsequencing and polymerase chain reaction technologies has been responsible for the identification of additional members of this family.

Brain Derived Neurotrophic Factor

Barde and coworkers identified a neurotrophic activity with a specificity of action different from NGF. Brain derived neurotrophic factor (BDNF) supports the survival of neurons in both nodose ganglia and DRG in culture, but does not support sympathetic neurons (Barde, 1989). In the DRG, BDNF acts upon a distinct subpopulation of sensory neurons which are insensitive to NGF (Lindsay, et al., 1985). The addition of exogenous BDNF in the developing embryo reduces naturally occurring cell death of neurons in both the DRG and nodose ganglia (Hofer and Barde, 1988).
BDNF is important in the CNS, in addition to its role in the periphery. BDNF is widely expressed in the mammalian brain in the cortex, cerebellum, spinal cord, midbrain, and olfactory bulb (Hofer, 1990). Like NGF, BDNF is expressed in the hippocampus, but at levels 50-fold higher than NGF. BDNF, like NGF, supports the survival of the NGF-responsive, basal cholinergic neuronal population selectively lost in Alzheimer's disease (Alderson, et al., 1990). This trophic factor may be involved in the progression of the disease as levels of BDNF mRNA are decreased in the hippocampus of AD patients (Phillips, et al., 1991). BDNF may also be involved in Parkinson's disease as it acts as a trophic factor for degenerating mesencephalic dopaminergic neurons (Hyman, et al., 1991). In addition, the administration of BDNF prevents the death of neurons of the facial nucleus after axotomy in newborn rats (Sendtner, et al., 1992), suggesting that it plays a role in motor neuron survival.

*Neurotrophin-3 and Neurotrophin-4/5*

The purification and cloning of BDNF revealed significant homology between BDNF and NGF (Leibrock, et al., 1989). The use of PCR and oligonucleotides corresponding to conserved regions of these proteins resulted in the cloning of additional neurotrophin family members. The specific actions of the neurotrophins upon neuronal populations *in vitro* is presented in Table 1. The significant homology of these family members and crossreactivity with anti-NGF antibodies has forced reevaluation of the neuronal populations that require NGF (Acheson, et al., 1991), as determined by the *in vivo* anti-NGF and autoimmunization experiments.
NT-3 is a trophic factor for both neural crest and placode-derived neurons (Rosenthal, et al., 1990). This neurotrophin is widely expressed in non-neuronal tissues including heart, skin, and gut, and is expressed centrally in both the hippocampus and cerebellum (Hohn, et al., 1990). The expression pattern of NT-3 suggests that it may play a larger role in development of the nervous system than NGF and BDNF. NT-3 mRNA is highly expressed in immature regions of the nervous system, and expression decreases with maturation, opposite to that of the BDNF expression pattern (Maisonpierre, et al., 1990). Moreover, NT-3 expression in the brain is maximal just after birth, well before maximal expression of NGF and BDNF (Emsfors, et al., 1990).

An additional neurotrophin, NT-4/5, was more recently identified in both Xenopus and mammals (Berkemeier, et al., 1991; Hallbook, et al., 1991; Ip, et al., 1992). This neurotrophin is expressed in non neuronal tissues including ovary, prostate, and thymus, and at very low levels in the brain (Ip, et al., 1992). NT-4/5 is a trophic factor for DRG and nodose ganglia neurons, but does not support survival of sympathetic neurons (Hallbook, et al., 1991). The biological actions of this molecule have yet to be extensively investigated.

Other Neurotrophic Factors

Additional proteins with neurotrophic actions but no homology to the neurotrophin family have been identified. An example is the fibroblast growth factor family. The expression of members of this growth factor family is developmentally-regulated in the brain (Wilcox and Unnerstall, 1991). FGF can support the survival of populations of CNS neurons (Barde, 1989; Hefti, et
al., 1989), in addition to its actions as a mitogen in a variety of tissues. Moreover, FGF may play a role in the generation of neurons in the embryonic nervous system. Exposure of neuronal precursor cells to FGF primes these cells to produce a mitogenic response following subsequent NGF treatment (Cattaneo and McKay, 1990). FGF may also have a protective role in the nervous system, as demonstrated by FGF prevention of retinal cell death following optic nerve transection (Sievers, et al., 1987). In addition, FGF rescues forebrain cholinergic neurons after lesion of the fimbria fornix (Anderson, et al., 1988).

CNTF is a trophic factor for the parasympathetic neurons of the ciliary ganglia (Hefzi, et al., 1989). It also induces the survival of other peripheral neuronal populations including sympathetic ganglia, in addition to central populations such as motor neurons (Hefzi, et al., 1989). CNTF is distantly related to leukemia inhibitory factor (LIF), another neurotrophic factor for DRG and motor neurons, and cytokine in the immune system (Korsching, 1993).

Other mitogenic factors have been shown to have neurotrophic activities. Insulin, insulin like growth factor-I (IGF-I), insulin like growth factor-II (IGF-II), and receptors for these proteins are distributed throughout the brain (Baskin, et al., 1988; Bohannon, et al., 1988; Hill, et al., 1986; Mendelsohn, 1987; Rotwein, et al., 1988), and have been shown to promote neuronal survival and neurite extension in cultures of brain cells (Bhat, 1983). Epidermal growth factor (EGF), a mitogen in a variety of tissues, stimulates dopamine uptake by embryonic mesencephalic cells in culture (Knusel, et al., 1990). However, the trophic effects of these factors in some cases may be secondary to their actions upon non neuronal cells.
II. Neurotrophin Receptors

The ligand-receptor interactions between NGF and the cell surface of sensory neurons revealed two binding sites with distinct affinities (Sutter, et al., 1979). The high affinity or slowly dissociating component has a $K_d$ for NGF of $10^{-11}$ M, while the low affinity, rapidly dissociating component, responsible for 90% of the NGF binding sites, has a $K_d$ of $10^{-9}$ M. The NGF dose dependence for cellular responses is consistent with the affinity of NGF for the high affinity receptor, indicating that the physiological actions of NGF are mediated by the high affinity receptor (Raffioni, et al., 1993).

The establishment of the clonal rat pheochromotoma cell line, PC12, has been extraordinarily valuable in the analysis of the molecular events that mediate NGF signal transduction. This cell line responds to NGF treatment by ceasing division, extending neurites, and taking on a sympathetic neuronal-like phenotype (Greene, et al., 1987). Like sensory neurons, PC12 cells express both low and high affinity NGF receptors in similar ratios to those detected in sympathetic neurons (Raffioni, et al., 1993), and have proven an excellent system for examination of signal transduction processes unique to either NGF or mitogens due to the response of these cells to both classes of stimuli (Greene, et al., 1987).
Low Affinity NGF Receptor

The low affinity NGF receptor (LANGFR), or p75, was cloned several years ago from both PC12 cells and A875 melanoma cells (Johnson, et al., 1986; Radeke, et al., 1987). This protein contains a ligand binding domain as well as a single transmembrane sequence and short cytoplasmic tail compared to the size of this domain in other catalytic receptors. Unlike other growth factor receptors such as the EGF and insulin receptors, there was no tyrosine kinase domain within the cytoplasmic domain, so it was unclear how this protein transduced the NGF signal. Moreover, the expression of this protein in non neuronal cell lines resulted in only low affinity NGF binding and did not confer NGF responsiveness to the cells (Bothwell, 1991), suggesting additional proteins are required to initiate the NGF signal transduction process.

The LANGFR is a promiscuous neurotrophin receptor. It is widely expressed in both neuronal and non-neuronal cells and binds all neurotrophins with the same, low affinity (Rodriguez-Tebar, et al., 1990; Squinto, et al., 1991). Whether LANGFR is a component of the high affinity, signal transducing neurotrophin receptor remains controversial (see below). Genetic inactivation of the LANGFR gene in the mouse did not produce a loss of sympathetic neurons (Lee, et al., 1992). This finding was unexpected given the presumption that LANGFR was a functional component of the signal transduction machinery. Yet, a loss of sensory neurons and subsequent sensory deficits were evident in the adult. LANGFR is homologous to a number of cytokine receptors that mediate cell death including the CD40,
CD30, and Fas receptors. Recent evidence demonstrates that when unbound, LANGFR induces cell death (Rabizadeh, et al., 1993), suggesting that LANGFR may regulate naturally occurring cell death *in vivo.*

**The Trks - Initiators of the Neurotrophin Response**

TrkA, also termed p140<sup>C-trk</sup>, consists of a ligand binding domain distinct from that of the LANGFR, a single transmembrane sequence, and a cytoplasmic sequence containing a tyrosine kinase domain. The binding of NGF to TrkA stimulates its intrinsic tyrosine kinase activity and initiates signal transduction events (Raffioni, et al., 1993). Controversy still exists regarding the requirement of LANGFR, along with TrkA, for high affinity NGF binding. The view promoted by Chao and his colleagues is that the LANGFR and TrkA form a high affinity NGF receptor as heterodimers, while TrkA or LANGFR monomers or homodimers alone have low affinity (Kaplan, et al., 1991b). This was demonstrated by binding studies using fragmented cell membranes from COS cells expressing either TrkA, LANGFR, or both. Equilibrium binding studies on whole cells by Barbacid and his colleagues showed that the expression of TrkA alone conferred high affinity binding to NIH3T3 cells (Klein, et al., 1991). However, recent kinetic and equilibrium binding analysis and inability to identify TrkA/LANGFR heterodimers within the cell strongly suggest that TrkA is responsible for high affinity binding (Jing, et al., 1992). Moreover, binding to only TrkA is sufficient to elicit NGF responsiveness. NGF treatment of oocytes expressing TrkA induces their maturation (Nebreda, et al., 1991). Similar results were obtained in NIH3T3 cells.
expressing TrkA, where NGF stimulates a mitogenic response (Loeb, et al., 1991). In a particularly telling experiment, NGF mutants were developed which eliminated the ability of NGF to bind to LANGFR while retaining its ability to bind to TrkA resulted in neurite outgrowth in PC12 cells, survival of sympathetic neurons in culture, and high affinity interactions between NGF and the TrkA receptor (Ibanez, et al., 1992).


\textit{Trk Distribution and Expression}

The expression of trk mRNA is consistent with the trophic action of the neurotrophins on the neuronal populations examined thus far. TrkA is expressed in primarily NGF-responsive peripheral ganglia, with limited expression in the CNS (Raffioni, et al., 1993). TrkB is expressed in regions throughout the brain and spinal cord (Barbacid, et al., 1991; Parada, et al., 1992). In the periphery, trkB expression is detected in both ganglia derived from the neural crest as well as the neuroepithelium. Low levels of trkB expression are also evident in non neuronal tissues including lung, muscle, spleen, testis, and ovaries (Barbacid, et al., 1991; Middledmas, et al., 1991).
Expression of trkC is detected throughout regions of the CNS both overlapping and distinct from regions of trkB expression (Parada, et al., 1992). Expression of trkC in the peripheral nervous system is coincident with trkB expression. TrkC transcripts have also been detected in non neuronal tissues including the artery walls and lining of the gut, suggesting that TrkC and NT-3 may play a role in the vascular and enteric nervous system (Raffioni, et al., 1993).

Multiple protein products are produced by all trk genes as a result of alternative splicing. Two TrkA proteins are produced which differ by a 6-amino acid insertion in their extracellular domains (Barker, et al., 1993). An effect of this insertion upon ligand binding or signal transduction has not yet been demonstrated. These trkA isoforms show tissue specific expression. trkA with the insert is expressed in neuronal tissues, with expression of the other isoform primarily in non neuronal tissues.

Two isoforms of TrkB are expressed (Klein, et al., 1990; Klein, et al., 1989; Middlemas, et al., 1991). The full length p145C-trkB contains the same structural motifs as TrkA. The other isoform, p95C-trkB, acts as a receptor, but lacks the catalytic domain, and does not function in signal transduction. The functional significance of the p95C-trkB isoform is not clear, however, it would be expected to inhibit the actions of neurotrophins in a dominant negative fashion. Astrocytes, peripheral nerve, and non neuronal tissues express only the truncated isoform of TrkB (Raffioni, et al., 1993).

Multiple protein products of the trkC gene are also expressed. These include TrkC proteins containing the structural motifs present in TrkA, and TrkC isoforms lacking the tyrosine kinase domain (Tsoufas, et al., 1993;
Valenzuela, et al., 1993). As is the case for truncated TrkB, astrocytes,
peripheral nerve, and non neuronal tissues contain only truncated trkC
transcripts (Raffioni, et al., 1993). TrkC protein with a 14-amino acid (TrkC-
ki14) and 39-amino acid insert (TrkC-ki39) within the kinase domain is also
expressed (Tsoufas, et al., 1993; Valenzuela, et al., 1993), and these kinase
inserts alter the biological activity of TrkC. NT-3 treatment of PC12 cells
expressing TrkC induces neurite outgrowth. While TrkC-ki14 and TrkC-ki39
undergo autophosphorylation in response to NT-3, TrkC-ki14 and TrkC-ki39
do not mediate NT-3-stimulated differentiation of PC12 cells. Again, the role
these splice variants play in the nervous system is not clear.

III. Signal Transduction Mechanisms of NGF

NGF elicits biological responses in PC12 cells through a complex
series of signal transduction events initiated by receptor binding and
propagated through the cytoplasm into the nucleus primarily by regulated
protein phosphorylation (Fig. 1). The resulting changes in gene transcription
are responsible for both the morphological and biochemical differentiation of
these cells (see below). Until recently, the mechanisms that transduced NGF
binding into initiation of a signal transduction cascade were enigmatic. The
discovery that TrkA mediates NGF signal transduction through tyrosine
phosphorylation of itself and other protein substrates solved this mystery
(Raffioni, et al., 1993). However, a paradox has arisen from these studies.
Activation of the tyrosine kinase activity of growth factor receptors results in
the stimulation of a nearly identical series of signaling events, although these
receptors elicit phenotypic responses as diverse as differentiation and proliferation (Chao, 1992; Schlessinger and Ullrich, 1992). We are just beginning to understand how these general molecular elements are transduced into a specific cellular response. However, the similarity between mitogenic and differentiation-induced signaling cascades has aided in the identification of the molecular elements involved in NGF signal transduction and description of signaling pathways.

**Receptor-Associated Events**

The binding of NGF to TrkA in PC12 cells initiates a signal transduction cascade through the dimerization of the receptors and activation of the intrinsic tyrosine kinase activity (Kaplan, et al., 1991a; Raffioni, et al., 1993). This results in the extensive autophosphorylation of Trk (Kaplan, et al., 1991a), by an intermolecular reaction between Trk dimers (Jing, et al., 1992). The phosphotyrosine residues and their flanking sequences create binding sites which are specifically recognized by proteins containing conserved motifs termed src homology 2 (SH2) domains (Koch, et al., 1991). Recruitment of normally cytosolic proteins to the receptor brings them in proximity to the tyrosine kinase, inducing the phosphorylation of these receptor substrates and regulation of their activity. The activation of TrkA induces membrane ruffling presumably through the GTP-binding protein, rac (Connolly, et al., 1979; Ridley, et al., 1992). An influx of extracellular Ca\(^{2+}\) is also stimulated by NGF treatment, however, the mechanism that mediates this rapid event is not understood (Pandiella-Alsonso, et al., 1986).
Interactions between receptor autophosphorylation sites and SH2 domains on cellular proteins disseminates the growth factor signal into multiple pathways. An SH2-mediated association between TrkA and both phospholipase C-γ (PLC-γ) and phosphoinositol 3-kinase (PI3K) following NGF treatment has been described (Raffioni, et al., 1993). The association of PLC-γ with the receptor results in its tyrosine phosphorylation (Kim, et al., 1991; Vetter, et al., 1991). Growth factor stimulated phosphorylation induces the activation of PLC-γ, catalyzing phosphoinositide breakdown to biologically active products, including diacylglycerol and inositol phosphates (Contreras and Guroff, 1987; Fantl, et al., 1993).

PI3K kinase is responsible for the phosphorylation of phosphoinositides produced by PLC-γ (Carpenter and Cantley, 1991). This inositide kinase is a dimer composed of an 85kDa regulatory subunit and a 110kDa catalytic subunit. An interaction through the SH2 domains of both TrkA and the regulatory p85 subunit results in its tyrosine phosphorylation and enzymatic activation of PI3K (Soltoff, et al., 1992). The interactions of PLC-γ and PI3K with TrkA occur at distinct sites on the receptor (Obermeier, et al., 1993). An association between the tyrosine kinase of TrkA and a GTPase activating protein p120GAP of p21ras and its subsequent tyrosine phosphorylation have been demonstrated (Ohmichi, et al., 1991a; Ohmichi, et al., 1991b). This modification is presumed to enhance GAP activity, however, this has not been formally demonstrated.

Growth factor receptors may also regulate other membrane associated, non receptor tyrosine kinases, such as Src, and these kinases might be responsible for some of the tyrosine phosphorylation events that
follow receptor activation. Although NGF-induction of Src activity has not been demonstrated, Kremer et al. showed that injections of anti-Src antibodies inhibited NGF-stimulated neurite outgrowth (Kremer, et al., 1991). Moreover, expression of a constitutively activated Src stimulates neurite outgrowth in untreated PC12 cells by a Ras-dependent mechanism (Alema, et al., 1985; Kremer, et al., 1991).

An interaction between the SH2 domains of receptors and phosphotyrosine phosphatases has been demonstrated (Feng, et al., 1993; Vogel, et al., 1993). These proteins are postulated to terminate the receptor signaling events through the dephosphorylation of tyrosine phosphates of the receptor, thus eliminating SH2 binding sites. TrkA signaling is likely regulated by such a mechanism, however, a direct association between TrkA and a tyrosine residues has not yet been described.

**Adapter Proteins - Connection to Ras**

The GTP-binding protein p21\textsuperscript{ras} is an obligatory participant in NGF signal transduction, as well as other growth factor-stimulated signaling cascades (Feig and Cooper, 1988; Szeberenyi, et al., 1990; Thomas, et al., 1992; Thomas, et al., 1991; Wood, et al., 1992). The importance of Ras in NGF signal transduction was demonstrated by the induction of neurite outgrowth in PC12 cells following injection of activated Ras (Bar-Sagi and Feramisco, 1985; Noda, et al., 1985), and the inhibition of NGF-induced differentiation with the introduction of anti-Ras antibodies (Hagag, et al., 1986). Similarly, the use of a dominant negative mutant of Ras interrupts

Ras contains a GTP/GDP binding site and possesses an intrinsic GTPase activity (Lowy and Willumsen, 1993). When active, GTP is bound, and Ras is coupled to downstream effectors. In its inactive state, GDP is bound to Ras (Fig. 2). Ras inactivates itself through its intrinsic GTPase, and the regulation of this cycle is controlled by interactions between ras and cellular proteins. GTPase activating proteins (GAPs) activate the ras GTPase, leading to the inactivation of ras. A number of cellular proteins exhibit GAP activity including p120\textsuperscript{GAP} and NF-1, the defective gene product responsible for von Recklinghausen's neurofibromatosis (Lowy, and Willumsen, 1993). Furthermore, GAPs appear to act as downstream effectors of Ras in some systems, (Fantl, et al., 1993; Lowy, and Willumsen, 1993). Ras is activated by guanine nucleotide releasing proteins (GNRPs), which induce GDP for GTP exchange (Fantl, et al., 1993). Like GAPs, GNRPs contain SH2 and SH3 domains, and are activated by indirect association with growth factor receptors (see below). The regulation of Ras by GNRPs may be complex due to the action of multiple exchange proteins.

Recently, a class of proteins has been identified that is responsible for the coupling of Ras, and potentially other signaling mechanisms, to the receptor. The adapter proteins such as Crk, Grb2, and Shc are small proteins comprised of SH2 domains and another src homology motif, SH3 domains (Fantl, et al., 1993; Koch, et al., 1991). These adapters can act as intermediaries between GNRPs and the receptor to induce GNRP activation
and subsequent activation of Ras (Buday and Downward, 1993; Egan, et al., 1993; Gale, et al., 1993; Li, et al., 1993; Lowenstein, et al., 1992; Rozakis-Adcock, et al., 1993; Skolnik, et al., 1993). It is postulated that large signaling complexes form through binding such adapter molecules to the receptor, and the proximity of these signaling entities drives growth factor signal transduction. Consistent with this hypothesis, overexpression of Shc in PC12 cells induces neurite outgrowth in the absence of NGF by a Ras-dependent mechanism (Rozakis-Adcock, et al., 1992). Moreover, the formation of downstream signaling complexes is important for subsequent signaling actions of Ras (see below).

Activation of Cytosolic Protein Kinases

The activation of non-membrane-associated protein kinases follows the receptor-associated signaling events. Protein serine/threonine kinases and dual specificity kinases are activated in response to NGF, and these enzymes are the chief elements in the longest leg of the signal transduction journey in the transmission of signals through the cytosol and into the nucleus (Vetter, et al., 1991; Fig. 1). A number of protein kinases activated by NGF treatment have been characterized (Raffioni, et al., 1993), however, these are undoubtably a small subset of the enzymes that mediate the effects of NGF. A current challenge in the study of growth factor signal transduction is to identify both the upstream regulators of these enzymes and their downstream effectors, and ultimately to determine where these kinases fit in relation to
previously described signaling elements. Presently, very few kinases can be described in such detail.

*Mitogen Activated Protein Kinases*

MAP2 kinase was identified as a rapidly activated, insulin-sensitive serine/threonine kinase that phosphorylated microtubule-associated protein 2 (MAP2) *in vitro* (Ray and Sturgill, 1988). MAP2 kinase phosphorylated and partially activated ribosomal S6 kinase II, also known as p90rsk, *in vitro* (Sturgill, et al., 1988), demonstrating the first example of a growth factor stimulated kinase cascade. MAP2 kinase activity required phosphorylation on both threonine and tyrosine residues (Anderson, et al., 1990), and it was hypothesized that MAP2 kinase was an integration point between cytosolic serine/threonine kinases and receptor tyrosine kinases. However, phosphorylation of MAP2 kinase by receptor tyrosine kinases has not been demonstrated (Blenis, 1993). MAP2 kinase activation was observed in a variety of systems in response to a number of stimuli including NGF stimulation of PC12 cells (Landreth, et al., 1990), where MAP2 kinase is responsible for the phosphorylation of a cellular protein, pp250.

The cloning of MAP2 kinase revealed that this kinase is a member of a family of kinases (Boulton, et al., 1991), subsequently termed mitogen-activated protein kinases (MAPK) or extracellular stimulus regulated protein kinases (ERKs). Three members of this family have been identified. MAP kinases are most highly related to the cdc2 family of protein kinases that function in cell cycle regulation (Crews, et al., 1992c). MAP kinases autoactivate through autophosphorylation on both serine/threonine and
tyrosine residues (Seger, et al., 1991), demonstrating their possible function *in vivo* as dual specificity kinases. It was hypothesized that autophosphorylation of MAP kinases was responsible for their activation, however, this reaction proceeds at an inconsequential rate.

A number of *in vitro* MAPK substrates, in addition to p90RSK, have been identified which are phosphorylated in vivo. Among these are phospholipase A2 (PLA2), an enzyme responsible for growth factor-stimulated phospholipid metabolism (Davis, 1993). In addition, MAPK activation of p90RSK leads to phosphorylation of protein phosphatase-1 (PP-1). This modification of PP-1 results in an increase in glycogen synthase phosphatase activity (Dent, et al., 1992).

The activation of MAP kinases may lead to structural alterations in the cell through microtubule reorganization. Several microtubule-associated proteins and cytoskeletal proteins are substrates of MAP kinases (Landreth, et al., 1990), and their modification can change the dynamic state of tubules (Nishida and Gotoh, 1992). Even more exciting is the multi-site phosphorylation of the microtubule-associated protein, tau (Drewes, et al., 1992). Phosphorylation of tau stabilizes the polymerized state of microtubules. Hyperphosphorylated tau and polymerized tubules are components of the senile plaques in brains of individual with Alzheimer's disease (Kosik, 1989), suggesting that MAPK activation may contribute to the pathology of the disease.

MAP kinases may act in the regulation of gene transcription through phosphorylation of transcription factors. The transcriptional regulators c-Jun, c-Myc, and p62Tcf (Elk-1) are phosphorylated by MAP kinases at potential
regulatory sites (see below). Consistent with a possible role in the regulation of transcription in vivo, MAP kinases are translocated to the nucleus following growth factor stimulation (Chen, et al., 1992). p90RSK may perform similar transcriptional regulatory functions through phosphorylation of the serum response factor and likely c-Fos (Rivera, et al., 1993). P90RSK is also translocated to the nucleus following growth factor treatment (Chen, R., et al., 1992).

A MAP kinase activator has recently been characterized and cloned (Crews, et al., 1992a). This dual specificity protein kinase, MEK (MAPK or ERK kinase), activates MAP kinases through phosphorylation at both threonine and tyrosine residues. Like MAP kinases, MEK is rapidly activated by growth factor stimulation (Ahn, et al., 1991), including NGF treatment of PC12 cells (Gomez and Cohen, 1991; Jaiswal, et al., 1992). MEK is also a component of a family of related kinases, and two members have been identified thus far (Zheng and Guan, 1992; Otsu, et al. 1993). The MEK/MAPK cascade shows evolutionary conservation. A MEK homologue has been identified in yeast, and genetic analysis has positioned it upstream of yeast MAPK homologues (Blenis, 1993; Crews, et al., 1992a; Crews, et al., 1992b; Crews and Erikson, 1992c).

The mechanisms of MEK activation are not completely understood and may be cell type specific. In response to a variety of stimuli, MEK activation requires only serine/threonine phosphorylation (Kosako, et al., 1992; Shirakabe, et al., 1992). However, MEK activation by NGF in PC12 cells requires serine/threonine and tyrosine phosphorylation (Jaiswal, et al., 1992). Two potential MEK activators have been identified, and their actions may be
responsible for these differences. One of these, the serine/threonine kinase Raf-1, is growth factor activated in a variety of systems (Rapp, et al., 1988), including NGF treatment of PC12 cells (Wood, et al. 1992), and will phosphorylate and activate MEK \textit{in vitro} (Kyriakis, et al., 1992). Moreover, expression of activated Raf-1 (v-Raf) in NIH3T3 cells induces the activation of MEK and MAPK (Kyriakis, J., et al., 1992). However, expression of the same v-Raf in PC12 cells (Wood, et al., 1992) and Rat-1a cells (Gupta, et al., 1992) does not result in MAPK activation. Lange-Carter et al. have recently identified another MEK kinase, MEKK, based upon its homology to protein kinases in yeast that activate the MEK homologue (Lange-Carter, et al., 1993). MEKK is expressed in a variety of tissues and cell lines, including PC12 cells, where MAPK activation is not stimulated by Raf.

