AN ANALYSIS OF NERVE GROWTH FACTOR-DEPENDENT DIFFERENTIATION AND NEURITE OUTGROWTH IN THE PC12 CELL MODEL SYSTEM

by

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CERTIFICATE OF APPROVAL

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Abstract

The pheochromocytoma-12 cell (PC12) model system has been used to study neuronal differentiation since the cell line was first established in 1976. These cells, cloned from a solid pheochromocytoma tumor of the rat adrenal medulla, respond to nerve growth factor (NGF) by acquiring a phenotype resembling sympathetic neurons. Upon treatment with NGF, these cells exit the cell cycle and begin to extend neuronal-like processes from the cell body. These neurites are the hallmark of PC12 differentiation. Before this cell line was established, studying sensory and sympathetic neurons was difficult because these neurons were dependent on NGF for survival. This made controls for experiments virtually impossible because cells that were not given NGF died. Not only did PC12 cells allow for experimental controls concerning the biochemical events of NGF signaling, but they also provided a practically unlimited amount of cells to work with. Thus, for roughly 30 years, scientists have used these cells as a great tool to uncover the molecular events associated with NGF-dependent neuronal differentiation.

The hallmark of PC12 differentiation is neurite outgrowth. The NGF-dependent signaling pathways that are required for this effect has been under intense study. What pathways are necessary or sufficient for neurite outgrowth? The work provided here used both a pharmacological and molecular approach to address this question. The results shown here that NGF uses both extracellular signal-regulated kinase-dependent (ERK) and ERK-independent pathways to mediate neurite outgrowth in PC12 cells. Inhibitors against both phosphoinositide-3 kinase (PI3K) and Src family kinase (SFK) pathways reduced NGF-dependent outgrowth. In addition, we developed a system to analyze the effects of the ERK pathway on neurite outgrowth in isolation. The results showed that

sustained ERK pathway activation is required for maximal neurite outgrowth. Thus, these studies help us better understand how NGF dictates differentiation and neurite outgrowth in the PC12 model system.

NGF activates a number of transcription factors that are required to initiate the genetic program of differentiation. In fibroblasts, it has been shown that activation of extracellular signal-regulated kinases (ERKs) are required to induce proliferation through regulation of the transcription factor c-fos. Does NGF use similar molecular mechanisms in neuronal cells? Work in this thesis analyzes the role of ERK-dependent regulation of the transcription factor c-fos by NGF. The results shown here that ERKs can regulate both c-fos stability and activation in PC12 cells. The kinetics of ERK activation was important, as sustained activation of ERKs was required for NGF's effects. Specifically, ERK-dependent phosphorylation of residues Thr325 and Thr331 are required for maximal NGF-dependent transactivation of c-fos. Protein stability of c-fos is controlled by ERK-dependent phosphorylation of Ser374, while phosphorylation of Ser362 induces structural changes in the protein. In addition, c-fos transactivation is also dependent on an intact ERK binding site, known as a DEF domain. Thus, the results presented here document specific molecular events that are required for NGF regulation of c-fos transactivation. NGF requires proper c-fos function for PC12 differentiation and this work aids in our understanding the mechanisms underlying this effect.

Chapter 1

The history of the discovery of nerve growth factor

1.1 Rita Levi-Montalcini & Viktor Hamburger team up

In 1986, the Nobel Prize for Physiology or Medicine was awarded in part to Rita Levi-Montalcini for her contribution to the discovery of nerve growth factor (NGF). As with most scientific bodies of work, many people contributed over the years. The most influential people were Stanley Cohen, who received the award along with her, and Viktor Hamburger, who persuaded her to work in his lab at Washington University in St. Louis, MO to study the developing nervous system. Many people in the field of developmental neurobiology felt that the Nobel committee had failed to recognize how instrumental Hamburger was in helping to understand the nerve growth-promoting factor that was eventually named NGF. However, those who are well aquainted with the history and importance of NGF know of Hamburger's pivotal involvement.

Rita Levi-Montalcini was born to a Jewish family in 1909 in Turin, Italy. At the time of this writing, she is the oldest living Nobel laureate. After graduating from the University of Turin with a degree in medicine in 1936, Levi-Montalcini lived during a very tumultuous time in which Mussolini prohibited Jews from studying and teaching in state schools. This dictator's decree did not dissuade her from following her curiosity in developmental neurobiology. In 1940, she read a paper written by Viktor Hamburger describing the effects of wing bud removal on the development of the central nervous system in chick enbryos. It was published in 1934, and in that paper, Hamburger described the arrested development of spinal nerves after wing bud extirpation (Hamburger 1934). Levi-Montalcini went to the extent of setting up a makeshift lab in her house in order to examine Hamburger's results for herself. She acquired eggs from

local farms and kept them in an incubator that she constructed. She also built her own surgical instruments and fixed the embryos for histological study. These rudimentary beginnings would be a key event in how we study neuronal development.

Levi-Montalcini introduced a new approach to studying neuronal development. She pioneered the use of silver staining to allow better visualization of neurons and their developmental state. Her undeniable expertise, along with the silver staining method, allowed her to not only to expand on Hamburger's studies, but also to reinterpret them. Hamburger had posited that the loss of neuronal cells (spinal ganglia) secondary to the loss of peripheral innervations (wing buds) was due to the failure to proliferate or differentiate (hypoplasia). Levi-Montalcini observed similar results but demonstrated that the degeneration of neurons was from the death of mature (differentiated) cells. Thus, decreased survival of differentiated cells was the reason for the decrease in cell numbers (Levi-Montalcini and Levi 1942, 1943). This thorough reexamination of Hamburger's work prompted him to invite Levi-Montalcini to be part of his laboratory at Washington University. She arrived to St. Louis, MO in 1947.

1.2 Sarcoma 180

Levi-Montalcini and Hamburger had scientific strengths that complimented each other and were instrumental in their unified success. Hamburger was trained as an experimental embryologist, an area where Levi-Montalcini had little knowledge. Levi-Montalcini had extensive understanding of the anatomy of the nervous system because of her medical training. This training, along with her expertise in the silver staining method, allowed them to push forward their investigation of the developing nervous system.

From their initial publication together, it became apparent they were moving towards the theory of a chemical growth factor (Hamburger 1949). They had postulated that the observed neuronal degeneration could be explained by a breakdown in "a metabolic exchange between the growing neurite and the substrate on which it grows." (Cowan 2001).

A former student of Hamburger's, Elmer Bueker, went on to do a series of experiments that were of particular interest. Bueker transplanted a piece of tumor, known as sarcoma 180, into chick embryos near the developing spinal cord (Bueker 1948). He discovered that sensory neurons, in particular, were growing into the implanted tumor. This finding supported the theory that the tumor itself had promoted the growth for a specific class of neurons. Levi-Montalcini and Hamburger had tried a similar approach previously substituting various tissues in place of a limb, but the results were inconclusive and the work was never published (Cowan 2001). Once they were aware of Bueker's findings, they also started to experiment with transplanting sarcomas. With the use of silver staining, they were able to observe both sensory and sympathetic ganglia growing into the tumors. This growth promoting effect, however, was not a general phenomenon and seemed to affect only a subset of neurons.

1.3 The famous halo effect

Levi-Montalcini noted that many of the sympathetic neurons were getting bigger despite never making direct contact with the sarcoma tumors. This suggested that whatever was causing the neurons to respond to the sarcomas must be a diffusible factor secreted by the tumor cells.

Their first attempts at recreating the effect of the tumor transplants were done by injecting tumor extracts and dried tumor pellets into developing chick embryos (Levi-Montalcini 1987). These experiments were unsuccessful and Levi-Montalcini had to resort to another method. She then designed an experiment to see if she could recreate the growth promoting effect of the sarcomas *in vitro*. As chance would have it, her friend, Hertha Meyer, was director of a state of the art tissue culture facility at the University of Brazil in Rio de Janeiro, which opened up a realm of possibilities. In the fall of 1952, Levi-Montalcini traveled to Rio de Janeiro with 2 mice in her purse, each of which were harboring sarcoma transplants (Levi-Montalcini 1987).

Once in Brazil, she conducted experiments in which explants of both sensory and sympathetic ganglia were cultured with fragments of the sarcomas in close proximity. Nerve fibers from the ganglia grew with the greatest vigor on the side facing the tumor. In addition, outgrowth was reduced as distance between ganglia and sarcoma was increased, providing more evidence of a diffusible factor at work. The neuronal outgrowth had a characteristic halo appearance (Fig. 1.1). These results were described in illustrations of the famous halo *in vitro* bioassay and were published shortly after Levi-Montalcini's arrival in Rio de Janeiro (Levi-Montalcini et al. 1954).

Figure 1.1

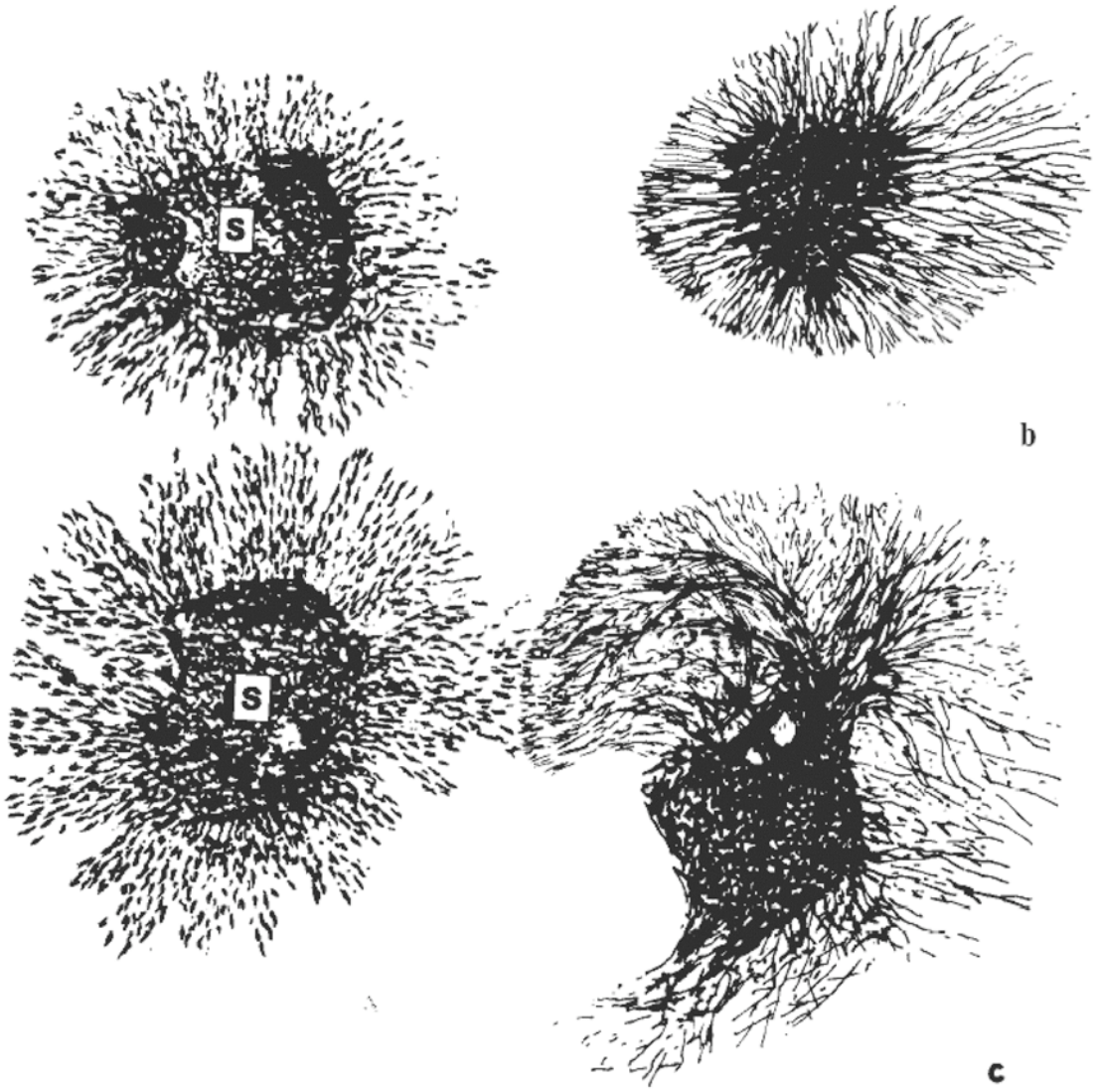

Fig 1.1. Examples of the ink drawings sent by Rita Levi-Montalcini to Viktor Hamburger while Levi-Montalcini was working at the University of Brazil in Rio de Janeiro. These pictures show the characteristic "halo" effect on 8-day chick embryo sensory ganglia cultured in the presence of sarcoma 180 (s). In (b), drawings were made at 24 hours. In (c), drawings were made at 48 hours.

1.4 Stanley Cohen: the biochemist

In his quest to isolate the growth-promoting factor, Hamburger invited a promising young scientist, Stanley Cohen, who was just completing his post-doctoral training to join the project. They were successful in isolating NGF as a nucleoprotein, a mixture of DNA and protein, from the mouse tumors (Cohen et al. 1954). At this point however, the growth factor was not yet known as NGF. Cohen was not convinced that the nucleic acid that he had isolated along with the protein was required for biological activity. In order to test this hypothesis, he treated his isolated preparation with snake venom, which contains high levels of phosphodiesterase, which would degrade nucleic acids. If the preparation treated with snake venom continued to have biological activity in the halo assay, he would then know the nucleic acid was not required. Not only did they discover that the nucleic acid was not required for biological activity, but the snake venom actually increased the growth of the ganglia. The surprises did not stop there. When snake venom was added by itself, the same effect was observed. They then theorized that the venom also contained the growth promoting factor or something similar. Cohen went on to isolate the factor from the venom which he determined was a heat-labile protein that had a molecular weight close to 20kDa (Cohen and Levi-Montalcini 1956). This snake venom NGF had a specific activity that was one thousand times greater than the NGF isolated form the mouse tumors.

The mammalian equivalent of the glands that produce snake venom are the salivary glands. Again, Cohen was able to purify a protein factor that had growth promoting activity in large quantities from the mouse submaxillary gland (Cohen 1960).

Much of the NGF purchased today is still purified from this source. As with the factor isolated from snake venom, the submaxillary factor was sensitive to heat and proteases. Cohen also developed antibodies against the submaxillary factor and demonstrated their ability to block the growth-promoting factor in the halo assay. This same antiserum also worked against the factor isolated from the snake venom suggesting the two proteins shared homology (Cowan 2001). Stanley Cohen's involvement was indispensable in the purification of the growth-promoting factor that eventually becomes known as NGF. He noticed that mice given a semi-purified preparation of the submaxillary factor experienced certain developmental events earlier than their control littermates. Namely, they opened their eyes at younger ages and had their incisor teeth appear earlier. Cohen isolated a novel growth factor, known as epidermal growth factor (EGF), that was responsible for these events (Cohen 1962). This was one of the main reasons he was recognized with the Nobel Prize along with Levi-Montalcini in 1986.

1.5 Early Classic NGF Experiments

It has been known for decades that NGF is able to promote growth and permit survival of sympathetic and sensory neurons. In fact, many seminal experiments were conducted years before mouse NGF was sequenced (Angeletti and Bradshaw 1971) or cloned (Scott et al. 1983). In 1960, Levi-Montalcini had the isolated growth factor that she could test *in vitro* in multiple systems (Levi-Montalcini and Booker 1960a). In this report, sensory and sympathetic neurons were used from human fetuses that were terminated. The cultures were exposed to NGF isolated from the sarcomas, snake venom, and the submaxillary gland. The neurons responded to all of the preparations. In addition, both

newborn and adult mice are also used. Mice were injected with NGF and then were sacrificed at varying times. All of the ganglia treated with NGF were found to be larger. Serum collected from the mice was also found to contain naturally occurring NGF. Male mice had higher levels than females which correlates well with sympathetic neurons in males generally being larger. As a result, this study showed that NGF not only acted on chicken neurons but also had the same effects on mammals, including fetal human neurons.

The development of NGF antiserum was an important tool that allowed for testing its requirement for the survival and growth of developing neurons. Levi-Montalcini was able to theorize about what happens to the sympathetic nervous system when NGF is taken away from a developing mouse (Levi-Montalcini and Booker 1960b). In that study, mice of various ages were injected with NGF antibodies for different time periods and their sympathetic neurons were isolated and analyzed. It was found that the experimental animals had fewer and smaller neurons than the controls. The greatest effects were seen with the youngest animals. This practice of killing off the sympathetic neurons with NGF antiserum was referred to as “immunosympathectomy”.

Although Hamburger did not directly participate in the work leading to the isolation of NGF, he was able to contribute significantly to the literature years later. In a study published in 1979, he labeled NGF with I^{125} and attached it to gel pellets. These pellets were implanted subcutaneously in the leg of chicken embryos. These embryos were harvested 8 hours later and NGF incorporation was analyzed by autoradiography. It was found that sensory neurons in the dorsal root ganglia were labeled, thus showing that the neurons were actively taking up the NGF and transporting it retrogradely toward the

cell body. Retrograde transport of growth factors is an intense area of study today and this experiment was done in a very elegant fashion (Brunso-Bechtold and Hamburger 1979).

Years later, Hamburger published another study providing further evidence that NGF was the naturally produced survival factor for sensory neurons *in vivo*. NGF was injected daily into the yolk sac of chick embryos and then the number of dying cells was counted in different ganglia. The results showed that neurons, which usually died at specific time points, were kept alive by the NGF injections. It was concluded that exogenous NGF can prevent neurons from dying a "natural" death (Hamburger et al. 1981).

Once NGF was discovered, the next challenge for scientists was to identify which receptors were responsible for transmitting the survival and growth effects of NGF. In addition, much work has been done on studying the signaling pathways that are required for NGF's effects. Many different signaling proteins participate in NGF signaling in pheochromocytoma-12 cells (PC12 cells). The following chapter discusses NGF-dependent signaling pathways and their role in neurite outgrowth in the PC12 model system.

Chapter 2

The role of NGF activated signaling pathways in neurite outgrowth in PC12 cells

2.1 The low affinity NGF receptor: p75

The identification of NGF receptors took much longer than the discovery of the ligand itself. The first receptor to be identified was the low affinity NGF receptor known as p75 (Chao et al. 1986; Radeke et al. 1987). This receptor is a nonselective neurotrophin receptor as it binds NGF, BDNF, NT-3, and NT4/5 with similar affinities. In addition to binding mature processed forms of the neurotrophin family, p75 has also been shown to bind immature pro-neurotrophin isoforms (Lee et al. 2001). Recent work has shown that proNGF is involved in promoting cell death in neurons by binding p75 (Hempstead 2002; Harrington et al. 2004; Pedraza et al. 2005). This receptor has also been implicated in a number of other roles including: acting as a coreceptor with TrkA to induce high affinity ligand binding (Hempstead et al. 1991), release of acetylcholine from sympathetic neurons (Yang et al. 2002), and transmiting the signal to inhibit neurite outgrowth in dorsal root ganglion neurons (Yamashita et al. 2002). Subsequently, although p75 is an important protein for many biological processes, it is not the focus of this dissertation.