Activation of MAPK, p90\textsuperscript{rsk}, and Raf-1 is dependent upon Ras, as demonstrated through the action of a dominant negative Ras mutant (Thomas, et al., 1992; Wood, et al., 1992), suggesting that MEK is also Ras dependent. Moreover, it appears that Ras may mediate activation of these kinases by driving the assembly of a protein complex. MEK, Raf-1, and MAPK from brain homogenate form a complex with Ras \textit{in vitro} (Moodie, et al., 1993). In addition, the interaction of Ras, Raf, and MEK has been demonstrated through the 2-hybrid system in yeast (Van Aelst, et al., 1993). The yeast homologue of MEKK also interacts with Ras within the cell, and its activation is Ras-dependent (Wang, et al., 1991), suggesting that MEKK, like Raf, is Ras-dependent.
Activation of Other Protein Kinases

Additional NGF-stimulated protein kinases have been identified, but, the details surrounding their participation in the signal transduction cascade are currently limited. For example, protein kinase C (PKC) is rapidly stimulated following NGF treatment of PC12 cells (Heasley and Johnson, 1989). The influx of extracellular Ca\(^{2+}\) (Pandiella-Alsonso, et al., 1986) and the diacylglycerol produced through the action of PLC-\(\gamma\) (Contreras and Guroff, 1987) are responsible for induction of this kinase activity. However, a downstream effector of PKC has not been identified. Stimulation of PKC with phorbol esters activates MAPK by a mechanism partially-dependent upon Ras (Thomas, et al., 1992), but PKC does not appear to be responsible for MAPK activation by NGF. Elimination of the phorbol ester-sensitive PKC isoforms through proteolytic degradation has no effect upon NGF induction of MAPK activity (L.K.T. and G.E.L., unpublished observations). Moreover, in the absence of PKC, NGF induction of neurite outgrowth remains unaffected (Reinhold and Neet, 1989), indicating that PKC activation is not a required element of the NGF signaling cascade. It should be noted that the position of PKC within growth factor signaling cascades is highly variable and may be a function of cell type and ligand used (de Vries-Smits, et al., 1992; Thomas, et al., 1992).

p70s6k is another example of a growth factor-activated serine/threonine kinase (Erikson, 1991). This protein kinase is required for cell cycle progression and at least some mitogen-induced changes in gene transcription (Lane, et al., 1993), and p70s6k is also responsible for the phosphorylation of ribosomal S6 protein in vivo (Chung, et al., 1992).
Phosphorylation of this ribosomal protein is hypothesized to increase the protein translation rate, however, this has not been formally demonstrated. p70s6k is regulated by serine/threonine phosphorylation (Ferrari, et al., 1992), but the responsible kinase(s) has not yet been identified. Activation of this kinase is independent of Ras and MAP kinases (Ballou, et al., 1991; Blenis, et al., 1991; J. Blenis, pers. comm.), although it has been reported that MAP kinases will phosphorylate the C-terminal portion of p70s6k in vitro (Mukhopadhayay, et al., 1992). Growth factor activation of p70s6k is specifically inhibited by the immunophilin, rapamycin (Chung, J., et al., 1992), as a result of dephosphorylation of the kinase (Ferrari, et al., 1993). In yeast, the target of this drug is a phospholipase similar to PLC-γ (Kunz, et al., 1993), suggesting that PLC-γ may lie upstream of p70s6k in growth factor signaling cascades, although this relationship is presently speculative.

Additional NGF-stimulated protein kinases including protein kinase N and b-Raf have been described (Oshima, et al., 1991; Rowland, et al., 1987; Stephens, et al., 1992; Volonte and Greene, 1992), however, the knowledge of actions upstream and downstream of these kinases is currently extremely limited, making it difficult to know where to place them within the NGF signaling cascade. Continued identification, characterization, and positioning of protein kinases within signal transduction pathways will allow the elucidation of signal transduction mechanisms which mediate the growth factor dependence. Considering the wide spread use of protein phosphorylation in signal transduction, it is probable that only a subset of growth factor stimulated kinases have been identified.
NGF-Specific Signal Transduction Events

The signal transduction events discussed thus far are common to growth factors inducing differentiation and proliferation (Chao, 1992). To date, only a few differentiation-specific events have been described. These include prolonged activation of MAPK in response to NGF compared to EGF stimulation of the kinase (Peraldi, et al., 1993; Traverse, et al., 1992), and phosphorylation of EIF4, a component of the translational machinery, at NGF specific sites (Frederickson, et al., 1991). In addition, the differentiation-induced tyrosine phosphorylation of a newly identified protein, SNT, has been described. SNT was identified as a result of its interaction with p13^{SUC}, a subunit of the cell cycle regulated protein kinase p34^{cdc2}, suggesting that SNT is related to cdc2. In PC12 cells, SNT is tyrosine phosphorylated in response only to agents that induce differentiation, including NGF and pp60^{v-src} (Rabin, et al., 1993), while mitogenic stimuli are without effect. Although SNT co-precipitates with kinase activity, this activity cannot yet be attributed to SNT. Recently, it has been appreciated that growth factor signaling specificity may be generated through the association of specific adapters with receptors. Suen et al. have demonstrated that the SH2/SH3 adapter, Grb2, associates with the EGF receptor but not TrkA (Suen, et al., 1993). These data indicate that receptor-specific signals are a result of differences between growth factor receptor structures, and are manifested through interaction with specific members of the SH2/SH3 adapter family.
IV. Transcriptional Regulation in Response To Growth Factors

Growth factor signal transduction ultimately transduces its actions in the nucleus with the alteration of gene transcription, leading to the eventual expression of cellular phenotype. The rapid transcription of a unique class of genes upon growth factor stimulation, termed immediate early genes (IEGs), does not require new protein synthesis (Sheng and Greenberg, 1990). Growth factor-activated IEG transcription is induced by modification of the existing transcriptional machinery, most commonly by phosphorylation of these proteins (Hunter and Karin, 1992). Transcription of IEGs is transient, although new protein synthesis is generally required to stop their transcription, as demonstrated by the superinduction of IEGs obtained using the protein synthesis inhibitor cycloheximide (Milbrandt, 1988). Examples of NGF-stimulated IEGs are provided in Table 2.

Regulation of IEG Transcription in the Nervous System

In the nervous system, transynaptic stimulation can induce plasticity or alter a neuron's response to excitation or trauma. These effects are reflected in the alteration of IEG expression (Sheng and Greenberg, 1990). Rapid and transient activation of a number of IEGs, including c-fos, fra-1, egr-1, NGF-IB, jun-B, and c-jun, has been detected in CNS neurons following a variety of stimuli, including seizure (Morgan and Curran, 1991), light/dark cycles (Yoshida, et al., 1993), and those stimuli which elicit long term potentiation (Cole, et al., 1989).
The regulation of IEGs by transsynaptic stimulation has been studied using depolarization of the PC12 cell as a model system. Growth factor treatment and synaptic action induce quantitatively different transcriptional responses (Sheng and Greenberg, 1990). For example, c-jun is not induced by depolarization, however, its transcription is stimulated by growth factors. The stimulation of egr-1 transcription is more robust in response to growth factors than depolarization, while the opposite correlation is true for NGF-IB. The different signaling cascades induced in response to growth factors and depolarization are also manifested in the regulation of c-fos transcription and post-translational modification of the resulting protein (see below).

Regulation of IEG Transcription by Phosphorylation

Regulated protein phosphorylation, while primarily responsible for transmission of growth factor receptor signals through the cytoplasm, is also in large part responsible for regulation of IEG transcription and function (Hunter and Karin, 1992). Phosphorylation of transcription factors has been shown to alter their function at three levels, regulation of subcellular localization, DNA binding activity, and transcriptional activation or repression by the transcription factor. Examples of transcription factors whose actions are regulated by phosphorylation are presented in Table 3.

Regulation of fos transcription by phosphorylation

Perhaps the best understood example of growth factor-regulated gene expression is that of the proto-oncogene c-fos, and involves post-translational
modification of transcription factors both in activation and termination of its transcription. The regulation of c-Fos transcription by growth factors occurs over the serum response element (SRE; Sheng, et al., 1988; Sheng and Greenberg, 1990). Two DNA binding proteins, the serum response factor (SRF) and p62\textsuperscript{tcf}, interact with the SRE to mediate transactivation (Graham and Gilman, 1991; Shaw et al., 1989), and phosphorylation of both proteins enhances this process. Formation of a ternary complex between p62\textsuperscript{tcf}, SRF, and the SRE is stimulated by phosphorylation of p62\textsuperscript{tcf} by MAP kinases (Gille, et al., 1992). The phosphorylation p62\textsuperscript{tcf} alters the electrophoretic migration of the ternary complex, similar to the alteration in electrophoretic mobility seen following growth factor stimulation of the cell (Marias, et al., 1993). The interaction of SRF with the SRE is also stimulated through SRF phosphorylation (Rivera, et al., 1993). Growth factors induce SRF phosphorylation \textit{in vivo} at a site phosphorylated by p90\textsuperscript{rsK} and calcium/calmodulin dependent (CaM) kinases \textit{in vitro}. Phosphorylation at this site induces the DNA binding activity of SRF. In addition to its transactivation function, c-Fos is able to repress transcription from some promoters, including its own. Transrepression likely requires the phosphorylation of c-Fos, and may be responsible for the termination of c-fos transcription (see below).

The induction of c-fos transcription by depolarization also involves phosphorylation. In PC12 cells, transcription of c-Fos is induced by the cAMP/Ca\textsuperscript{2+} response element (CRE/CaRE; Sheng and Greenberg, 1990). cAMP treatment or depolarization of PC12 cells induces the phosphorylation of the transcription factor CREB at ser-133, a site modified by cAMP dependent kinase and CaM kinases \textit{in vitro} (Gonzales and Montminy, 1989;
Replacement of ser-133 with an alanine inhibits cAMP-induced c-Fos transcription from the c-fos promoter (Gonzales and Montminy, 1989), strongly suggesting that phosphorylation of this site increases transcriptional activation by CREB. The rapid increase in c-fos transcription shows a similar time dependence regardless of stimulus, however, c-fos transcription following depolarization is prolonged compared to growth factor stimulation, suggesting that, like the onset, c-fos transcription is terminated by stimulus specific mechanisms (Bartel, et al., 1989).

Transcriptional Activation by IEGs

The products of most IEGs are transcription factors, and are hypothesized to mediate the transcription of later genes, secondary response genes (Sheng and Greenberg, 1990). Transcriptional activation of this class of genes is generally reflective of the final cellular phenotype, and is dependent upon new protein synthesis. The downstream transcriptional regulation by the IEG products Fos and Jun are the best described. Fos family members form heterodimers with members of the Jun family through parallel association of their leucine zipper domains, while the Jun family has also been shown to form homodimers through similar interactions (Distel and Speigelman, 1990). These dimers interact with AP-1 promoter elements through their respective DNA binding domains and activate transcription, although this transactivation event can be inhibited by an interaction between this complex and the glucocorticoid receptor (Konig, et al., 1992). More
recently, dimerization of Fos and Jun with members of the CREB and ATF transcription factor families has been demonstrated in vitro (Ziff, 1990). The formation of these complexes alters the DNA binding specificity of Fos and Jun, resulting in divergence in the regulation of target gene transcription.

The promoters of secondary response genes contain AP-1 elements, and transcriptional regulation of some genes through these sites has been demonstrated. For example, growth factor stimulation of collagenase transcription is regulated through an AP-1 promoter element (Schonthal, et al., 1988). The transcription of tyrosine hydroxylase, the enzyme that regulates the rate limiting step in catecholamine synthesis, is also regulated by an AP-1 site (Gizang-Ginsberg and Ziff, 1990). Another NGF-regulated secondary response gene, transin, contains an AP-1 site, however, NGF regulation of transin through this promoter element has not yet been demonstrated (Machida, et al., 1989). Other NGF-stimulated IEGs, such as egr-1, are likely to regulate gene transcription, however, their targets have not yet been identified.

_Growth factor-specific transcriptional regulation_

Growth factor stimulation induces the transcription of a similar set of IEGs in a variety of cells, yet specific secondary response gene products and cellular phenotypes result. The formation of alternative heterodimers between Fos, Jun, and other leucine zipper proteins, including CREB and ATF family members may be cell type specific. Moreover, subtle differences in the amount of the IEG product may contribute to the induction of a specific cellular phenotype, as exemplified by depolarization and NGF stimulation.
Functional Regulation of IEG Products by Phosphorylation

Regulated protein phosphorylation plays an important role in growth factor signal transduction activity to regulate the function of some IEG products. c-Myc is phosphorylated in vivo within its activation domain at ser-62 and thr-58 (Gupta, et al., 1993). In vitro, ser-62 is phosphorylated by MAPK (Alvarez, et al., 1991), and phosphorylation at both ser-62 and thr-58 is important for transactivation by Myc (Gupta, et al., 1993). Phosphorylation also regulates the activity of c-Jun (Hunter and Karin, 1992). Transactivation by c-Jun is stimulated by phosphorylation of ser-63 and 73 in the activation domain of this transcription factor by a Ras-induced kinase (Binetruiy, et al., 1991), potentially by MAPK (Pulverer, et al., 1991). Jun is also phosphorylated at 3 sites at the N-terminal side of the DNA binding domain (Boyle, et al., 1991). Phosphorylation at these sites, potentially by casein kinase II or glycogen synthesis kinase-3, inhibits the DNA binding of Jun. However, there is substantial controversy over these findings and the issue remains unresolved. Phorbol ester treatment stimulates dephosphorylation of Jun at these sites, and a subsequent increase in DNA binding activity results.

Regulation of c-Fos Function through Phosphorylation

c-Fos, and other Fos family members, are dual function transcriptional factors that activate transcription through AP-1 sites as heterodimers with the Jun family members, but can also represses transcription from some promoters, including its own. It has been hypothesized that transcriptional
repression by c-Fos is responsible for the transient nature of c-fos transcription, as a result of transcriptional termination induced by newly made c-Fos. Like growth factor activation of c-fos transcription, transrepression occurs over the SRE (Guis, et al., 1990). However, this function is likely mediated by a higher order protein complex as c-Fos does not directly interact with this promoter element. The transrepressive function of c-Fos does not require the leucine zipper or DNA binding domains, but does require the C-terminus of c-Fos (Guis, et al., 1990; Wilson and Treisman, 1988). v-Fos, the viral cognate of c-Fos, differs from c-Fos in its C-terminal 48-amino acids due to a deletion-induced frameshift (Curran, et al., 1984). While v-Fos retains its transactivation potential, it is unable to repress transcription (Guis, et al., 1990; Lucibello, et al., 1989; Ofir, et al., 1990), and elimination of the transrepressive function is postulated to contribute to the transforming potential of this oncogene.

Contained within the C-terminus of c-Fos are potential phosphorylation sites that are absent in v-Fos (Barber and Verma, 1987). Phosphorylation at these sites is serum-stimulated, and a variety of data suggests that this modification is important for the regulation of the transrepressive function of c-Fos. Mutation of serines at any of 3 potential phosphorylation sites at the C-terminus of c-Fos (ser-362-364, ser-368-369, or ser-373-374) to alanines creates c-Fos mutants that are unable to repress transcription (Ofir, et al., 1990). Moreover, replacement of alanines at positions 362-364 in this c-Fos mutant with glutamic acids, creating a net negative charge over the C-terminus, partially restores the transrepressive function to the c-Fos mutant. Mutation of ser-362-364 to alanines also creates a c-Fos protein with a
transforming potential equivalent to v-Fos (Tratner, et al., 1992), strongly suggesting that the transrepressive function of c-Fos is responsible for the regulation of the transforming potential of this proto-oncogene.

The phosphorylation state of c-Fos is dependent upon the stimulus. In CHO cells, while the phosphorylation of the C-terminus of c-Fos is serum stimulated, cAMP does not induce this phosphorylation (Barber and Verma, 1987). In PC12 cells, NGF stimulation induces the production of a hyperphosphorylated c-Fos protein, however, c-Fos produced in response to depolarization is hypophosphorylated (Curran and Morgan, 1986). Taken together, these data strongly suggested the existence of an NGF-stimulated Fos kinase that acts at a potentially important regulatory site in the C-terminus of c-Fos. It was this observation that provoked us to perform a directed search for such a kinase.

This thesis describes the identification of a novel NGF-regulated, depolarization-insensitive Fos kinase in PC12 cells as well as in epidermal, fibroblast, and lymphatic cell lines, and extensive characterization of this enzyme in PC12 cells. The mechanism of Fos kinase activation was examined in NGF-treated PC12 cells in order to position it within the NGF signal transduction cascade. Finally, the discussion will explore the possible functions of Fos kinase in growth factor signal transduction and transcriptional regulation in order to determine the possible biological relevance of this unique protein kinase.
Figure 1. **NGF signal transduction mechanisms.** This figure details characterized pathways which function initially in the NGF signal transduction cascade. NGF signaling is initiated by TrkA, followed by the subsequent regulation of protein kinase activation and transmission of the signal into the nucleus.
The Ras Cycle

Nucleotide Exchange

GDP to GTP by GNP

GTPase Activation

P120 GAP

Protein Kinase Activation
**Figure 2. Regulation of p21ras.** This schematic depicts the mechanisms that control Ras activity and the cellular proteins which influence this cycle. Inactive Ras, in its GDP-bound state, is activated by GTP binding, and this exchange can be accelerated by guanine nucleotide releasing proteins (GNRPs). Activated Ras is responsible for transmitting the growth factor signal. Ras is inactivated by GTP hydrolysis by its intrinsic GTPase, and this enzymatic activity is enhanced by GTPase activating proteins such as p120GAP.
### Table I - Neurotrophin Family

<table>
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<th>Neuronal population</th>
<th>NGF&lt;sup&gt;a-g&lt;/sup&gt;</th>
<th>BDNF&lt;sub&gt;a-h,k&lt;/sub&gt;</th>
<th>NT-3&lt;sub&gt;g,h,l&lt;/sub&gt;</th>
<th>NT-4/5&lt;sub&gt;m,n&lt;/sub&gt;</th>
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<td>--</td>
<td>+</td>
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a. (Lindsay, 1988)
b. (Katz, et al., 1990)
c. (Levi-Montalcini, 1987)
d. (Eckenstein, et al., 1990)
e. (Greene, et al., 1987)
f. (Schwab, et al., 1979)
g. (Knusel, et al., 1991)
h. (Maisonpierre, et al., 1990)
i. (Squinto, et al., 1991)
j. (Alderson, et al., 1990)
k. (Hyman, et al., 1991)
l. (Rosenthal, et al., 1990)
m. (Hallbook, et al., 1991)
n. (Berkemeier, et al., 1991)
Table 2 - Summary of Immediate Early Genes

<table>
<thead>
<tr>
<th>Immediate Early Gene</th>
<th>Structure</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>fos family</strong></td>
<td>contains leucine zipper and basic DNA binding domain</td>
<td>forms heterodimeric transcription complexes with members of jun family and bind to AP-1 consensus sites</td>
</tr>
<tr>
<td><strong>c-fos</strong></td>
<td></td>
<td>functions as both a transactivator and transrepressor</td>
</tr>
<tr>
<td><strong>fra-1</strong></td>
<td></td>
<td>acts as transactivator and transrepressor</td>
</tr>
<tr>
<td><strong>fos-B</strong></td>
<td></td>
<td>function unknown</td>
</tr>
<tr>
<td><strong>jun family</strong></td>
<td>contains leucine zipper and basic DNA binding domain</td>
<td>forms heterodimers or homodimers with members of fos and jun families, recognizing AP-1 sites</td>
</tr>
<tr>
<td><strong>c-jun</strong></td>
<td></td>
<td>dimers act as transcriptional activators at AP-1 sites</td>
</tr>
<tr>
<td><strong>jun-B</strong></td>
<td></td>
<td>has transcriptional repressor function in dimers with Fos</td>
</tr>
<tr>
<td><strong>jun-D</strong></td>
<td></td>
<td>constitutively expressed jun family member</td>
</tr>
<tr>
<td><strong>egr-1</strong> (NGF-1A or zif/268)</td>
<td>contains zinc fingers</td>
<td>binds to consensus sequence -GCGGGGCGGGC-</td>
</tr>
<tr>
<td><strong>NGF-1B</strong> (nur/77)</td>
<td>homologous to steroid hormone receptor family, ligand is unknown</td>
<td>stimulates transcription from DNA sequence related to estrogen and thyroid hormone response elements^b^</td>
</tr>
<tr>
<td><strong>NGF-1C</strong></td>
<td>structure similar to egr-1</td>
<td>recognizes same consensus sequence as egr-1^c^</td>
</tr>
<tr>
<td><strong>c-myc</strong></td>
<td>contains both helix-loop-helix and leucine zipper domains</td>
<td>binds to -CAGGTG- as heterodimers with Max^d^</td>
</tr>
</tbody>
</table>

a. (Guis, et al., 1990)
b. (Wilson, et al., 1991)
c. (Crosby, et al., 1991)
d. (Blackwood, et al., 1991)

<table>
<thead>
<tr>
<th>Mechanism of Regulation</th>
<th>Protein</th>
<th>Effect of Phosphorylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regulation of nuclear translocation</td>
<td>NF-kB</td>
<td>Phosphorylation of IκB regulatory subunit triggers nuclear import</td>
</tr>
<tr>
<td></td>
<td>ISGF3</td>
<td>Tyrosine phosphorylation induces nuclear import&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Regulation of DNA binding</td>
<td>c-Jun</td>
<td>Phosphorylation of one or more sites in Thr-231-Ser-249 by GSK3 or CKII inhibits binding</td>
</tr>
<tr>
<td></td>
<td>SRF</td>
<td>Phosphorylation by p90&lt;sup&gt;rsk&lt;/sup&gt; or CaM kinase stimulates DNA binding&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Regulation of transactivation</td>
<td>CREB</td>
<td>Phosphorylation of ser-133 by PKA or CaM kinases stimulates transactivation</td>
</tr>
<tr>
<td></td>
<td>c-Jun</td>
<td>Phosphorylation of ser-63/73 stimulates transactivation</td>
</tr>
<tr>
<td></td>
<td>p62&lt;sup&gt;tcf&lt;/sup&gt;</td>
<td>Phosphorylation enhances ternary complex formation&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Stimulation of transrepression</td>
<td>c-Fos</td>
<td>Phosphorylation of one or more sites in C-terminus required for transrepression</td>
</tr>
</tbody>
</table>

<sup>a</sup> (Shuai, et al., 1992)  
<sup>b</sup> (Rivera, et al., 1993)  
<sup>c</sup> (Gille, et al., 1992)  

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protein kinase phosphorylation site in the c-Fos protein augments its

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cascade may be required for differentiation of PC12 cells. Biochem. J.


CHAPTER 2

Identification of a Nerve Growth Factor and Epidermal Growth Factor-Regulated Protein Kinase Which Phosphorylates the Proto-Oncogene Product c-Fos
**Introduction**

The proto-oncogene c-fos is one member of a class of immediate early genes which are rapidly and transiently induced upon treatment of rat pheochromocytoma (PC12) cells with nerve growth factor (NGF) or a number of other agents (Greenberg, et al., 1985). The c-fos gene product is a transcriptional regulator, mediating transactivation by interacting with AP-1 sites as a heterodimer with c-Jun (Chiu, et al., 1988; Curran and Franza, 1988; Distel, et al., 1987; Lucibello, et al., 1988; Rausher, et al., 1988; Schonthal, et al., 1988). Two N-terminal domains are necessary for transactivation, a basic DNA binding domain and a leucine zipper domain through which c-Fos associates with Jun (Kouzarides and Ziff, 1988; Sassone-Corsi, et al., 1988; Turner and Tjian, 1989). In addition to its role as a transactivator, c-Fos can repress transcription from its own promoter as well as those of other immediate early genes (Guis, et al., 1990; Sassone-Corsi, et al., 1988; Schonthal, et al., 1988; Wilson and Treisman, 1988). This transrepressive function of c-Fos is independent of the basic region and leucine zipper and is mediated by the C-terminus of this protein (Guis, et al., 1990; Wilson and Treisman, 1988). A c-Fos mutant lacking the C-terminal 27 amino acids (residues 356-381) cannot repress transcription (Guis, et al., 1990). v-Fos, the oncogenic viral cognate of c-Fos, contains both the leucine zipper and DNA binding domains, and maintains the ability to transactivate over AP-1 sites. However, v-Fos differs from c-Fos due to a deletion-induced frameshift resulting in 48 unrelated amino acids at the C-terminus (Curran, et al., 1984; Van Beveren, et al., 1983). Like c-Fos
mutants lacking the C-terminus, the v-Fos protein does not exhibit 
transrepressive activity (Lucibello, et al., 1989; Ofir, et al., 1990). c-Fos 
contains potential phosphorylation sites at its C-terminus which are absent 
in v-Fos, one of which is phosphorylated \textit{in vivo} (Barber and Verma, 1987; 
Tratner, et al., 1992). Phosphorylation of c-Fos at sites in the C-terminal 
region is increased upon serum stimulation of CHO cells (Barber and 
Verma, 1987).

NGF treatment of PC12 cells results in the stimulation of the Trk 
protein tyrosine kinase (Kaplan, et al., 1991a; Kaplan, et al., 1991b), 
initiating a signal transduction cascade and the serial activation of a number 
of protein kinases (Gomez and Cohen, 1991; Heasley and Johnson, 1989; 
Landreth, et al., 1990; Mutoh, et al., 1988). As a result, many proteins show 
increased levels of phosphorylation, including c-Fos (Curran and Morgan, 
1986). Curran and Morgan reported that NGF treatment of PC12 cells 
resulted in the hyperphosphorylation of newly synthesized c-Fos protein 
relative to cells in which Fos synthesis was stimulated by membrane 
deropolarization, suggesting the existence of an NGF-regulated Fos kinase 
(Curran and Morgan, 1986). This observation provoked us to search for an 
NGF-regulated protein kinase which phosphorylates c-Fos. We describe 
here a rapidly activated, NGF-sensitive protein kinase which phosphorylates 
the C-terminus of c-Fos, a locus shown to be involved in the transrepressive 
activity of this molecule (Ofir, et al., 1990). We have identified the 
phosphorylated residue within the peptide substrate as that corresponding 
to Ser$^{362}$. 
Experimental Procedures

*Materials* - NGF was prepared by the method of Smith et al. (Smith, et al., 1968). Radiolabeled ATP was synthesized using Gamma Prep A (Promega, Madison, WI). The Fos peptide, comprising residues 359-370 (RKGSSSNEPSSD) was synthesized at facilities at the Medical University of South Carolina (Charleston, SC) and by Coast Scientific (La Jolla, CA). c-Fos and Fos ΔF proteins, a gift of Dr. Tom Curran (Hoffman-LaRoche Inc., Nutley NJ), were expressed in *E.coli* as a His6 fusion protein as described by Abate et al. (Abate, et al., 1990).

*Fos Kinase Isolation* - PC12 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% horse serum and 5% fetal bovine serum in an atmosphere of 10% CO2. The cells were collected by trituration in phosphate buffered saline containing 1 mg/mL BSA and 1 mg/mL glucose, resuspended at a concentration of 2X10^6 cells/mL, and treated as described for the indicated period of time. After collection by centrifugation, the cells were lysed by sonication in a buffer containing 20mM Tris, pH 7.4, 1mM EGTA, pH 7.4, 100mM sodium orthovanadate (lysis buffer) and 10mM p-nitrophenyl phosphate (PNP), essentially as described by Smith et al. (Smith, et al., 1989). The cellular lysate was centrifuged at 100,000 X g and the supernatant collected. The soluble cell lysate was applied to a Mono Q HR 5/5 column (Pharmacia, Piscataway, NJ) in lysis buffer and developed with a 0 - 0.4M NaCl gradient. The peak of activity eluting at 0.18M NaCl was collected, pooled, and dialyzed in 20mM Hapes, pH6.8, 1mM EGTA, and 100μM sodium orthovanadate. The sample was then applied to a Mono
S HR 5/5 column equilibrated in the same buffer and developed with a 0-0.4M NaCl gradient.

Kinase Assays- Kinase reactions were performed in triplicate using 1μg protein per sample or 40μL of column fractions, in lysis buffer containing 0.5mM Fos peptide, 1.2mM DTT, 20μM [γ^{32}P]ATP (6.6 cpm/fmole), and 10mM MgCl₂ at 22°C for 30 min. Reactions were stopped by addition of trichloroacetic acid to 3.7% and 10μg BSA. The samples were cooled to 4°C for 15 min and centrifuged for 5 min. The supernatants were applied to P81 paper in triplicate, washed 4 times for 10 min in 75mM phosphoric acid (Casnellie, 1991), and incorporated radioactivity measured. Fos protein phosphorylation was evaluated by incubation as described above except [γ^{32}P]ATP specific activity was 13.2 cpm/fmole and reactions were stopped by the addition of 3X Laemmli's sample buffer. Products were resolved by SDS-PAGE on a 10% gel followed by autoradiography.

Sequencing of Radiolabeled Fos Peptide - The c-Fos peptide (25nmoles) was incubated with Fos kinase under the same reaction conditions as previously described except the [γ^{32}P]ATP specific activity was 528 cpm/fmole. After 1 hr, the reaction mix was diluted into 2mL of 0.05% trifluoracetic acid (TFA) and loaded onto a Pharmacia Super-Pac Pep-S HPLC column. The column was washed with 5 column volumes of 0.05%TFA and the peptide eluted in 20% acetonitrile and 0.05%TFA. The radiolabelled peptide was covalently attached to arylamine-derivatized polyvinylidene difluoride membranes (Sequalon-AA, Millipore Corp., Milford, MA) according to the manufacturer's instructions using water soluble carbodiimide. The membranes were analyzed by sequencing in a Model
473A automatic protein sequencer (Applied Biosystems, Foster City, CA). Cycles were modified to extract the anilinothiazolinone derivatives with liquid trifluoroacetic acid followed by n-butyl chloride/ethyl acetate (1:1, v:v) containing 0.01% (v:v) phosphoric acid. The amino acid derivatives at each cycle were collected directly into scintillation vials, mixed with 10mL liquid scintillant (National Diagnostics Co.) and measured in a scintillation counter.

Results

NGF treatment of PC12 cells resulted in the activation of a protein kinase which phosphorylated a synthetic Fos peptide corresponding to the C-terminus of c-Fos (residues 359-370). NGF maximally activated the enzyme within 5 minutes and the activity remained elevated at least 2-fold up to 2 hours later (Fig. 1). Fos kinase activity was elevated throughout the period during which c-Fos is actively synthesized (Curran and Morgan, 1986), consistent with phosphorylation of the nascent protein by this enzyme.

The Fos kinase was activated in response to NGF and epidermal growth factor (EGF) to approximately the same levels (Fig. 2). Basic fibroblast growth factor (bFGF), dibutyl cAMP (dbcAMP), 12-tetradecanoyl-phorbol 13-acetate (TPA) or depolarization of the cells with KCl had little or no effect upon kinase activation. The finding that Fos kinase activity was not stimulated following depolarization is consistent with the previous observation of Morgan and Curran that the c-Fos protein produced in
response to depolarization was hypophosphorylated relative to that
generated following NGF treatment (Curran and Morgan, 1986).

A major concern was that the Fos kinase represented another known
protein kinase which exhibited activity toward the Fos peptide substrate. We
have attempted to rule out the involvement of other protein kinases based on
assays for their activities or immunological cross-reactivity (Taylor and
Landreth, in dissertation). Fos kinase was partially purified by ion exchange
chromatography on a Mono Q column. The NGF-stimulated Fos kinase
eluted at 0.18M NaCl, and was well resolved from p42 mitogen activated
protein kinase (MAPK), otherwise known as extracellular signal-regulated
kinase 1 (ERK1), and p44 MAPK (ERK2) which eluted at 0.15 and 0.22M
NaCl, respectively. Similarly, Fos kinase did not coelute with the major peak
of Kemptide kinase activity. Immunoblot analysis using anti-p90Rsk
antibodies demonstrated that this enzyme eluted from the column in a peak
prior to Fos kinase. Chromatography on the Mono S column allowed clear
resolution of Fos kinase from kinases phosphorylating the S6 peptide
substrate, as well as contaminating Kemptide kinase activity. These
enzymes were of concern due to sequence similarity between these peptide
substrates and the Fos peptide. We estimate a 2000-fold purification of the
Fos kinase following Mono S chromatography.

The partially purified, NGF-stimulated, Fos kinase phosphorylated
authentic c-Fos protein. c-Fos was phosphorylated when combined with Fos
kinase obtained from NGF treated, but not untreated cells (Fig. 3, lane b). To
verify that Fos kinase phosphorylated Fos principally at the C-terminus, a
deletion mutant lacking the C-terminal 80 amino acids, Fos ΔF, was assayed
(Abate, et al., 1990). The Fos ΔF protein was not significantly phosphorylated (Fig 3, lane c). Chromatography of the enzyme on the Mono S ion exchange column demonstrated coelution of kinase activity phosphorylating both the peptide substrate and c-Fos protein (Fig.4). The Fos ΔF substrate was phosphorylated at very low levels by a contaminating kinase that demonstrated a different elution profile than the c-Fos kinase. We consistently observe a 90kDa phosphoprotein following an in vitro kinase reaction using the partially purified Mono S fraction. While the molecular weight of this species is similar to the M_r of 90kDa determined by gel filtration of Fos kinase, we have not established that this 90kDa species possesses a kinase activity (data not shown).

The site at which Fos kinase modified the peptide substrate was determined by microsequencing of the phosphorylated peptide. Fos kinase phosphorylated the c-Fos peptide at Ser^{362} as demonstrated by release of radioactivity in cycle 4 (Fig. 5). The radioactivity released in cycle 1 was due to residual [γ^{32}P]ATP. The radioactivity present in cycle 5 was likely due to incomplete peptide bond cleavage in the previous cycle.
Discussion

An increase in c-Fos transcription is a common response in a number of cell types to a variety of stimuli. The c-fos gene product can participate in both the activation and repression of transcription. The ability of Fos to differentially regulate gene expression is central to its capacity to affect cell growth, differentiation, and transformation. It is not presently clear how these multiple effects of c-Fos are achieved, however, phosphorylation of Fos and other transcription factors is likely to be a prevalent mechanism through which their activities are regulated. The protein kinases responsible for these regulatory phosphorylations are important in the hormonal control of cellular phenotype.

We have identified a novel NGF-regulated Fos kinase in PC12 cells. Fos kinase was distinct from other previously described NGF-stimulated kinases. Fos kinase was not cAMP dependent kinase, as it was not inhibited by IP20, (at concentrations up to 1μM), a specific peptide inhibitor of this enzyme, and cAMP dependent kinase displays different chromatographic behavior (Landreth and Taylor, in dissertation; Cheng, et al., 1986). Although c-Fos is phosphorylated in vitro at its C-terminus by cAMP dependent kinase (Abate, et al., 1991), dbcAMP treatment of PC12 cells did not result in increased Fos kinase activity toward the peptide substrate. As previously indicated, Fos kinase eluted separately from MAP (ERK) protein kinases on ion exchange chromatography and did not phosphorylate their substrates, myelin basic protein and microtubule associated protein 2 (MAP2). The PSSD motif in the Fos peptide has been identified as a
potential casein kinase II (CKII) site due to its similarity to the CKII phosphorylation site in serum response factor (SRF; Manak and Prywes, 1991). However, Fos kinase was not inhibited by heparin up to 1mM, indicating that Fos kinase was not casein kinase II. This is consistent with the recent observations of Abate et al. showing that CKII does not phosphorylate c-Fos in vitro (Abate, et al., 1991). Fos kinase was distinct from protein kinase C as it was not stimulated by TPA and had no requirement for Ca^{2+}. Similarly, the absence of a dependence upon Ca^{2+} also indicated that calmodulin kinases were not responsible for this activity. Antibodies to S6II kinase family failed to precipitate Fos kinase activity (Taylor and Landreth, manuscript in preparation). Moreover, the activation kinetics and chromatographic behavior of Fos kinase were inconsistent with previously reported S6 kinases in PC12 cells (Heasley and Johnson, 1989). While Fos kinase is implicated in c-Fos phosphorylation, given its rapid activation, it is also likely to phosphorylate other substrates.

The C-terminus of c-Fos is essential for the transcriptional repression exhibited by this protein (Guis, et al., 1990; Wilson and Treisman, 1988). Ofir et al. have recently provided evidence that phosphorylation of serine residues contained within the peptide employed in the present study are necessary for transrepression (Ofir, et al., 1990). Substitution of alanine residues within the GSSS sequence (residues 362-364) inhibited the transrepressive activity of c-Fos, which was restored upon introduction of negatively charged residues into this motif (Ofir, et al., 1990). The phosphorylation site at the C-terminus of c-Fos has been identified as Ser^{362}, corresponding to the phosphorylation site within the Fos peptide.
substrate of the NGF-activated Fos kinase described here (Tratner, et al., 1992). The requirement for the presence of this phosphorylation site for expression of transrepressive activity strongly suggests that phosphorylation of c-Fos by a kinase with the specificity of Fos kinase regulates this event. The potential functional importance of phosphorylation at this locus is demonstrated by the enhanced transforming capacity of Fos mutants lacking serine residues 362-364 (Tratner, et al., 1992).

The mechanism through which c-Fos is phosphorylated at its C-terminus may be both cell type and ligand specific. It has been suggested that A-kinase mediates the phosphorylation of Fos within this motif, based on the ability of A-kinase to phosphorylate this site in vitro (Abate, et al., 1991) and the observation that Fos becomes phosphorylated following forskolin treatment of JEG3 cells (Tratner, et al., 1992). However, dbcAMP treatment of CHO cells had no effect on Fos phosphorylation (Barber and Verma, 1987). NGF does not cause an increase in cAMP levels in PC12 cells (Race and Wagner, 1985), suggesting cAMP dependent kinase is unlikely to be responsible for the resulting phosphorylation of c-Fos. It is likely that multiple, alternative mechanisms exist that activate the transrepressive function of this transcription factor.

The mechanism(s) through which c-Fos exerts its transrepressive activity is presently unclear. The CarG elements of the serum response element (SRE) in the Fos promoter are required for transrepression. However, c-Fos does not directly interact with this DNA motif (Guis, et al., 1990). A protein complex of SRF and p62\textsuperscript{Gf} binds to the SRE and is responsible for increased c-fos transcription following growth factor or serum
stimulation (Graham and Gilman, 1991; Rivera, et al., 1990; Shaw, et al., 1989; Treisman, 1986). It is hypothesized that a protein-protein interaction between newly synthesized c-Fos and the SRF/p62^tf complex down regulates c-fos transcription. The phosphorylation of c-Fos at its C-terminus by Fos kinase is thought to render it able to participate in such an interaction. Consistent with involvement of newly synthesized c-Fos protein, on going protein synthesis is required for the repression of c-fos transcription following growth factor stimulation (Muller, et al., 1984). Depolarization results in the transient transcription of c-Fos through the cAMP/Ca^{2+} responsive element (CRE/CaRE), but the period of transcriptional activation is considerably longer (Bartel, et al., 1989). This pathway is mechanistically distinct from growth factor-induced transcriptional events mediated by the SRE (Sheng, et al., 1988), suggesting that depolarization-induced c-Fos transcription may be down regulated by alternative means. Importantly, cellular stimuli which act through the CRE/CaRE to modulate transcription fail to activate Fos kinase.

Identification of a hormonally-stimulated Fos kinase provides an additional link in the chain of events through which the cell is able to rapidly alter the pattern of gene transcription and subsequently modulate its response to signals impinging upon it. The Fos kinase identified here may have a wider role in transcriptional regulatory events and in the cascade of protein kinase activation following NGF receptor occupancy.
Fig. 1  Time course of NGF-activation of Fos kinase.  PC12 cells were treated with NGF (50 ng/mL) for the indicated time.  Fos kinase activity was measured following partial purification by Mono Q chromatography. The data are expressed as mean cpm of $^{32}$P incorporated into Fos peptide ($\pm$ s.d.) measured in triplicate.
**Fig. 2** Ligand specificity of Fos kinase. PC12 cells were treated with NGF (50 ng/mL), EGF (5 ng/mL), bFGF (30 ng/mL), dbcAMP (1 mM), KCl (50 mM), or TPA (50 ng/mL) for 5 min. Fos kinase activity was assayed as described following Mono Q chromatography. The data are expressed as the mean cpm incorporated into Fos peptide (±s.d.) of triplicate measurements.
Fig. 3. Phosphorylation of c-Fos. Fos kinase (approximately 6ng) was incubated alone (lane a), with 1.8pM c-Fos (lane b), or with 1.8pM Fos ΔF (lane c) and the phosphorylation reaction carried out for 60 min. Proteins were fractionated by SDS-PAGE on a 10% gel. Positions of the molecular weight standards are shown.
Fig. 4. Cation exchange chromatography of Fos kinase. NGF-stimulated Fos kinase activity obtained following chromatography on a Mono Q column was applied to a Mono S column and developed with a 0-0.4M NaCl gradient. Column fractions were then assayed alone (inset, lane a), or in the presence of 1.8pM Fos (inset, lane b) or 1.8pM Fos ΔF (inset, lane c). The proteins were separated by SDS-PAGE on a 10% gel. Fos peptide kinase assays were performed in parallel with fractions obtained from control (---) or NGF-treated (-----) cells. The data are expressed as the mean (+s.d.) of triplicate determinants.
Fig. 5. Sequence analysis of the phosphorylated Fos peptide.

Fos kinase isolated by Mono Q and Mono S chromatography was incubated with c-Fos peptide, and the phosphorylated peptide subject to microsequencing as described. The data is expressed as cpm released from the peptide per cycle.
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CHAPTER 3

*Isolation and Characterization of a Nerve Growth Factor-Regulated Fos Kinase from PC12 Cells*
Introduction

Nerve growth factor (NGF), the prototypic member of the neurotrophin family, acts as a trophic factor for sympathetic neurons, and for some sensory and central nervous system neurons (Barde, 1989). NGF appears to elicit its biological actions through binding to Trk, resulting in the activation of the intrinsic tyrosine kinase of this receptor, initiating a complex series of signaling events (Gomez and Cohen, 1991; Heasley and Johnson, 1989; Kaplan, et al., 1991a; Kaplan, et al., 1991b; Landreth, et al., 1990; Mutoh, et al., 1988; Schlessinger and Ullrich, 1992). It is not well understood how NGF binding to Trk and cell signaling are coordinated, nor are all elements of the signal transduction pathway known. However, the generation of binding sites on the receptor for adapter proteins and the capacity of these molecules to mediate the activation of \( p21^{ras} \) has recently been appreciated (Lowenstein, et al., 1992; Qui and Green, 1991; Rozakis-Adcock, et al., 1993; Rozakis-Adcock, et al., 1992). An important facet of this signaling cascade is the serial activation of serine/threonine kinases and their subsequent phosphorylation of cellular substrates. One substrate of NGF-regulated serine/threonine kinases is the transcriptional regulator c-Fos. Curran and Morgan have reported that NGF induces the phosphorylation of nascent c-Fos at levels much greater than that found associated with c-Fos produced following depolarization of rat pheochromocytoma (PC12) cells (Curran and Morgan, 1986). We therefore reasoned that NGF must activate a protein kinase which directly phosphorylates c-Fos.

The proto-oncogene c-fos is one of several immediate early genes whose mRNA and protein levels are rapidly, but transiently induced upon
treatment of PC12 cells with NGF and a variety of other stimuli (Greenberg, et al., 1985). The c-fos gene product, like the protein products of other immediate early genes, is a DNA binding protein that acts as a transcriptional regulator (Morgan and Curran, 1991). c-Fos is a 380 amino acid protein and contains both a DNA binding domain and leucine zipper that forms heterodimers with members of the Jun family through parallel association of their leucine zipper domains. These protein complexes associate in a sequence specific manner with AP-1 promoter elements and mediate transcriptional regulation. c-Fos is an important regulator of cellular responses to stimuli as a consequence of regulation of the transcription of other hormonally-responsive genes, and is important for the development and function of a variety of tissues (Gizang-Ginsberg and Ziff, 1990; Johnson, et al., 1992; Schonthal, et al., 1988).

c-Fos also has the capacity to repress transcription from some promoters, including its own (Guis, et al., 1990; Sassone-Corsi, et al., 1988; Schonthal, et al., 1988; Wilson and Treisman, 1988). The transient nature of fos transcription following stimulation of the cells has been postulated to be a consequence of negative autoregulation of its own transcription. The leucine zipper and DNA binding domains of c-Fos required for transcriptional activation are not necessary for c-Fos mediated transrepression; however, the extreme C-terminus of c-Fos is essential for this function (Guis, et al., 1990; Wilson and Treisman, 1988). The transrepressive activity of c-Fos provides a clear functional distinction from its oncogenic viral cognate, v-Fos, which does not possess this activity (Guis, et al., 1990; Lucibello, et al., 1989; Ofir, et al., 1990). The inability of v-Fos to
repress transcription is due to a deletion-induced frame shift in v-fos resulting in an altered C-terminal protein sequence over a length of 48 amino acids, with the elimination of potential phosphorylation sites (Barber and Verma, 1987; Curran, et al., 1984). Moreover, mutation of sites within this region of c-Fos confers transforming activity to this protein (Tratner, et al., 1992).

C-Fos undergoes regulated phosphorylation in response to a variety of conditions. NGF induces hyperphosphorylation of c-Fos in PC12 cells (Curran and Morgan, 1986). Serum stimulation results in the enhanced phosphorylation of c-Fos, but not v-Fos, indicating that serum activates a kinase that phosphorylates the C-terminus of c-Fos (Barber and Verma, 1987). The replacement of serines within potential phosphorylation sites with alanine residues creates a mutant c-Fos protein unable to repress transcription from its own promoter (Ofir, et al., 1990). Taken together, these data strongly suggest that phosphorylation of the C-terminal region regulates the transrepressive function of c-Fos.

We initiated a search for an NGF-regulated Fos kinase in PC12 cells responsible for the hyperphosphorylation of this protein and which may regulate the transrepressive function of this transcription factor. PC12 cells have proven to be a valuable model system for the investigation of NGF function and growth factor signal transduction (Greene, et al., 1987). Using a peptide corresponding to the C-terminus of c-Fos containing potential phosphorylation sites as a substrate (residues 359-370), we have identified a novel growth factor-activated Fos kinase which was rapidly activated upon treatment with NGF or epidermal growth factor (EGF; Taylor, et al., 1993).
This kinase was not stimulated by depolarization of the cells, as predicted by Curran and Morgan (Curran and Morgan, 1986). The activation of this kinase was rapid, reaching maximal levels within 5 min and remained active throughout the period of c-Fos synthesis. Fos kinase phosphorylated the peptide substrate at a site corresponding to Ser^{362}, and phosphorylated c-Fos at a site near the C-terminus. Phosphorylation at this site is likely to be important for the expression of the transrepressive function of this protein. We describe here the molecular properties of Fos kinase that establish it as a novel protein kinase and an intermediary in the NGF and EGF signal transduction cascades.

**Materials and Methods**

*Materials* - NGF was prepared by the method of Smith et al. (Smith, et al., 1968). EGF was purchased from Upstate Biotechnology (Stoughton, MA). Radiolabeled $[\gamma^{32}P]ATP$ was synthesized using Gamma Prep A (Promega, Madison, WI). The Mono Q HR5/5, Mono S HR5/5, Mono P HR5/5, and Superose 12 HR10/30 columns were obtained from Pharmacia LKB (Piscataway, NJ), as were the Fast Flow S, Fast Flow Q resins, Polybuffer 74, and Polybuffer 96. Fractogel EMD DEAE-650 resin was obtained from EM Separations (Gibbstown, NJ). The Fos peptide, comprising residues 359-370 of c-Fos (RKGSSSNEPSSD), as well as several mutant c-Fos peptides, were synthesized by solid-phase methods using an ABI 430A peptide synthesizer. The peptides, which were prepared with a C-terminal amine group, were purified by reverse-phase HPLC (Longo, et al., 1990). The native Fos peptide substrate used in these studies was also obtained from Coast Scientific (La Jolla, CA). The peptide
corresponding to the phosphorylation site in ribosomal S6 protein (RRRLSSLRA) was obtained from UBI (Lake Placid, NY). Kemptide (LRRASLG) and syntide (PLARTSLVAGLPBK) peptides were obtained from Sigma (St. Louis, MO). The Fos 2.2 antibody, c-Fos and FosΔF proteins were gifts of Dr. Tom Curran. Antibodies to p70s6k, p90rsk, and b-Raf were gifts of Drs. Joseph Avruch, John Blenis, and Gordon Guroff, respectively. The TrpE-serum response factor (SRF) fusion proteins were provided by Drs. Ravi Misra and Michael Greenberg.

**Lysate preparation** - PC12 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% horse serum and 5% fetal calf serum in an atmosphere of 10% CO₂ at 37°C. To prepare the cell lysates, PC12 cells were collected by trituration and suspended (3 X 10⁶ cells/mL) in phosphate buffered saline (PBS), pH 7.4 containing 1mg/mL bovine serum albumin (BSA) and 1mg/mL glucose, and treated as indicated. Following centrifugation, the cells were resuspended in TEV buffer (20mM Tris, pH 7.4, 1mM EGTA, and 100μM sodium orthovanadate) plus 10mM p-nitrophenyl phosphate (PNP) and sonicated. The cell lysate was centrifuged in a microfuge for 5 min at 4°C to remove the particulates. All subsequent procedures were carried out at 4°C.

**Chromatographic characterization of Fos kinase** - The soluble fraction of PC12 cell lysates was centrifuged at 100,000 X g for 30 min. Anion exchange chromatography was carried out by loading high speed supernatants onto a Pharmacia FPLC Mono Q HR5/5 column, previously equilibrated in TEV. The column was run at a flow rate of 1mL/min and
developed with a discontinuous 0-0.4M NaCl gradient, and 0.5mL fractions were collected.

Hydrophobic interaction chromatography was carried out by applying Fos kinase activity isolated by Mono Q chromatography to a phenyl Sepharose column (2 X 1.5cm) equilibrated in TEV plus 0.2M NaCl. The column was washed extensively with TEV buffer containing 30% ethylene glycol and developed with a 30% - 80% ethylene glycol gradient in TEV, pH 7.4 at a flow rate of 1 mL/min, and 1mL fractions were collected.

Cation exchange chromatography of Fos kinase was performed with a Pharmacia FPLC Mono S HR5/5 column. Fos kinase-containing Mono Q fractions were first dialyzed against 20mM Hepes, pH 6.8, 1mM EGTA, and 100μM vanadate (HEV) then loaded onto the Mono S column at a flow rate of 1mL/min. The column was developed using a discontinuous 0-0.4M NaCl gradient, and 1mL fractions collected.

Mono S column fractions containing Fos kinase activity were loaded onto a Pharmacia FPLC Mono P HR5/5 column for chromatofocusing. Following a 6-fold dilution in 25mM Bis-Tris, pH 7.1 and 1mM EGTA (buffer A), Fos kinase activity was serially applied to the Mono P column previously equilibrated in buffer A. The column was developed over the range of pH 5.5 - pH 7.1, using ampholytes as described by the manufacturer, at a flow rate of 1mL/min, and 1mL fractions were collected.

Mono P fractions containing Fos kinase activity were then applied to a Mono Q HR5/5 column. The sample was first diluted 6-fold in TEV, pH 7.4 and serially loaded onto the Mono Q column equilibrated in TEV at a flow
rate of 1mL/min. A linear 0-0.25M NaCl gradient was used to develop the
column, and 1mL fractions were collected.

Finally, Mono Q fractions containing Fos kinase activity were subjected
to gel filtration chromatography. The sample was loaded onto a Pharmacia
FPLC Superose 12 HR10/30 column in TEV containing 0.2M NaCl at a flow
rate of 0.5 mL/min, and 0.25mL fractions were collected. Immunoglobulin G
(IgG; 158kDa), BSA (68kDa), ovalbumin (45kDa), soybean trypsin inhibitor
(STI; 20kDa), and cytochrome C (12.4kDa) were used as standards. Blue
dextran and $^{32}$P$_{i}$ were used to determine the exclusion and inclusion
volume of the column, respectively. Gel filtration was also performed with
Fos kinase activity isolated by a single Mono Q chromatography step.

*Western blotting of p70$^{S6k}$ and p90$rsk* - Cell lysates from 6 X 10$^{7}$
NGF-stimulated PC12 cells were chromatographed on a Mono Q HR5/5
column. Proteins in the column fractions were precipitated by the addition of
deoxycholate to 150μg/mL and trichloroacetic acid to 6%, and the proteins
separated on a 9% SDS-PAGE gel. The proteins were blotted onto PVDF
membranes at 300mA for 3 hr in 15mM Tris, pH 8.3, 100mM glycine, and 9%
methanol. The blots were probed with p90$rsk$ and p70$^{S6k}$ antibodies at a
1:500 dilution and detected by enhanced chemiluminescence as
recommended by the manufacturer (Amersham, Arlington Heights, IL).