2.2 The high affinity NGF receptor: TrkA

In 1987, ^{125}I-NGF was crosslinked to a 130kd protein on the cell surface in PC12 cells (Hosang and Shooter 1987). This publication demonstrated that the radio-labeled protein was internalized upon NGF binding. Shortly thereafter, similar cross-linking methods were used to show that antibodies against phosphotyrosine reacted with the 130kd protein, but various antibodies against p75 did not. This finding led authors to conclude

that “tyrosine phosphorylation may be one of the initiating events in the NGF-induced signal transduction cascade” (Meakin and Shooter 1991). These initial reports allowed for several labs to help identify TrkA as the high affinity NGF receptor (Martin-Zanca et al. 1989; Kaplan et al. 1991). TrkA was first discovered in a cancer cell line where tropomyosin was fused to an unknown tyrosine kinase domain (Martin-Zanca et al. 1986). The wild-type gene, tropomyosin-related kinase (trk), was shown to be a transmembrane protein highly expressed in the nervous system. Whereas the tyrosine kinase domain resides on the intracellular side of the plasma membrane, the extracellular domain of TrkA was crystallized and shown to contain the ligand binding motif (Perez et al. 1995; Ultsch et al. 1999).

There is a variant strain of PC12 cells called PC12 nnr cells that are unable to differentiate in response to NGF. In addition, these cells express no detectable TrkA. Introduction of exogenous TrkA into this mutant cell line renders them responsive to NGF and they in turn behave like wild-type PC12 cells in response to NGF (Loeb et al. 1991). This report showed that TrkA was indeed the receptor required for NGF-dependent differentiation in this neuronal cell line. Overexpression of exogenous TrkA in wild-type PC12 cells can also increase the onset of neurite outgrowth (Hempstead et al. 1992). This provides evidence that regulation of TrkA expression levels is of crucial importantance for neuronal differentiation. PC12 cells can also be induced to differentiate with FGF, however FGF-dependent differentiation requires its cognate receptor, FGFR-1. The PC12 subclone, fnr-PC12 cells, fails to differentiate in response to FGF due to reduced expression levels of FGFR-1 (Lin et al. 1996).

It has been demonstrated that the intracellular domain of the TrkA receptor is required for differentiation by growth factors. Most of the work providing support for this notion comes from data using chimeric receptors. Experiments using the extracellular domain of the TNF receptor and the intracellular domain of TrkA show that PC12 cells transfected with this chimera will differentiate in response to TNF (Rovelli et al. 1993). In addition, if the p75 intracellular domain is used in place of TrkA the cells are unresponsive. Researchers have taken advantage of the fact that PC12 cells do not express the PDGF receptor. PC12 cells expressing a chimera of the extracellular PDGF domain fused to the intracellular FGFR domain also differentiate in response to PDGF (Foehr et al. 1998). These studies show the requirement for the tyrosine kinase domain of growth factor receptors involved in differentiation.

2.3 Basics of TrkA signaling

The β subunit of NGF forms a dimer, which is the biologically active ligand that binds TrkA. Ligand binding to TrkA triggers dimerization of the receptors (Jing et al. 1992). Receptor dimerization activates the tyrosine kinase domain and each receptor phosphorylates one another *in trans* on multiple residues (Kaplan and Stephens 1994). Phosphorylation of TrkA is required for two very important functions. Firstly, tyrosine phosphorylation at multiple residues is required for maximal catalytic activity of the kinase domain (Cunningham et al. 1997). Secondly, phosphorylation provides binding sites to intracellular proteins that recognize phosphorylated tyrosine residues. Proteins that contain Src homology 2 domains (SH2) bind phosphorylated tyrosine residues

(Pawson and Gish 1992). Proteins that bind in this manner propagate the signaling cascade upon ligand binding.

NGF generates a complex signal that is able to activate multiple signaling cascades. Upon NGF binding, many proteins are recruited to TrkA and activated by tyrosine phosphorylation (Fig. 2.1). PLCγ1 is recruited to tyrosine 785 (human TrkA) which is located in the carboxy terminus on the intracellular side of the cell (Obermeier et al. 1993b; Loeb et al. 1994). Upon recruitment, PLCγ1 is activated via phosphorylation. This allows for the production of diacyl glycerol (DAG) and inositol (1,4,5) triphosphate (IP3) from membrane lipids (Rhee 1991). DAG is able to activate protein kinase C (PKC). Some forms of PKC are required for NGF-dependent neurite outgrowth in PC12 cells (Coleman and Wooten 1994; Corbit et al. 1999; Santos et al. 2007). In addition, overexpression of a PKC isoform enhances neurite outgrowth and survival in response to NGF in PC12 cells (Wooten et al. 1999). IP3 production is capable of releasing calcium from intracellular stores by binding the IP3 receptor located predominately on the endoplasmic reticulum (Foskett et al. 2007). Calcium release is able to activate Calmodulin (Cam) kinases and some isoforms have been linked to modulation of ERK activation in PC12 cells (Egea et al. 2000; Agell et al. 2002). Another study creating a point mutation at Y785 of TrkA shows its requirement for binding to PLCγ1 in PC12 cells (Loeb et al. 1994). Loeb and company went on to show that Y785 was required for NGF-dependent induction of peripherin, an intermediate filament protein. However, PC12nnr cells transfected with the Y785F mutant exhibited neurite outgrowth similar to wild-type cells in response to NGF. Thus, the PLCγ1 arm of the NGF activated TrkA

pathway is important for ERK activation and several hallmarks of PC12 differentiation outside of neurite outgrowth (Fig. 2.1).

Another binding site on activated TrkA is Y490, which has been shown to bind the adaptor class of proteins Shc (Fig. 2.1) (Obermeier et al. 1993a). Once recruited and activated by tyrosine phosphorylation, Shc can bind another linker protein called Grb-2 (Rozakis-Adcock et al. 1992; Suen et al. 1993; Ohmichi et al. 1994). Grb-2 is able to bind the Ras nucleotide exchange factor Son of Sevenless (SOS) (Egan et al. 1993; Quilliam et al. 2002), which is able to activate the MAPK pathway, a signaling pathway covered in greater detail later. Grb-2 also recruits and binds Gab-1. The p85 subunit of phosphoinositide 3-kinase (PI3K) can be activated by binding to Gab-1. In addition, the small GTPase Ras has also been shown to activate PI3K directly (Downward 1998). PI3K pathways have long been implicated in pro-survival events in many cell types, including neurons (Franke et al. 1997; Brunet et al. 2001).

Figure 2.1

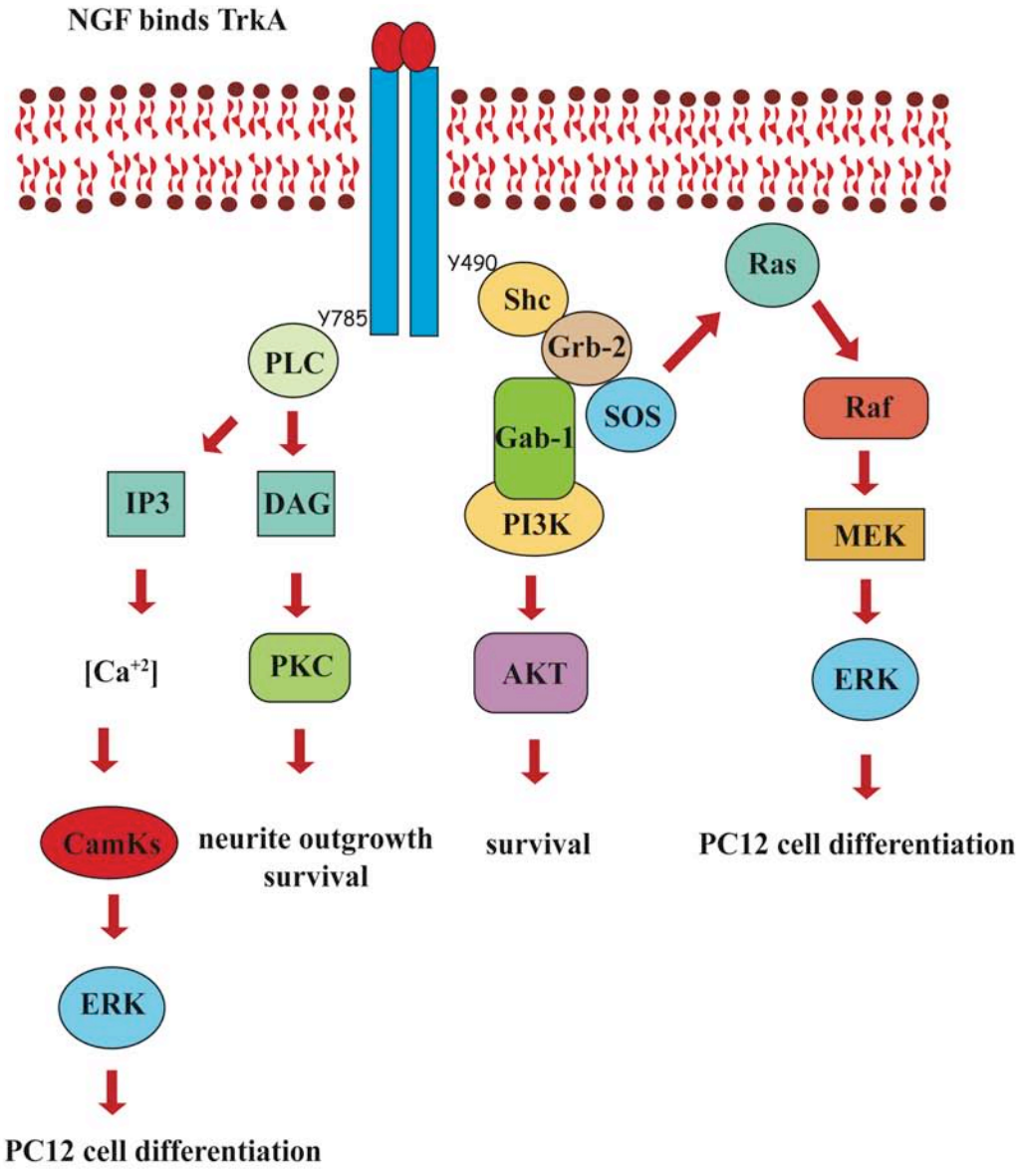
NGF binds TrkA
Y490
Y785
Shc
Grb-2
SOS
Ras
PLC
Gab-1
Raf
IP3
DAG
PI3K
MEK
[Ca+2]
PKC
AKT
ERK
CamKs
neurite outgrowth
survival
survival
PC12 cell differentiation
ERK
PC12 cell differentiation

Fig 2.1. NGF is a complex stimulus that can ultimately lead to activation of multiple cellular signaling pathways in PC12 cells. Upon NGF binding, PLCγ1 binds pY785 on TrkA and initiates the production of both IP3 and DAG from membrane lipids. DAG is a well-known activator of PKC isoforms, which have been implicated in neurite outgrowth and survival in PC12 cells. IP3 can promote the release of Ca^{+2} from intracellular stores in order to activate Calmodulin-dependent protein kinases (CamKs). CamKs have been shown to activate ERK1/2 in PC12 cells. Phosphorylation of Y490 in TrkA can recruit and bind the Shc family of adaptor proteins. Grb-2 can bind Shc and mediate Ras activation through activation of the exchange factor SOS. Activation of Ras can lead to stimulation of the ERK cascade and many elements of PC12 cell differentiation. Grb-2 can also bind the adaptor Gab-1, which results in activation of the PI3K-AKT pathway. Activation of this pathway has been well documented in promoting cell survival.

The Shc family members are not interchangeable and there are several major distinctions between them. ShcA binds the adaptor Grb-2 with more affinity than ShcC. While both of these isoforms are expressed in the nervous system, ShcA is found predominantly in precursor neural cells, while ShcC is more prevalent in differentiated neurons (Conti et al. 2001). Interestingly, ShcC is better able to bind the proteins Crk and C3G, which mediate activation of the small G-protein Rap1 (Fig. 2.2). This is due to the fact that ShcC contains more binding sites for Crk than it has for to Grb-2 (Nakamura et al. 2002). Rap1 is important for sustained activation of ERK1/2 and required for PC12 differentiation (York et al. 1998). Hence, ShcC is an important molecule for ERK signaling and it is not surprising that overexpression of Shc causes neurite outgrowth in PC12 cells (Rozakis-Adcock et al. 1992).

FGF Receptor Substrate-2 (Frs-2) is another important adaptor molecule in TrkA signaling. This molecule competes for the same docking site as Shc adaptor proteins. This protein was originally shown to be tyrosine phosphorylated in response to NGF in PC12 cells. It was named suc-associated neurotrophic factor-induced tyrosine-phosphorylated target (SNT) (Rabin et al. 1993). It was purified, cloned, and characterized in PC12 cells later by another group who renamed it Frs-2 (Kouhara et al. 1997). As its name suggests, it is required for FGF-dependent differentiation of PC12 cells (Hadari et al. 1998). Frs-2 is a versatile protein that contains a lipid anchor and can bind various other signaling molecules including Grb-2 and Crk (Fig. 2.2). Mutation of the Crk binding site in Frs-2 causes Rap1 activation to be blocked and sustained ERK signaling to be impaired in PC12 cells in response to NGF (Kao et al. 2001).

2.4 ERK/MAPK signaling

Extracellular signal-regulated kinases (ERKs) were originally called microtuble-associated protein-2 kinases (MAPKs) because there were able to phosphorylate both MAP-2 and myelin basic protein (MBP) (Ray and Sturgill 1987; Rossomando et al. 1989). The proteins were eventually renamed mitogen-activated protein kinases (MAPKs) when it was shown they were important for proliferation. For convenience, the acronym was not changed. It is now well known that ERKs are important for many more diverse biological events that include differentiation, survival, synaptic plasticity, learning and memory, and axon elongation (Chao 1992; Grewal et al. 1999; Atwal et al. 2000; Thiels and Klann 2001; Chen et al. 2006). Long before they were cloned, they were identified a decade earlier as tyrosine phosphorylated proteins in transformed chicken embryos (Martinez et al. 1982). Both ERK1 and ERK2, 44kDa and 42kDa respectively, were cloned in the early 1990s and were given their new names because so many different stimuli could activate these two kinases (Boulton and Cobb 1991; Boulton et al. 1991). Technically speaking, the ERKs are part of a large family of kinases termed MAPKs, that also include p38 and c-Jun N-terminal kinases (JNKs) (Roux and Blenis 2004).

Figure 2.2

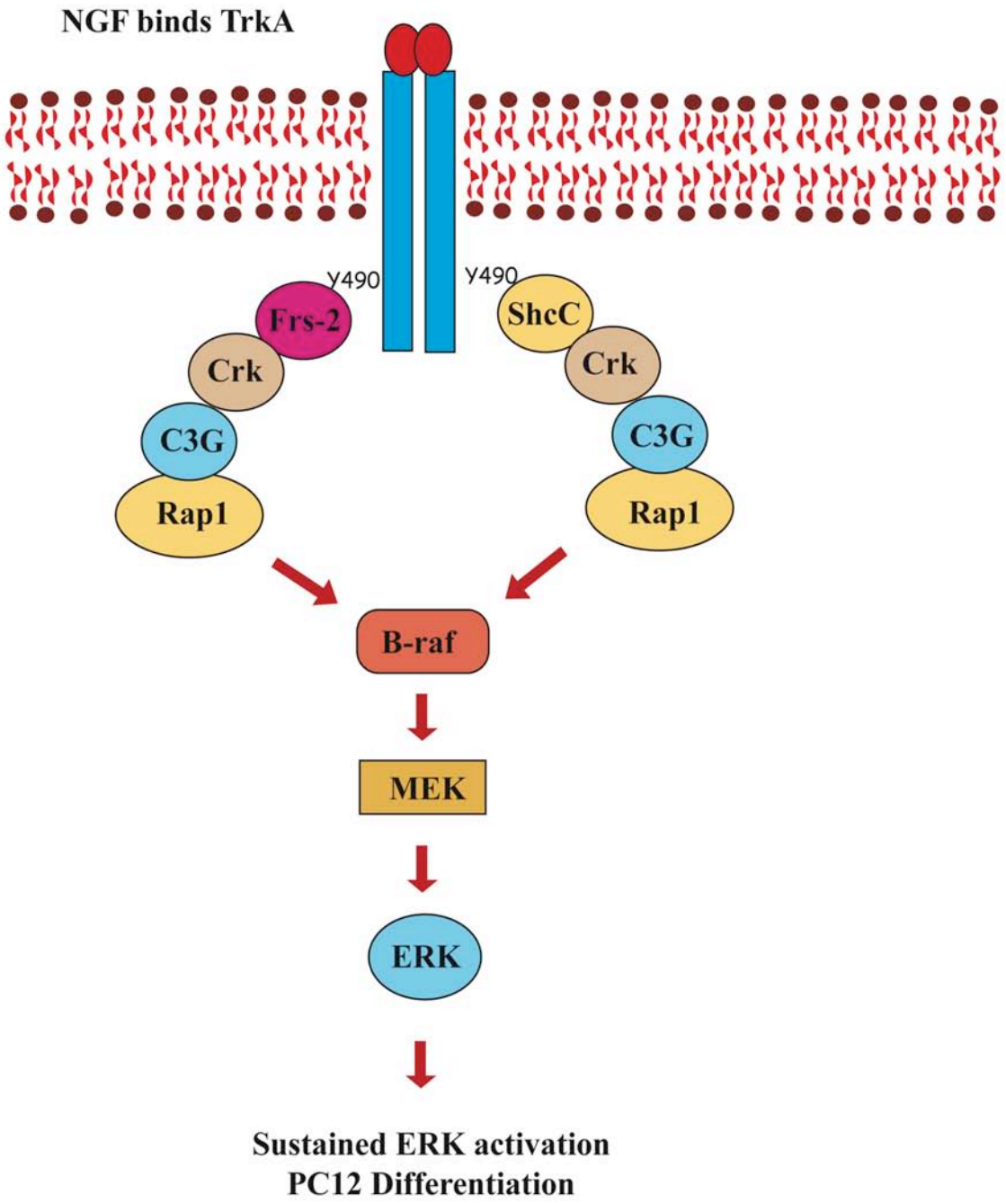
NGF binds TrkA
Y490
Y490
Frs-2
Crk
C3G
Rap1
ShcC
Crk
C3G
Rap1
B-raf
MEK
ERK
Sustained ERK activation
PC12 Differentiation

Fig 2.2. Upon NGF binding, tyrosine 490 is one of the sites that become phosphorylated on TrkA. This provides a docking site for adaptor proteins such as ShcC and Frs-2. ShcC is an adaptor that preferentially bonds the Crk-C3G complex, which allows for activation of the small G protein Rap1. The adaptor protein Frs-2 also binds phosphorylated tyrosine 490 on TrkA. Frs-2 becomes tyrosine phosphorylated itself and can also bind the Crk-C3G complex. A mutant Frs-2 (Y436A) unable to bind Crk has been shown to impair Rap1 activation in response to NGF in PC12 cells (Kao et al. 2001).