*Purification of Fos kinase* - The characterization of the chromatographic
behavior of Fos kinase allowed the design of procedures to scale up Fos
kinase purification from PC12 cells. One purification paradigm involved four
sequential chromatographic steps, Fractogel EMD DEAE 650, phenyl
Sepharose, Fast Flow S, and Mono Q chromatography. At the completion of
each chromatographic step, fractions were assayed for Fos kinase activity and fractions containing peak activity were loaded onto the next column.

PC12 cells (2.5 X 10^9 cells) were collected in PBS containing 1mg/mL each of BSA and glucose (5 X 10^6 cells/mL) and treated for 5 min with 50ng/mL NGF. The cells were then collected by centrifugation and resuspended in TEV containing 10mM PNP, 10μg/mL leupeptin, 50nM okadaic acid, 0.01% aprotinin, 1mM phenylmethylsulfonyl fluoride (PMSF), and 10μg/mL pepstatin (lysis buffer). High speed supernatant was collected from cells that were disrupted by sonication, centrifuged at 10,000 X g for 10 min at 4°C, and centrifuged again at 100,000 X g for 30 min at 4°C. The high speed supernatant was loaded onto a Fractogel EMD DEAE-650 column (5 X 1.5 cm) equilibrated in TEV. The column was run at a flow rate of 1.5mL/min, developed with a 0.1-0.3M NaCl gradient, and 2mL fractions were collected. Fos kinase activity eluted in fractions from this anion exchange resin with approximately 0.15M NaCl. These fractions were separated by hydrophobic interaction chromatography on a phenyl Sepharose column (8 X 1.5cm) as described previously. The column was run at a flow rate of 1.5mL/min and 2mL fractions collected. Fos kinase activity eluted with 80% ethylene glycol in HEV, pH 7.4. Fos kinase-containing fractions were next applied to a Fast Flow S cation exchange column (3 X 1.5cm) at pH 6.8. This column was run under the same conditions as previously described and developed with a 0-0.5M NaCl gradient. Finally, Fast Flow S fractions which contained Fos kinase were further separated by anion exchange chromatography with a Mono Q HR5/5 column as previously described. Protein yield from each column step was
quantitated by the Bradford method (lysat, DEAE, phenyl Sepharose) or by silver stain (Fast Flow S, Mono Q).

In a second round of Fos kinase purification, soluble protein was obtained from 37.5g of untreated PC12 cells (about 1 x 10^{10} cells, a gift of Drs. S. Feinstein and M. Radeke) by 2 freeze-thaw cycles in 20mM HEPES, pH 7.4, 150mM NaCl, 1mM EDTA, 1mM PMSF, 1μg/mL pepstatin, and 1μg/mL leupeptin. The soluble fraction was first centrifuged at 30,000 X g for 30 min at 2°C followed by centrifugation at 100,000 X g at 4°C for 30 min. This high speed supernatant was diluted 3-fold in TEV, pH 7.4. PC12 cells (1 X 10^{9} cells) were treated with NGF as described previously and lysed by sonication in the lysis buffer. The high speed supernatants of the control and NGF-treated PC12 cells were combined, then purified successively over a Fractogel EMD DEAE 650 (6 x 2cm) column, phenyl Sepharose column, and a Fast Flow S column, as previously described. Fos kinase eluting from the Fast Flow S column at 0.15M NaCl was diluted and serially loaded onto the Mono P HR5/5 column and developed with a pH 5.5 - 7.1 gradient. Finally, Fos kinase containing fractions eluting at approximately pH 6.0 were applied to a Mono Q HR5/5 column, and anion exchange chromatography was performed as described.

**Kinase assays** - Fos kinase activity of the chromatographic fractions was assayed in a buffer containing 10μM [γ^{32}P]ATP (13.2 cpm/fmole), 10mM MgCl_{2}, 1.2mM dithiothreitol, and 0.25mM Fos peptide for 30 min at 20°C, unless otherwise indicated. Reactions were stopped by the addition of trichloroacetic acid at a final concentration of 3.5% and 10μg BSA. After 15 min at 4°C, samples were centrifuged to pellet precipitated proteins and
25µL aliquots of the supernatants were spotted in triplicate onto P81 paper (Casnellie, 1991). The P81 papers were washed 4 times (10 min each) in 75mM phosphoric acid, and incorporated radioactivity was measured (Smith, et al., 1989). Assays for kinase activities phosphorylating the S6 peptide and Kemptide substrates were carried out under the same conditions, except that the peptide concentration in each case was 0.05mM.

Assays for kinase activities toward protein substrates were conducted under similar reaction conditions with 0.5µM [γ³²P]ATP (264 cpm/fmole) and 2µg of substrate, unless otherwise indicated. These reactions were stopped by the addition of 3X Laemmli’s sample buffer, and the products were resolved by SDS-PAGE followed by autoradiography.

Immunokinase assays were performed with Fos kinase isolated by Mono Q and Mono S chromatography by incubation with the indicated antibodies in 20mM Hepes, pH 7.4, 1% Triton X-100, 10% glycerol, 50mM NaF, 1mM PMSF, and 1mM sodium orthovanadate for 1 hr. Protein A agarose was then added, and the incubation continued for 30 min at 4°C. The supernatant was saved and the agarose pellet was washed 4 times in 20mM Hepes, pH 7.5, 150mM NaCl, 0.1% Triton X-100, and 10% glycerol. Pellets were resuspended in 20mM Hepes, pH 7.5 and 150mM NaCl, and both the supernatant and pellet were assayed for Fos kinase activity, as described previously. As a positive control for the immunoprecipitations and immunokinase assays, PC12 whole cell lysates were used for precipitation of p90rsk and b-Raf, and the precipitated kinase activity was assayed using the S6 peptide and casein substrates, respectively.
**Subcellular fractionation of PC12 cells** - PC12 cells (2.4 X 10^7 cells) were suspended in PBS containing 1mg/mL BSA and glucose (4 x 10^6 cells/mL), and incubated in the absence or presence of 50ng/mL NGF for 5 min. Cells were collected by centrifugation, resuspended in 10mM PNP, and placed on ice and allowed to swell for 10 min. The cell suspension was passed through a 27g needle several times. Buffer (TEV) was added, and the lysate was layered onto a 1M sucrose cushion as described by Chen et al. (Chen, et al., 1992). Following centrifugation at 1600 X g for 10 min, the crude nuclear pellet was isolated and washed again through a 1M sucrose cushion. The soluble fraction and the sucrose cushions were subjected to centrifugation at 100,000 X g for 30 min to yield soluble proteins in the supernatant and membrane fraction in the pellet. The crude nuclear pellet and the membrane pellets were solubilized in TEV with 10mM PNP and 1% Triton X-100, and the respective fractions were applied to a Fast Flow Q resin. The column was washed with TEV plus 0.15M NaCl, and Fos kinase was eluted stepwise with 0.3M NaCl in TEV. Fos kinase assays for each subcellular fractionation were conducted as described, using 5μg of cellular protein and the Fos peptide as a substrate.

**Immunoprecipitation of c-Fos** - PC12 cells (4 X 10^6 cells) were incubated in methionine-free DMEM containing the indicated stimuli for 30 min, then ^35^S-methionine was added (250μCi/mL) for an additional 45 min. The cells were harvested in 200μL of boiling 50mM Tris, pH 8, 0.5% SDS, and 5mM dithiothreitol, as described by Barber and Verma (Barber and Verma, 1987). Samples were then collected and boiled for 5 min. After the addition of 800μL of RIPA buffer (10mM Tris, pH 7.4, 1% deoxycholate, 1%
NP-40, 150mM NaCl, 0.001% aprotinin, 0.25mM PMSF, and 20mM PNP), lysates were subjected to centrifugation at 37,000 X g for 90 min. The Fos 2.2 antibody was incubated with the supernatant for 2 hr at 4°C. Protein A agarose beads were added and the incubation continued for 30 min at 4°C. Pellets were washed 3 times in RIPA containing 0.1% SDS and twice in 50mM Tris, pH 8 prior to resuspension in Laemmli's sample buffer and boiling for 5 min. The immunoprecipitated proteins were then separated by 10% SDS-PAGE, followed by autoradiography.

**Results**

*Chromatographic behavior of Fos kinase* - Soluble protein obtained from cellular lysates of unstimulated and NGF-treated PC12 cells were applied to a Mono Q HR5/5 anion exchange column. Two major peaks of NGF-stimulated Fos peptide kinase activity were detected, using the C-terminal Fos peptide substrate, in fractions eluting at 0.08M and 0.18M NaCl (Fig. 1A). However, use of full length c-Fos as a substrate demonstrated that the major peak of activity phosphorylating the protein eluted at 0.18M NaCl and was coincident with the principal peak of activity phosphorylating the peptide substrate (Fig. 1B). Importantly, the activity which eluted at 0.18M NaCl did not phosphorylate a C-terminally truncated Fos protein, FosΔF. The kinase activity in these fractions, designated Fos kinase, was stimulated 20-fold in PC12 cells by a 5 min treatment with NGF (Fig. 1A). Western blot analysis demonstrated that this activity was resolved away from both p90rsk and p70S6k, which eluted at 0.08M NaCl and 0.3M NaCl, respectively (Fig. 1C). Additionally, Fos kinase did not coelute with mitogen activated protein
(MAP) kinases (assayed using myelin basic protein, MBP), or with major Kemptide, syntide, and S6 kinase activities (Fig. 2).

Fos kinase was further resolved from contaminating S6 and Kemptide kinase activities by subjecting the peak Fos kinase containing Mono Q fractions to hydrophobic interaction chromatography using a phenyl Sepharose column. Fos kinase bound to the column and was eluted in approximately 60% ethylene glycol (Fig. 3). An interesting feature of the behavior of Fos kinase on the phenyl Sepharose column was the elevated level of Fos kinase activity detected in fractions obtained from unstimulated PC12 cells. Apparent activation of the kinase has been consistently observed, with a recovery of approximately 400-600% of the input activity from the phenyl Sepharose resin. We recovered approximately equivalent levels of activity from control samples as from NGF-treated samples. The nature of this effect is not presently understood.

Fos kinase first partially purified by Mono Q chromatography eluted from a Mono S HR5/5 cation exchange column at approximately 0.2M NaCl (Fig. 4). Like phenyl Sepharose chromatography, Mono S chromatography was an effective step in the purification of Fos kinase, allowing separation of Fos kinase from an unidentified contaminating S6 kinase (Fig. 4).

Chromatofocusing was conducted to determine the pI of Fos kinase. Fos kinase was partially purified by Mono Q chromatography from NGF-treated PC12 cells and applied to a Mono P HR5/5 column. Fos kinase eluted from the pH gradient between 5.9 and 6.0, indicating the enzyme has a pI within this range (Fig. 5).
We observed unusual chromatographic behavior of Fos kinase on the Mono Q anion exchange column when the column was used to resolve the activity at later stages of the purification scheme. Fos kinase was initially found to elute at 0.18M NaCl on this column, however, following sequential Mono Q, Mono S, and Mono P steps, this activity eluted from the same column at 0.075M NaCl (Fig. 6). This proved to be an effective means of purification, as Fos kinase activity was separated from the majority of contaminating protein which was eluted from this column at 0.18M NaCl. Again, the peak Fos kinase containing fractions were free of contaminating p90rsk and p70s6k, as these fractions did not contain protein which was immunoreactive with antibodies to these enzymes (data not shown).

The apparent $M_r$ of Fos kinase was determined by gel filtration chromatography on a Superose 12 HR10/30 column. Fos kinase that had been partially purified by Mono Q chromatography eluted from the gel filtration column between the IgG (158kDa) and BSA (68kDa) standards, with an apparent $M_r$ of approximately 100,000 (Fig 7A). However, further purification of Fos kinase altered its apparent $M_r$. Fos kinase purified by sequential Mono Q, Mono S, Mono P, and Mono Q chromatography had an apparent $M_r$ of 30,000, eluting between the ovalbumin (45kDa) and STI (20kDa) standards (Fig 7B).

**Purification of Fos kinase** - Analysis of the chromatographic behavior of Fos kinase allowed us to design a strategy for the scale up for purification of Fos kinase. Fos kinase was purified from approximately $2.5 \times 10^9$ cells (Table I and Figure 8A). Briefly, soluble cell lysates were applied to a Fractogel EMD DEAE column. This anion exchange resin was chosen as
the initial purification step due to its a high protein binding capacity. Fos kinase activity eluted from this anion exchanger at 0.15M NaCl yielding a 13-fold purification. Fractions containing Fos kinase activity were pooled and applied to a phenyl Sepharose column at 0.2M NaCl. This hydrophobic interaction resin allowed significant purification of Fos kinase from contaminating S6 kinase activities resulting in an approximate 6-fold purification (Fig. 3). Fos kinase activity was eluted with 80% ethylene glycol, and corresponding fractions were further purified by cation exchange chromatography on Fast Flow S. Fos kinase activity was efficiently recovered from this column with a net increase in specific activity of 15-fold. The Fos kinase activity eluting from the Fast Flow S column at 0.2M NaCl was applied to an FPLC Mono Q HR5/5 column. Although the yield from this second Mono Q column was low, the addition of a second anion exchange procedure proved to be an efficient step, yielding a 20-fold purification (24,000-fold overall) due to the high resolution of the column and the altered chromatographic behavior of Fos kinase upon extensive purification (Fig. 6). Silver staining revealed that the major protein in the peak fractions had an apparent molecular weight of 60kDa.

Because the apparent Mr of Fos kinase determined by gel filtration (30,000) did not correspond to the molecular weight of the principal protein in these fractions, as determined by SDS-PAGE (60kDa), we thought that Fos kinase might be of too low abundance to be detected by silver staining from a preparation of this scale. Therefore, the purification was repeated on a larger scale starting approximately 1 \times 10^{10} PC12 cells (1.2g protein). The bulk of soluble protein was obtained from untreated PC12 cells which
was combined with lysates prepared from NGF-treated PC12 cells to allow us to follow Fos kinase activity in the initial purification steps. This approach was taken since we have not detected differential chromatographic behavior of the stimulated and unstimulated Fos kinase activities. Fos kinase was purified by Fractogel EMD DEAE, phenyl Sepharose, Fast Flow S, Mono P, and Mono Q essentially as described for the previous preparation with the addition of chromatofocusing chromatography on a Mono P HR5/5 column between the Fast Flow S and Mono Q column steps. Silver staining of Fos kinase containing fractions obtained from the final Mono Q step revealed the presence of a 37kDa protein coincident with elution of Fos kinase activity from Mono Q chromatography at 0.075M NaCl (Fig. 8B, lane 2). Importantly, the 60kDa species previously seen in peak fractions was resolved from the 37kDa protein or Fos kinase activity with the additional column step, and appeared in adjacent fractions.

Substrate specificity of Fos kinase - The site at which Fos kinase phosphorylated the native c-Fos protein was localized to the C-terminus, as the enzyme phosphorylated the full length c-Fos, but failed to phosphorylate FosΔF, a mutant Fos protein lacking 66 C-terminal residues. Importantly, the phosphorylation of c-Fos by Fos kinase was competitively inhibited in the presence of the Fos peptide substrate (Fig. 9A). These data suggest that Fos kinase modified c-Fos in the 359-370 region. We have previously demonstrated that Fos kinase phosphorylates the peptide substrate at the serine residue corresponding to Ser362. These data also demonstrated that phosphorylation of c-Fos by Fos kinase altered its electrophoretic mobility resulting in an apparent increase in molecular weight from 53 to 60kDa (Fig.
9A). The capacity of Fos kinase to lower the electrophoretic mobility of c-Fos was consistent with the enhanced phosphorylation and altered apparent size of Fos isolated from NGF-treated PC12 cells (Curran and Morgan, 1986; Fig. 14).

Fos kinase phosphorylated the protein substrates histone IIS, histone V1S, histone VIIIS, and MBP, and the phosphorylation of these substrates was competed with 1mM Fos peptide (Fig. 9B). Fos kinase also phosphorylated a recombinant TrpE-SRF fusion protein (residues 46-244) at a site near the N-terminus, likely at Ser$^{103}$, as indicated by its inability to phosphorylate a mutant TrpE-SRF protein in which Ser$^{103}$ was replaced by an alanine (Fig 9C). Fos kinase did not phosphorylate casein, histone IIIS, the Raf-1 peptide, and c-Jun (data not shown).

Fos kinase phosphorylated the S6 peptide and Kemptide peptide substrates (Fig. 6, data not shown). We previously had not detected this activity in a less highly purified preparation (Taylor, et al., 1993).

The identification of elements of the consensus recognition site of Fos kinase was investigated by the synthesis of mutant peptides in which either serines 362-364 (RKGAAANEPSSEC), or serines 368-369 (RKGSSSNPEPAAD) were replaced with alanines. Fos kinase showed little activity toward either mutant peptide (Fig. 10). The inability of Fos kinase to phosphorylate the RKGAANEPSSD peptide was not surprising since the phosphorylation site of Fos kinase has been identified as Ser$^{362}$ (Taylor, et al., 1993). The RKGSSSNPEPAAD peptide is a poor substrate for Fos kinase as well as for cAMP dependent kinase (data not shown), suggesting the
necessity of serines 368-369 in determining the recognition sequence for both of these protein kinases.

Comparison of properties of Fos kinase with other known kinases - We have performed a series of experiments to allow the discrimination of Fos kinase from other characterized protein kinases. IP20, a specific peptide inhibitor of cAMP dependent kinase, had no effect on Fos kinase activity (Cheng, et al., 1986; data not shown). Similarly, heparin, which specifically inhibits casein kinase II, was also without effect (Hathaway, et al., 1982). These data are consistent with chromatographic data indicating that Fos kinase can be resolved from these well characterized enzymes.

A major effort was made to distinguish Fos kinase from the growth factor regulated S6 kinases, p90\textsuperscript{rsk} and p70\textsuperscript{S6k}. This was of particular concern due to the capacity of Fos kinase to phosphorylate the S6 peptide substrate. We have directly tested for immunological crossreactivity of Fos kinase with p90\textsuperscript{rsk} and p70\textsuperscript{S6k}. Immunoprecipitation from Fos kinase-containing fractions with antisera to p90\textsuperscript{rsk} demonstrated that Fos kinase was not recognized by this antisera (data not shown). Western blot analysis confirmed that the S6 kinases and Fos kinase were not immunologically related (Fig. 1C). b-Raf is a 90kDa kinase that is activated in response to NGF treatment of PC12 cells with a time course similar to Fos kinase (Oshima, et al., 1991). However, b-Raf antibodies also failed to immunoprecipitate Fos kinase activity (data not shown), demonstrating that b-Raf and Fos kinase are distinct.

Kinetic Properties of Fos Kinase - The NGF-activated Fos kinase had a $K_m$ of 120\textmu M for the peptide substrate (Table II). The $K_m$ ATP of Fos kinase
was determined to be 12µM, and was not altered by NGF treatment. However, the \( V_{\text{max}} \) was elevated 10-fold in NGF-treated samples relative to controls.

*Cation Dependence of Fos Kinase* - Fos kinase activity was dependent upon divalent cations, showing a preference for \( \text{Mg}^{2+} \) over \( \text{Mn}^{2+} \)(Fig. 11A). This kinase activity was extremely sensitive to \( \text{NaCl} \) concentration. Fos peptide phosphorylation was inhibited by 50% in the presence of 0.5M \( \text{NaCl} \), with complete inhibition at 1M \( \text{NaCl} \) (Fig. 11B). Fos kinase exhibited a broad pH optimum between 6.8 and 8.0 (data not shown).

**NGF dose required for Fos kinase activation** - The EC\(_{50}\) of NGF required to activate Fos kinase in PC12 cells after a 5 min incubation was 0.4nM (Fig. 12). This EC\(_{50}\) for NGF is similar to that required to activate other protein kinases such as MAP kinases (Miyasaka, et al., 1990) and protein kinase N (Rowland, et al., 1987), and is consistent with occupancy of the high affinity NGF receptor.

**Subcellular localization of Fos kinase** - Subcellular fractionation of PC12 cells was carried out using both control and NGF-treated PC12 cells. As shown in Figure 13, Fos kinase activity was located both within the cytoplasm (50%) and nucleus (33%). NGF treatment of PC12 cells did not significantly affect its subcellular distribution.

**In vivo phosphorylation of c-Fos** - The selective activation of Fos kinase by NGF and EGF but not other ligands lead us to verify that Fos kinase activation was correlated with the phosphorylation state of the protein *in vivo* induced by the same ligands. NGF or EGF treatment of PC12 cells resulted in synthesis of c-Fos which was highly phosphorylated, as reflected by the
reduced electrophoretic mobility of the protein (Curran and Morgan, 1986; Fig. 14). Treatment of the cells with 12-tetradecanoyl phorbol 13-acetate (TPA), dibutyryl cAMP (dbcAMP), depolarization with KCl resulted in detection of Fos protein distributed into two bands of greater mobility than that detected in growth factor-treated cells, reflective of lower levels of phosphorylation of the protein. These findings confirm those of Curran and Morgan and provide evidence that neither TPA nor dbcAMP can elicit PC12 cells to produce highly phosphorylated Fos species (Curran and Morgan, 1986).

**Discussion**

The capacity of growth factors to initiate intracellular signaling events ultimately regulating cellular proliferation and phenotype has been extensively studied. The constituents of these signaling cascades and their relationship to one another have been the focus of considerable interest. It has become clear that receptor-initiated tyrosine phosphorylation is responsible for generation of sites recognized by a class of adapter proteins bearing SH2 and SH3 domains (Lowenstein, et al., 1992; Rozakis-Adcock, et al., 1993; Rozakis-Adcock, et al., 1992). This group of proteins then directs the dissemination of intracellular signals throughout the cell. A central element of this scheme is the serial activation of protein serine/threonine kinases located at the distal extensions of these pathways which are likely to mediate the specific biochemical changes which typify growth factor action. A good deal less is known about these latter kinases and their protein substrates. The present study was initiated in an effort to identify one such protein kinase which was responsible for the growth factor-
induced hyperphosphorylation of c-Fos. We report here the purification of Fos kinase to near homogeneity and the enzymatic characterization of this enzyme.

During the purification process, we noticed that Fos kinase reproducibly displayed unusual chromatographic behavior. The NGF-activated Fos kinase eluted from a Mono Q column at 0.18M NaCl when isolated directly from cell lysates; however, after extensive purification, Fos kinase eluted from the same Mono Q column at 0.075M NaCl (Fig. 1A, 6). This alteration in behavior was elicited by prior purification with either phenyl Sepharose or Mono P chromatography. The apparent $M_r$ of Fos kinase was also observed to change during purification. Fos kinase isolated by a single Mono Q chromatography step had an apparent $M_r$ of approximately 100,000 as estimated by its behavior on gel filtration. However, upon subsequent purification by sequential Mono Q, Mono S, Mono P, and Mono Q chromatography, Fos kinase had an apparent $M_r$ of 30,000. These data suggest a multi-subunit composition for Fos kinase that is altered upon purification of the kinase activity, resulting in the observed chromatographic alterations. While the molecular weight of Fos kinase determined by gel filtration analysis differs slightly from the 37kDa determined by SDS-PAGE, this is not atypical. For example, glycogen synthase kinase 3 yielded an apparent $M_r$ of 75,000 on gel filtration, 51kDa by SDS-PAGE, and the sequence obtained from its cDNA predicted a 47kDa product (Woodgett, 1992).

Of particular interest was the observation that upon application of Fos kinase obtained from untreated PC12 cells to a phenyl Sepharose column,
we reproducibly recovered 400-600% of the activity initially loaded on to the column (Fig. 3). The apparent activation of Fos kinase from untreated cells by chromatography on phenyl Sepharose may be explained by the presence of a regulatory subunit that is retained or dissociated from the catalytic subunit by the hydrophobic resin. This hypothesis is consistent with the changes in apparent molecular weight and behavior on anion exchange chromatography.

Fos kinase is clearly distinct from several additional growth factor regulated kinases. Fos kinase was not precipitated by antibodies to p90rsk (data not shown), and did not coelute from anion exchange chromatography with p90rsk as detected by Western blot analysis (Fig. 1C). Further, no protein immunoreactive with p90rsk antibodies was present in highly purified preparations of Fos kinase (data not shown). As evaluated by Western blotting, Fos kinase did not coelute on anion exchange chromatography with either isoform of p70s6k (Fig. 1C), and in highly purified Fos kinase preparations contained no material which was immunoreactive with anti-p70s6k antibodies.

There are similarities between the Fos peptide sequence (RKGSSSNEPSSD) and the consensus site for cAMP dependent kinase (RRXS*) and a known phosphorylation site for casein kinase II (PSSD), however, the Fos kinase described here is distinct from these enzymes (Barber and Verma, 1987; Kemp and Person, 1990). Fos kinase was not inhibited by IP20, a specific inhibitor of cAMP dependent kinase (Cheng, et al., 1986; data not shown). Fos kinase activity was not inhibited by heparin, indicating that Fos kinase was not casein kinase II (Hathaway, et al., 1982;
data not shown). Moreover, Fos kinase did not coelute on anion exchange chromatography with MAP kinases (Fig. 2) and was not immunoreactive with anti-MAP kinase antibodies (data not shown). The possible identity of Fos kinase as other growth factor regulated serine/threonine kinases such as protein kinase N, Raf-1, b-Raf, and protein kinase C has been eliminated based upon its ligand activation, substrate specificity, immunokinase assays, or cation dependence (Kishimoto, et al., 1983; Oshima, et al., 1991; Rowland-Gagne and Greene, 1990; T. Roberts, pers. comm.).

Although we have no direct evidence that Fos kinase phosphorylates c-Fos in vivo, the present data are consistent with this hypothesis. We have demonstrated that the activation of Fos kinase is achieved only upon treatment of the cells with NGF and EGF, while other stimuli including dbcAMP, phorbol esters, and depolarization are without effect. Importantly, we (and others) have shown that the hyperphosphorylation of Fos is observed with identical specificity (Curran and Morgan, 1986, Fig. 14). The data also demonstrate that phosphorylation of Fos by Fos kinase results in the reduced electrophoretic mobility of the protein and of a magnitude similar to that observed in vivo following growth factor treatment (Fig. 9A). We have also provided evidence suggesting that Fos kinase phosphorylates c-Fos at a site which is modified in vivo (Taylor, et al., 1993; Fig. 1,9). Taken together, these data suggest Fos kinase may mediate the phosphorylation of c-Fos and functionally regulate its biological actions. It remains to be determined at what cellular locus Fos phosphorylation occurs, and the detection of Fos kinase activity both in the nucleus and cytoplasm suggests
that this NGF-regulated kinase could act in either cellular compartment (Fig. 13).