ERKs are part of a classical tiered signaling cascade that has been studied extensively (Fig. 2.3). Many growth factor receptors, including TrkA, activate small GTPases, such as Ras and Rap1, upon ligand binding. This leads to the recruitment and activation of mitogen activated kinase kinase kinase (MAPKKK). MAPKKKs include the Raf isoforms, B-Raf, Raf-1, and A-Raf. B-Raf is the kinase most strongly activated by NGF in PC12 cells (Kao et al. 2001). Raf kinases activate mitogen activated protein kinase kinases (MAPKKs) or, as they are more commonly called, MAPK/ERK kinases (MEKs). MEK1/2 are dual specificity kinases that phosphorylate and activate ERK1/2. MEK 1 and MEK2 are thought to be interchangeable in their ability to activate ERK1/2. Recently, both MEK1 and MEK2 have been knocked out in both human and mouse epidermis. Loss of only one of the isoforms demonstrated no phenotype. Ablation of both proteins, however, resulted in loss of proliferation, skin defects, apoptosis, and death (Scholl et al. 2007). Therefore each isoform is able to compensate for the loss of the other. MEK1/2 phosphorylate ERK1/2 in the activation loop motif, Thr-Glu-Tyr (TEY), and the phosphorylation of both threonine and tyrosine residues are required for ERK1/2 maximal activity (Anderson et al. 1990; Posada and Cooper 1992). ERK1/2 catalyze the phosphorylation of target serine and threonine residues that are followed by a proline (Segal 2003). ERK1/2 has a number of both cytoplasmic and nuclear substrates (Pearson et al. 2001). Given that ERK1/2 are 75% similar at the amino acid level, it was initially thought that ERK2 could compensate for the loss of ERK1 as ERK1 knock out mice are essentially normal and fertile, with only a minor defect in thymocyte differentiation (Pages et al. 1999; Pouyssegur et al. 2002). However, recent reports have implicated ERK1 in adipocyte differentiation and a role in behavior plasticity and addiction

(Mazzucchelli et al. 2002; Bost et al. 2005). In contrast, ERK2 knock out mice are embryonic lethal and die early in development before the implantation stage (Saba-El-Leil et al. 2003).

Figure 2.3

Classical mitogen activated protein kinase cascade

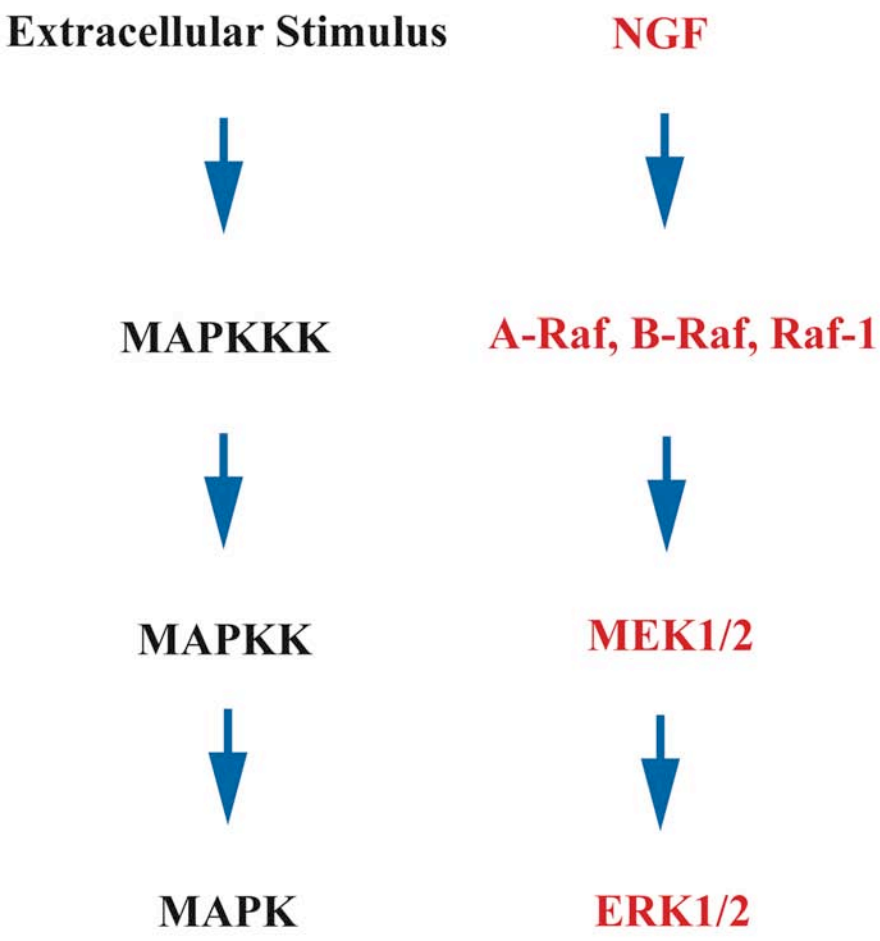

Fig 2.3. The ERK1/2/MAPK signaling cascade is initiated upon many extracellular stimuli including NGF. In this case, NGF-dependent activation of TrkA stimulates phosphorylation and activation of Raf family members (MAPKKK). B-Raf is the isoform most strongly activated by NGF in PC12 cells. MEK1/2 (MAPKK) are then phosphorylated and activated by B-Raf. This leads to phosphorylation and activation of ERK1/2 (MAPK). ERK1/2 have many cytoplasmic and nuclear substrates that are required for NGF's effects, namely PC12 cell differentiation.

2.5 Membrane scaffold: KSR

Components of the ERK1/2 kinase cascade are organized by their association with scaffolds. As the term suggests, these scaffolding proteins allow multiple members of the cascade to be positioned correctly for activation. Kinase suppressor of Ras (KSR) resides in the cytoplasm where it is inactive (Fig. 2.4). KSR is prevented from activating signals by being bound to 14-3-3 and to another protein, impedes mitogenic signal propogation (IMP). In its inactive form, IMP and MEK1/2 are complexed to KSR and upon growth factor stimulation, the KSR complex is released and recruited to the plasma membrane where active Ras is able to activate the ERK1/2 cascade (Kolch 2005). In fibroblasts, expression of KSR with mutations at distinct phosphorylation sites causes an increase in sustained ERK1/2 activation and increased proliferation by growth factors (Razidlo et al. 2004). B-KSR is a splice variant and is highly expressed in neurons. Overexpression of B-KSR in PC12 cells enhances neurite outgrowth in response to NGF. In addition, EGF is transformed from a mitogenic stimulus to an agent of differentiation characterized by neurite outgrowth. Overexpression of B-KSR allows EGF to extend the duration of ERK activation. This effect is inhibited by pharmacological blockade of ERK1/2 (Muller et al. 2000). Thus, B-KSR is able to alter the kinetics of ERK activation and change the biological fate of the cell.

Figure 2.4

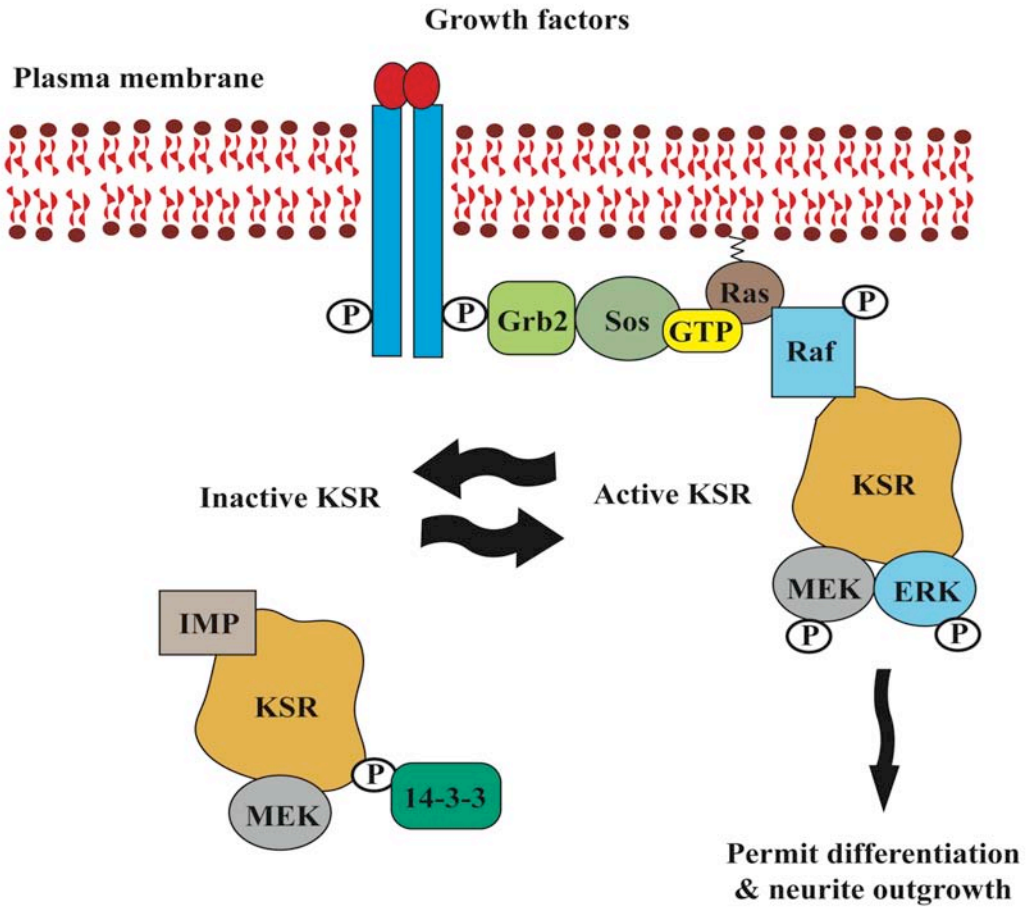

Fig 2.4. The kinase suppressor of Ras (KSR) is a scaffold for the ERK1/2 pathway. KSR can bind many, if not all, members of the ERK pathway. However, KSR binds MEK constitutively. Before activation, KSR is also bound to 14-3-3 proteins via serine phosphorylated residues. In addition, impedes mitogenic signal propagation (IMP) is an E3 ubiquitin ligase that is degraded in a Ras-dependent manner upon activation of KSR. In a stimulus-dependent fashion, KSR is recruited to the plasma membrane where the both Raf-1 and ERK1/2 can bind KSR. Overexpression of specific KSR isoforms have been implicated in PC12 differentiation and neurite outgrowth in response to NGF.

2.6 An Endosomal Scaffold: MP-1

MEK partner-1 (MP-1) is a scaffold that is specific to ERK1 and MEK1 (Fig. 2.5). It binds a protein called p14 to the cytoplasmic surface of endosomes (Schaeffer et al. 1998). Overexpression of MP-1 can enhance ERK nuclear signaling by increasing Elk-1-dependent transcription in a reporter system. In addition, knockdown of either p14 or MP-1 using siRNA causes inhibition of sustained ERK1/2 activation (Teis et al. 2002). More importantly, mislocalization of the p14/MP-1 complex only alters sustained ERK1/2 activation in endosomes, leaving transient ERK1/2 signaling at the plasma membrane unchanged. This complex has also been shown to be required for proper development and early embryogenesis through studying a conditional knock out of p14 (Teis et al. 2006). This shows the importance of MP-1 in regulating sustained ERK1/2 signaling in a specified compartment of the cell. It would suggest that this scaffolding complex would also be important for PC12 differentiation.

Figure 2.5

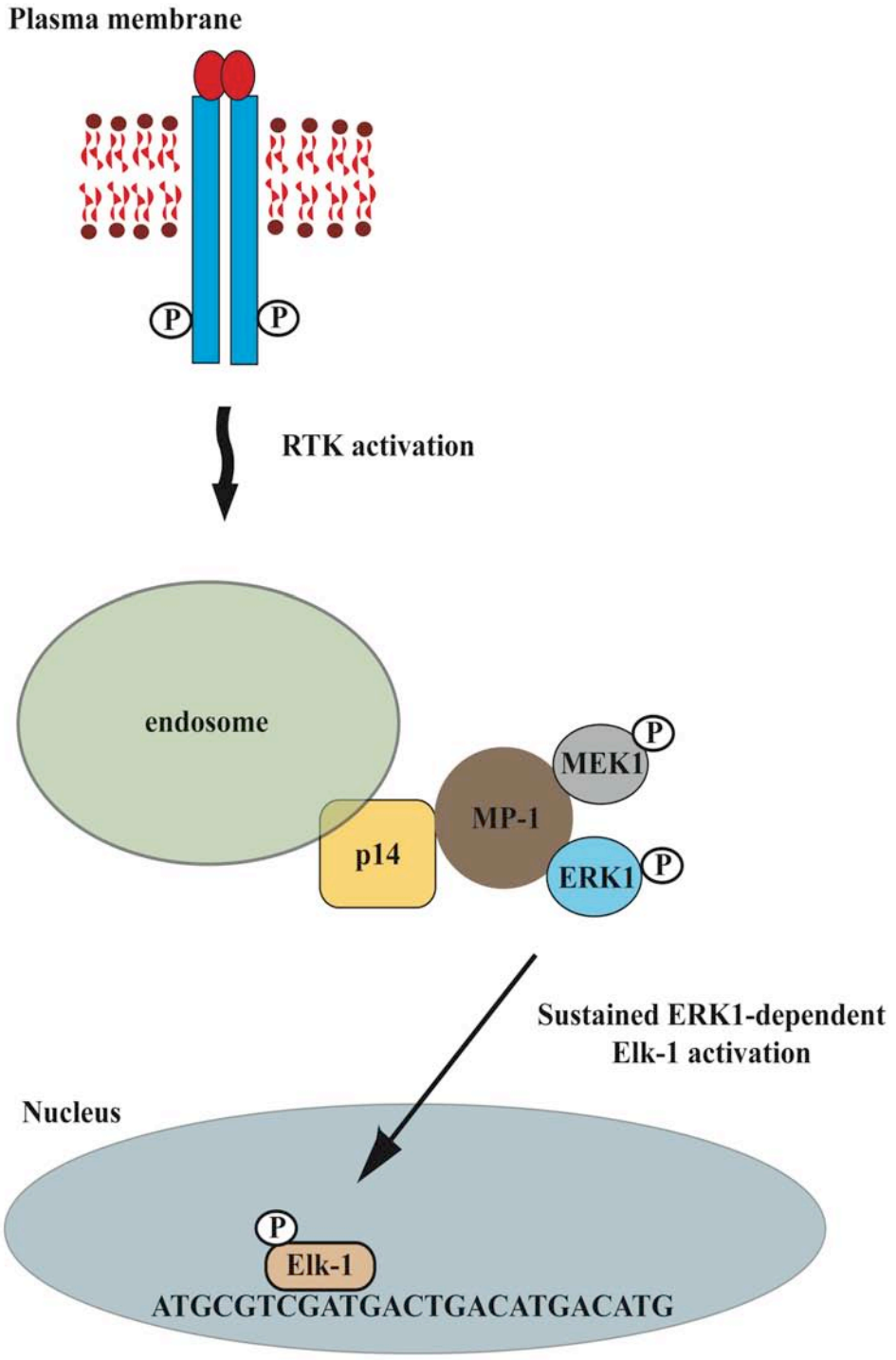
Plasma membrane
P
P
RTK activation
endosome
P
MEK1
MP-1
p14
ERK1
P
Sustained ERK1-dependent
Elk-1 activation
Nucleus
P
Elk-1
ATGCGTCGATGACTGACATGACATG

Fig 2.5. MP1 is a scaffold that is ubiquitously expressed and specifically regulates MEK1/ERK1 signaling. MP1 binds p14, which localizes this signaling complex to endosomal compartments. Reduced expression of MP1 can inhibit the duration of ERK1 activation. Moreover, targeting this MP1 complex to the plasma membrane, as opposed to endosomes, blocks ERK1 signaling. As a result, MP1 is a scaffold that positively regulates ERK1 signaling in a specified compartment of the cell, the endosome.

2.7 A Golgi Apparatus anchor: Sef

Like sustained ERK1/2 activation, nuclear translocation of ERK1/2 is required for NGF-dependent differentiation in PC12 cells (Boglari et al. 1998). The protein Sef (similar expression to fgf genes) was originally identified in zebrafish as a feedback inhibitor of the ERK1/2 pathway induced by FGF (Furthauer et al. 2002; Tsang et al. 2002). Further study showed that Sef localizes MEK-ERK complexes to the Golgi (Torii et al. 2004). Interestingly, Sef permits activation of ERK1/2 in the golgi compartment of the cytoplasm, but restricts translocation of ERK1/2 into the nucleus (Fig. 2.6). Sef permits ERK1/2 activation of cytoplasmic substrates, but nuclear targets, such as c-fos and Elk-1, are not phosphorylated. In PC12 cells, stable expression of human Sef inhibits both FGF and NGF-dependent differentiation (Xiong et al. 2003). This lends further evidence to the notion that ERK1/2 nuclear translocation is required for neuronal differentiation.