However, Fos kinase is not the only kinase which can phosphorylate c-Fos. cAMP dependent kinase phosphorylates c-Fos in vitro, probably at Ser362 (Abate, et al., 1991), and consensus phosphorylation site comparisons predict that p90rsk will phosphorylate c-Fos at this site (Kemp and Person, 1990). Fos phosphorylation and transrepression are likely controlled by a number of kinases specific to the stimulus and cell type.

Fos is an important transcriptional regulator in mammalian cells. An increase in c-Fos protein levels is a common response to a number of stimuli including growth factors, where c-Fos mediates transcription of secondary response genes (Gizang-Ginsberg and Ziff, 1990; Greenberg, et al., 1985; Schonthal, et al., 1988). However, the transcriptional transactivation controlled by c-Fos is temporally constrained due to the transient period of transcription and short half life of the protein. This regulation is postulated to be critical for prevention of the transformed phenotype induced by its viral cognate, v-Fos. Fos protein levels are controlled by transcription as well as by mRNA stability (Guis, et al., 1990; Lucibello, et al., 1989; Ofir, et al., 1990; Shaw and Kamen, 1986; Treisman, 1985). The transient expression of c-Fos may be partially a consequence of the ability of Fos to negatively autoregulate transcription from its own promoter.

The ability of c-Fos to repress transcription as well as its capacity to cause cellular transformation is regulated by phosphorylation at the C-terminus of c-Fos (Ofir, et al., 1990; Tratner, et al., 1992). Mutation of the serines to alanines within the putative phosphorylation sites yields a c-Fos
protein, that like v-Fos, is phosphorylated at low levels and is unable to repress transcription (Ofir, et al., 1990). However, when glutamic acid is substituted for serine residues 362-364 creating a net negative charge at this site, the transrepressive activity of c-Fos is largely restored (Ofir, et al., 1990). Ser^{362} in c-Fos is most probably a site phosphorylated in vivo, and a c-Fos mutant in which serines 362-364 have been replaced by alanines exhibits a transforming potential equal to that of v-Fos (Tratner, et al., 1992). The ability of Fos kinase to phosphorylate c-Fos, likely at Ser^{362}, suggests that the kinase may play a role in vivo regulating the transrepressive function of c-Fos synthesized in response to NGF or EGF, allowing c-Fos to control its own synthesis and inhibit transformation.

Fos kinase might also play a role in the regulation of c-Fos transcription through the phosphorylation of SRF. In vitro, Fos kinase phosphorylates SRF at Ser^{103} (Fig. 9C), a regulated in vivo phosphorylation site in growth factor responsive cells (Rivera, et al., 1993). Phosphorylation at this site stimulates binding of SRF to the serum response element (SRE).

Growth factor stimulation of cells results in the induction of a signaling cascade which induces the regulated phosphorylation of proteins and ultimately the control of gene expression. The novel Fos kinase characterized in this study is likely to function as part of the growth factor signaling cascade in PC12 cells. Its rapid activation upon growth factor treatment and its ability to phosphorylate other transcription factors such as SRF suggest that Fos kinase is likely to have a broad range of actions in addition to its putative role in regulating the transrepressive functions of c-Fos. The identification of a new component in the growth factor regulated
intracellular signaling cascades is of some importance, particularly in view of its potential role in the direct regulation of transcription.
Figure 1. Mono Q profile of Fos kinase. Cell lysates (5mg) were isolated from untreated PC12 cells or cells treated for 5 min with 50ng/mL NGF and applied to a Mono Q HR5/5 column. The column was developed with a discontinuous 0-0.4M NaCl gradient. A. Fos kinase activity was measured in 40µL aliquots of the column fractions and results are expressed as mean $^{32}$P cpm incorporated into the c-Fos peptide (±s.d.), measured in triplicate. B. Fos kinase activity was measured in 40µL aliquots of column fractions using both authentic c-Fos and FosΔF with 10µM [$^{32}$P]ATP (13.2 cpm/fmole). Results are expressed as $^{32}$P cpm incorporated into the proteins as determined by Cerevkov counting. C. The proteins in the indicated Mono Q fractions were precipitated, resolved by SDS-PAGE, and Western blotted. The respective blots were probed with p90rsk or p70s6k antibodies and visualized by Enhanced Chemi-Illuminescence.
Figure 2. Mono Q profiles with other peptide substrates. Cell lysates (5mg) were prepared and fractionated as described in Figure 1 from NGF treated PC12 cells. A. Kinase activity was assayed across the Mono Q column (40μL/assay) with S6 peptide and Kemptide substrates. Results are expressed as mean {superscript}32P cpm incorporated into the peptides (±S.D.) measured in triplicate. B. Kinase activities phosphorylating MBP and syntide were assayed as described. The results from the syntide phosphorylation are expressed as mean {superscript}32P cpm incorporated into the peptides (±S.D.) measured in triplicate. The MBP reaction products were resolved by SDS-PAGE, and radioactive incorporated into MBP was measured by excision of the labeled band followed by Cerenkov counting.
Figure 3. Profile of Fos kinase from phenyl Sepharose chromatography. Fos kinase activity obtained following Mono Q chromatography of cell lysates from untreated and NGF-treated PC12 cells (5mg) was loaded onto a phenyl Sepharose column at 0.2M NaCl, and the column was developed with a 30% - 80% ethylene glycol gradient. Fos, S6, and Kemptide kinase activities are expressed as mean cpm incorporated into the peptides determined by triplicate measurements (±s.d.) from assays of 40μL aliquots of the fractions.
Figure 4. Mono S profile of Fos kinase. Fos kinase activity obtained following Mono Q chromatography of cell lysates (15mg) was dialyzed and loaded onto a Mono S HR5/5 column at pH 6.8, and the column developed with a discontinuous 0-0.4M NaCl gradient. Kinase assays were conducted as described with 10μM[γ-32P]ATP (5.9 cpm/fmole). Fos and S6 kinase activities are expressed as mean cpm incorporated into the peptides determined by triplicate measurements (±s.d.) from assays using 40μL aliquots of the column fractions.
Figure 5. Mono P profile of Fos kinase. Fos kinase purified by Mono Q and Mono S chromatography from 12.4mg of cell lysates was diluted 6-fold into 25mM Bis-Tris, 1mM EGTA, pH 7.1 and serially loaded onto a Mono P column. The column was developed with a pH 7.1 to pH 5.5 gradient, and Fos kinase activity was measured using the Fos peptide and 40μL aliquots of each column fraction. Results are expressed as mean cpm incorporated into Fos peptide from triplicate measurements (±s.d.).
Figure 6. Mono Q chromatography of purified Fos kinase.

Following initial Mono Q, Mono S, and Mono P chromatography of Fos kinase from 9mg of cell lysates, fractions containing Fos kinase activity were pooled and reapplied to a Mono Q HR5/5 column at pH 7.4. The column was developed with a linear 0-0.25M NaCl gradient. Kinase activity was assayed as described with the Fos or S6 peptide, using 40µL aliquots of each column fraction, and 1µM[γ\textsuperscript{32}P]ATP (132 cpm/fmole). The data are expressed as mean 32P cpm incorporated into the peptides, measured in triplicate (±s.d.).
Figure 7. Gel filtration chromatography. A. Fos kinase was isolated by Mono Q chromatography. The sample was then applied to a Superose 12 HR10/30 column, and kinase activity was measured. Results are expressed as the mean cpm incorporated into the Fos peptide (+s.d.). B. Fos kinase was isolated from NGF treated PC12 cells and purified by Mono Q, Mono S, Mono P, and Mono Q chromatography. The sample was applied to a Superose 12 HR10/30 column, and kinase activity was measured. Results are expressed as the mean $^{32}$P cpm incorporated into the Fos peptide (+s.d.).
**Figure 8. Purification of Fos kinase.** A. Aliquots of the peak fraction obtained at each chromatographic step in the initial purification were separated on 10% SDS-PAGE. Each lane represents the protein present in the peak of Fos kinase activity from 1) Fractogel EMD DEAE 650 chromatography (7mU; U=nmol/min/mg), 2) phenyl Sepharose chromatography (9mU), 3) Fast Flow S chromatography (40mU), and 4) Mono Q chromatography (50mU, representing 50% of total Fos kinase activity isolated). Lanes 1 and 2 are Coomassie stained while lanes 3 and 4 are silver stained. B. Fos kinase preparation was further scaled up and purified by Fractogel EMD DEAE-650, phenyl Sepharose, Fast Flow S, Mono P, and Mono Q chromatography. The peak of Fos kinase activity eluting from the Mono Q column (lane 2, representing 40% of total Fos kinase activity isolated) and adjacent fractions were separated by SDS-PAGE on a 10% gel. The protein was visualized by silver staining. The gel was torn during processing.
Figure 9. Substrate specificity of Fos kinase. A. Fos kinase purified by Mono Q, Mono S, Mono P, and Mono Q chromatography was assayed alone (enzyme only), or with 2μg of c-Fos or FosΔF. The specificity of phosphorylation was evaluated by inclusion of Fos peptide (1mM) as indicated, as a competitive substrate. B. Fos kinase purified by Fractogel EMD DEAE-650, phenyl Sepharose, Fast Flow S, Mono P, and Mono Q chromatography was assayed alone (enzyme only) or with 2μg of histone II*S, histone VI*S, histone VIII*S, or MBP. Fos peptide (1mM) was added as indicated as a competitive substrate. C. Fos kinase purified by Mono Q, Mono P, and Mono Q chromatography was assayed in the absence of substrate (lane 1), with approximately 2μg of TrpE-SRF fusion protein (residues 46-244; lane 2) or the same fusion protein with an Ser to Ala mutation at 103 (lane 3).
Figure 10. **Required elements in the Fos kinase recognition sequence.** Fos kinase isolated following sequential Mono Q, hydrophobic interaction, and Mono S chromatography was assayed against 1mM of each peptide. The results are expressed as the mean $^{32}$P cpm incorporated into the c-Fos peptide measured in triplicate determinants ($\pm$ s.d.). The random peptide (SSRSEDSPGSKN) is a randomized arrangement of the amino acids in the c-Fos peptide.
Figure 11. Physical properties of Fos kinase. Fos kinase isolated following sequential Mono Q and Mono S chromatography was measured as described with the indicated concentration of Mg\(^{2+}\) or Mn\(^{2+}\)(A) or NaCl (B). Results are expressed as the mean \(^{32}\)P cpm incorporated into the c-Fos peptide, measured in triplicate (±s.d.).
Figure 12. The dose response relationship between NGF and Fos kinase. PC12 cells were treated with the indicated concentration of NGF for 5 min. Fos kinase activity was measured following Mono Q chromatography. These data are expressed as the mean cpm of $^{32}$P incorporated into the c-Fos peptide substrate (±s.d.) measured in triplicate.
Figure 13. Subcellular fractionation of Fos kinase. Cytosolic, nuclear, and membrane fractions were isolated as described in Materials and Methods. Fos kinase activity was assayed as described. The data are expressed as mean cpm incorporated into the c-Fos peptide(±s.d.).
Figure 14. Immunoprecipitation of c-Fos. PC12 cells remained untreated, or were treated with NGF(50ng/mL), EGF(5ng/mL), TPA(50ng/mL), dbcAMP(1mM), or KCl(50mM) in methionine-free DMEM for 30 min. 35S-methionine was added at this time and the incubation continued for an additional 45 min. c-Fos was immunoprecipitated as described and the products resolved by SDS-PAGE on a 10% SDS-PAGE gel followed by autoradiography.
<table>
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<th>Protein</th>
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<th>Column</th>
<th>Column</th>
<th>Total Protein</th>
<th>Specific Activity</th>
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FOLD PURIFICATION
Table I. Purification of Fos kinase. Soluble protein was isolated from 2.5 X 10^9 PC12 cells following 5 min treatment with 50ng/mL NGF and applied to the Fractogel EMD DEAE column, followed by chromatography on phenyl Sepharose, Fast Flow S, and Mono Q columns.
Table II. Kinetic Properties of Fos Kinase

<table>
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<th>$K_m$ ATP</th>
<th>$K_m$ Fos peptide</th>
<th>$V_{max}$</th>
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<td>n.d.</td>
<td>0.469nmole/min/mg</td>
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<td>NGF-activated</td>
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Table II. Kinetic Properties of Fos kinase. Fos kinase isolated following sequential Mono Q, hydrophobic interaction, and Mono S chromatography was assayed for 1 hr varying either the concentration of ATP or Fos peptide, and mean value of cpm incorporated into the c-Fos peptide determined from triplicate measurements.
References


APPENDIX - CHAPTER 3

Inhibition of Fos Kinase In Vitro by Protein Kinase Inhibitors
The alkaloid-like compound K252a has been a valuable material for the study of NGF signal transduction. Studies by Guroff and colleagues demonstrated that this molecule, previously described as a kinase inhibitor, specifically inhibited NGF-stimulated neurite outgrowth in PC12 cells as well as other biological effects, while having no effect upon EGF-induced mitogenesis or FGF-stimulated differentiation of the cells (Koizumi, et al., 1988). Initially, the target(s) of K252a were unknown. These studies were conducted to determine if the in vitro sensitivity of Fos kinase to K252a, and its structural homologue staurosporine (Str), was consistent with the hypothesis that Fos kinase might be a target for these inhibitors, and the inactivation of this enzyme may contribute to K252a inhibition of NGF-stimulated neurite outgrowth. However, the primary site of action for K252a has recently been identified as the Trk tyrosine kinase, accounting for the ability of this inhibitor to block all NGF-stimulated signaling events (Berg, et al., 1992).

The effect of K252a and Str upon the activity of partially purified Fos kinase was assayed. Str inhibits Fos kinase with an IC50 of 10nM (Fig. 1). K252a has a similar inhibitory effect upon Fos kinase with an IC50 near 25nM (Fig. 2). Moreover, sensitivity towards K252a and Str can be used to discriminate kinase activities. The effects of Str and K252a upon Fos kinase activity demonstrate that this enzyme is distinct from ERK1, which is inhibited in vitro by K252a (IC50=50nM; Smith, et al., 1989), but not by Str (L.K.T. and G.E.L., unpublished observations).
Figure 1. **Staurosporine inhibition of Fos kinase activity.** Fos kinase was partially purified by Mono Q and Mono S fractionation of cell lysates from NGF-treated PC12 cells. Kinase reactions were conducted as described in the "Materials and Methods" with 1 μM \( \gamma^{32}\text{P} \text{ATP} \) (138 cpm/fmole) in the presence of the indicated concentration of Str. Fos kinase activity is presented as the mean \( 32\text{P} \) cpm incorporated into the Fos peptide (± s.d.).
Figure 2. **K252a inhibition of Fos kinase activity.** Fos kinase isolated as described above and assayed as described above with 1μM [γ\(^{32}\)P]ATP (138 cpm/fmole) and the indicated concentration of K252a. Fos kinase activity is presented as the mean \(^{32}\)P cpm incorporated into the Fos peptide (± s.d.).
References


CHAPTER 4

Identification of a growth factor-stimulated Fos kinase in NIH3T3 cells and A431 cells
Introduction

Growth factors are responsible for the regulation of cell growth and differentiation. The effects of growth factors are elicited following binding to membrane-spanning receptors through the stimulation of the intrinsic tyrosine kinase activity of the receptor (Schlessinger and Ullrich, 1992). Autophosphorylation of the receptor generates binding sites for SH2/SH3 adapter proteins, and results in the tyrosine phosphorylation of a number of these receptor-associated proteins including phosphoinositol-3 kinase, phospholipase C-γ, and effectors of p21ras (Fantl, et al., 1993). The formation of large signaling complexes through interactions of SH2/SH3 adapter proteins and Ras mediates the transmission of signals, eventually leading to the activation of dual specificity kinases and serine/threonine kinases (Blenis, 1993; Crews and Erikson, 1993; Fantl, et al., 1993; Moodie, et al., 1993; Van Aelst, et al., 1993). The cascade of regulated protein phosphorylation and kinase activation induces the transcription of immediate early genes, often through phosphorylation of transcription factors (Hunter and Karin, 1992). Many immediate early gene products are themselves transcription factors which go on to regulate the expression of secondary response genes whose gene products ultimately dictate the biological response (Sheng and Greenberg, 1990). As a result, there has been considerable interest in the discovery and characterization of protein kinases which act directly to regulate gene expression through their capacity to alter transcription factor function.

The proto-oncogene c-fos is an immediate early gene whose transcription is rapidly, but transiently induced upon growth factor stimulation
(Distel and Speigelman, 1990). c-Fos acts as a transcriptional regulator by forming heterodimers with Jun family members, and binding in a sequence specific manner to AP-1 sites (Curran and Franza, 1988). Transcriptional activation requires the leucine zipper and DNA binding domains of both Fos and Jun (Kouzarides and Ziff, 1988; Turner and Tjian, 1989). Fos also exhibits a transrepressive function through which it is able to repress transcription from some promoters, including its own (Guis, et al., 1990; Sassone-Corsi, et al., 1988; Schonthal, et al., 1989; Wilson and Treisman, 1988). This transrepressive activity accounts for the transient nature of the transcription of this gene in response to growth factors. Transrepression requires the C-terminus of c-Fos and is independent of the leucine zipper and DNA binding domains (Guis, et al., 1990; Wilson and Treisman, 1988). The C-terminus of c-Fos contains a number of potential phosphorylation sites, and there is substantial evidence that these are required for expression of the transrepressive function of this protein (Ofir, et al., 1990). This view is reinforced by the observation that v-Fos, which differs from c-Fos in its final 48-amino acids (Barber and Verma, 1987; Curran, et al., 1984; Lucibello, et al., 1989), lacks these sites and is unable to repress transcription (Ofir, et al., 1990). The inability of v-Fos to transrepress has been postulated to contribute to the transforming potential of this oncogene product (Tratner, et al., 1992).

The functional importance of the putative phosphorylation sites at the C-terminus of c-Fos was shown by Barber and Verma who demonstrated that c-Fos became highly phosphorylated in response to serum or phorbol ester treatment of CHO cells (Barber and Verma, 1987). In contrast, v-Fos was
poorly phosphorylated. Similarly, in rat pheochromocytoma (PC12) cells, a model system for the study of the mechanisms of nerve growth factor (NGF) action, NGF treatment induces the synthesis of a hyperphosphorylated c-Fos protein. However, the phosphorylation of c-Fos is stimulus specific, as depolarization of these cells produces hypophosphorylated c-Fos (Curran and Morgan, 1986).

The regulated phosphorylation of c-Fos, and specifically the ability of NGF to stimulate this modification, suggested the existence of a growth factor-activated Fos kinase. We synthesized a peptide corresponding to residues 359-370 in c-Fos, containing potential C-terminal phosphorylation sites, to screen for such a kinase. In PC12 cells, we identified a rapidly activated, NGF and epidermal growth factor (EGF)-stimulated Fos kinase that phosphorylated the C-terminus of c-Fos, likely at Ser362 (Taylor, et al., 1993a). Fos kinase is a novel serine/threonine kinase, and it is postulated to function in growth factor signal transduction and transcriptional regulation (Taylor, et al., 1993b). The present study was directed at establishing if Fos kinase was expressed in other cell types, and if it could be induced by other ligands. A431 cells, a human epidermal cell line, and NIH3T3 cells, a mouse fibroblast cell line, were chosen because of their diverse origins and previously characterized growth factor signaling responses. We report here the identification of a growth factor-regulated Fos kinase from both A431 cells and NIH3T3 cells that was chromatographically similar to Fos kinase from PC12 cells. These data indicate that Fos kinase may function in multiple mammalian tissues in response to a variety of growth factors and in the regulation of the transrepressive function of c-Fos.
Materials and Methods

Materials - EGF was purchased from Upstate Biotechnology (Waltham, MA). Recombinant c-Fos and FosΔF were provided by Dr. Tom Curran. The Fos peptide, residues 359-370 (RKGSSSNEPSSD) in c-Fos, was synthesized by Coast Scientific (LaJolla, CA). The Mono Q, Mono S, and Mono P HR5/5 columns were purchased from Pharmacia LKB (Piscataway, NJ), as were the Fast Flow Q resin and polybuffers.

Lysate preparation - A431 and NIH3T3 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) in an atmosphere of 5% CO₂ at 37°C. The cells were serum starved for 48 hrs prior to the experiment by lowering the serum concentration to 0.5%. A431 cells or NIH3T3 cells were treated for 5 min as described in phosphate buffered saline (PBS), pH 7.4 with 1mg/mL bovine serum albumin (BSA) and 1mg/mL glucose at 37°C, or remained untreated. The cells were scraped from the plate in 5mL cold PBS, pelleted, and resuspended in 2mL 20mM Tris, pH 7.4, 1mM EGTA, 100μM sodium orthovanadate (TEV) with 10mM p-nitrophenyl phosphate (PNP). The cells were lysed by sonication and the centrifuged in a microfuge for 5 min to pellet the particulates. High speed supernatants were prepared by centrifugation of the soluble fraction at 100,000 X g for 30 min.

Anion exchange chromatography - High speed supernatants from both A431 cells (1.6 X 10⁷ cells) and NIH3T3 cells (5 X 10⁷ cells) were fractionated by anion exchange chromatography on a Pharmacia FPLC Mono Q HR5/5 column. The column, previously equilibrated in TEV, was developed with a discontinuous 0-0.4M NaCl gradient at a flow rate of 1mL/min, and
0.5mL fractions were collected. Fos kinase activity from both cell lines eluted from this initial Mono Q column at 0.18M NaCl.

**Cation exchange chromatography** - The peak fractions of Fos kinase activity from NIH3T3 cells obtained from the anion exchange step were pooled and dialyzed against 20mM Hepes, pH 6.8, 1mM EGTA, and 100µM sodium orthovanadate (HEV) to exchange the buffer. The sample was then applied to a Pharmacia FPLC Mono S HR5/5 column, previously equilibrated in HEV. The column was developed with a discontinuous 0-0.4M NaCl gradient at a flow rate of 1mL/min, and 1mL fractions were collected.

**Chromatofocusing and further anion exchange chromatography** - Fos kinase activity from A431 cells isolated by anion exchange chromatography was diluted 5-fold in 25mM Bis-Tris and 1mM EGTA, pH 6.6. The sample was then serially loaded onto a FPLC Mono P HR5/5 chromatofocusing column previously equilibrated in 25mM Bis-Tris, 1mM EGTA, pH 6.6. The column was developed with a pH 6.5-5.5 gradient with ampholytes as recommended by the manufacturer, at a flow rate of 1mL/min, and 1mL fractions were collected. The pH of each fraction was adjusted to pH 7.4 prior to performing the kinase assays. Fractions containing Fos kinase activity eluting at pH 6.2 from the Mono P column were pooled, diluted 5-fold in TEV, and serially loaded onto the Mono Q HR5/5 column at a flow rate of 1mL/min. This second Mono Q column step was performed by equilibration of the column in TEV, and was developed with a linear 0-0.25M NaCl gradient, and 1mL fractions were collected.

**Rapid Fos kinase preparations** - A431 cells (2 X 10^6 cells/condition) were incubated in 5mL DMEM for 2 hr and treated as indicated. NIH3T3 cells
(1 X 10^6 cells/condition), previously incubated in DMEM with 0.5% FCS for 48 hr, were treated as indicated. Following stimulation, the media was removed and replaced with 1mL PBS containing BSA and glucose, and the cells were scraped from the plate and pelleted. After resuspension in 0.5mL TEV with 0.05M NaCl and 10mM PNP, the cells were sonicated, and the particulates were removed by centrifugation in a microfuge for 5 min. The supernatant was loaded onto 100μL of Fast Flow Q resin equilibrated in TEV with 0.05M NaCl. The sample was allowed to bind to the resin for 5 min at 4°C. We have previously demonstrated that p90fsK will not bind to Fast Flow Q resin under these conditions (Nel, et al., 1993). The resin was then washed with TEV with 0.15M NaCl, and Fos kinase was eluted in 200μL of TEV with 0.25M NaCl.

**Fos kinase assays** - Fos peptide kinase assays were conducted with column fractions or eluents from rapid kinase preparations with 10μM[γ^32P]ATP (13.2 cpm/fmole), 10mM MgCl₂, 0.25mM Fos peptide, and 1.2mM dithiothreitol in TEV for 30 min at 22°C, unless otherwise indicated. Reactions were stopped by the addition of trichloroacetic acid to 3.5% and 10μg BSA, and the proteins were precipitated on ice for 15 min. After centrifugation in a microfuge for 5 min, the supernatants were spotted in triplicate to P81 papers (2.25cm²; Casnellie, 1991). The papers were washed 4 times (10 min each) in 75mM phosphoric acid and incorporated radioactivity measured.

Kinase assays using recombinant Fos and FosΔF were conducted with partially purified Fos kinase and 0.5μM[γ^32P]ATP (528 cpm/fmole), 10mM MgCl₂, and 1.2mM dithiothreitol in TEV for 1 hr at 22°C. Samples were fractionated by SDS-PAGE on a 10% gel and processed for autoradiography.
Results

Activation of Fos kinase in NIH3T3 and A431 cells - Fos kinase activity was detected in both NIH3T3 cells and A431 cells, and this activity was rapidly activated in response to growth factors. Fos kinase activity from NIH3T3 cells was maximally activated following 5 to 20 min of serum treatment, exhibiting a 5-6 fold stimulation (Fig. 1A). Fos kinase activity then declined, however, the enzyme activity remained 2-3 fold above basal levels as late as 2 hrs after stimulation. Similarly, the activity of the A431 enzyme peaked at 10 min following EGF stimulation, and also remained elevated after 2 hr (Fig. 1B).

The Fos kinase activity in NIH3T3 cells was also activated upon treatment with 12-tetradecanoyl phorbol 13-acetate (TPA) or EGF to a similar levels as that observed with FCS (Fig. 2A). Insulin and bradykinin treatment resulted in only a slight elevation of Fos kinase activity, while dibutyryl cAMP (dbcAMP) was without effect.

In contrast, the ligand-specific activation of Fos kinase in A431 cells was distinctly different from that observed with NIH3T3 cells. In A431 cells, EGF was the only effective kinase activator (Fig. 2B). TPA treatment only modestly enhanced Fos kinase activity. Insulin, bradykinin, and dbcAMP had little or no effect upon the activity of this enzyme.

Chromatographic identification of Fos kinase from NIH3T3 and A431 cells - The chromatographic behavior of the growth factor-stimulated Fos kinase activity was characterized to verify that these activities were distinct from other enzymes which might utilize this substrate. Moreover, the chromatographic behavior of Fos kinase allowed the unequivocal
identification of the activity based on the similarity to Fos kinase activity purified from PC12 cells (Taylor, et al., 1993b).

Lysates from NIH3T3 cells were isolated from untreated and serum-stimulated NIH3T3 cells and fractionated by anion exchange chromatography on a Pharmacia FPLC Mono Q HR5/5 column. Fractions were assayed for Fos kinase activity using the c-Fos peptide (Fig. 3A). There were two major peaks of peptide kinase activity. The initial peak that eluted at approximately 0.1M NaCl had been previously identified as p90fSk (Nel, et al., 1993; Taylor, et al., 1993b). The second peak of kinase activity eluted at approximately 0.18M NaCl, and corresponded to the elution position of this enzyme isolated from PC12 cells (Taylor, et al., 1993b). Fos kinase obtained from Mono Q chromatography was pooled and applied to a Pharmacia FPLC Mono S HR5/5 column for cation exchange chromatography and was eluted at approximately 0.2M NaCl (Fig.3B).

Similarly, cell lysates prepared from untreated and EGF treated A431 cells were fractionated by chromatography on a Mono Q HR5/5 column, and an EGF-stimulated Fos kinase activity eluting at the same position from this anion exchange column (0.18M NaCl) was detected (Fig 4A). The chromatographic behavior of this kinase activity was further characterized by chromatofocusing on a Mono P column. The elution position of Fos kinase indicated that this enzyme had a pI of 6.2 (Fig. 4B).

In summary, the chromatographic behavior of the A431 and NIH3T3 enzymes resembles that of the rat pheochromocytoma Fos kinase upon Mono Q (eluting at 0.18M NaCl), Mono S (eluting at 0.2M NaCl), and chromatofocusing (pI of 6.0) chromatography. While the pI of the A431 Fos
kinase was slightly different from Fos kinase in PC12 cells, this may result from species differences between the rat and human enzymes. Importantly, Fos kinase from A431 cells exhibited a characteristic shift in elution position on the second Mono Q column (eluting at 0.075M NaCl), significantly different from its original elution position of 0.18M NaCl (Fig. 4C). This behavior is identical to Fos kinase isolated from PC12 cells (Taylor, et al., 1993b), and likely to be a consequence of subunit dissociation upon chromatofocusing (Taylor, et al., 1993b).

_Fos kinase from NIH3T3 and A431 cells phosphorylated c-Fos at its C-terminus._ Fos kinase was partially purified from NIH3T3 cells by Mono Q and Mono S chromatography and incubated with recombinant c-Fos to determine if it phosphorylated this transcription factor. Fos kinase activity was serum-stimulated as reflected in increased phosphorylation of c-Fos (Fig. 5A). Moreover, Fos was phosphorylated at the C-terminus of c-Fos as FosΔF, a C-terminal deletion mutant of c-Fos lacking the final 66 amino acids, was phosphorylated at a significantly lower level than c-Fos. A similar contaminating FosΔF kinase activity was detected in PC12 cells after Mono Q and Mono S isolation of Fos kinase (Taylor, et al., 1993a). Fos kinase isolated from A431 cells following purification by Mono Q, Mono P, and a second Mono Q chromatographic step also phosphorylated c-Fos (Fig. 5B). The EGF stimulation of Fos kinase resulted in enhanced phosphorylation of c-Fos, although the basal levels were significantly higher than detected in NIH3T3 cells. Fos kinase phosphorylated c-Fos at its C-terminus, as indicated by its inability to phosphorylate FosΔF at significant levels.
Discussion

Engagement of growth factor receptors initiates an intracellular signaling cascade that transmits signals to the nucleus, inducing changes in gene expression that control the cellular phenotype (Chao, 1992; Fantl, et al., 1993; Schlessinger and Ullrich, 1992). The serial activation and appropriate regulation of protein kinases is a necessary element of signal transduction cascades. Protein kinases appear to play roles in both the propagation of signals from growth factor receptors to targets in the cytoplasm (Blenis, 1993; Fantl, et al., 1993) and in transcriptional regulation (Hunter and Karin, 1992).

We have previously identified a growth factor regulated kinase, Fos kinase, in PC12 cells that may function in both capacities (Taylor, et al., 1993a; Taylor, et al., 1993b). This rapidly activated protein kinase has been purified to near homogeneity and is a 37kDa protein with a pI of 6.0. Fos kinase phosphorylates c-Fos at its C-terminus, likely at Ser\(^{362}\), a site required for the expression of the transrepressive function of this transcription factor, as well as another transcription factor, serum response factor, at a site which stimulates its binding to, and potentially transactivation from, the serum response element. Here we extend these findings to demonstrate that Fos kinase is expressed in other cell types, and may perform similar functions in response to other ligands.

Both A431 cells and NIH3T3 cells possessed a growth factor-stimulated Fos kinase activity which was chromatographically and enzymatically indistinguishable from Fos kinase isolated from PC12 cells (Taylor, et al., 1993b; Fig. 3 and 4). The presence of Fos kinase in cell lines of neural crest, epidermal, and fibroblast origins demonstrated that Fos kinase
may be present in a variety of tissues. Consistent with this hypothesis, we have recently shown that a chromatographically similar Fos kinase was present in Jurkat T-lymphocytes (Nel, et al., 1993). In the cell types studied, Fos kinase activity showed a similar time course following stimulation. The activity was rapidly activated, with maximal activity detected between 5 and 20 min, and it then subsequently declined (Nel, et al., 1993; Taylor, et al., 1993a; Fig. 1). Importantly, Fos kinase activity remained well above basal levels for the next 2 hrs. This period corresponds to that of growth factor-stimulated c-Fos protein synthesis, and we postulate that Fos kinase acts to phosphorylate the nascent protein. Extensive characterization of the PC12 enzyme has shown Fos kinase to be distinct from any previously described protein kinases (Taylor, et al., 1993b).

Fos kinase was activated by EGF treatment of both A431 and NIH3T3 cells, and was not activated by dbcAMP in either cell type (Fig. 2). The inability of dbcAMP to activate Fos kinase is consistent with results obtained from Jurkat T-lymphocytes and PC12 cells (Nel, et al., 1993; Taylor, et al., 1993a). However, the ability of some agents to activate Fos kinase depended upon the cell type. Although both A431 and NIH3T3 cells respond to insulin and bradykinin (Corps and Brown, 1988; Leeb-Lundberg and Song, 1993; Sommercom, et al., 1987; Wheeler, et al., 1990) these agents only modestly activated Fos kinase in NIH3T3 cells, while having no effect on Fos kinase in A431 cells. The stimulation of protein kinase C by TPA effectively activated Fos kinase in NIH3T3 cells (Fig. 2A) and in Jurkat T-lymphocytes (Nel, et al., 1993), and induced hyperphosphorylation of c-Fos in CHO cells (Barber and Verma, 1987). However, TPA had little or no effect on Fos kinase in both
A431 cells and PC12 cells (Taylor, et al., 1993a; Fig. 2B), and failed to activate it or induce the hyperphosphorylation of c-Fos in PC12 cells (Taylor, et al., 1993b). The differential response of Fos kinase to the PMA and other ligands suggests that Fos kinase may be regulated by multiple upstream mechanisms which vary with the cell type. The rapid activation of Fos kinase suggests that, like other kinases with similar rapid stimulation such as MAP kinases (Blenis, 1993), it may be a participant within a signal transduction cascade.

Fos kinase may also regulate transcription through its phosphorylation of c-Fos was well as other transcription factors. We have previously demonstrated that Fos kinase phosphorylated serum response factor in vitro at a site which stimulates the DNA binding capacity of this transcription factor (Taylor, et al., 1993b). Fos kinase may also affect transcriptional repression through its phosphorylation of the C-terminus of c-Fos. Ofir et al. have demonstrated potential phosphorylation sites at the C-terminus of c-Fos are required for expression of its transrepressive function (Ofir, et al., 1990). One of these potential phosphorylation sites, Ser\textsuperscript{362}, is likely phosphorylated in vivo (Tratner, et al., 1992). Mutation of Ser\textsuperscript{362-364} to alanine residues destroys this phosphorylation site, and creates a c-Fos protein that is unable to repress transcription from its own promoter (Ofir, et al., 1990). This molecule has been reported to have a transforming potential equal to v-Fos (Tratner, et al., 1992). We have demonstrated that Fos kinase in PC12 cells phosphorylates the Fos peptide, and likely the c-Fos protein, at a site equivalent to Ser\textsuperscript{362} (Taylor, et al., 1993a). The same Fos kinase described here in A431 and NIH3T3 cells may regulate both the transrepressive and
transforming potential of c-Fos through phosphorylation of Ser^{362}. It is unlikely that Fos kinase is the only enzyme that acts at this site, as other kinases such as cAMP dependent kinase and p90^{rsk} may phosphorylate c-Fos at this same site (Abate, et al., 1991; Kemp and Person, 1990).

We have identified a growth factor-stimulated kinase activity in both A431 and NIH3T3 cells that is indistinguishable from the behavior of the Fos kinase activity previously described in PC12 cells and Jurkat T-lymphocytes (Nel, et al., 1993; Taylor, et al., 1993a; Taylor, et al., 1993b). The time dependence and substrate specificity of this novel Fos kinase suggests that it is one of an ever-growing number of protein kinases which directly function both in the propagation of signals through the cytoplasm in signaling cascades (Blenis, 1993) and in the regulation of gene transcription (Hunter and Karin, 1992). Regulated protein phosphorylation and eventual transcriptional regulation are necessary elements of these signaling pathways. The widespread distribution and multiple functions of Fos kinase indicate that this novel protein kinase is likely to have a broad range of actions in growth factor signal transduction.
Figures

A

![Graph A]

B

![Graph B]
Figure 1. Time course of Fos kinase activation. A. NIH3T3 cells were treated with 15% FCS for the indicated time, and Fos kinase was isolated by the rapid purification protocol described in the "Materials and Methods" and assayed with 2.2μg of protein using the Fos peptide substrate. B. A431 cells were treated for the indicated time with 20ng/mL EGF, and Fos kinase was isolated by the same rapid purification method. Fos kinase activity was assayed using 4.3μg of protein. Kinase activity is expressed as mean 32P cpm incorporated in the Fos peptide (+s.d.).
Figure 2. Ligand activation of Fos kinase. **A.** NIH3T3 cells were treated with 15% FCS, TPA (100ng/mL), dbcAMP (1mM), insulin (10μg/mL), or bradykinin (10nM) for 5 min or remained untreated. Fos kinase was partially purified using the rapid purification protocol (see Materials and Methods). Fos kinase activity was assayed using 9.3μg of protein. **B.** A431 cells were treated for 5 min with EGF (20ng/mL), TPA (100ng/mL), dbcAMP (1mM), insulin (10μg/mL) or bradykinin (10nM), or remain untreated. Fos kinase was isolated by the rapid purification method, and activity was assayed with 1.4μg of protein. Results are expressed as mean $^{32}$P cpm incorporated into the Fos peptide substrate (+s.d.).
Figure 3. Chromatographic behavior of Fos kinase from NIH3T3 cells.  
A. Lysates were prepared from untreated NIH3T3 cells or from cells after 5 min treatment with 15% FCS, and applied to a Mono Q HR5/5 column. The column was developed with a discontinuous 0-0.4M NaCl gradient. Fos kinase activity was measured with 10μL aliquots of column fractions, and results are expressed as mean $^{32}$P cpm incorporated into the Fos peptide (+s.d.), measured in triplicate.  
B. Fos kinase activity isolated by Mono Q chromatography, fractions 44-48, was dialyzed and loaded into a Mono S HR5/5 column. The column was developed with a discontinuous 0-0.4M NaCl gradient. Fos kinase activity was assayed with 40μL aliquots of column fractions, and the results are expressed as mean $^{32}$P cpm incorporated into the Fos peptide (+s.d.).
Figure 4. Chromatographic characterization of Fos kinase from A431 cells. A. Lysates were prepared from untreated A431 cells or cells following a 5 min treatment with 20ng/mL EGF, and applied to a Mono Q HR5/5 anion exchange column. The column was developed with a discontinuous 0-0.4M NaCl gradient, and Fos kinase activity was measured with 40μL aliquots of column fractions. The results are expressed as mean $^{32}$P cpm incorporated into the Fos peptide (±s.d.). B. Fos kinase activity from the Mono Q column, fractions 44-48, was pooled and applied to a Mono P HR5/5 chromatofocusing column at pH 6.7. The column was developed with a pH 6.5-5.5 gradient, and Fos kinase activity was assayed with 40μL aliquots of fractions as described in the Materials and Methods with 0.5μM[$^{32}$P]ATP (268 cpm/fmole). Results are expressed as mean $^{32}$P cpm incorporated into the Fos peptide. C. Fos kinase activity eluting from the Mono P column, fractions 30-32, was pooled and applied again to the Mono Q column at pH
7.4. The column was developed with a linear 0-0.25M NaCl gradient. Fos kinase activity was assayed with 40μL aliquots of fractions as described with 0.5μM[γ32P]ATP (268 cpm/fmole), and results are expressed as mean 32P cpm incorporated into the Fos peptide (±s.d.).
Figure 5. Phosphorylation of c-Fos by Fos kinase from NIH3T3 and A431 cells. Fos kinase partially purified from A. untreated or serum stimulated NIH3T3 cells by Mono Q and Mono S chromatography or B. untreated or EGF stimulated A431 cells by Mono Q, Mono P, and subsequent Mono Q chromatography, was assayed alone, or with 2μg of recombinant c-Fos or FosΔF under reaction conditions described in the Materials and Methods. Reaction products were visualized by autoradiography following SDS-PAGE.
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APPENDIX - CHAPTER 4

*Identification of Fos Kinase in Jurkat T-Lymphocytes*
Introduction

Lymphocyte activation by the T-cell antigen receptor (TCR) regulates various aspects of the immune response (Weiss and Imboden, 1987). These antigen receptors initiate signaling cascades which transduce extracellular signals into a series of biological actions. Intracellular signaling is a complex process propagated primarily by regulated protein phosphorylation. TCR signaling is initiated by a receptor associated tyrosine kinase, and leads to serine and threonine phosphorylation through the activation of serine/threonine kinases, and eventual changes in gene transcription (Nel, et al., 1990; Patel, et al., 1987; Weiss and Imboden, 1987). The regulated changes in gene expression are responsible for the acquisition and maintenance of cellular phenotype.

c-fos is an immediate early gene whose transcription is transiently induced by a variety of agents in numerous systems including ligation of lymphocyte antigen receptors (Muller, et al., 1984; Reed, et al., 1987; Reed, et al., 1986; Shipp and Reinherz, 1987). The product of this proto-oncogene is a bifunctional transcription factor (Chiu, et al., 1988; Curran and Franza, 1988; Distel, et al., 1987; Sassone-Corsi, et al., 1988; Schonthal, et al., 1988). c-Fos regulates transcriptional activation by forming heterodimers with Jun family members through leucine zippers and binding to DNA (Chiu, et al., 1988; Curran and Franza, 1988; Distel, et al., 1987; Kouzarides and Ziff, 1988; Sassone-Corsi, et al., 1988; Schonthal, et al., 1988; Turner and Tjian, 1989). c-Fos is also able to repress transcription from some promoters, including its own, and may therefore regulate the magnitude of the early response (Guis, et al., 1990; Lucibello, et al., 1989; Ofir, et al., 1990; Wilson
and Treisman, 1988). The transrepressive function requires the extreme C-terminus of c-Fos, and phosphorylation within this region is likely important for expression of this function (Ofir, et al., 1990; Tratner, et al., 1992).

In a collaboration with Dr. Andre Nel (UCLA), we examined T-lymphocytes for the presence of a T-cell receptor-stimulated Fos kinase activity. Utilizing the Fos peptide substrate, we have identified a CD3 and phorbol ester-inducible Fos kinase activity in Jurkat cells. This kinase is similar, if not identical, to a growth factor-regulated Fos kinase in rat pheochromocytoma cells based upon the chromatographic behavior of these enzymes (Taylor, et al., 1993a; Taylor, et al., 1993b). These studies are part of a larger manuscript describing a rapidly stimulated, CD2 and CD3-sensitive Fos kinase in both Jurkat and normal human T-lymphocytes (Nel, et al., 1993). Fos kinase was also identified in B-cell lines, and its activity was stimulated by α-IgM or interleukin-6 treatment. In all lines examined, Fos kinase was activated by TPA stimulation, but was insensitive to dibutyryl cAMP treatment, indicating that protein kinase C, but not cAMP dependent kinase, was likely upstream of Fos kinase in a signaling cascade. Fos kinase partially purified from Jurkat T-cells phosphorylated the C-terminus of c-Fos, and this phosphorylation was competed with the peptide. Moreover, the Jurkat Fos kinase, like the comparable enzyme in PC12 cells, is a novel protein kinase based upon chromatographic behavior, Western blot analysis, and sensitivity to kinase inhibitors. Considering its substrates, cellular distribution, and rapid activation, Fos kinase may function in transcriptional regulation as well as in a variety of receptor-mediated signal transduction events in lymphocytes and other cell types.
Materials and Methods

**Materials** - OKT-3 (anti-CD3) was obtained from Ortho Pharmaceutical (Raritan, NJ). Rabbit antisera to p90^f^sk and p70^s^6k were generously provided by Dr. John Blenis (Department of Cellular and Molecular Physiology, Harvard Medical School, Boston, MA) and Dr. Joseph Avruch (Massachusetts General Hospital, Harvard University, Charlestown, MA), respectively. Mono Q HR5/5 and Mono P HR5/5 columns were obtained from Pharmacia LKB (Piscataway, NJ) as were polybuffers 74 and 96. The Fos peptide, comprising residues 359-370 of c-Fos (RKGSSNEPSSD) was synthesized by Coast Scientific (La Jolla, CA).

**Cell stimulation and purification of Fos kinase** - The Jurkat T-lymphocyte clone, E-6.1 (CD2^+, CD3^+, CD4^+) was obtained from Dr. A. Weiss (Department of Medicine, University of California, San Francisco) and was grown in RPMI 1640 supplemented with 10% fetal calf serum and antibiotics. Jurkat cells (2 X 10^8 cells/condition) were stimulated with 1μg/mL OKT-3 or 100ng/mL TPA at a density of 1.6 X 10^7 cells/mL for 5 min at 37°C or left untreated. The cells were lysed by sonication in 2mL TEV, pH 7.4 with 10mM p-nitrophenol phosphate, and soluble lysate was isolated by centrifugation in a microfuge for 5 min at 4°C followed by centrifugation at 100,000 X g for 30 min at 4°C. The high speed supernatants were loaded onto a Mono Q HR5/5 column, and the column was developed at a flow rate of 1mL/min with a discontinuous 0-0.4M NaCl gradient in TEV, pH 7.4, collecting 0.5mL fractions. The peak of Fos kinase activity eluting at 0.18M NaCl (fractions 46-52, 2mL total) was pooled and diluted 3-fold in 25mM Bis-Tris, 1mM EGTA, pH 6.5. The sample was serially loaded onto a FPLC Mono
P HR5/5 column at 1 mL/min, and 1mL fractions were collected. The column was developed with a pH 6.5-5.5 gradient as directed by the manufacturer. The pH of the fractions was adjusted to 7.4 prior to assaying for Fos kinase. For a second anion exchange step, Fos kinase activity from Mono P chromatography was pooled, diluted 3-fold in TEV, pH 7.4, and loaded onto a FPLC Mono Q HR5/5 column at a flow rate of 1mL/min. The column was developed with a linear 0-0.25M NaCl gradient in TEV, pH 7.4, and 1mL fractions were collected.

Kinase assays - Fos peptide kinase reactions were performed in TEV, pH 7.4 with 10mM p-nitrophenol phosphate containing 0.5mM Fos peptide, 1.2mM dithiothreitol, 10μM[γ32P]ATP (14 cpm/fmole), and 10mM MgCl2 in a final reaction volume of 50μL, unless otherwise indicated. Reactions were incubated at room temperature for 30 min and stopped by the addition of 35μL 10% trichloroacetic acid and 10μg BSA. Samples were cooled to 4°C for 15 min and centrifuged for 5 min. Supernatants were applied to Whatman P81 paper in triplicate, washed 4 times for 10 min in 75mM phosphoric acid (Casnellie, 1991), and incorporated radioactivity was measured as Cerenkov counts. MAP kinase assays were conducted under similar conditions with 1μg MBP, 2mM MnCl2, and 10μM[γ32P]ATP (14 cpm/fmole). Reactions were terminated by the addition of 25μL of 3X sample buffer, boiled, and fractionated by SDS-PAGE on a 12% gel. Phosphorylation was quantitated by Cerenkov counting of MBP protein. Fos protein phosphorylation was evaluated by incubation as described above with 0.5μM[γ32P]ATP (440 cpm/fmole) for 1 hr, and reactions were stopped by the addition of 3X sample
buffer. Products were resolved by 10% SDS-PAGE and visualized by autoradiography.

**Western blotting of p70S6K and p90RSK** - Protein collected by Mono Q chromatography was precipitated in 6% trichloroacetic acid and 150µg/mL sodium deoxycholate at 4°C. The precipitants were solubilized in 1X sample buffer, resolved by SDS-PAGE on a 9% gel, and transferred onto Immobilon P membranes at 300mA for 3 hrs in 15mM Tris, pH 8.3, 100mM glycine, and 9% methanol. The membranes were blocked with 6% BSA in TBS, pH 7.6, and overlaid with 1:500 dilution of rabbit antisera to p70S6K or p90RSK. The blots were then processed as recommended for enhanced chemiluminescence.

**Results**

**Chromatographic characterization of Fos kinase from Jurkat T-cells** - Cell lysates from untreated and anti-CD3-stimulated Jurkat T-cells were applied to a FPLC Mono Q HR5/5 anion exchange column and developed with a discontinuous 0-0.4M NaCl gradient (Fig 1A). Mono Q chromatography resolved two peaks of Fos kinase activity. Peak I eluted around 0.1M NaCl (fractions 20-28), while peak II eluted at 0.18M NaCl (fractions 46-54). While untreated cells had discernible peak I activity, which increased further upon anti-CD3 treatment (1.3-2 fold), peak II was absent and increased >20-fold upon anti-CD3 stimulation. TPA-treated cells also contained two peaks of activity which eluted in identical fractions as the CD3-activated kinases (Fig. 1A). The behavior of peak II is consistent with the Fos kinase previously
identified in rat pheochromocytoma cells (Taylor, et al., 1993b), as well as A431 and NIH3T3 cells, and will be referred to as Fos kinase.

Additional chromatographic characterization of the Jurkat enzyme was consistent with the behavior of Fos kinase from pheochromocytoma cells and A431 cells (Taylor, et al., 1993b). Anion exchange-purified Fos kinase from Jurkat T-cells was applied to a Mono P chromatofocusing column, and eluted from this column at pH 6.1 (Fig. 1B). Reapplication of this Fos kinase to the Mono Q column indicated that purification of Fos kinase altered its behavior on this anion exchange resin. Fos kinase eluted at 0.075M NaCl, a significant shift from its original elution position of 0.18M NaCl (Fig. 1C).

**Chromatographic comparison of Fos kinase to other CD3-inducible serine/threonine kinases** - The behavior of Fos kinase on anion exchange chromatography indicated that Fos kinase was distinct from other CD3-stimulated protein kinases. MAP kinase activity eluted from the Mono Q column as two peaks in fractions 24-35 and 52-62, respectively (Fig. 1D), and did not co-elute with Fos kinase. Immunoblotting with an antiserum to p90rsk indicated that Mono Q peak I co-eluted with this serine/threonine kinase (Fig. 1E) and distinguished Mono Q peak II, Fos kinase, from p90rsk. Moreover, Fos kinase did not co-elute with p70s6k (Fig. 1F), again indicating that Fos kinase is distinct from this previously identified protein kinase.

**Fos kinase phosphorylated authentic c-Fos** - Fos kinase sequentially purified from Jurkat T-cells by Mono Q, Mono P, and a second round of Mono Q chromatography, phosphorylated recombinant c-Fos protein *in vitro* (Fig. 2, lane 2). When the same reaction was performed in the presence of Fos peptide, phosphorylation of c-Fos was inhibited by >50% (Fig. 2, lane 3). To
further demonstrate that Fos kinase principally phosphorylates the C-terminal region of c-Fos, a deletion mutant lacking the C-terminal 66 amino acids, Fos ΔF, was used as a substrate (Abate, et al., 1990). This protein was not phosphorylated to any significant degree (Fig. 2, lane 4). These results establish that Fos kinase phosphorylates authentic c-Fos protein near its C-terminus.

Discussion

An increase in c-fos gene transcription constitutes an immediate early gene response to stimulation by a variety of receptors in different cell types. Increased expression of c-fos commences almost immediately in T-lymphocytes with anti-CD3 stimulation (Reed, et al., 1987; Reed, et al., 1986; Shipp and Reinherz, 1987). The c-Fos gene product participates in the activation as well as repression of gene transcription, and is therefore critical in the regulation of immediate early gene responses (Guis, et al., 1990; Schonthal, et al., 1988). It is not clear how these multiple effects of c-Fos are achieved, but phosphorylation of this and other transcription factors is likely to be the prevalent mechanism by which their activities are regulated (Hunter and Karin, 1992). We report here the identification of a novel serine/threonine kinase in lymphocytes activated by the CD3 receptor that phosphorylates the C-terminus of c-Fos.

The transrepressive activity of c-Fos has been correlated with the phosphorylation of this protein at C-terminal sites (Ofir, et al., 1990). Interestingly, the viral cognate, v-Fos, does not exhibit transrepressive activity due to a deletion-induced frameshift resulting in 48 unrelated C-terminal
amino acids compared to c-Fos (Curran, et al., 1984). The loss of potential phosphorylation sites in the C-terminus is likely responsible for the inability of v-Fos to repress transcription and is postulated to contribute to the oncogenicity of this protein (Ofir, et al., 1990; Tratner, et al., 1992). Phosphorylation of the C-terminus is likely to be essential for transrepression as a serine to alanine substitutions within these sites abolish the transrepressive activity of this protein (Ofir, et al., 1990). In pheochromocytoma cells, we have shown that NGF activates a serine/threonine kinase which phosphorylates a peptide derived from the C-terminus of c-Fos (residues 359-370; Taylor, et al., 1993a; Taylor, et al., 1993b). In Jurkat T-cells, we now provide evidence that CD3 and TPA activate a chromatographically identical kinase which phosphorylates the Fos peptide and authentic c-Fos protein (Fig. 1 and 2). Moreover, the Fos peptide inhibits c-Fos phosphorylation, and Fos ΔF, a c-Fos mutant lacking residues 315-381, is not phosphorylated (Fig. 2). These data strongly suggest that Fos kinase phosphorylates c-Fos at its C-terminus in vitro and may be responsible for c-Fos phosphorylation in intact T-cells.