Figure 2.6

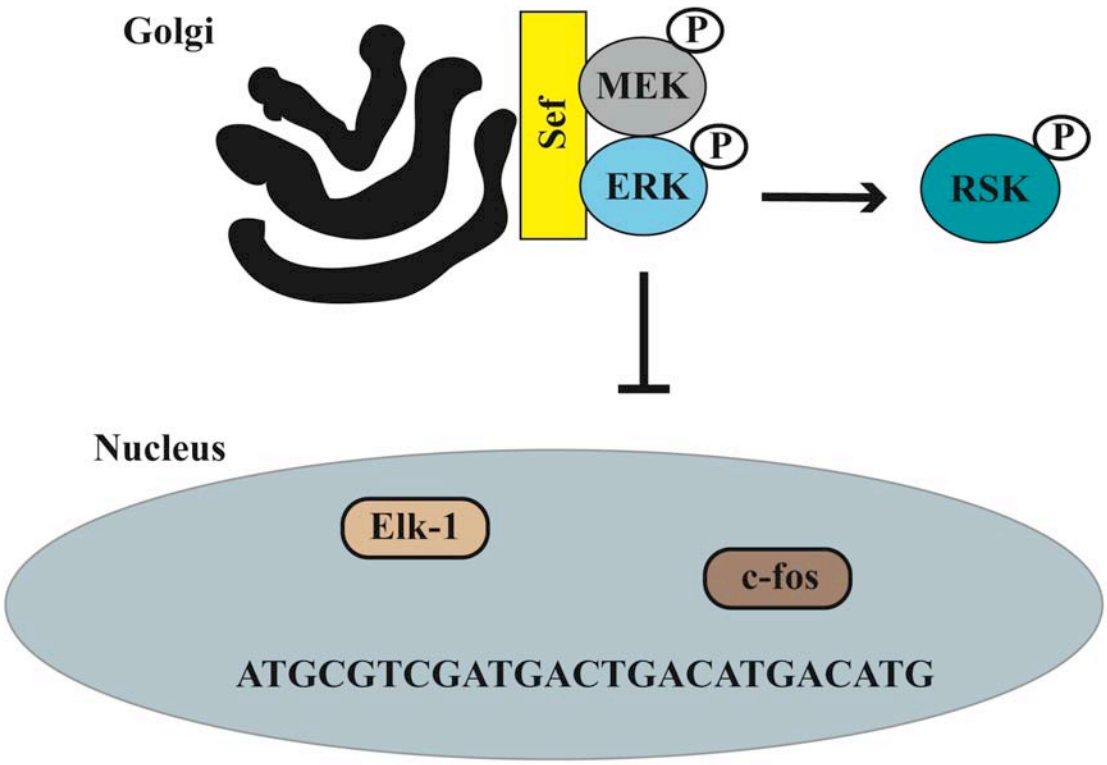

Fig 2.6. Sef is a transmembrane protein that resides at the golgi apparatus and negatively regulates ERK1/2 signaling. Although it is currently controversial about how Sef regulates ERK1/2, it has been shown Sef blocks ERK1/2 nuclear translocation without affecting its ability to activate cytoplasmic substrates, such as RSK. However, phosphorylation of nuclear substrates, like the transcription factors c-fos and Elk-1, are inhibited by Sef. Overexpression of Sef blocks both FGF and NGF-dependent PC12 differentiation. Thus, Sef is a strong negative regulator of proper ERK1/2 signaling propagating to the nucleus in response to growth factors.

2.8 ERK-dependent PC12 cell differentiation: Dogma revisited

Soon after ERK1/2 were discovered and cloned, they were implicated in PC12 cell differentiation and NIH 3T3 cells proliferation (Cowley et al. 1994). In this important study, ERK1/2 were postulated to be both necessary and sufficient for PC12 cell differentiation. As with all scientific reports, these results are open to interpretation. Cowley and company made different mutants of MEK1 that acted as both constitutively active and dominant negative proteins. They then used microinjection techniques to express these mutants in different sublines of PC12 cells. Their best results come from using the PC12-HER cell line which overexpresses the EGFR. This permits the cells to differentiate in response to both NGF and EGF (Traverse et al. 1994). The authors decided to use this PC12-HER cell line because only a low percentage of wild-type PC12 cells were responding to NGF as measured by neurite outgrowth. The authors reported that wild-type PC12 cells were "making results more difficult to interpret". In any case, they were able to use the dominant negative MEK1 to block 80% of EGF treated PC12-HER cells from differentiating. In addition, they went on to show that ERK1/2 is essential for this effect. This publication is likely the report cited most often when there is discussion regarding ERK1/2 being required for differentiation of PC12 cells. It is important to note that NGF-dependent neurite outgrowth was not directly examined. This serves as an example of where reexamination of the dogma will allow us to interpret the current data better and begin to entertain the idea that other signaling pathways may also be involved in differentiation as well (Morooka and Nishida 1998; Xiao and Liu 2003; Sanchez et al. 2004; Eriksson et al. 2007).

2.9 Sustained ERK1/2 activation and differentiation: initial reports

PC12 cells were shown to respond to both NGF and EGF when they were first reported (Greene and Tischler 1976; Greene 1978). It was known that both NGF and EGF activated the ERK1/2 signaling cascade in PC12 cells and that these growth factors did share some biological effects (Huff et al. 1981; Boonstra et al. 1983; Greenberg et al. 1985). However, the ultimate biological response differed vastly. NGF treatment resulted in exit from the cell cycle and neurite outgrowth while EGF treatment resulted in mitogenesis. It was once postulated that maybe another unknown kinase could be required for differentiation induced by NGF (Gomez et al. 1990). However, since the differences in the kinetics of ERK1/2 activation between NGF and EGF were discovered, the notion of sustained ERK1/2 activation and its requirement for NGF-dependent PC12 cell differentiation has been intensely investigated (Gotoh et al. 1990b; Qui and Green 1992; Traverse et al. 1992; York et al. 1998).

2.10 A requirement for nuclear translocation of ERK1/2

Traverse and company identified another very important correlation between sustained ERK1/2 activation and nuclear translocation of ERK1/2 in an early study (Traverse et al. 1992). They postulated that sustained activation of ERK1/2 may be required for entry into the nucleus to initiate the genetic program for differentiation. In PC12 cells, insulin is mitogenic like EGF. However, overexpression of insulin receptors causes the cells to differentiate and have extensive translocation of ERK1/2 into the nucleus (Dikic et al. 1994). Very similar experiments have also been conducted overexpressing the EGFR and the results are similar (Traverse et al. 1994). Another study shows nuclear

translocation of ERK1/2 to be required for PC12 neurite outgrowth (Boglari et al. 1998). Boglari and company used dominant negative Ras mutants and expressed them in PC12 cells stimulated with NGF. High expressers inhibit ERK1/2 activation, nuclear translocation, and differentiation. Low expressers allowed a transient ERK1/2 activation that had a similar magnitude as control cells. However, nuclear translocation and differentiation were not observed. They concluded that both sustained ERK1/2 activation and nuclear translocation were required for NGF-dependent neurite outgrowth in PC12 cells. Other studies in PC12 cells also suggest nuclear translocation of ERK1/2 is required for PC12 differentiation (Nguyen et al. 1993; Camps et al. 1998). The requirement for nuclear translocation of ERK1/2 would suggest that the pathway is needed for gene transcription to initiate the differentiation program.

2.11 Evidence for ERK1/2 in neurite outgrowth using PD98059

In 1995, Pang and company published a study where they utilized the MEK1/2 inhibitor PD98059 to block NGF-dependent ERK1/2 activation in PC12 cells (Pang et al. 1995a). In this report, they conclude that ERK1/2 are required for NGF-dependent PC12 cell differentiation. Although the authors provided a phase-contrast light microscope photograph for each condition that was compelling (Control, NGF, and PD + NGF), unfortunately that was the extent of the evidence they provided. There was no quantification of the data. In addition, they show approximately a 50% reduction in MEK1/2 activity using 20μm PD98059 employing *in vitro* kinase assays. In the assays for NGF-dependent neurite outgrowth, they use 10μm PD98059 and report "the compound completely blocked NGF-induced neurite formation…". Therefore, although

some evidence is provided to support the author's claim, it may be important to look more closely at the requirement for ERK1/2 activity in all aspects of neurite outgrowth in PC12 cells.

2.12 PC12D cells: ERK1/2-independent neurite outgrowth

PC12D cells are a subline of wild-type PC12 cells that respond to NGF by differentiating and extending neurites (Katoh-Semba et al. 1987; Sano et al. 1988). However, unlike wild-type PC12 cells, which respond by extending neurites over the course of days, PC12D cells extend neurites within hours. NGF-dependent neurite outgrowth can occur in the presence of both transcription and translational inhibitors (Sano and Kitajima 1996). Thus, it came as no surprise that ERK nuclear translocation or activity was not required for differentiation in PC12D cells because no new protein synthesis was needed (Sano et al. 1995; Sano and Kitajima 1998). Interestingly enough, studies were also done with enucleated PC12D cells and NGF induced neurite outgrowth in these cells as well (Sano and Iwanaga 1996). It is thought that these PC12D cells behave as "primed" PC12 cells. "Primed" PC12 cells are cells that have been pre-treated with NGF for enough time to allow transcription-dependent events to occur. The cells are then replated and can respond to NGF (extend neurites) much more quickly because transcriptional events have already taken place. Recently, a PI3K inhibitor, wortmannin, was used to block NGF-dependent neurite outgrowth in PC12D cells (Jin et al. 2007). In this report, they inhibited Ras by using a dominant negative version of Ras and saw corresponding inhibition of neurite outgrowth. The MEK1/2 inhibitor PD98059 had no effect on these cells. Upon stimulation with NGF, membrane ruffling precedes neurite outgrowth.

Wortmannin or dominant negative Ras did not inhibit membrane ruffling. However, ruffling was inhibited by dominant negative mutants of Rac1 and Cdc42. Clearly, the proteins required for neurite outgrowth in PC12D will be somewhat different than wild-type PC12 cells due to the lack of a transcriptional requirement, but this report shows that multiple signaling pathways can contribute to neurite outgrowth by NGF. In fact, expression of a nuclear localized Ca^{+2}/calmodulin-regulated kinase II (CamKII) can inhibit NGF-dependent neurite outgrowth of wild-type PC12 cells through an unknown pathway (Kutcher et al. 2003).

2.13 "Signaling endosome hypothesis"

Neurons are unique cells that are faced with challenges of relaying signaling events from an axon or dendrite tip, to the nucleus of the cell. NGF must relay its survival signal to the nucleus after binding TrkA at the plasma membrane. William Mobley attempted to explain this mechanism with a model he called the "signaling endosome hypothesis"(Beattie et al. 1996; Grimes et al. 1996). In this model, neurotrophins (NGF) bind Trks and the receptor-ligand complex is internalized into an endocytic vesicle that is capable of transporting the signal. This model provides a mechanism for transcriptional activation in response to distal axon stimulation in neurons (Watson et al. 1999). PC12 cells were used as a source to purify these signaling endosomes after stimulation with NGF (Grimes et al. 1997). Signaling endosomes are retrogradely transported from the synaptic terminal to the cell body. Later studies showed that other signaling proteins were found in these signaling endosomes in PC12 cells as well including Shc, PLCγ, ERK1/2, and the small GTPase Rap1 (Howe et al. 2001; Wu et al. 2001). Treatment of

PC12 cells with brefeldin A (BFA) disrupts the endosomal compartment of the cell and inhibits the sustained activation of ERK1/2. In addition, inhibitors of clathrin-mediated endocytosis can also block sustained ERK1/2 activation in PC12 cells (York et al. 2000). Another report using NGF that was covalently cross-linked to beads showed that ERK1/2 activation was blocked when TrkA internalization was inhibited in rat sympathetic neurons (MacInnis and Campenot 2002). These results show that the mechanisms explaining the "endosomal hypothesis" may also help explain NGF-dependent sustained ERK1/2 activation.

2.14 Requirement of BMK1/ERK5 in retrograde transport-mediated survival

One recent study investigating retrograde signaling demonstrated that the location of the stimulus is important in the survival of sensory neurons (Watson et al. 2001). In this report, they used compartmentalized cultures of dorsal root ganglion (DRG) neurons in order to stimulate the distal axons and cell bodies separately. When cell bodies were stimulated with neurotrophin, ERK1/2 were activated and translocated into the nucleus. Survival of the cells was ERK1/2-dependent. However, when distal axons were stimulated, no ERK1/2 was activated or retrogradely transported to the nucleus. It was determined that a related family member, big MAP kinase (BMK1), or ERK5, was activated, translocated into the nucleus, and mediated the survival of the sensory neurons via a CREB-dependent mechanism (Watson et al. 2001) (Fig 2.7).

Figure 2.7

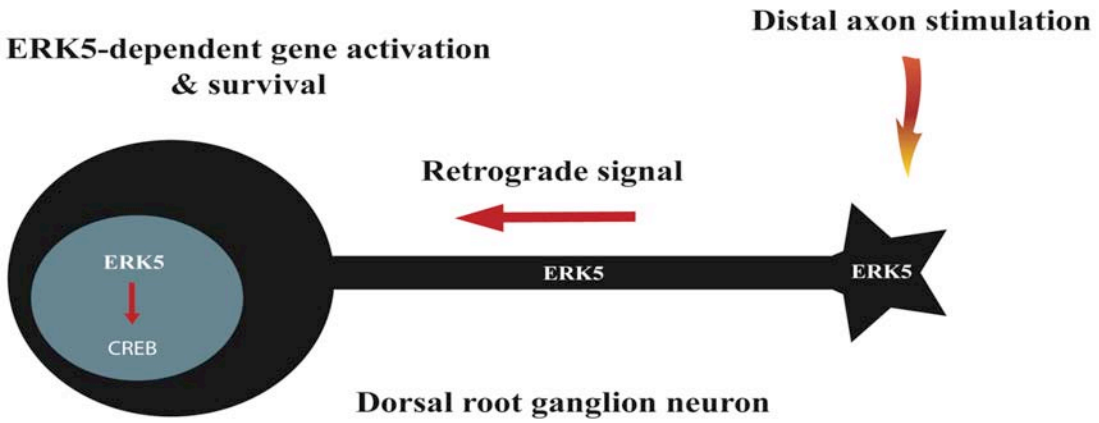

Fig 2.7. It has been demonstrated that the location of neurotrophin stimulation can dictate the survival response in sensory neurons. If distal axons are stimulated, BMK1, also known as ERK5, is activated and retrogradely transported down the axon. ERK5 is transported into the nucleus where it is required for CREB activation and the resulting survival response.

Figure 2.8

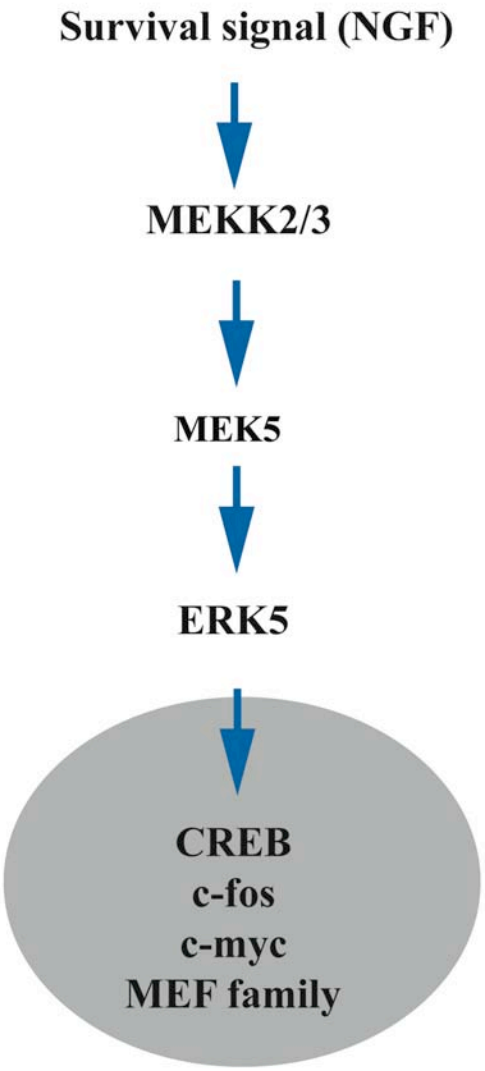

Fig 2.8. It has been demonstrated that growth factors, like NGF, can activate the BMK1/ERK5 cascade. MEKK2/3 (MAPKKK) is able to activate MEK5 (MAPKK). MEK5 then phosphorylates and activates ERK5 (MAPK). ERK5 then is able to phosphorylate and activate many transcription factors including CREB, c-fos, c-myc, and MEF family members. As a result, ERK5 signaling can mediate the survival signal in response to NGF in some neuronal cells.

Like ERK1/2, ERK5 contains an activation loop sequence TEY motif. However, ERK5 is activated by MEK5, a tyrosine/threonine kinase (Kato et al. 1998) (Fig 2.8). MEK5 is activated by MEK2/3 serine/threonine kinases (Chao et al. 1999). Initially, ERK5 known to be activated by hyperosmolarity and oxidative stress (Nishimoto and Nishida 2006). However, further study showed that ERK5 could be activated by growth factors, including NGF (Kamakura et al. 1999). ERK5 has been shown to phosphorylate and activate a number of transcription factors including c-Myc, MEF2 family members, CREB, and c-fos (Kato et al. 1997; English et al. 1998; Yang et al. 1998; Watson et al. 2001; Terasawa et al. 2003; Sasaki et al. 2006) (Fig 2.8). Interestingly, ERK1/2 has also been shown to phosphorylate c-fos, but the sites of phosphorylation seem to be distinct (Murphy et al. 2002; Monje et al. 2003). Although both ERK1/2 and ERK5 positively regulate c-fos activation, ERK1/2 promote stabilization and increased transcriptional activity while ERK5 inhibits nuclear exit of c-fos. Accordingly, both ERK5 and ERK1/2 can positively regulate c-fos-dependent gene expression in different ways. It has been shown that MEK1/2-specific inhibitors, PD98059 and U0126, also are able to inhibit MEK5 (Mody et al. 2001). PD184352 is another compound that inhibits each pathway separately (ERK5 & ERK1/2) by adjusting the dose. Thus, data that has been acquired using these inhibitors must be carefully reviewed to determine whether the effects seen are attributed to ERK1/2 or ERK5. Recently, a nonpeptide compound called Nobiletin was reported to induce neurite outgrowth in PC12D cells. This effect was shown to be sensitive to U0126. Importantly, they showed the effect to be ERK1/2-dependent and ruled out any activation of ERK5 or TrkA (Nagase et al. 2005). Therefore, unlike NGF,

sustained ERK1/2 activation is required for Nobiletin-dependent neurite outgrowth in PC12D cells.

2.15 Ca+2/cAMP response element binding protein (CREB): MAPK-dependent survival factor

CREB is a transcription factor that can be activated by a number of stimuli including growth factors and neurotransmitters. In the nervous system, CREB has been implicated in a number of cellular processes including synaptic plasticity, growth, survival, and neuroprotection (Lonze and Ginty 2002). CREB can be activated and phosphorylated on serine 133 by the ERK-dependent RSK and MSK family members (Xing et al. 1996; De Cesare et al. 1998; Xing et al. 1998; Wiggin et al. 2002). In sympathetic neurons, CREB is required for NGF-dependent survival by regulating expression of the pro-survival factor Bcl-2 (Riccio et al. 1997; Riccio et al. 1999). In PC12 cells, CREB has been implicated in neurite outgrowth and survival. Constitutively active CREB mutants have been shown to rescue ERK1/2 inhibition of NGF-dependent neurite outgrowth (Cheng et al. 2002). In this report, cAMP and PI3K were shown to activate CREB and induce neurite outgrowth in an ERK-independent manner as well. In PC12 cells, NGF can stimulate CREB-dependent transcription in many genes including VGF, tyrosine hydroxylase (TH), transin, and c-fos (Matrisian et al. 1985; Harrington et al. 1987; Hawley et al. 1992; Ginty et al. 1994). Unlike EGF, NGF can induce CREB phosphorylation at serine 133 for a sustained period in PC12 cells that may reflect the sustained activation of ERK1/2 (Bonni et al. 1995).

In PC12 cells and dorsal root ganglion neurons, CREB is required for NGF-dependent survival (Watson et al. 2001). In this report, internalization and retrograde transport of TrkA is required for survival upon stimulation of distal axons with NGF. This leads to ERK5 activation and translocation into the nucleus for CREB-dependent survival. If cell bodies are stimulated, ERK1/2 activation is sufficient to mediate CREB-dependent survival. Hence, although both ERK5 and ERK1/2 can mediate CREB-dependent survival, these kinases also have their own specific transcriptional targets (Nishimoto and Nishida 2006). This demonstrates that specific gene expression can be dependent on location of stimulation even though survival of the cells may be a common response.

2.16 Sustained ERK1/2 activation regulates the immediate early gene (IEG) c-fos

Much of the evidence discussed thus far has made a case for sustained ERK1/2 activation playing a role in neurite formation in response to NGF in wild-type PC12 cells. It is clear that other stimuli that activate ERK1/2 transiently, such as EGF, do not induce differentiation in these cells. The question that arises is how the kinetics of ERK1/2 activation provides the signal for a PC12 cell to either differentiate or proliferate. Recent work in fibroblasts has shown that the transcription and post-translational activation of IEGs can depend on the sustained activation of ERK1/2 (Murphy et al. 2002; Monje et al. 2003; Murphy et al. 2004; Mackeigan et al. 2005). In these reports, activation of these transcription factors requires sustained ERK1/2 activation in order to initiate a proliferative response. Transient ERK1/2 is not sufficient for proper regulation of IEGs, such as c-fos, Egr-1, and Fra-2 leaving cells unable to enter S-phase. This model can

help explain how sustained activation of ERK1/2 can lead to transcription factor activation and initiation of a biological program, whereas transient ERK1/2 activation is insufficient. Phosphorylation by ERK1/2 at specific residues can result in stabilization and transactivation of the transcription factor c-fos in response to NGF in PC12 cells. Chapter 4 will analyze the role of sustained ERK1/2 activation in transcription factor regulation using the IEG c-fos as an example in the PC12 model system.

2.17 Ras: role in neurite outgrowth & differentiation

The activation of Ras proteins has been linked with activation of the ERK cascade and differentiation of PC12 cells for many years. Microinjection of activated Ras was shown early on to induce PC12 cell differentiation (Bar-Sagi and Feramisco 1985). Indeed, it was demonstrated shortly thereafter that Ras was required for differentiation of PC12 cells by microinjection of antibodies to p21 Ras (Hagag et al. 1986). Recently, it has been demonstrated that Ras activation at the Golgi apparatus can induce differentiation of PC12 cells as well (Bivona et al. 2003). Expression of constitutively active N-Ras using an inducible system also promotes PC12 neurite outgrowth (Guerrero et al. 1986). Constitutive activation of Ras and differentiation was eventually linked to immediate early gene transcription (Sassone-Corsi et al. 1989). AP-1 family members, c-fos and c-jun, were shown to be upregulated. This link was further explored by the establishment of stable PC12 lines that expressed dominant negative mutants of H-Ras. Dominant negative H-Ras mutants were shown to inhibit both NGF and FGF-dependent PC12 neurite outgrowth (Szeberenyi et al. 1990; Robbins et al. 1992). In the study by Szeberenyi and company, both high expressers and low expressers exhibited this effect.

Moreover, only high expressers of H-Ras mutants blocked IEGs like c-fos and c-jun. Low expressers of H-Ras mutants permitted transcription of these IEGs. However, delayed response genes, such as Transin and SCG10, were blocked by all of the H-Ras mutant cell lines. Although this same group revisited the Ras-dependent induction of IEGs and found similar results, other groups more recently have shown the requirement for c-fos and AP-1 activation in PC12 differentiation (Szeberenyi 1998; Gil et al. 2004; Eriksson et al. 2007). Consequently, it may seem that Ras activation is necessary and sufficient for NGF-dependent outgrowth in PC12 cells, however, recent studies suggest that c-fos and AP-1-dependent gene activation may also be required.

A report in the early 1990's compared NGF and EGF signaling in PC12 cells (Qui and Green 1992). In this study, NGF caused a sustained elevation of Ras activity while EGF caused a transient activation of Ras activity. Sustained Ras activation led to sustained ERK1/2 activation and differentiation. Transient activation of ERK1/2 by EGF was insufficient for neurite outgrowth and differentiation. However, although many would argue that sustained ERK/12 is required for differentiation, some groups would argue that sustained activation of Rap1, and not Ras, is required for sustained ERK1/2 activation in PC12 cells (York et al. 1998; Kao et al. 2001). However, other Ras family proteins have been implicated in sustained activity in response to NGF in PC12 cells (Sun et al. 2006). In this report, NGF induces M-Ras activation for as long as 24 hours. K-Ras, N-Ras, and H-Ras were all analyzed and found to be activated transiently (<10 minutes). In addition, they find that constitutively active Rap1 is not sufficient for ERK1/2 activation or neurite outgrowth. However, dominant negative Rap1, along with dominant negative M-Ras and H-Ras, all inhibited NGF-dependent neurite outgrowth in

these cells. R-Ras, another Ras family member, has also been implicated in neurite formation of embryonic retinal neurons and PC12D cells (Ivins et al. 2000; Iwashita et al. 2007). Thus, it seems that activity of many Ras family proteins may be required for a complex event like neurite outgrowth in PC12 cells.

The standard MEK1/2 inhibitor, U0126, has been used to inhibit NGF-dependent neurite outgrowth (Sun et al. 2006). In their hands, NGF caused about 65% of the cells to undergo differentiation. U0126 caused about a 50% reduction in neurite outgrowth. The interesting point is that the reduction is the same regardless of whether the cells were pretreated for 30 minutes with U0126 or if it were added 1 day later. Although it isn't surprising they did not see a complete inhibition of neurite outgrowth when the cells were pretreated with U0126, it was surprising there was the same amount of inhibition if U0126 was added 24 hours later. This data would suggest that there are no ERK1/2-dependent transcriptional events associated with neurite outgrowth in PC12 cells in response to NGF. These results would be expected for NGF-primed PC12 cells or PC12D cells since it has been reported that these cells can undergo complete ERK1/2-independnent neurite outgrowth in response to NGF (Wood et al. 1993; Sano et al. 1995). Results may have been different if they had used a higher concentration of U0126 (10μM). However, an alternative explanation may be that other signaling pathways are able to compensate for the loss of ERK1/2 signaling when inducing neurite outgrowth in PC12 cells.

2.18 The role of Rap1 in ERK activation and neurite outgrowth

Rap1 is a member of the Ras superfamily of small G proteins (Stork 2003). Rap1 was originally called K-rev for its ability to revert Ki-Ras transformed fibroblasts by antagonizing Ras signaling. Reports have shown that Rap1 is able to inhibit the activation of Raf-1 by Ras (Cook et al. 1993). This is due to the fact that Rap1 can bind Raf-1 without facilitating the necessary phosphorylations that are needed to activate it (Bos et al. 2001; Carey et al. 2003). As a result, in certain cells, Rap1 can inhibit ERK1/2 signaling (Carey et al. 2000). However, it has been reported that activation of Rap1 can stimulate the activity of B-Raf and ERK1/2 to the same extent as Ras (Ohtsuka et al. 1996). In cells that express B-Raf, like PC12 cells, Rap1 has been shown to be an activator of ERK1/2 (Vossler et al. 1997; York et al. 1998; York et al. 2000; Guo et al. 2001; Gendron et al. 2003). This is consistent with the fact that greater than 90% of NGF-dependent ERK1/2 activation is due to B-Raf (Kao et al. 2001). Indeed, cell-type specific expression of B-Raf determines whether Rap1 is able to activate the ERK1/2 signaling cascade (Stork 2003).

In PC12 cells, Rap1 was shown to be essential for the late phase of ERK1/2 activation in response to NGF. Interestingly, Ras activation was shown to be responsible for the early phase of ERK1/2 activation (York et al. 1998). In this report, activation of Rap1 was required for two markers of differentiation by NGF: electrical excitability and expression of the neural specific gene, Transin. The Crk adaptor proteins and the Rap1GEF, C3G, mediated Rap1 activation. However, although dominant negative mutants of Rap1 inhibited sustained ERK1/2 activation by NGF, neurite outgrowth was not blocked. An earlier report by the same group demonstrated that constitutively active

mutants of Rap1 were able to activate ERK2 and induce neurite outgrowth in PC12 cells (Vossler et al. 1997). However, dominant negative Rap1 mutants were also shown to be insufficient in blocking NGF-dependent neurite outgrowth. Dominant negative Ras was able to block NGF-dependent neurite outgrowth. Interestingly, Ras mutants were able to partly block the neurite outgrowth induced by the Rap1 mutants (Vossler et al. 1997). Another recent report had somewhat different findings (Sun et al. 2006). This group showed that Rap1 was required for neurite outgrowth in PC12 cells using dominant negative mutants of Rap1. However, constitutively active mutants of Rap1 were not sufficient for promoting neurite outgrowth. A role for Rap1-dependent activation of integrins was proposed as a requirement for neuritogenesis (Sun et al. 2006). In addition, NGF-dependent neurite outgrowth can be inhibited by blocking Rap1 activation via endocannabinoids (Rueda et al. 2002). Therefore, the exact role of Rap1 and sustained ERK1/2 activation in NGF-dependent neurite outgrowth isn't entirely clear. However, it is clear that mechanisms involving both Ras and Rap1-dependent activation of ERK1/2 are being utilized to induce neurite outgrowth by NGF in PC12 cells (Marshall 1998).

Phosphorylation and activation of TrkA, ERK1/2, and Akt are all maintained over basal levels when PC12 cells are bathed in NGF. This persistent kinase activity can be blocked by either NGF withdrawl or the TrkA inhibitor, K252a (Chang et al. 2003). NGF stimulation has also been shown to activate Rap1 persistently (hours) (Wu et al. 2001). In this report, many members of the ERK1/2 cascade are found in endosomal fractions. These proteins include Rap1, TrkA, B-Raf, MEK1, and ERK1/2. Interestingly, pharmacological disruption of the endosomal compartments inhibits NGF-induced Rap1 activation and sustained ERK1/2 activity. Ras-dependent ERK1/2 activity

at early time points was unchanged. Supporting evidence for this model comes from another report where TrkA internalization is required for activation of Rap1 by NGF (York et al. 2000). These studies support the model of sustained Rap1-dependent ERK1/2 signaling in endosomal compartments in response to NGF. This component of NGF signaling is accompanied by transient Ras-dependent ERK1/2 activity at the plasma membrane. Together, the data shows that the endosome appears to be an active signaling complex essential for NGF-dependent PC12 differentiation.

2.19 The role of ARMS and Rap1 in neurite outgrowth

The ankyrin-rich membrane-spanning protein (ARMS) was shown to associate with TrkA upon NGF stimulation in PC12 cells (Arevalo et al. 2004). This association was specific for TrkA and was not observed with the EGFR following EGF treatment. This report showed that ARMS is rapidly tyrosine phosphorylated and provides a docking site for the Crk-L-C3G complex. As a result, activation of ARMS results in Rap1-dependent sustained ERK1/2 activity. Small interfering RNAs (siRNA) were used against ARMS and sustained ERK1/2 activation was inhibited. Ras-dependent transient ERK1/2 activation was unaffected. In addition, dominant negative mutants of ARMS (truncated forms) inhibited NGF-dependent neurite outgrowth as well. Recently, the same group has identified the phosphorylation site on ARMS required for Crk-L binding and Rap1 activation (Y1096) (Arevalo et al. 2006). They demonstrated that a mutant ARMS (Y1096A) that is unable to be phosphorylated specifically blocked sustained ERK1/2 activation and reduced NGF-dependent neurite outgrowth. As expected, transient ERK1/2 in response to NGF was unchanged by the Y1096A mutant. Using density

gradient centrifugation, the authors demonstrated that ARMS, TrkA, Rap1, and C3G were all present in endosomal membranes in PC12 cells and cortical neurons. Thus, these studies provide further insight as to how signaling through TrkA can result in sustained activation of ERK1/2 via Rap1. It will be exciting to learn more about the role ARMS plays in neuronal signaling as it is further studied.

A recent report has shed even more light of the role of sustained activation of Rap1 and ERK1/2 in neurite outgrowth (Hisata et al. 2007). In PC12 cells, the authors identified a tetrameric complex formed by ARMS, TrkA, PDZ-GEF1, and synaptic scaffolding molecule (S-SCAM) (Fig. 2.9). This complex was found in late endosomes after NGF treatment. PDZ-GEF1 is a GEF specific for Rap1. S-SCAM is a synaptic molecule that contains PDZ domains as well. Disruption of this tetrameric complex inhibited NGF-dependent neurite outgrowth. Using siRNA, the authors showed that PDZ-GEF1 was required for Rap1 activation and sustained ERK1/2 activation. Knockdown of PDZ-GEF1 also inhibited NGF-dependent neurite outgrowth. It was also shown that early Rap1 activation does not contribute to early ERK1/2 activation. Interestingly, C3G was found specifically in early endosomes while PDZ-GEF1 was found in late endosomes. Rap1 was found in both endosomal compartments. Knockdown of either Rap1 GEF modestly inhibited neurite outgrowth by NGF. Knockdown of both Rap1 GEFs had an additive effect. Hence, C3G and PDZ-GEF1 work cooperatively to persistently activate Rap1 in response to NGF and are involved in neurite outgrowth. This report provides evidence linking NGF-dependent Rap1 activation with both sustained ERK1/2 and neurite outgrowth (Fig. 2.9). It also shows the importance of compartmentalized signaling whereby Rap1 is activated in different areas of the cell by different Rap1 GEFs.

In addition, it provides further support for the “signaling endosome hypothesis” demonstrating that signaling complexes are formed in specific cellular compartments upon growth factor stimulation and are required for carrying out biological fates like differentiation.

Figure 2.9

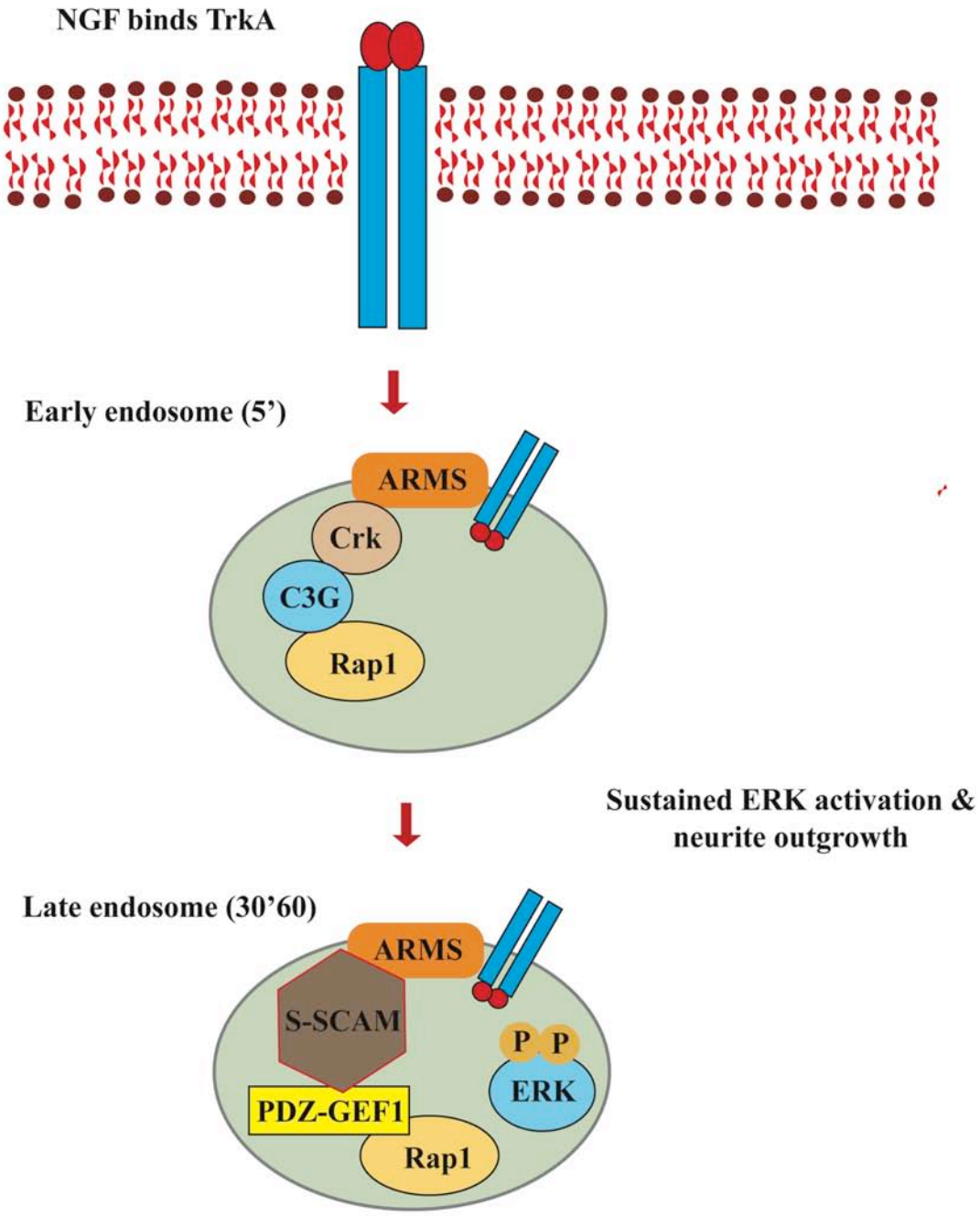
NGF binds TrkA
Early endosome (5')
ARMS
Crk
C3G
Rap1
Sustained ERK activation & neurite outgrowth
Late endosome (30'60)
ARMS
S-SCAM
P P
ERK
PDZ-GEF1
Rap1

Fig 2.9. NGF activates Rap1 signaling in distinct endosomal compartments leading to neurite outgrowth in PC12 cells. NGF stimulation causes Rap1 activation in early endosomes at 5'. C3G is the Rap1 specific exchange factor found in this compartment and mediates Rap1 activation. At 30'-60', sustained activation of Rap1 is mediated by PDZ-GEF1, another GEF specific for Rap1. In late endosomes, the tetrameric complex of TrkA, S-SCAM, PDZ-GEF1, and ARMS induces sustained activation of Rap1 and ERK resulting in neurite outgrowth. So, C3G and PDZ-GEF1 are both involved in the NGF-dependent activation of Rap1 and neurite outgrowth in PC12 cells.