Fos kinase purified from T-lymphocytes appears to differ from previously described CD3- or TPA-inducible serine/threonine kinases (Fig. 1D-F). Fos kinase was clearly distinct from MAP kinases upon anion exchange chromatography (Fig 1D). Although p90rsk can phosphorylate the Fos peptide in vitro, and Fos kinase can phosphorylate the S6 peptide, p90rsk clearly chromatographed as a peak distinct from Fos kinase on Mono Q chromatography (Fig. 1E). Western blot analysis also distinguished Fos kinase from p70S6k (Fig. 1F). Additionally, Fos kinase is distinct from cAMP
dependent protein kinase, casein kinase II, and \( \text{Ca}^{2+} \)-dependent protein kinases (Nel, et al., 1993).

In summary, we have identified a novel Fos kinase in Jurkat T-lymphocytes (Nel, et al., 1993) chromatographically similar to a protein kinase activity previously isolated in pheochromocytoma cells (Taylor, et al., 1993b), A431 cells, and NIH3T3 cells. The rapid activation of Fos kinase by a variety of receptors in multiple cell populations suggests that this enzyme may have broad effects upon transcription and signal transduction in the intact animal.
Figure 1 - Chromatographic behavior of Fos kinase and other serine/threonine kinases. A. Mono Q chromatography. 2 X 10^8 Jurkat cells were used for each of the control (unstimulated), anti-CD3 (1µg/mL OKT-3 for 5 min) or TPA (100ng/mL for 5 min) treated samples. High speed supernatants were loaded onto a FPLC Mono Q HR5/5 column and developed with a discontinuous 0-0.4M NaCl gradient. Fractions of 0.5 mL were collected and 10µL aliquots were assayed for Fos kinase as described. Data are expressed as mean cpm incorporated into the Fos peptide (±s.d.).
B. Mono P chromatography. The peak Mono Q fractions from anti-CD3 treated Jurkat cells were pooled, diluted 3-fold in 25mM Bis-Tris, 1mM EGTA, pH 6.6, and loaded onto a FPLC Mono P HR5/5 column. The column was developed with a 6.5-5.5 linear pH gradient and 1mL fractions were collected. Fos kinase was assayed as described with 40μL aliquots of fractions and 0.5μM[γ^{32}P]ATP (220 cpm/fmole), and data are expressed as mean cpm incorporated into the Fos peptide (± s.d.).
C. Mono Q chromatography. The peak of Fos kinase activity from anti-CD-3 stimulated Jurkats eluted from the Mono P column at pH 6.1. This activity was pooled and diluted 3-fold in TEV, pH 7.4 and loaded onto the FPLC Mono Q column, previously equilibrated in TEV, pH 7.4. The column was developed with a linear 0-0.25M NaCl gradient at a flow rate of 1mL/min, and 1mL fractions were collected. Fos kinase was assayed as described following Mono P chromatography.
D. MAP kinase assays. Cell lysates from anti-CD-3 treated Jurkats were chromatographed as described in Fig. 1A. MAP kinase assays were conducted as directed in the Materials and Methods with MBP, and fractionated by SDS-PAGE. Results were quantitated by Cerenkov counting of MBP.
E. Western blot for p90\textsuperscript{rsk}. Mono Q fractions collected from anti-CD3 treated Jurkat cells, generated in Figure 1A, were precipitated with 6% trichloroacetic acid and 150μg/mL deoxycholate. Proteins were resolved by SDS-PAGE on a 9% gel and transferred to Immobilon P membranes. Membranes were overlaid with rabbit antiserum to p90\textsuperscript{rsk}. Blots were then processed as recommended for enhanced chemi-luminescence.

F. Western blot for p70\textsuperscript{S6K}. Fractions from the Mono Q column presented in Figure 1A were precipitated as described above, fractionated by SDS-PAGE, and transferred to Immobilon P membranes as described above. The membranes were overlaid with rabbit antiserum to p70\textsuperscript{S6K}, and the blots were processed as recommended for enhanced chemi-luminescence.
**Figure 2. Fos kinase phosphorylated authentic c-Fos.** Fos kinase was incubated alone (lane 1), with 36pM recombinant c-Fos (lanes 2, 3) or with 36pM Fos ΔF (lanes 4, 5). The phosphorylation reaction was carried out for 1 hr. Fos peptide (1mM) was included in reactions shown in lanes 3 and 5. Proteins were fractionated by SDS-PAGE on a 10% gel and visualized by autoradiography.
References


Chapter 5

Mechanisms which Regulate Nerve Growth Factor Stimulation of Fos Kinase Activation in PC12 cells
Introduction

Nerve growth factor (NGF) acts as a trophic factor for sympathetic neurons as well as some sensory and central nervous system neurons, and is the prototypic member of the neurotrophin family (Barde, 1989). Much of what is known about the events that follow NGF receptor binding has been gained through the use of rat pheochromocytoma (PC12) cells, which has proven to be a valuable model system for study of NGF action (Greene, et al., 1987). PC12 cells respond to NGF by acquisition of a sympathetic neuron-like phenotype, including development of electrical excitability and the elaboration of neurites. The biological actions of NGF are initiated through the interaction of NGF with its receptor, TrkA (Jing, et al., 1992; Kaplan, et al., 1991a; Kaplan, et al., 1991b; Klein, et al., 1991). The binding of NGF to TrkA induces the influx of extracellular Ca^{2+} (Pandiella-Alsonso, et al., 1986) as well as the activation of the intrinsic tyrosine kinase activity of this receptor (Kaplan, et al., 1991a). The resulting receptor autophosphorylation generates binding sites for a number of proteins which associate with the receptor through src homology 2 (SH2) motifs. SH2 domains are protein sequences that recognize phosphotyrosine residues and flanking sequences. Receptor-associated proteins include GTPase activating protein (GAP; Ohmichi, et al., 1991a; Ohmichi, et al., 1991b), an effector of the GTP binding protein, p21\textsuperscript{ras}, phospholipase C-γ (PLC-γ; Vetter, et al., 1991), phosphoinositol 3-kinase (PI3K; Soltoff, et al., 1992), and SH2/SH3 adapter proteins such as Shc (Buday and Downward, 1993; Gale, et al., 1993; Rozakis-Adcock, et al., 1993). These adapter molecules have also been shown to mediate the interactions between ras and its
effectors; GAP and guanine nucleotide releasing proteins (GNRPs; Buday and Downward, 1993; Egan, et al., 1993; Gale, et al., 1993; Li, et al., 1993; Rozakis-Adcock, et al., 1993; Skolnik, et al., 1993a; Skolnik, et al., 1993b). Through its interaction with these proteins, the NGF receptor stimulates multiple signaling pathways which function in concert to comprise the NGF signal transduction cascade. This cascade propagates the NGF signal through the cytoplasm and into the nucleus, ultimately resulting in morphological and biochemical differentiation of these cells (Halegoua, et al., 1991).

Three signaling pathways emanating from the NGF receptor have been described which regulate protein kinases. The serine/threonine kinase, protein kinase C (PKC), is stimulated by NGF treatment of PC12 cells (Heasley and Johnson, 1989). PKC exists as a family of protein kinase isoforms (Parker, et al., 1989). The activation of the α, β, δ, and ε isoforms is sensitive to diacylglycerol and exogenous application of phorbol esters. However, only the classical PKC family members, the α and β isoforms, requires Ca$^{2+}$. Diacylglycerol and Ca$^{2+}$ may be supplied through the NGF-induced influx of this cation (Pandiella-Alsonso, et al., 1986) and phospholipid turnover and Ca$^{2+}$ release generated in response to PLC-γ stimulation (Altin and Bradshaw, 1990; Contreras and Guroff, 1987; Vetter, et al., 1991). However, activation of phorbol ester-sensitive isoforms of PKC is neither necessary nor sufficient to induce neurite outgrowth in PC12 cells (Reinhold and Neet, 1989).

A second pathway mediates the activation of p70$^{60k}$, a growth factor-regulated serine/threonine kinase that is postulated to function in the
regulation of protein translation rate through phosphorylation of the ribosomal S6 protein (Erikson, 1991; Sweet, et al., 1990). p70S6k activity is required in the cell cycle for the G1 to S phase transition induced by serum (Lane, et al., 1993). This pathway has been dissected through selective inhibition of its activation by the immunophilin, rapamycin. Growth factor activation of p70S6k is inhibited by this immunophilin, resulting in decreased ribosomal S6 phosphorylation, reduced cell division rates, and delayed DNA replication in response to growth factors (Chung, et al., 1992). The targets of rapamycin have not been identified although it appears to affect protein phosphatase activity (Ferrari, et al., 1993) and a yeast PI3K homologue (Kunz, et al., 1993).

dependent upon Ras (Wood, et al., 1992); a similar dependence has not yet been demonstrated for MEKK. Like its activators, MAPK participates in a cascade of growth factor regulated protein phosphorylation through its phosphorylation of multiple substrates (Davis, 1993), including the phosphorylation and activation of the serine/threonine kinase p90rsk (Ahn and Krebs, 1990; Sturgill, et al., 1988).

The activation of MAPK by PKC is only partially dependent upon ras in PC12 cells (Thomas, et al., 1992), however, PKC is not essential for growth factor activation of MAPK (L.K.T. and G.E.L., unpublished data), indicating that growth factor stimulation of MAPK proceeds through an alternate mechanism. MAPK and p70s6k lie on distinct signaling pathways as p70s6k is not dependent upon ras (Ballou, et al., 1991; J. Blenis, pers. comm.). The activation of serine/threonine kinases is an important, common element of the distal signal transduction pathways which mediate the specific biochemical events associated with growth factor action, and in concert, produce eventual alterations in cellular phenotype.

Fos kinase is a novel serine/threonine kinase that is rapidly activated by NGF treatment of PC12 cells (Taylor, et al., 1993a; Taylor, et al., 1993b). This kinase was identified by its ability to phosphorylate c-Fos in vitro at a C-terminal site likely to regulate the transrepressive function of this transcription factor (Ofir, et al., 1990). Fos kinase also phosphorylates serum response factor in vitro (Taylor, et al., 1993b), and this phosphorylation may regulate the DNA binding capacity of this transcription factor (Rivera, et al., 1993). Because of its rapid activation, it is likely that Fos kinase likely plays a wider role in NGF signal transduction, potentially as a component of a
protein kinase cascade. The objectives of this study were to identify mechanisms responsible for Fos kinase activation and to determine the relationship between Fos kinase and other participants in the NGF signaling cascade. We report here that the NGF-mediated activation of Fos kinase requires phosphorylation of the enzyme at serine/threonine residues and functional p21ras, but is activated through a pathway distinct from MAPK activators and effectors.

**Materials and Methods**

*Materials* - NGF was prepared by the method of Smith et al. (Smith, et al., 1968). Radiolabeled ATP was synthesized using Gamma Prep A (Promega, Madison, WI) and $^{32}$P$_i$ from ICN (Irvine, CA). The Mono Q HR5/5, Mono S HR5/5, and Mono P HR5/5 columns were obtained from Pharmacia LKB (Piscataway, NJ), as were Polybuffer 74 and Polybuffer 96. The Fos peptide, comprising residues 359-370 of c-Fos (RKGSSSNEPSSD), was synthesized by Coast Scientific (La Jolla, CA). Okadaic acid was obtained from Kamiya Biochemical Company (Thousand Oaks, CA). Recombinant rat brain protein tyrosine phosphatase-1 (rmbPTP-1) and protein phosphatase 2A (pp2A) were gifts of Dr. J. Dixon (University of Michigan) and Dr. M. Mumby (University of Texas), respectively. Rapamycin was provided by Dr. A. Nel (University of California, Los Angeles). The ERK2 cDNA clone was kindly provided by Dr. M. Cobb (University of Texas), and MEK1 cDNA clone was provided by Drs. C. Crews and R. Erikson (Harvard University). Both MEK and MAPK were produced as hexa-histidine fusion proteins and purified by affinity chromatography on Ni$^+$ resin (Gentz, et al., 1989). NGF-activated MEK was purified from PC12 cells as described by Jaiswal and
Landreth (Jaiswal and Landreth, 1993). The cDNA clone of truncated p90rsk was provided by Dr. J. Blenis (Harvard University) and recombinant protein was isolated from inclusion bodies (Chung, et al., 1991).

_Fos kinase preparation_- PC12 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% horse serum and 5% fetal calf serum in an atmosphere of 10% CO2 at 37°C. PC12 cells were collected by trituration and suspended in phosphate buffered saline (PBS), pH 7.4 containing 1mg/mL bovine serum albumin (BSA) and 1mg/mL glucose (3 x 10^6 cells/mL), and treated as indicated. To eliminate extracellular Ca^2+, PC12 cells were suspended in Ca^2+/Mg^2+ free PBS containing 1mg/mL BSA, 1mg/mL glucose, and 1mM EGTA for 30 min at 37°C prior to treatment. The cells were pelleted and resuspended in 20mM Tris, pH 7.4, 1mM EGTA, and 100mM sodium orthovanadate (TEV) plus 10mM p-nitrophenol phosphate (PNP) followed by sonication. The cell lysate was centrifuged in a microfuge for 5 min at 4°C to remove the particulates, and high speed supernatant was collected after centrifugation at 100,000 X g for 30 min at 4°C. Anion exchange chromatography of the high speed supernatant was carried out with a Pharmacia FPLC Mono Q HR5/5 column. The column was previously equilibrated in TEV and developed with a discontinuous 0-0.4M NaCl gradient at a flow rate of 1mL/min, and 0.5mL fractions were collected.

_Fos kinase was further purified by chromatofocusing_. Fos kinase activity eluting from the Mono Q column was diluted 4-fold in 25mM Bis-Tris, pH 6.5 and 1mM EGTA and serially loaded onto a Pharmacia FPLC Mono P HR5/5 chromatofocusing column. The column was developed with a pH 6.5-
5.5 gradient at a flow rate of 1mL/min, as described by the manufacturer, and 1mL fractions were collected. Fos kinase isolated by Mono P chromatography was diluted 3-fold in TEV and reapplied to a Mono Q HR5/5 column. The column was developed with a linear 0-0.25M NaCl gradient at a flow rate of 1mL/min, and 1mL fractions were collected.

*Fos kinase assays*- Fos kinase activity was assayed in TEV containing 10μM [γ³²P]ATP (13.2 cpm/fmole), 10mM MgCl₂, 1.2mM dithiothreitol, and 0.5mM Fos peptide for 30 min at 22°C, unless otherwise indicated. Reactions were stopped by the addition of trichloroacetic acid to a final concentration of 3.5% and 10μg bovine serum albumin. After 15 min at 4°C, precipitated proteins were pelleted and supernatants spotted in triplicate on P81 paper (Casnelli, 1991). The papers were washed four times (10 min each) in 75mM phosphoric acid, and incorporated radioactivity was measured.

Kinase assays using recombinant MEK1 (2μg), ERK2 (2μg), p90rsk (20μg), and c-Fos (2μg) were carried out under similar conditions for 1 hr with 0.5μM [γ³²P]ATP (528 cpm/fmole). Autophosphorylation reactions were carried out under the same conditions in the absence of Fos kinase. Reactions were stopped by addition of Laemmli's sample buffer and boiled. Samples were fractionated by SDS-PAGE on a 10% gel followed by autoradiography.

*MEK and MAP kinase activation assays*- To determine the ability of MEK1 to activate Fos kinase, Fos kinase was partially purified from untreated PC12 cells by Mono Q and Mono P chromatography and was then combined with MEK1 purified from NGF-treated PC12 cells in a buffer
containing 10μM ATP, 10mM MgCl₂, and 2mM MnCl₂ in TEV for 30 min at 22°C. Fos kinase activity was then assayed using the Fos peptide substrate as described above. To determine if Fos kinase is activated by ERK2, recombinant ERK2 was first activated by incubation with MEK1 in 10μM ATP, 10mM MgCl₂, and 2mM MnCl₂ in TEV for 1 hr at 22°C, and was then combined with partially purified Fos kinase from untreated PC12 cells under these same reaction conditions for 30 min. Fos kinase activity was assayed with the peptide substrate as described.

**Phosphatase treatment of Fos kinase**—Fos kinase isolated by Mono Q chromatography from NGF-treated PC12 cells was dialyzed against 20mM Tris, pH 7.4, 1mM EGTA, and 1mM dithiothreitol to remove any residual PNP, and incubated with 2U/mL of agarose-conjugated alkaline phosphatase in the presence or absence of 20mM PNP for 15 min at 30°C. PNP (20mM) was then added to stop the reaction, and the alkaline phosphatase was separated from Fos kinase by centrifugation. Fos kinase reactions were carried out as previously described. Fos kinase, isolated as described above, was incubated with pp2A (20mU/mL) in 2mM MgCl₂ in the presence or absence of 2.5μM okadaic acid at 30°C for the indicated time. Okadaic acid (2.5μM) was added to inhibit the phosphatase activity, and Fos kinase assays were conducted as described.

Fos kinase isolated by Mono Q chromatography was subjected to extensive dialysis against 20mM Tris, pH 7.4, 1mM EGTA, 1mM dithiothreitol, and 0.025% Triton X-100 to remove the sodium orthovanadate. The tyrosine phosphatase reactions were carried out at 22°C for 30 min in the presence or absence of rrbPTP-1 (1.2 X 10⁶U/mL), a recombinant
truncated form of rat brain PTP-1 (Hallbook, et al., 1991). Sodium orthovanadate (2.5mM) was added to stop the reaction. Fos kinase activity was assayed as described above.

Results

Regulation of Fos kinase activity by phosphorylation- We tested whether Fos kinase activity was regulated by phosphorylation of the enzyme. Incubation of NGF-activated Fos kinase with alkaline phosphatase inactivated the kinase. Inactivation of the enzyme was prevented by coinubcation with PNP, an inhibitor of alkaline phosphatase, indicating that the activation of Fos kinase required its phosphorylation (Fig. 1A). However, the identity of the phosphorylated residue(s) could not be deduced as alkaline phosphatase acts on phosphoserine and -threonine, as well as phosphotyrosine residues (Swarup, et al., 1981).

We therefore initiated studies to identify the phosphorylated residues responsible for Fos kinase activity using the serine/threonine-specific, phosphatase, pp2A, or the phosphotyrosine-specific phosphatase, rrPPTP-1. Incubation of the NGF-activated Fos kinase with pp2A resulted in the complete inactivation of the kinase (Fig. 1B). Control incubations with pp2A and okadaic acid, a specific inhibitor of this enzyme, blocked the inactivation and established the requirement for serine/threonine phosphorylation for expression of enzymatic activity. However, incubation with the tyrosine specific phosphatase rrPPTP-1 was without effect, indicating that Fos kinase was not regulated through tyrosine phosphorylation of the enzyme (Fig. 1C).

A cell permeant phosphatase inhibitor, okadaic acid, was used to determine if a similar dependence of Fos kinase activity upon
phosphorylation was observed in vivo. Okadaic acid acts to selectively inhibit the serine/threonine phosphatases pp2A and protein phosphatase 1 (pp1). Indeed, okadaic acid alone stimulated Fos kinase activity, and in the presence of NGF induced the synergistic activation of the enzyme (Fig. 2). These data indicate that Fos kinase activity is subject to chronic negative regulation by a serine/threonine phosphatase. It is not clear if the phosphatase acts directly on Fos kinase or an upstream activator, but the requirement for serine/threonine phosphorylation is consistent with the notion that Fos kinase is a direct phosphatase target. The tyrosine independence of Fos kinase activity was further supported by the inability of sodium orthovanadate, a tyrosine phosphatase inhibitor, to activate Fos kinase (data not shown).

Fos kinase activation did not require an influx of Ca$^{2+}$ - PC12 cells were incubated in a calcium-free buffer containing EGTA. The elimination of extracellular Ca$^{2+}$ resulted in only a small diminution of NGF-stimulated Fos kinase activity (Fig. 3). The independence of Fos kinase activation from Ca$^{2+}$ influx demonstrates that activation of this enzyme does not require this NGF-mediated signaling event, and suggests that a Ca$^{2+}$-dependent protein kinase is not an upstream regulator of Fos kinase.

Fos kinase activation was protein kinase C independent - Protein kinase C is a serine/threonine kinase that is rapidly activated by NGF treatment of PC12 cells (Heasley and Johnson, 1989). To determine if PKC played a role in Fos kinase activation, NGF induction of Fos kinase activity in PC12 cells was examined following chronic treatment with 12-tetradecanoyl phorbol 13-acetate (TPA). This treatment results in depletion, or
downregulation, of all isoforms of this kinase family in PC12 cells (M. Wooten, pers. comm.), except the phorbol ester-insensitive ζ isoform, through the action of a protease (Ballester and Rosen, 1985). Elimination of phorbol ester-sensitive PKC had no effect on the basal or NGF-stimulated activity of Fos kinase, indicating that TPA-sensitive isoforms are not required for Fos kinase activation (Fig. 4).

_Fos kinase activation did not require a rapamycin-sensitive pathway_. Rapamycin, an inhibitor of a signaling cascade leading to growth factor activation of p70S6k (Chung, et al., 1992), was used to determine if Fos kinase was regulated by this pathway. PC12 cells were treated with rapamycin, and its effect on Fos kinase activity was examined. Rapamycin had no effect upon basal levels or NGF activation of Fos kinase (Fig. 5). These data strongly suggest that Fos kinase is regulated through a mechanism distinct from p70S6k activation.

_Fos kinase activation was dependent upon functional p21ras_. We were interested in the possible role of the proto-oncogene p21ras, an obligatory intermediary in NGF signal transduction, in the regulation of Fos kinase activation. The actions of ras can be inhibited in a dominant negative fashion by mutation of Ser^17 to an Asp in Ha-ras, generating a biologically inactive mutant which preferentially binds GDP over GTP (Feig and Cooper, 1988). Ras signaling was inhibited using a PC12 cell line expression this ras dominant negative mutant, under the control of the MMTV promoter, stably integrated into genome (GSrasDN6; Thomas, et al., 1991). Induction of expression of the mutant p21ras by dexamethasone treatment of the GSrasDN6 cells has been previously shown to inhibit the NGF-induced
morphological and biochemical differentiation of PC12 cells, and to specifically block the activation of MAP kinases and p90rsk (Robbins, et al., 1992; Thomas, et al., 1992; Thomas, et al., 1991; Wood, et al., 1992). GSrasDN6 cells were incubated with dexamethasone for 24 hr to induce expression of the mutant p21ras, followed by treatment with NGF. NGF activation of Fos kinase in these cells was observed when incubated in the absence of dexamethasone (Fig. 6). However, in dexamethasone-treated cells, the ability of NGF to stimulate Fos kinase activity was abolished, indicating that ras-dependent signaling events are essential for Fos kinase activation.

Relationship of Fos kinase to other kinases in the ras-regulated protein kinase cascade- The observed dependence of Fos kinase activation upon ras suggested that this kinase might be a component of the MAPK cascade. To test this hypothesis, Fos kinase isolated from control PC12 cells was assayed following preincubation with active MEK1, or activated recombinant ERK2, and Mg2+ATP. Fos kinase was not activated in vitro by either MEK1 or ERK2, indicating that neither enzyme functions as an immediate upstream activator of Fos kinase (Fig. 7A).

Conversely, we wished to determine if Fos kinase could potentially serve as an upstream regulator of MEK1, ERK2, or p90rsk, and tested if these proteins were substrates for Fos kinase. MEK1 and ERK2 exhibited autophosphorylation activity, however, inclusion of Fos kinase in these reactions failed to increase the level of phosphorylation (Fig. 7B) indicating that these enzymes were not phosphorylated by Fos kinase. Recombinant p90rsk was not catalytically active, as previously reported (Chung, et al.,
1991), and Fos kinase did not phosphorylate this potential substrate. These
data demonstrate that Fos kinase does not phosphorylate these enzymes
and is not likely to directly interact or regulate these components of the
MAPK cascade.

Discussion

Growth factors transduce signals through their interaction with specific
receptors and the activation of intrinsic or receptor-associated tyrosine
kinases (Fantl, et al., 1993). However, the sequence of the events that follow
receptor engagement have only recently been understood in any detail, and
clearly, not all of the elements of these signal transduction cascades are
known (Ullrich and Schlessinger, 1990). The challenge now is to identify
new participants in growth factor signaling cascades and position these
proteins within signaling pathways. We have identified a novel, NGF-
stimulated protein kinase, Fos kinase, which was detected by its ability to
phosphorylate the C-terminus of c-Fos at an important regulatory site
(Taylor, et al., 1993a; Taylor, et al., 1993) The rapid activation of Fos kinase
suggests that it may have a broad range of functions, in addition to its
putative role in transcriptional regulation. This study was conducted to
explore the regulation and actions of Fos kinase and to determine its
position in relation to other NGF signal transduction events. These studies
have allowed us to establish that Fos kinase activation, while reliant upon
ras activity, lies outside previously identified pathways.

Fos kinase activation requires serine/threonine phosphorylation of the
enzyme (Fig. 1B), and is consistent with its regulation by a protein kinase
cascade. The importance of serine/threonine phosphorylation to Fos kinase
regulation was further demonstrated in vivo through the use of okadaic acid, a specific inhibitor of serine/threonine phosphatases including PP1 and pp2A at concentrations less than 1µM. Treatment of PC12 cells with okadaic acid alone resulted in the activation of Fos kinase, and treatment with both okadaic acid and NGF induced the synergistic activation of Fos kinase (Fig. 2). These data indicate that both the basal and NGF-stimulated levels of Fos kinase are suppressed through the constitutive action of serine/threonine phosphatases. While tyrosine kinase activity of TrkA is responsible for the induction of NGF signal transduction (Kaplan, et al., 1991a), and the cytoplasmic tyrosine kinase, Src, has been reported to be required for some aspects of NGF action (Kremer, et al., 1991), neither of these tyrosine kinases directly regulate Fos kinase activation as indicated by the observation that tyrosine phosphorylation of Fos kinase is not required for its activity (Fig. 1C). Moreover, treatment of PC12 cells with sodium orthovanadate prior to Fos kinase isolation had no effect upon the basal or NGF-stimulated levels of activity of this protein kinase, indicating that Fos kinase activity is not regulated through a tyrosine phosphatase (data not shown).