2.20 The small GTPases Rit and Rin

Rit is a member of the Ras super family of small GTPases (Andres et al. 2005). Recent work has been done analyzing the role Rit plays in NGF-dependent outgrowth in PC6 cells. PC6 cells are a subline of PC12 cells that become dependent on NGF upon treatment. If the cells have been exposed to NGF, they undergo a transcription-dependent cell death following its removal (Pittman et al. 1993). This is unlike wild-type PC12 cells, which can proliferate if NGF is withdrawn if they are cultured in serum-containing media. In PC6 cells, constitutive active Rit can induce neurite outgrowth that is ERK1/2-dependent. In addition, dominant negative Rit can block NGF-dependent neurite outgrowth in these cells (Spencer et al. 2002). Moreover, siRNA to Rit alters NGF-mediated ERK activation and neurite outgrowth in PC6 cells (Shi and Andres 2005). Interestingly, the authors state that the neuritic processes are distinct from those produced by a constitutively active Ras mutant (increased branching via Rit). Rit has properties that are similar to the related family member Rap1. Like Rap1, Rit selectively activates B-Raf, and not Raf-1 (Shi et al. 2005). In addition, evidence has shown that Rit is also required for NGF-dependent sustained activation of ERK1/2 in PC6 cells (Shi et al. 2006). In a recent study, Rit has been implicated in both axonal and dendritic growth in both hippocampal and sympathetic neurons (Lein et al. 2007). The evidence shows that Rit is acting via ERK1/2 to promote these biological events in these primary cells. In view of this, Rit has been shown to play a role in mediating ERK1/2-dependent neurite outgrowth in different neuronal cell types.

A related molecule to Rit is the GTPase Rin. Rin is highly expressed in the nervous system and has been shown to bind calmodulin (Lee et al. 1996; Hoshino and Nakamura 2003). In PC6 cells, NGF activates Rin is a Ras-dependent manner (Spencer et al. 2002). Although constitutively active Rin has no effect on neurite outgrowth, dominant negative Rin can block NGF-dependent neurite outgrowth in PC6 cells. Rin is unable to activate Raf-1, but can potently stimulate B-Raf activation in PC6 cells (Shi et al. 2005). Moreover, downregulation of Rin can block neurite outgrowth in these cells. Inhibition of Rin activity blocks B-Raf and p38 activation induced by NGF. However, ERK1/2 activation remains unaffected. The authors postulate that the NGF-dependent neurite outgrowth may be the result of B-Raf-mediated p38 activation. Although Rin is clearly involved in NGF-dependent PC6 cell differentiation, its exact role needs further investigation.

2.21 NGF effects require other proteins

NGF stimulation activates a signaling cascade that involves many proteins required to induce differentiation in PC12 cells. A viral form of the adaptor Crk can induce neurite outgrowth and differentiation in PC12 cells when microinjected. This effect is also dependent on Ras (Tanaka et al. 1993). In fact, stable cell lines expressing v-crk in PC12 cells have been shown to enhance NGF's effect of neurite outgrowth. Interestingly, these cells also respond to the mitogen EGF by extending neurites as well (Hempstead et al. 1994). The nonreceptor tyrosine kinase Src has also been shown to play a role in neurite outgrowth in PC12 cells (Thomas et al. 1991). In this study, a constitutively active form of the protein, v-Src, was expressed by establishing a temperature-sensitive PC12 cell

line. This cell line showed extensive neurite outgrowth in a temperature-inducible manner. Like NGF-mediated outgrowth, it was reversible. However, unlike NGF, v-Src-induced extension of neurites was not blocked at high cell densities. In a more recent report, Src activation was shown to be required for both NGF-dependent Rap1 and ERK activation in PC12 cells (Obara et al. 2004). However, PP2, an inhibitor of Src family kinases (SFKs), was unable to block NGF-dependent neurite outgrowth in this study. Thus, both Crk and Src are proteins that are activated by NGF in PC12 cells and have been demonstrated to play a role in neurite outgrowth.

PC12 cells do not express platelet-derived growth factor receptors (PDGFRs) (Thompson et al. 1997). This property has been exploited in order to better understand which proteins are important for PC12 differentiation. Receptor chimeras were made that have the ligand binding domain of PDGF and the intracellular signaling components of TrkA (Obermeier et al. 1994). This allows for PDGF to only activate the transfected receptors and then to analyze TrkA signaling. Mutants were created that were unable to bind Shc, PLCγ and PI3K. The data showed that Shc is required for Trk-mediated neurite outgrowth (Obermeier et al. 1994). However, elimination of PLCγ or PI3K binding sites did not drastically affect neurite outgrowth. The data regarding PI3K has been verified by another group and shows that NGF-induced PI3K activity is sufficient to initiate neurite outgrowth, but failed to stably maintain it (Ashcroft et al. 1999). PI3K activity was able to mediate a survival response in response to NGF. However, a more recent report showed PLCγ was required for activation of ERK1/2 in PC12 cells (Choi et al. 2001). The transient and sustained activation of ERK1/2 was inhibited by blocking NGF-induced stimulation of PLCγ. Neurite outgrowth was not analyzed in this study. Another

recent study used pharmacological inhibitors of both phospholipase C and the inositol-triphosphate-receptor (U73122 and Xestospongin C, respectively) to block NGF-induced neurite outgrowth. They claim the effects are Ras-independent, but ERK1/2 activation is not analyzed (Kiss et al. 2006). Inhibition of PLC signaling does not block all aspects of differentiation, as induction of IEGs, such as c-fos, is not altered. These pathways have also been studied in primary neurons. In rat cortical neurons, a direct pathway is reported demonstrating that PLCγ-mediated ERK1/2 activity leads to neurite outgrowth (Zhai et al. 2005). Therefore, all of the studies on PLC signaling and neurite outgrowth don't all have the same findings. However, the recent studies seem to provide convincing evidence that PLCγ plays a role in contributing to ERK activation and neurite outgrowth.

2.22 NGF: ERK-dependent & ERK-independent pathways

Many groups have provided evidence that NGF-induced sustained ERK1/2 activation are required for neurite outgrowth in PC12 cells (Heasley and Johnson 1992; Qui and Green 1992; Traverse et al. 1992; Nguyen et al. 1993; Marshall 1995; Harada et al. 2001; Willard et al. 2007). Boglari and company used a MEK1/2 inhibitor to block ERK1/2-dependent outgrowth by NGF (Boglari and Szeberenyi 2001). They examined wild-type PC12 cells after six days of NGF treated and then scored cells as positive that had neurites twice as long as the cell body. Roughly 90% of cells treated with NGF were scored positive. Treatment with either a membrane permeable analogue of cAMP (dbcAMP) or a calcium ionophore (ionomycin) alone had no increase in neurite outgrowth. If cells were pretreated with PD98059 (MEK1/2 inhibitor) prior to NGF treatment, about 5% were positive. If cells were co-treated with either cAMP or Ca^{+2}

along with NGF, no further neurites were seen. However, PD98059 pretreatment with cells treated with NGF + cAMP or NGF + Ca^{+2} still had 75% of cells score positive. Interestingly, this effect was still dependent on TrkA activation because pretreatment with a TrkA inhibitor (K252a) completely inhibited neurite outgrowth in response to the combination treatments (NGF+cAMP or NGF+Ca^{+2}). As a result, the second messenger analogues turned NGF from and ERK-dependent stimulus to a primarily ERK1/2-independent stimulus (Table 2.1). The authors go on to show that the effect they observe with NGF alone is entirely Ras-dependent by using stable cells that express a dominant negative mutant of Ras (PC12-M17). Surprisingly, combination treatments (NGF+cAMP or NGF+Ca^{+2}) can still induce neurite outgrowth in 55% of the cells. PD98059 pretreatment is only able to "weakly" inhibit neurite outgrowth in PC12-M17 cells following the combination treatments. Although statistics are not done, the effect looks to be insignificant. Again, activation of TrkA is required for this effect as K252a completely inhibits neurite outgrowth in response to all combination treatments in PC12-M17cells. This study demonstrates that NGF can induce neurite outgrowth in PC12 cells in both an ERK-dependent and ERK-independent manner (Table 2.1). The exact nature and contribution of cAMP and Ca^{+2}-dependent signaling in promoting neurite outgrowth is still unclear and warrants further study.

Table 2.1

2nd messenger analogues render NGF-dependent neurite outgrowth ERK-independent

	Neurite Outgrowth	
	PC12 (WT)	**PC12 (M17)**
NGF	+	−
cAMP	−	−
Ca^{+2}	−	−
NGF/cAMP	+	+
NGF/Ca^{+2}	+	+
NGF/PD	−	**NA**
NGF/cAMP/PD	+	+
NGF/Ca^{+2}/PD	+	+

*** K252a blocks all stimulated neurite outgrowth**

Table 2.1. This is a summary of the data from the report entitled "NGF in combination with 2nd messenger analogues causes neuronal differentiation of PC12 cells expressing a dominant inhibitory Ras protein without inducing activation of ERKs" by Boglari and Szeberenyi (2001). The +/- indicate the presence (+) or absence (-) of neurite outgowth upon stimulation/treatment. cAMP= dibutyryl cyclic AMP, Ca^{+2}= ionomycin, PD=PD98059 (MEK1/2 inhibitor), and K252a=Trk inhibitor. The cells used to perform these experiments were wild-type PC12 cells (WT) and PC12 cells expressing a dominant negative Ras (M17).

2.23 Neurite outgrowth: p38, JNK, and PKC

There have been other kinases and proteins reported as being necessary for NGF-dependent neurite outgrowth in PC12 cells. An inhibitor of the MAPK family member p38, SB203580, was shown to significantly inhibit NGF-dependent neurite outgrowth in PC12 cells even at low doses (Morooka and Nishida 1998). They also use a constitutively active MEK that results in activation of ERK1/2, p38, and neurite outgrowth. If these transfectants are treated with SB203580, p38 activation is abolished and sustained ERK1/2 activation is maintained. However, neurite outgrowth is blocked even if the presence of sustained ERK1/2 activation. Interestingly, sustained p38 activation is not sufficient to induce neurite outgrowth. However, if sustained activation of p38 (arsenic treatment) is coupled with a transient ERK1/2 activator like EGF, neurite outgrowth is observed. Thus, the authors postulate that sustained activation of p38 is required for neurite outgrowth in this model. Another report contradicts these results and showed that treatment of PC12 cells with EGF and the p38 inhibitor, SB203580, caused neurite outgrowth through a mechanism involving prolonged activation of B-Raf (Yoon et al. 2004). However, these results are not clearly understood.

It has been shown that transient c-jun N-terminal kinase (JNK) activation occurs upon NGF stimulation of PC12 cells (Leppa et al. 1998). The AP-1 family member, c-jun, is a target of JNKs and phosphorylation increases its transcriptional activity (Kyriakis and Avruch 1996). Leppa and company have shown that constitutively active c-jun is sufficient to promote neurite outgrowth in PC12 cells (50% of cells positive) (Leppa et al. 1998). In addition, a dominant negative c-jun can inhibit PC12

differentiation induced by a constitutively active form of MEK (65% to 25%). However, activation of JNK by itself is not sufficient for PC12 differentiation unless exogenous c-jun is provided as well. Consequently, the authors propose a model whereby ERK1/2 activation is required for transciption of c-jun in response to NGF. The c-jun protein is then phosphorylated and activated by JNK. C-jun proteins are able to dimerize with AP-1 family members in order to promote NGF-dependent gene transcription. However, c-jun has been shown to be a substrate for ERK1/2 and the kinetics of c-jun protein phosphorylation and JNK activation do not overlap in the NGF-stimulated PC12 cells. In view of this, it is possible that ERK1/2 are sufficient for regulation of c-jun and neurite outgrowth in PC12 cells in response to NGF (Fig. 2.10).

Figure 2.10

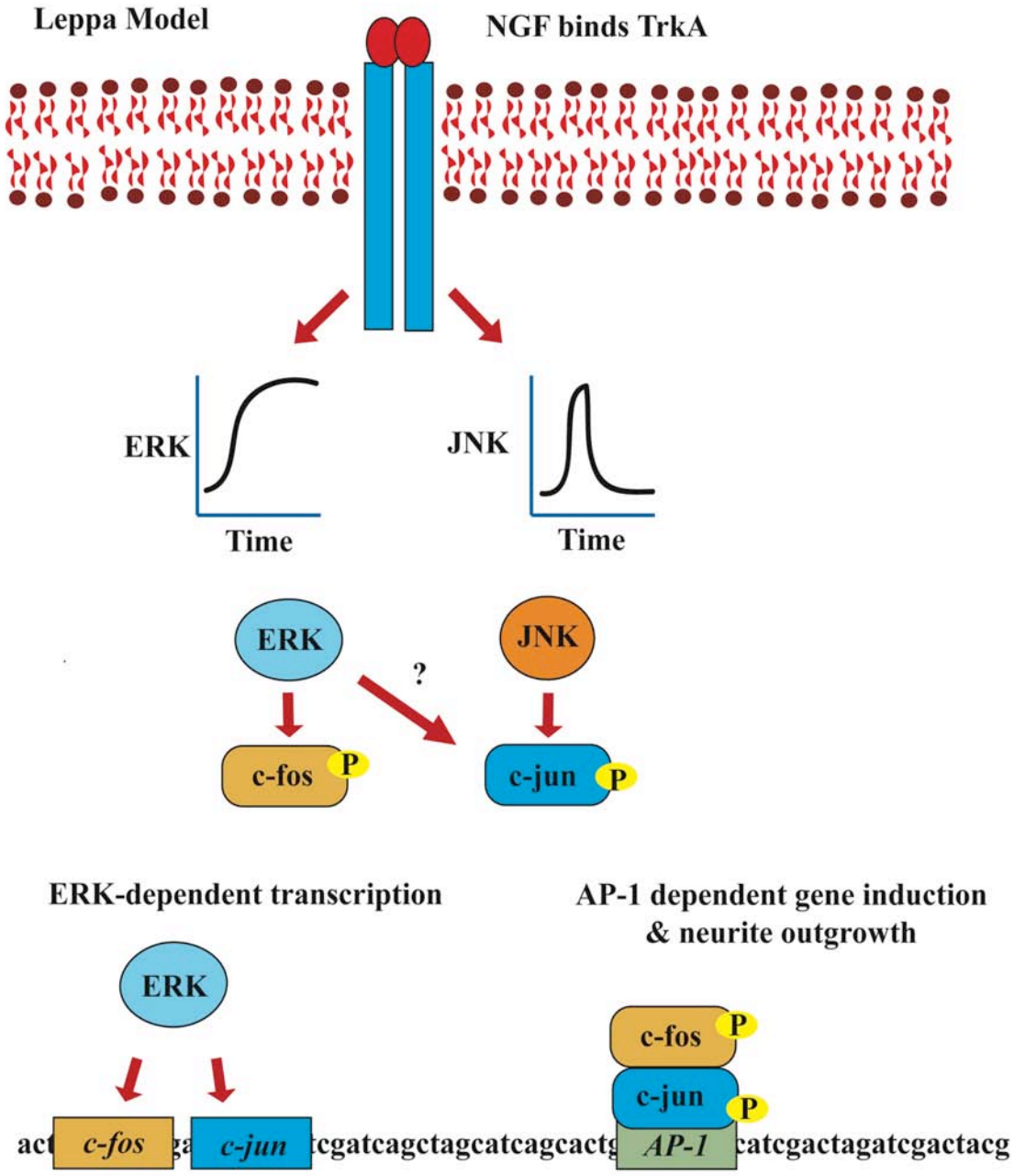
Leppa Model
NGF binds TrkA
ERK
Time
JNK
Time
ERK
?
JNK
c-fos
P
c-jun
P
ERK-dependent transcription
AP-1 dependent gene induction
& neurite outgrowth
ERK
c-fos
c-jun
tcgatcagctagcatcagcactg
c-fos
P
c-jun
P
AP-1
catcgactagatcgactacg

Fig 2.10. c-fos and c-jun are required for NGF-induced neurite outgrowth in PC12 cells. NGF stimulation causes activation of both ERK and JNK signaling pathways. ERKs are activated for a sustained period of time while JNKs are activated transiently. NGF causes a persistent AP-1 DNA binding activity that is required for neurite outgrowth. Using siRNA technology, it is demonstrated that both AP-1 family proteins, c-fos and c-jun, are required for NGF-dependent neurite outgrowth in PC12 cells. In this model, it is proposed that the expression and activation of the AP-1 proteins c-fos and c-jun are dependent on ERK and JNK signaling. ERK activation induces transcription of both c-fos and c-jun. Phosphorylation and activation of c-fos is ERK-dependent. c-jun can be phosphorylated and activated by JNKs. It is also proposed that NGF may involve ERKs in phosphorylation of c-jun as well due to the overlap in the kinetics of ERK and c-jun activation (Leppa et al. 1998).

Another report also provides a role for both ERK1/2 and JNK in NGF-treated PC12 cells (Xiao and Liu 2003). PC12-N1 (N1) cells are a variant cell line that exhibits spontaneous neurite extension (Xiao et al. 2002). These cells have elevated levels of activated JNK and c-jun (Xiao and Liu 2003). Interestingly, pharmacological inhibition of JNK, using SP600125, had no effect on spontaneous neurite outgrowth. Surprisingly, PD98059 (MEK1/2 inhibitor) treatment inhibits the spontaneous outgrowth in a dose-dependent fashion. The authors conclude that ERK1/2 is required for the early stage of neuritogenesis, initiation. However, inhibiting JNKs can block late stages of neuritogenesis, including an increase in cell body size and branching. Thus, the authors provide different roles for both ERK1/2 and JNK in neurite outgrowth in N1 cells.

The protein kinase C (PKC) pathway has been implicated in neurite outgrowth in PC12 cells. PKC is activated upon NGF stimulation (Altin and Bradshaw 1990). Sphingosine, an inhibitor of PKC, blocked NGF-dependent neurite outgrowth in PC12 cells (Hall et al. 1988). In addition, microinjection of antibodies against PKC also inhibited differentiation in PC12 cells (Altin et al. 1992). Evidence against these reports comes from studies in which prolonged TPA stimulated down regulation of PKC had no effect on NGF-dependent neurite outgrowth in PC12 cells (Reinhold and Neet 1989). However, many different PKC isoforms are expressed in PC12 cells and the exact role PKC may play in this process might need further study.

A recent study has combined mathematical and experimental approaches to better understand the difference between NGF and EGF signaling in PC12 cells (Santos et al. 2007). In this report, they found that NGF stimulated a PKCδ-dependent feed forward postive feedback on the ERK1/2 cascade. This feedback allowed for sustained ERK1/2

activation that was specific to NGF stimulation. They showed that simultaneous EGF and PKCδ activation using phorbol ester allowed the cells to undergo differentiation instead of proliferation. These results support many reports in the literature that have shown EGF to synergize with other treatments to promote differentiation in PC12 cells (Isono et al. 1994; Mark et al. 1995; Wu and Bradshaw 1996; Mark and Storm 1997; Kiermayer et al. 2005). In addition, knockdown of PKCδ using siRNA prevented NGF from inducing the cells to differentiate. In fact, these cells entered the cell cycle and proliferated. By using mathematical analysis combined with siRNA technology, the authors have identified how PKCδ activation can help explain the differences between NGF and EGF signaling in PC12 cells.

As discussed above, many signaling pathways have been implicated in NGF-dependent neurite outgrowth. In the next chapter, we analyze the NGF activated signaling pathways that are required for neurite outgrowth in PC12 cells by taking a pharmacological approach. In addition, we take a molecular approach to specifically study the role the ERK1/2 pathway plays in neurite ougrowth in PC12 cells.

Chapter 3

Analysis of signaling pathways required for neurite outgrowth in PC12 cells

3.1 Abstract

Neuronal differentiation by nerve growth factor (NGF) has long been studied in the rat pheochromocytoma tumor cell line (PC12 cells). Cellular differentiation in this model system is a complex biological process that is characterized by the cessation of cell division, the induction of electrical excitability, enhanced gene expression, the ability to make synapses with muscle cells, and the formation of neurites. NGF generates a complex signal that activates many signaling pathways. The pathways involved in NGF-dependent neurite outgrowth have received much attention. Although many groups agree that the ERK1/2 pathway plays a prominent role in this process, many studies have provided evidence that ERK-independent signals are also important. In the following study, we investigate distinct NGF-dependent pathways and the role they play in neurite outgrowth of PC12 cells. We show that pharmacological blockade of the ERK pathway significantly inhibits NGF-dependent neurite outgrowth. In addition, we show that NGF-dependent neurite outgrowth utilizes both phosphatidylinositol-3 kinase (PI3K) and Src family kinase (SFK) pathways. Blockade of protein kinase C (PKC) had no effect on NGF-dependent neurite outgrowth. However, PKC-dependent pathways were required for neurite outgrowth induced by joint treatment with PMA and EGF. In addition, we developed an inducible system to study the role of ERK1/2 in isolation on neurite outgrowth using a ΔB-Raf-ER construct. We discovered that maximal neurite outgrowth requires sustained activation of ERK1/2. Thus, this study demonstrates that multiple pathways contribute to NGF-dependent neurite outgrowth in PC12 cells.

3.2 Introduction

PC12 cells have been used as a model for neuronal differentiation since they were first reported (Greene and Tischler 1976). In this report, PC12 cells were shown to respond to NGF by extending "varicose processes" that resembled those of primary sympathetic neurons in culture. Since then, neurite outgrowth has been considered the hallmark of differentiation and has been studied intensely. NGF-induced differentiation of PC12 cells is a biological response that also includes induction of neural-specific genes and electrical excitability (Dichter et al. 1977; Greenberg et al. 1985; deSouza et al. 1995; York et al. 1998). However, neurite outgrowth remains the most remarkable outcome of NGF signaling in PC12 cells. In addition, neurite outgrowth can be observed microscopically at low power and can be measured by eye. Consequently, not only is NGF-dependent neurite outgrowth the most striking characteristic of PC12 differentiation, but it is also a trait that is easily observed and quantified.

The signaling pathways responsible for NGF-dependent neurite outgrowth have been an area of intense investigation. A number of reports have implicated the ERK1/2 cascade as a signaling pathway instrumental for neurite outgrowth induced by NGF (Cowley et al. 1994; Pang et al. 1995a; Vaudry et al. 2002). Early reports demonstrate that both NGF and EGF activate the ERK1/2 cascade allowing serine and threonine phosphorylation on substrates *in vitro* (Gotoh et al. 1990a; Miyasaka et al. 1990). However, NGF causes a much more sustained activation of ERK1/2 and results in differentiation of PC12 cells characterized by neurite outgrowth. Another report postulates that sustained activation of ERK1/2 permits nuclear entry resulting in initiation of specific gene transcription necessary for neuronal differentiation (Traverse et al.

1992). Molecular techniques have been employed to create both constitutively active and dominant negative mutants of MEK1. These mutants were first used in PC12 cells that were overexpressing the EGF receptor (PC12-HER). This cell line responds to EGF, and NGF, by extending neurites (Cowley et al. 1994). In this report, caMEK1 mutants cause neurite outgrowth that is comparable to PC12-HER cells treated with EGF. In addition, dnMEK1 mutants inhibit growth factor mediated neurite outgrowth as well. Pretreatment with PD98059, a MEK kinase inhibitor, inhibited neurite outgrowth in response to NGF (Alessi et al. 1995; Pang et al. 1995a). Thus, the previous reports provide considerable evidence that the ERK1/2 cascade plays an important role in neurite outgrowth by growth factors.

Although there is extensive evidence that ERK1/2 activation by NGF contributes to differentiation of PC12 cells, another report suggests that NGF may be utilizing other signaling pathways as well (Marek et al. 2004). In this report, the authors use gene chip arrays to show that NGF requires ERK-independent pathways to induce genes that are characterized as markers of differentiation. For example, genes such as neurofilament light chain (NFLC) and transin require both ERK and c-Jun N-terminal kinase (JNK) signaling pathways for induction by NGF in PC12 cells. In addition, NGF-dependent induction of other genes, such as synapsin II, requires pathways that are independent of both ERK and JNK. These results indicate that NGF-dependent pathways, other than ERKs, may be contributing to neurite outgrowth and differentiation of PC12 cells.

As discussed previously, NGF generates a complex signal that results in phosphorylation and activation of many signaling pathways. The nonreceptor tyrosine kinase Src is activated by NGF in PC12 cells (Altun-Gultekin and A.Wagner 1996;

Lazarovici et al. 1997; Obara et al. 2004). Activation of Src by NGF has been shown to be involved in aspects of neuronal differentiation such as down regulation of voltage gated Na^+ currents (Hilborn et al. 1998). NGF-dependent induction of other neuronal markers such as transin, SCG10, and Thy-1 mRNAs all required Src activation (D'Arcangelo and Halegoua 1993). More importantly, constitutive activation of Src can induce neurite outgrowth that is comparable to NGF (Alema et al. 1985; Rausch et al. 1989). Although some aspects of differentiation are variable, such as the lack of EGF receptor down regulation by v-Src, neurite outgrowth is equivalent in both scenarios. It has also been demonstrated that expression of exogenous PDGF receptors (PDGFRs) in PC12 cells allows differentiation in response to PDGF (Heasley and Johnson 1992). Using this method, another report concluded that either Src or PLCγ pathways were required for differentiation and neurite outgrowth (Vaillancourt et al. 1995). Accordingly, these studies suggest that c-Src may be a crucial component of NGF-dependent neurite outgrowth in PC12 cells.

NGF has been shown to generate diacylglycerol (DAG) upon stimulation in PC12 cells (Altin and Bradshaw 1990). DAG is an activator of some isoforms of PKC. Antibodies against PKC have been demonstrated to block NGF-induced neurite outgrowth in PC12 cells (Altin et al. 1992). NGF stimulation is also able to cause release of arachidonic acid (AA) in PC12 cells (Zheng et al. 1996). AA is a lipid component that is able to activate a subset of PKC isoforms called atypical PKCs (aPKCs). Some studies have now shown that certain aPKCs are required for neurite outgrowth in PC12 cells (Coleman and Wooten 1994; Samuels et al. 2001). In addition, NGF-dependent ERK activation has been shown to require PKCδ (Corbit et al. 1999). Thus, the NGF-

dependent activation of aPKCs and their role in neurite outgrowth in PC12 cells warrants further study.

The phosphatidylinositol-3 kinase (PI3K) pathway has been implicated in NGF-induced survival of neuronal cells; including the PC12 cell line (Sofroniew et al. 2001). However, some reports have also implicated this pathway in aspects of NGF-dependent PC12 differentiation such as upregulation of acetylcholine (Ach) and choline acetyltransferase (ChAT) (Madziar et al. 2005). Another study has shown that PI3K is required for both sustained activation of ERK1/2 and internalization of TrkA in NGF treated PC12 cells (York et al. 2000). In addition, overexpression of the docking protein Gab-1 can stimulate neurite outgrowth in PC12 cells that is inhibited by blocking activation of PI3K (Korhonen et al. 1999). However, other studies have shown that the PI3K pathway antagonizes both branching and elongation of neurites (Ashcroft et al. 1999; Higuchi et al. 2003). As a result, the precise role of the PI3K pathway in neurite outgrowth remains controversial.

In the following study, we investigate the signaling pathways involved in NGF-dependent neurite outgrowth in PC12 cells. We find that inhibition of the ERK pathway partially inhibits NGF-dependent neurite outgrowth. Using a pharmacological approach, we show that NGF requires both PI3K and Src family kinase (SFKs) pathways for maximal neurite outgrowth. In addition, inhibition of NGF-dependent neurite outgrowth is not observed when PKC isoforms are blocked. Moreover, we use an inducible system to regulate only the ERK1/2 pathway without contribution from the other signaling pathways studied. We use a ΔB-Raf-ER construct that is activated by tamoxifen (4-OHT) and signaling downstream of B-Raf is turned off with U0126. Using this

approach, we are able to demonstrate that activation of the ERK1/2 pathway is sufficient for neurite outgrowth in PC12 cells. We also show that sustained activation of the ERK1/2 pathway is required for maximal neurite outgrowth in PC12 cells using this system.

3.3 MATERIALS & METHODS

Materials

PC12 cells were kindly provided by Patrick Casey (Duke University, Durham, NC). The MEK1/2 inhibitor, U0126, was purchased from Calbiochem (La Jolla, CA). Nerve growth factor was purchased from Roche (Indianapolis, IN). Epidermal growth factor, rat-tail collagen type I (C7661), agarose-coupled anti-Flag (M2) beads, hydroxytamoxifen (4-OHT), and anti-Flag (M2) antibody were purchased from Sigma (St. Louis, MO). The signaling pathway inhibitors PP2, LY294002, and Bisindolylmaleimide II were purchased from Calbiochem (San Diego, CA). Phosphorylation-specific ERK1/2 antibodies (Thr202/Tyr204) (#9101-polyclonal) was purchased from Cell Signaling Technology (Beverly, MA). LipofectAMINE 2000 was purchased from Invitrogen (Carlsbad, CA). All other chemical reagents were purchased from Sigma.

Cell culture

PC12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% horse serum and 5% fetal bovine serum at 37° C in 5% CO_2 (70%

confluence). Unless otherwise stated, cells were deprived of serum for 16 hours before being treated with various reagents for neurite outgrowth experiments and western blotting.

Plasmids and transfections

PC12 cells were transiently transfected with LipofectAMINE 2000 as per the manufacturers instructions. After transfection, cells were serum starved and treated with NGF (100ng/ml), EGF (50ng/ml), PP2 (10nM), LY294002 (20nM), Bisindolylmaleimide II (100nM), PMA (810nM), tamoxifen (1nM) and U0126 (20μM), unless otherwise stated. For western blotting experiments, the following amounts of DNA were used: 1 μg Flag-ERK2, 1 μg ΔB-Raf-ER, and 1 μg of pcDNA3 (vector) to make total amounts of transfected DNA equal. For neurite outgrowth experiments, the following amounts of DNA were used: 500ng GFP, 500ng ΔB-Raf-ER, 500ng Flag-RasV12, 500ng caMEK and 500ng of pcDNA (vector) to make total amounts of DNA equal.

Western blotting

Unless otherwise stated, cells were deprived of serum for 16 hours prior to treatment. Cells were lysed in RIPA buffer (1% Triton-X 100, 1% sodium deoxycholate, 0.2% sodium dodecyl sulfate, 125mM NaCl, 50mM Tris pH 8.0, 10% glycerol, 1mM EDTA, 25mM β-glycerolphosphate, 25mM NaF, 1mM sodium orthovanadate, 10μg/ml aprotinin, 10μg/ml leupeptin, 1mM phenylmethylsulfonyl fluoride) and equal amounts of lysate were sonicated in a bath sonicator (S-3000, Misonix INC., Farmingdale, NY) at level 6 for 30 seconds 3 times. Laemmli buffer was then added to the lysates and they were boiled for 2 minutes.

Proteins that were immunoprecipitated were incubated for 2 hours at 4°C with 12µl of Anti-FLAG (M2) agarose-coupled beads. Pellets were washed three times in lysis buffer and resuspended in 25µl 2x Laemmli buffer. Proteins were resolved by SDS-PAGE. Proteins were transferred onto nitrocellulose (Protran, Schleicher & Schuell, Keene, NH) and blocked in Odyssey™ blocking buffer (LI-COR biosciences INC, Lincoln, NE). Primary antibodies were diluted in Odyssey blocking buffer 1:1 with PBST, as per the manufacturers instructions. The appropriate fluorescently labeled secondary antibodies were diluted in Odyssey blocking buffer 1:1 with PBST, 0.01% SDS. Membranes were scanned with the Odyssey Infrared Imaging System (LI-COR biosciences, INC.).

Neurite Outgrowth Experiments

12 well plates were coated with 0.05mg/ml collagen solution (from rat tail) overnight at room temperature. The following day plates were washed 2x with sterile water and PC12 cells were seeded on the culture dishes. For these experiments, cells were 30% confluent. The following day, the cells are transfected in the morning and serum starved the same night. In all experiments, cells are transfected with green fluorescent protein (GFP) in order to better visualize neurite outgrowth. Cells are treated the next day. If signaling pathway inhibitors are used, cells are treated for 20 minutes prior to being stimulated with neurite outgrowth promoting agents. Forty-eight hours later, cells are scored positive for neurite outgrowth if they contain at least one neurite twice the length of the cell body. Nine random fields of view are scored for each experimental condition. Only GFP positive cells are scored for neurite outgrowth. The percentage of GFP positive neurite bearing cells over total GFP positive cells is recorded for each field of view. Images of living cells were obtained from a Leica DMIRE2 inverted fluorescence microscope from Leica Microsystems

Inc. (Bannockburn, IL). The statistical significance was calculated by a one-way analysis of variance (ANOVA) with a Newman-Keuls post test where indicated.

3.4 Results

3.4a U0126 partially blocks NGF-dependent neurite outgrowth

It is well known that NGF causes the induction of neurite outgrowth in PC12 cells. Using pharmacological inhibition of the ERK pathway, it has been shown that ERK1/2 is required for this effect (Pang et al. 1995a). Using a different pharmacological compound (U0126), we wanted to revisit this experimental paradigm. We pretreated PC12 cells with U0126, an inhibitor of the ERK kinase MEK. We then stimulated cells with NGF and measured neurite outgrowth 2 days later. Greater than 75% of the NGF-treated cells underwent neurite outgrowth (Fig. 3.1). Pretreatment of cells with U0126 causeed an inhibition of NGF-induced neurite outgrowth. Surprisingly, a proportion of these cells still exhibited neurite outgrowth when compared to untreated cells (Fig. 3.1). These data suggest that there may be other pathways besides the ERK pathway that are contributing to NGF-dependent neurite outgrowth in PC12 cells.

Figure 3.1

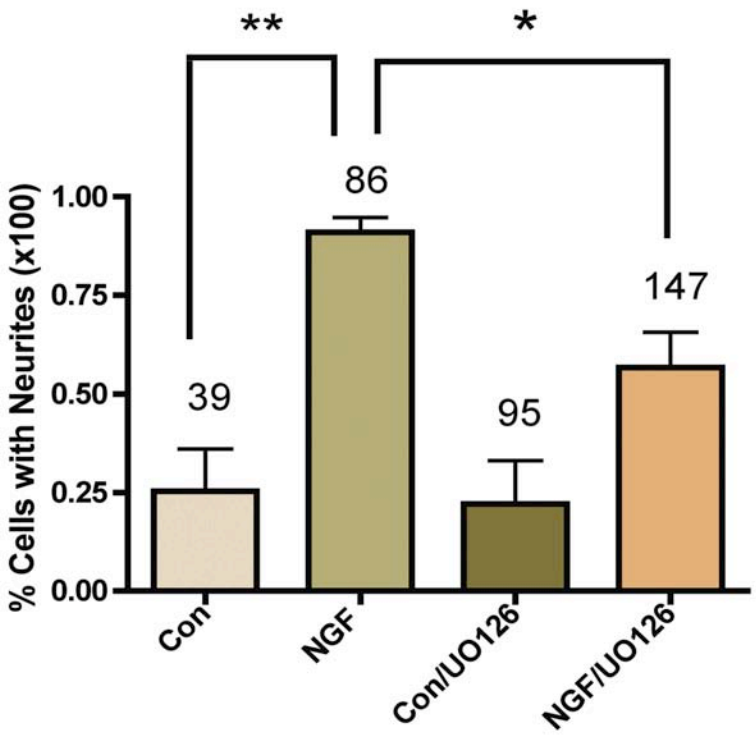

Fig. 3.1 NGF induced neurite outgrowth is not entirely dependent on ERKs. PC12 cells were transfected with GFP to better visualize neurite outgrowth as described in materials and methods. The numbers above each column indicate the numbers of cells scored for that condition. Cells were pretreated with U0126 or vehicle (DMSO) for 20 min prior to stimulation with NGF. The percentage of cells that scored positive for neurite outgrowth 48 hours after NGF treatment is shown. The data are means $\pm$ SE (n=9). Asterisks indicate significant differences. Two asterisks (**) compares control (1st column) versus NGF (2nd column); **$p < 0.001$. One asterisk (*) compares NGF (2nd column) versus NGF/U0126 (4th column); *$p < 0.01$. The statistical significance was calculated by a one-way ANOVA with a Newman-Keuls post test.

3.4b RasV12 induces ERK-dependent and ERK-independent neurite outgrowth

As discussed previously, NGF activates many signaling pathways (Kaplan and Stephens 1994; Sofroniew et al. 2001). In order to analyze the selective contribution of ERK1/2 signaling to neurite outgrowth, we transfected a constitutively active MEK1 mutant (caMEK1) into PC12 cells and measured neurite outgrowth (Fig. 3.2). Although cells transfected with caMEK1 were able to undergo some neurite outgrowth, neurite outgrowth was stimulated in NGF treated cells to a much greater extent. As expected, caMEK1-dependent neurite outgrowth was entirely ERK-dependent. Ras is a small G protein that has been shown to be involved in activation of many signaling pathways, including ERKs (Mitin et al. 2005). It has previously been shown that constitutively active Ras can induce neurite outgrowth in PC12 cells (Bar-Sagi and Feramisco 1985). We show that transfection of RasV12, a constitutively active mutant of Ras, into PC12 cells induced neurite outgrowth at a level comparable to PC12 cells that were treated with NGF (Fig. 3.2). U0126 partially blocks RasV12-induced neurite outgrowth. Consistent with the data in Fig 3.1, the data in Fig 3.2 support the notion that NGF-dependent neurite outgrowth in PC12 cells is mediated by ERK-dependent and ERK-independent pathways.