We next examined more specific NGF signaling events that might regulate Fos kinase activation. PKC is activated in response to NGF treatment of PC12 cells (Heasley, and Johnson, 1989). The α, β, δ, and ε isoforms of this kinase family are down regulated upon exposure to phorbol esters (Ballester, and Rosen, 1985; M. Wooten, pers. comm.), essentially eliminating these PKC isoforms. We have demonstrated that α, β, δ, and ε isoforms are not required for NGF-activation of Fos kinase (Fig. 4). This is
consistent with the failure of phorbol ester treatment to activate Fos kinase (Taylor, et al., 1993a). However, the ζ isoform of PKC is not activated or downregulated by TPA (Ways, et al., 1992), and its function in the regulation of Fos kinase and NGF signal transduction has not yet been determined. The influx of extracellular Ca\(^{2+}\) induced by NGF treatment of PC12 cells (Pandiella-Alsonso, et al., 1986) is hypothesized to play a role as a second messenger in NGF signal transduction through the activation of classical, Ca\(^{2+}\)-dependent isoforms of PKC (Parker, et al., 1989) as well as the activation of Ca\(^{2+}\)/calmodulin dependent kinases (Schulman and Lou, 1989). However, elimination of Ca\(^{2+}\) from the media resulted in only a small diminution of NGF stimulated Fos kinase activity (Fig. 3), indicating that Ca\(^{2+}\) influx is not required for Fos kinase stimulation.

Fos kinase activation was not inhibited by rapamycin indicating that Fos kinase is regulated by different mechanisms than p70s6k (Fig. 5). Chung et al. demonstrated that rapamycin-sensitive signaling events are responsible for ribosomal S6 subunit phosphorylation *in vivo* (Chung, et al., 1992). The insensitivity of Fos kinase to rapamycin indicates that Fos kinase is not responsible for this phosphorylation event. Rapamycin has been postulated to block p70s6k through regulation of phosphatase activities in mammalian cells (Ferrari, et al., 1993) and acts to inhibit a PI3K homologue in yeast (Kunz, et al., 1993). These data demonstrate that p70s6k is not required for NGF stimulation of Fos kinase activity.

Importantly, Fos kinase activation is dependent upon the GTP-binding protein p21\(^{ras}\). Inhibition of ras signaling through the use of a dominant negative inhibitory mutant of ras abolished the NGF-stimulated activation of
Fos kinase (Fig. 6). Fos kinase was not activated by ERK2 or its activator MEK1, nor were MEK1, ERK2, or p90\textsuperscript{Rsk} substrates for Fos kinase (Fig. 7). The data strongly suggest that Fos kinase does not stimulate the MAPK signaling cascade through direct activation of MEK1, ERK2, or p90\textsuperscript{Rsk}, nor is it regulated by these kinases. However, this conclusion remains tentative as it is likely that not all the constituents participating in this pathway have been identified.

The serine/threonine kinase Raf-1 is activated by growth factor stimulation in a ras-dependent manner (Wood, et al., 1992). Its viral cognate, v-Raf, will phosphorylate and activate MEK \textit{in vitro}, and in NIH3T3 cells the introduction of v-Raf leads to activation of MEK (Dent, et al., 1992; Howe, et al., 1992; Kyriakis, et al., 1992). These data suggest that Raf-1 can function as an activator of MEK \textit{in vivo}. However, this is not the case in PC12 cells, where the expression of v-Raf does not induce MAP kinase activation (Wood, et al., 1992). MEKK, another MEK activator, may be responsible for MEK activation in PC12 cells. MEKK is expressed in this cell line (Lange-Carter, et al., 1993), but its ras dependence has not yet been determined. The ability of Raf-1, MEKK, and ras to directly activate Fos kinase have not yet been tested.

A direct activator of Fos kinase has not yet been identified. We have performed a number of experiments employing a strategy that was successful for the isolation of MEK (Ahn, et al., 1991). In these studies, partially purified Fos kinase from untreated PC12 cells was incubated with chromatographically fractionated cell lysates from NGF-treated PC12 cells
and Mg\(^2\)+ATP, followed by Fos kinase assays, however, we did not detect any change in Fos kinase activity (data not shown).

Taken together, these data indicate that NGF stimulation of the tyrosine kinase activity of Trk elicits at least four distinct signaling pathways, as depicted in the signal transduction model presented in figure 8. The influx of Ca\(^2\)+ induced by NGF receptor activation (Pandiella-Alsonso, et al., 1986), represented by path A, is not required for NGF stimulation of Fos kinase activity, indicating that Ca\(^2\)+-dependent kinases, such as the calcium/calmodulin (CaM) kinase family (Schulman and Lou, 1989), do not lie upstream of Fos kinase in a signaling pathway. Fos kinase is also not regulated by phorbol ester-sensitive PKC isoforms (path B), demonstrated by the inability of TPA to activate Fos kinase (Taylor, et al., 1993a) and the continued NGF activation of Fos kinase in the absence of TPA downregulated PKC isoforms. This further demonstrates the Ca\(^2\)+-independence of Fos kinase stimulation, and suggests that PLC-\(\gamma\), responsible for production of diacylglycerol and Ca\(^2\)+ (Fantl, et al., 1993), does not participate in the regulation of this kinase. However, we cannot rule out the \(\zeta\) isoform in the activation of Fos kinase (Ways, et al., 1992).

Trk activation also induces the activity of p70\(^S6k\) (Matsuda, et al., 1986), potentially through PI3K (path C; Kunz, et al., 1993), or through the inhibition of a phosphatase (Ferrari, et al., 1993). The insensitivity of Fos kinase activation to the rapamycin sensitive events that regulate p70\(^S6k\) indicates that Fos kinase lies in an alternate signaling pathway. However, Fos kinase is clearly dependent upon functional p21\(^ras\), represented in path D. Importantly, these studies strongly suggest that Fos kinase participates in
a NGF-stimulated signaling pathway independent of MAPK and its direct activators or effectors. Moreover, it is highly unlikely that Fos kinase is activated by p90rsk or other events downstream of this kinase as growth factor activation of Fos kinase is significantly more rapid than stimulation of p90rsk activity (Erikson, 1991; Taylor, et al., 1993a). The potential direct or indirect regulation of Fos kinase activation by the MEK activators, MEKK or Raf-1, has not yet been examined.

The demonstration that Fos kinase is potentially regulated in parallel with the MEK/MAPK/p90rsk cascade introduces further complexity into growth factor signal transduction. Not only is there a network of participants in the MEK signaling pathway due to the expression of multiple MEK and MAPK family members (Boulton, et al., 1991; Otsu, et al., 1993; Zheng and Guan, 1993), but the lack of dependence of Fos kinase upon this kinase cascade establishes that other ras-dependent cascades exist. Identification of the in vivo activators of both Fos kinase and MEK will help to determine how these ras-dependent signaling cascades diverge. The evergrowing complexity of growth factor signal transduction with the appreciation of multiple, constantly diverging pathways reinforces the notion that signaling events must be considered in the context of a network rather than a linear mechanism.
Figures

A

![Graph showing CPM into FOS peptide under different conditions.]

- ALKALINE PTASE
- +PNP

B

![Graph showing CPM into FOS peptide over time with different treatments.]

- PP2A
- PP2A+OK ACID

TIME (MIN)
**Figure 1. Phosphatase effects on Fos kinase activity.** Fos kinase was isolated by Mono Q chromatography from PC12 cell lysates. A. Fos kinase (50µL) was incubated with alkaline phosphatase conjugated to agarose beads in the presence or absence of PNP to inhibit the phosphatase. The phosphatase was removed by centrifugation and Fos kinase activity was assayed. B. Fos kinase (50µL) was incubated with pp2A for the indicated period of time in the presence or absence of okadaic acid. Okadaic acid was then added to stop the phosphatase reaction, and Fos kinase activity was assayed. C. Fos kinase partially purified by Mono Q chromatography from PC12 cell lysates was incubated in the presence or absence of rrP-PTP-1 for 30 min at 30°C. The phosphatase reaction was stopped with sodium orthovanadate, and Fos kinase activity was assayed with 1µM $^{32}$P$_{$γ$}$ATP (132 cpm/fmole). Fos kinase activity is expressed as the mean cpm incorporated into the Fos peptide (±s.d.).
Figure 2. Okadaic acid stimulation of Fos kinase activity. PC12 cells were treated with 50ng/mL NGF for 5 min, 1μM okadaic acid for 15 min, or 1μM okadaic acid for 10 min followed by 5 min treatment with both okadaic acid and NGF. Fos kinase was partially purified from cell lysates by Mono Q chromatography and assayed. Fos kinase activity is expressed as mean cpm incorporated into the Fos peptide (±s.d.).
Figure 3. Ca$$^2+$$ independence of Fos kinase activation.

Extracellular Ca$$^2+$$ was removed from PC12 cells with their suspension in Ca$$^{2+}$$/Mg$$^{2+}$$ free PBS with 1mM EGTA. The cells were treated with NGF for 5 min, and Fos kinase was partially purified by Mono Q chromatography. Fos kinase activity was assayed as described, and is expressed as mean $$^{32}$$P cpm incorporated into the Fos peptide (± s.d.)
Figure 4. Effect of protein kinase C down regulation on Fos kinase activity. PC12 cells were treated with 1μM PMA for 24 hr prior to lysate preparation or remained untreated. Cells were then treated with 50ng/mL NGF for 5 min or remained untreated. Fos kinase was partially purified by Mono Q chromatography of cell lysates, and Fos kinase activity was assayed as described in the "Materials and Methods". Fos kinase activity is expressed as mean cpm incorporated into the Fos peptide (±s.d.).
Figure 5. Rapamycin resistance of Fos kinase activation. PC12 cells were treated with rapamycin (100ng/mL) for 30 min or remained untreated, followed by stimulation with NGF (50ng/mL, 5 min). Cell lysates were partially purified by Mono Q chromatography. Fos kinase activity was assayed and is expressed as mean cpm incorporated into the Fos peptide (±s.d.).
Figure 6. Ras dependence of Fos kinase activity. Prior to lysate preparation, GSrasDN6 cells, which express a dominant negative mutant of p21ras under control of the MMTV promoter, were treated with 2mM dexamethasone for 24 hr or remained untreated. Cells were then treated with 50ng/mL NGF for 5 min, and Fos kinase was partially purified by Mono Q chromatography from cell lysates. Fos kinase was assayed and is expressed as mean cpm incorporated into the Fos peptide (±s.d.).
Figure 7. MEK1, ERK2, and p90\textsuperscript{rsk} as Fos kinase substrates and activators. A. Fos kinase was partially purified from untreated PC12 cells by Mono Q and Mono P chromatography. Bacterially expressed ERK2 was activated by NGF-stimulated MEK1, and MEK1 and the activated ERK2 were incubated with Fos kinase and Mg\textsuperscript{2+}ATP. Fos kinase was then assayed with 0.25mM Fos peptide, and kinase activity in expressed as mean cpm incorporated into the Fos peptide (±s.d.). B. MEK1, ERK2, and p90\textsuperscript{rsk} were assayed as Fos kinase substrates by incubating the bacterially expressed proteins with Fos kinase isolated from PC12 cell lysates by Mono Q, Mono P, and Mono Q chromatography. Products were separated by SDS-PAGE and subjected to autoradiography.
Figure 8. Putative model for the NGF signal transduction cascade. We have demonstrated that Fos kinase activation is independent of Ca\textsuperscript{2+} and PLC-\gamma-regulated events as it is not activated by PKC (paths A and B). Fos kinase is independent of p70\textsuperscript{S6k} as Fos kinase is not inhibited by rapamycin (path C), a signaling pathway potentially regulated by PI3K or a related phosphoinositol kinase or a phosphatase. The activation of Fos kinase is dependent upon p21\textsuperscript{ras} (path D). Fos kinase is part of a divergent pathway, distinct from the MEK/MAPK/p90\textsuperscript{rsk} signaling cascade. While potential Fos kinase activators are depicted, possible actions of Fos kinase on MEKK and Raf-1 activity are not accounted for in this model. Abbreviations: GNRP - guanine nucleotide exchange protein, CaM kinases - calcium/calmodulin-dependent kinases.
References


CHAPTER 6

Discussion
The process through which a cell obtains information from its surroundings and generates appropriate cellular responses requires that signals be transduced into discrete biochemical changes. Among these influences on the cells, growth factors have been shown to play essential roles in the development and maintenance of multicellular organisms. The study of the signal transduction mechanisms that mediate the cellular response to this diverse group of polypeptides is an active and rapidly expanding area of biology. Stringent control of growth factor-stimulated signaling events is required, as their interruption can lead to inhibition of cellular responsiveness, and inappropriate control can lead to transformation (Fantl, et al., 1993; Karin, 1992).

Growth factors elicit a wide variety of cellular responses ranging from differentiation to mitogenesis. This spectrum of responses is induced by remarkably similar signaling events (Fantl, et al., 1993). Extracellular stimuli initiate signal transduction cascades by binding to their specific cell surface receptors activating the intrinsic or receptor-associated tyrosine kinase, resulting in receptor autophosphorylation. The tyrosine autophosphorylation initiates the dissemination of the signal into parallel pathways through the phosphorylation of other substrates by the receptor. Growth factor signals are then propagated through the cytosol primarily by regulated protein phosphorylation resulting from serial activation of kinases. The focus of growth factor signaling then moves from the cytosol into the nucleus with the induction of gene transcription, leading to the new protein synthesis responsible for the biological outcome (Sheng and Greenberg, 1990). At the initiation of this project, there was a gap in our understanding of signaling cascades, as it was not clear how cytosolic signals were transduced into
transcriptional events. The importance of phosphorylation in transcriptional regulation has recently been underscored with the recognition that protein kinases are the major component transducing cytosolic actions into nuclear events (Hunter and Karin, 1992).

The transcription of c-fos is rapidly, but transiently, induced in response to growth factors (Sheng and Greenberg, 1990). The protein product of the c-fos gene is a transcriptional regulator, inducing transactivation through its dimerization and subsequent DNA binding with a number of other transcriptional regulators, most notably c-Jun family members (Distel and Speigelman, 1990). The binding of c-Fos/c-Jun dimers to AP-1 promoter elements leads to the synthesis of additional, new proteins including collagenase (Schonthal, et al., 1988), tyrosine hydroxylase (Gizang-Ginsberg and Ziff, 1990), and ornithine decarboxylase (Wrighton and Busslinger, 1993).

Alternatively, c-Fos can act as a transcriptional repressor (Guis, et al., 1990; Sassone-Corsi, et al., 1988; Wilson and Treisman, 1988). This function is independent of the DNA binding and dimerization domains, but requires sequences at the C-terminus of this protein (Guis, et al., 1990; Wilson and Treisman, 1988). The transrepressive action of c-Fos is mediated through the serum response element (SRE; (Guis, et al., 1990). c-Fos down regulates its own transcription, as well as another immediate early gene, egr-1 through indirect interaction with SREs contained within these IEG promoters. It has been postulated that the transient nature of c-Fos transcription, as well as other IEGs, results from inhibition of transcription by the newly synthesized c-Fos protein.

c-Fos is a phosphoprotein, and its phosphorylation state is dependent upon the cellular stimulus (Barber and Verma, 1987; Curran and Morgan,
1986). Curran and Morgan demonstrated that NGF treatment of PC12 cells induced a hyperphosphorylated c-Fos protein, whereas c-Fos produced by depolarization was hypophosphorylated (Curran and Morgan, 1986). Stimuli that induced hyperphosphorylation of c-Fos did not induce similar modification of its viral cognate, v-Fos (Barber and Verma, 1987). Because c-Fos and v-Fos differ principally in their C-terminal 48 amino acids (Curran, et al., 1984), it is likely that c-Fos is phosphorylated at its extreme C-terminus, potentially within the transrepressive C-terminal domain. Based on these observations, it was hypothesized that NGF induced the activation of a Fos kinase in PC12 cells that phosphorylated the C-terminus of c-Fos. A peptide corresponding to sequences within the C-terminus of c-Fos (residues 359-370) was used to screen PC12 cells for an NGF-stimulated kinase activity. The previous chapters describe the identification and characterization of this enzyme. These data, along with important observations made by other labs during the progression of this project, have allowed speculation regarding the function of Fos kinase in vivo.

Fos kinase stimulation results from signaling from the high affinity NGF receptor, as indicated by its NGF dose dependence for activation. NGF stimulation of Fos kinase requires serine/threonine phosphorylation of the enzyme, but not tyrosine phosphorylation, and does not exhibit autophosphorylation activity, indicating that a serine/threonine or dual specificity kinase activates this enzyme. The enhanced activity of Fos kinase resulting from okadaic acid inhibition of serine/threonine phosphatases substantiates the importance of serine/threonine phosphorylation in the regulation of the activity of this kinase, and suggests that in resting cells, Fos kinase appears to be under the chronic regulation of a serine/threonine
phosphatase. The activity of Fos kinase also appears to be modulated at
another level, as demonstrated by the activation of Fos kinase from untreated
cells upon hydrophobic interaction chromatography, potentially by the
dissociation of a regulatory subunit. The decrease in apparent $M_r$ of this
protein upon purification also suggests that Fos kinase is a multisubunit
complex. However, the reagents are not currently available to further explore
the molecular composition of this kinase and role of additional subunits in the
regulation of Fos kinase.

Its rapid activation and regulation by phosphorylation suggests that Fos
kinase is both activated by and a participant in a kinase cascade, but neither
its upstream regulator nor a downstream kinase effector have been identified.
The dependence of Fos kinase activation upon p21$^{\text{ras}}$ suggests that Fos
kinase might participate in a previously identified, ras-dependent MAPK
signaling cascade (Thomas, et al., 1992; Wood, et al., 1992). However, Fos
kinase was not activated by ERK2, a MAPK isoform, or MEK1, an MAPK
activator (Crews, et al., 1992). Moreover, Fos kinase did not phosphorylate or
activate MEK1, ERK2, or p90$^{\text{rsk}}$ (Sturgill, et al., 1988). These data indicate
that Fos kinase is not a participant in the MAPK cascade downstream of
MEK. It has not been determined if Fos kinase is activated through a direct
interaction with ras or through an intervening kinase such as Raf-1 or MEKK,
Lange-Carter, et al., 1993), or by an unidentified kinase.

The widespread expression of Fos kinase suggests that the activity of
this enzyme may be important in multiple tissues in the animal. In addition to
its growth factor-stimulated activation in PC12 cells, a similar growth factor-
stimulated Fos kinase activity has been detected in A431 cells, an
epidermally-derived cell line, and NIH3T3 fibroblasts. Fos kinase has also been detected in cells of hematopoetic origin including T-lymphocytes and B-cells. In each of these cell types, Fos kinase showed a similar time course of activation, however, the mechanisms leading to its stimulation varied. In PC12 cells and A431 cells, Fos kinase was not activated by phorbol esters, indicating that protein kinase C does not lie upstream of this enzyme. Conversely, Fos kinase was activated by protein kinase C stimulation in T-lymphocytes and NIH3T3 cells.

Fos kinase is also likely to play another important regulatory role in growth factor signal transduction through phosphorylation of transcription factors. Phosphorylation of c-Fos within its C-terminus is likely required for the expression of the transrepressive ability of c-Fos (Ofir, et al., 1990), and may indicate why v-Fos, in which these sites are absent, does not have this capacity (Lucibello, et al., 1989; Ofir, et al., 1990). Ofir et al. have demonstrated that the mutation of serines to alanines within individual C-terminal phosphorylation sites generated mutant c-Fos proteins that are hypophosphorylated and cannot exhibit transrepressive activity (Ofir, et al., 1990). Generation of a net negative charge in Fos phosphorylation site mutants, through the replacement of alanines with glutamic acids, restored the transrepressive function to c-Fos. Moreover, expression of the transrepressive function of c-Fos is likely to be important for the inhibition of its transforming function as mutation of serine residues within one potential C-terminal phosphorylation site (Ser362-364) to alanines produced a c-Fos mutant with transforming capacity equivalent to v-Fos (Tratner, et al., 1992). These data provide significant support for the notion that Fos kinase is responsible for the regulatory phosphorylation of c-Fos in vivo. The growth
factor activation of Fos kinase is consistent with the appearance of a hyperphosphorylated c-Fos protein, whereas cAMP and TPA failed to activate Fos kinase or induce a hyperphosphorylated c-Fos. In addition, Fos kinase likely phosphorylates authentic c-Fos at Ser362. Tratner et al. have demonstrated stimulated phosphorylation \textit{in vitro} at this site (Tratner, et al., 1992).

The Fos kinase identified here is likely to account, at least in part, for the NGF-sensitive, depolarization-insensitive kinase activity detected by Curran and Morgan (Curran and Morgan, 1986), and is indicative of the very different signaling cascades induced in response to these stimuli. Growth factor stimulation of c-fos transcription is induced through the SRE in the c-fos promoter (Sheng, et al., 1988). The transcription factors which binds to this promoter, SRF and p62TCF, are responsible for transcriptional activation from the c-fos SRE promoter element (Fig. 1; Graham and Gilman, 1991; Shaw, et al., 1989). Depolarization of PC12 cells induces c-fos transcription through an alternate promoter element, the cAMP response element/Ca$^{2+}$ response element (CRE/CaRE; (Sheng, et al., 1988). Transcription from this sequence is stimulated by its binding of phosphorylated CREB, a substrate of a Ca$^{2+}$ dependent kinase (Sheng, et al., 1991). Transcriptional downregulation of c-fos in response to depolarization is not likely to occur by the autoregulatory mechanism stimulated by growth factors. c-fos transcription induced by depolarization shows a prolonged time course in comparison to growth factor stimulation (Bartel, et al., 1989), and the resulting c-Fos protein is hypophosphorylated (Curran and Morgan, 1986) and not likely to exhibit its transrepressive function. Thus, c-fos transcription is repressed by two
independent mechanisms, one of which involves the action of Fos kinase upon the SRE, not the CRE/CaRE.

If Fos kinase acts to regulate c-fos transrepression, then this modification must occur following synthesis of nascent c-Fos, as the amount of c-Fos in unstimulated cells is very low. c-Fos synthesis begins approximately 30 minutes after growth factor treatment, and Fos kinase is active during this period. However, the rapid activation of this enzyme strongly suggests that it acts upon other substrates early in the signaling cascade. The transcription factor SRF undergoes rapid growth factor stimulated phosphorylation in vivo (Rivera, et al., 1993), potentially due to the action of Fos kinase. In vitro, Fos kinase phosphorylates SRF at Ser103, a stimulated phosphorylation site in vivo. While these actions of Fos kinase remain speculative, we believe that this enzyme is likely to be a component of a signal transduction pathway.

Fos kinase may regulate both the induction and downregulation of c-fos transcription, through its phosphorylation of both SRF and c-Fos. As depicted in Figure 1, Fos kinase may help to drive growth factor-stimulated transcription from the SRE through SRF phosphorylation. Basal levels of transcription from this promoter are likely maintained through the competition between SRF and the transrepressor YY1 for binding to this common DNA sequence (Gualberto, et al., 1992). MAPK phosphorylation of p62tcf enhances complex formation at the SRE (Gille, et al., 1992), along with increased SRF binding as a result of its phosphorylation (Rivera, et al., 1993) by Fos kinase. This complex drives c-fos transcription, overcoming the inhibitory actions of YY1 (Gualberto, et al., 1992). c-Fos protein synthesis follows, and its phosphorylation by Fos kinase allows expression of its
transrepressive ability and autoregulation of c-fos transcription (Ofir, et al., 1990). The mechanism through which c-Fos induces transrepression is not understood, and likely involves an as yet unidentified higher order protein complex, as c-Fos does not directly interact with the SRE (Guis, et al., 1990). Fos kinase may regulate the transient stimulation of other SRE-regulated genes by this mechanism. Fos kinase is not the only protein kinase that phosphorylates SRF and c-Fos at regulatory sites (Abate, et al., 1991; Rivera, et al., 1993); other kinases may contribute to the growth factor regulation of c-fos transcription. It has recently been appreciated that an alternative promoter element, the sis-inducible element (SIE), may contribute to the stimulation of c-fos transcription (Hayes, et al., 1987). p91, a transcription factor which binds to the SIE, interacts with growth factor receptors or receptor-associated proteins through SH2 domains in p91, resulting in tyrosine phosphorylation of this transcription factor. Phosphorylation stimulates its translocation to the nucleus, DNA binding activity (Ruff-Jamison, et al., 1993; Sadowski, et al., 1993), and SIE dependent transcription (Fu and Zhang, 1993). This action is independent of p21^{ras} (Silvennoinen, et al., 1993). However, the role that the SIE plays in NGF stimulation of c-fos transcription is not clear as NGF stimulation of this gene requires only the SRE (Sheng, et al., 1988), and is dependent upon ras (Szeberenyi, et al., 1990).

The next step in this project is to examine the action of Fos kinase on growth factor signal transduction and transcriptional regulation in vivo. Such analysis requires functional elimination of Fos kinase, and the cDNA sequence of Fos kinase is necessary for such a manipulation. Purification of Fos kinase from PC12 cells and its identification as a 37kDa protein has been
essential for the design of a purification strategy from rabbit muscle, allowing the isolation of sufficient quantities of Fos kinase for peptide sequencing and eventual cloning. My work has resulted in the identification and characterization of a novel growth factor-regulated protein kinase with some potentially interesting substrates. However, the more exiting work is yet to come with the molecular and functional characterization of Fos kinase.
**Figure 1. Transcriptional regulation from the c-fos promoter.**

Depolarization and growth factor stimulation both activate and terminate c-fos transcription by alternative mechanisms. Growth factor stimulation of transcription occurs from the SRE through phosphorylation of SRF and p62tcf, and transcriptional repression occurs over this same promoter element, autoregulated by newly synthesized c-Fos. Fos kinase may act to both stimulate transcription through its phosphorylation of SRF, and later, repress transcription through its phosphorylation of nascent c-Fos and inducing this protein to exhibit transrepressive function. Depolarization induces transcription from the CRE/CaRE through phosphorylation of CREB. Termination of depolarization-induced c-fos transcription is regulated through an unidentified, alternate mechanism due to the production of hypophosphorylated, non-transrepressing c-Fos.
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CHAPTER 7

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