Figure 3.2

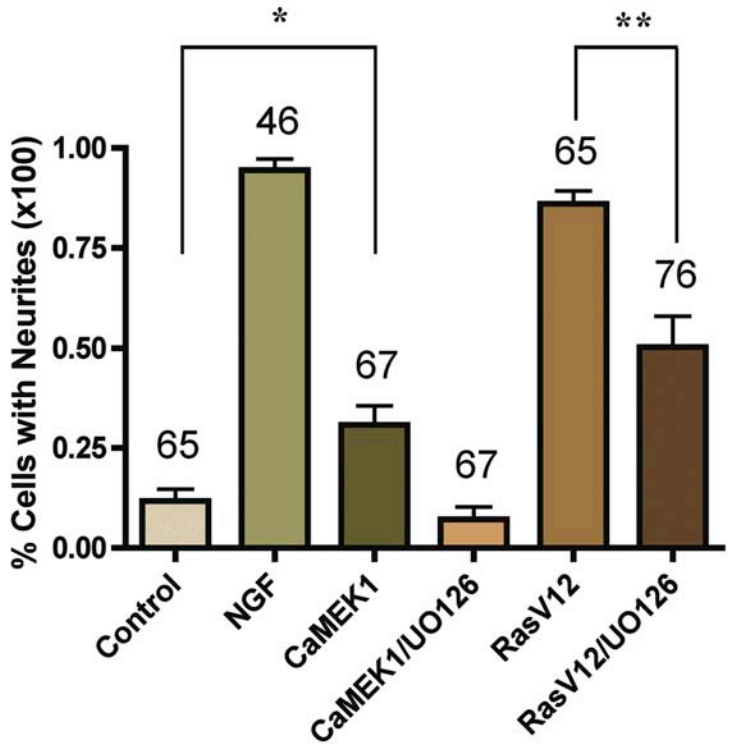

Fig 3.2 Specific ERK1/2 activation causes less neurite outgrowth than NGF. PC12 cells were transfected with GFP and the indicated plasmids (RasV12 or caMEK) to better visualize neurite outgrowth as described in materials and methods. The numbers above each column indicate the total number of cells scored for that condition. Cells were pretreated with U0126 or vehicle (DMSO) for 20 min prior to stimulation with NGF. The percentage of cells that scored positive for neurite outgrowth 48 hours after NGF treatment is shown. The data are means $\pm$ SE (n=9). Asterisks indicate significant differences. One asterisk (*) compares control (1st column) versus caMEK1 (3rd column); *p< 0.05. Two asterisks (**) compares RasV12 (5th column) versus RasV12/U0126 (6th column); **p< 0.001. The statistical significance was calculated by a one-way ANOVA with a Newman-Keuls post test.

3.4c Inhibition of SFKs and PI3K signaling partially inhibits NGF-dependent neurite outgrowth

Since the previous data suggest ERK-independent pathways may be contributing to neurite outgrowth in PC12 cells, we employed a series of pharmacological inhibitors of NGF activated proteins (Fig. 3.3a). Bisindolylmaleimide II, an inhibitor of PKC, had no effect on NGF-dependent neurite outgrowth. Both EGF and PMA had no effect on neurite outgrowth of PC12 cells by themselves (Fig. 3.3b). However, if EGF and PMA are used together, neurite outgrowth comparable to NGF was observed. This effect is inhibited by Bisindolylmaleimide II, demonstrating the requirement for PKC. Although PKC can promote neurite outgrowth when used with EGF, it was not involved in NGF-dependent neurite outgrowth (Fig. 3.3b).

3.4d PP2 and LY294002 show does-dependent inhibition of NGF-dependent neurite outgrowth

LY294002, a PI3K inhibitor, inhibited NGF-dependent neurite outgrowth. Similarly, PP2, a Src family kinase (SFK) inhibitor, inhibited NGF-dependent neurite outgrowth. However, neither inhibitor completely blocked neurite outgrowth by NGF (Fig. 3.3a). In order to more closely analyze this effect, we used decreasing amounts of both LY294002 and PP2 to inhibit NGF-dependent neurite outgrowth (Fig. 3.4a and 3.4b). Higher concentrations of both inhibitors blocked a greater percentage of neurite outgrowth induced by NGF. The dose-dependent effect we observed gave us confidence the effect was specific (Fig. 3.4a and 3.4b). Thus, these data suggest that both SFKs and PI3K are contributing to NGF-dependent neurite outgrowth in PC12 cells.

Figure 3.3

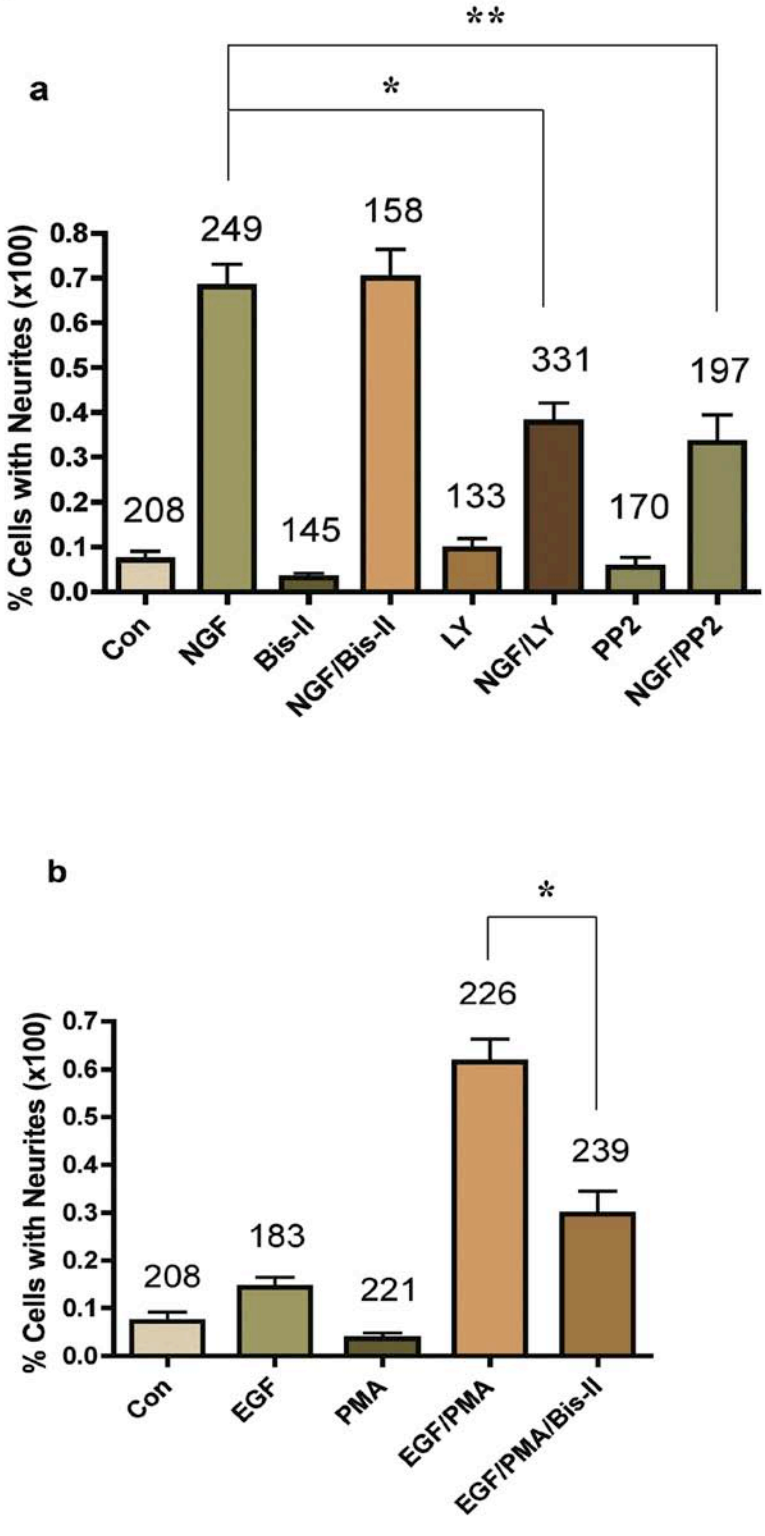
a
**
*
% Cells with Neurites (x100)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
208
249
145
158
133
331
170
197
Con
NGF
Bis-II
NGF/Bis-II
LY
NGF/LY
PP2
NGF/PP2
b
*
% Cells with Neurites (x100)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
208
183
221
226
239
Con
EGF
PMA
EGF/PMA
EGF/PMA/Bis-II

Fig 3.3 NGF stimulates neurite outgrowth via ERK-independent pathways. (a) PC12 cells were transfected with GFP to better visualize neurite outgrowth as described in materials and methods. The numbers above each column indicate the total number of cells scored for that condition. Cells were pretreated with Bis-II, LY294002, PP2, or vehicle (DMSO) for 20 min prior to stimulation with NGF. The percentage of cells that scored positive for neurite outgrowth 48 hours after NGF treatment is shown. Asterisks indicate significant differences. One asterisk (*) compares NGF (2^{nd} column) versus NGF/LY (6^{th} column); *p< 0.001. Two asterisks (**) compares NGF (2^{nd} column) versus NGF/PP2 (8^{th} column); **p< 0.001. The statistical significance was calculated by a one-way ANOVA with a Newman-Keuls post test. (b) PC12 cells were transfected with GFP to better visualize neurite outgrowth as described in materials and methods. The numbers above each column indicate the total number of cells scored for that condition. Cells were pretreated with Bis-II or vehicle (DMSO) for 20 min prior to stimulation with PMA, EGF, or the combination (EGF/PMA). The percentage of cells that scored positive for neurite outgrowth 48 hours after treatment is shown. The data are means $\pm$ SE (n=9). One asterisk (*) compares EGF/PMA (4^{th} column) versus EGF/PMA/Bis-II (5^{th} column); *p< 0.001. The statistical significance was calculated by a one-way ANOVA with a Newman-Keuls post test.

Figure 3.4

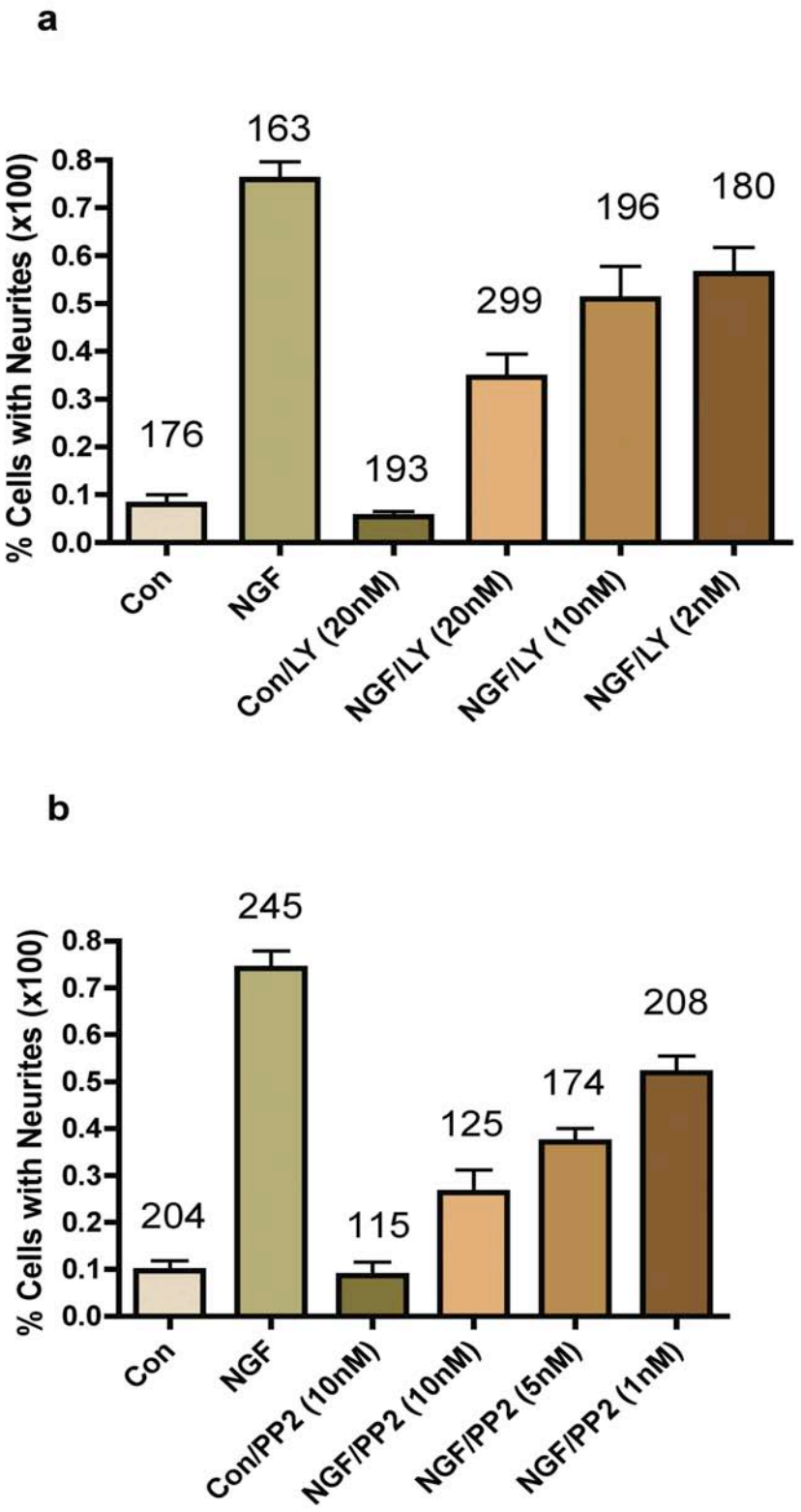
a
% Cells with Neurites (x100)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
176
163
193
299
196
180
Con
NGF
Con/LY (20nM)
NGF/LY (20nM)
NGF/LY (10nM)
NGF/LY (2nM)
b
% Cells with Neurites (x100)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
204
245
115
125
174
208
Con
NGF
Con/PP2 (10nM)
NGF/PP2 (10nM)
NGF/PP2 (5nM)
NGF/PP2 (1nM)

Fig 3.4 Inhibition of NGF-dependent ERK-independent neurite outgrowth is dose-dependent. (a) PC12 cells were transfected with GFP to better visualize neurite outgrowth as described in materials and methods. The numbers above each column indicate the total number of cells scored for that condition. Cells were pretreated with the indicated concentrations of LY294002 or vehicle (DMSO) for 20 min prior to NGF stimulation. The percentage of cells that scored positive for neurite outgrowth 48 hours after NGF treatment is shown. (b) PC12 cells were transfected with GFP to better visualize neurite outgrowth as described in materials and methods. The numbers above each column indicate the total number of cells scored for that condition. Cells were pretreated with the indicated concentrations of PP2 or vehicle (DMSO) for 20 min prior to NGF stimulation. The percentage of cells that scored positive for neurite outgrowth 48 hours after NGF treatment is shown. The data are means $\pm$ SE (n=9).

3.4e Sustained ERK signaling can be activated in isolation using the ΔB-Raf-ER construct

We were specifically interested in studying ERK-dependent neurite outgrowth more closely. Previously, we were using caMEK1 mutants to activate the ERK1/2 cascade. However, it is difficult to control the timing of ERK1/2 activation using this method. The beginning of ERK1/2 activation is difficult to determine and terminating ERK1/2 activation is not possible using this method. We decided to employ a method that allowed us better control of the ERK1/2 cascade. The ΔB-Raf-ER construct encodes for a chimeric protein that consists of the ligand binding domain of the estrogen receptor and the B-Raf kinase domain (Pritchard et al. 1995) (Fig. 3.5). The chimera is held in a complex with heat shock proteins, thereby distancing the kinase from its targets. The inhibition is alleviated by the addition of a synthetic steroid tamoxifen (4-OHT). In order to switch off the ERK1/2 cascade, U0126 was added to the cells to inhibit MEK1/2, a direct downstream target of B-Raf. Hence, this method introduces a controllable switch for the ERK1/2 cascade.

Figure 3.5

Fig 3.5 Schematic of ΔB-Raf-ER plasmid used in neurite outgrowth experiments. This figure was adapted from (Pritchard et al. 1995). The letters above the diagram predict the 6 N-terminal amino acids that begin the fusion protein. The arrow indicates the start of the actual B-Raf sequence. This sequence is the conserved region 3 domain (CR3) where the catalytic domain is located. ΔB-Raf is fused to the hormone binding domain of the HE14 allele of the hbER (human estrogen receptor). This fusion protein responds to the synthetic steroid tamoxifen (4-OHT). A mutation in the hbER (G525R) destroys the response to estradiol and phenol red.

We transfected cells with ΔB-Raf-ER and a Flag tagged ERK2 reporter construct. PC12 cells were then treated with 4-OHT over a 3-hour time-course and activation of Flag-ERK2 was measured using a phospho-specific antibody to ERK1/2 (Fig. 3.6a). Flag-ERK2 was activated over basal levels at all time points measured (Fig. 3.6a, top panel). Expression levels of Flag-ERK2 are shown (Fig. 3.6a, bottom panel). This experiment showed that we were able to activate the ERK1/2 cascade using 4-OHT for a sustained time period (3hrs). Next, we compared the kinetics of ERK2 activation in cells that were expressing the chimera to those that were treated with NGF. Cells were stimulated with either NGF or 4-OHT over the course of 80 minutes and Flag-ERK2 activation was analyzed by using phospho-specific antibodies to pERK1/2 (Fig. 3.6b, top panel). Cells that were pretreated with U0126 showed no activation of Flag-ERK2 over basal levels. In addition, similar levels of sustained Flag-ERK2 activation were measured for both 4-OHT and NGF stimulated Flag-ERK2 at the 80-minute time point (Fig. 3.6b, top panel). Expression levels of Flag-ERK2 are shown (Fig. 3.6b, bottom panel). As a result, tamoxifen-stimulated ΔB-Raf-ER causes sustained activation of the ERK1/2 cascade that is comparable to NGF stimulation. In addition, U0126 can be used to inhibit tamoxifen-induced activation of ERK1/2.

Figure 3.6

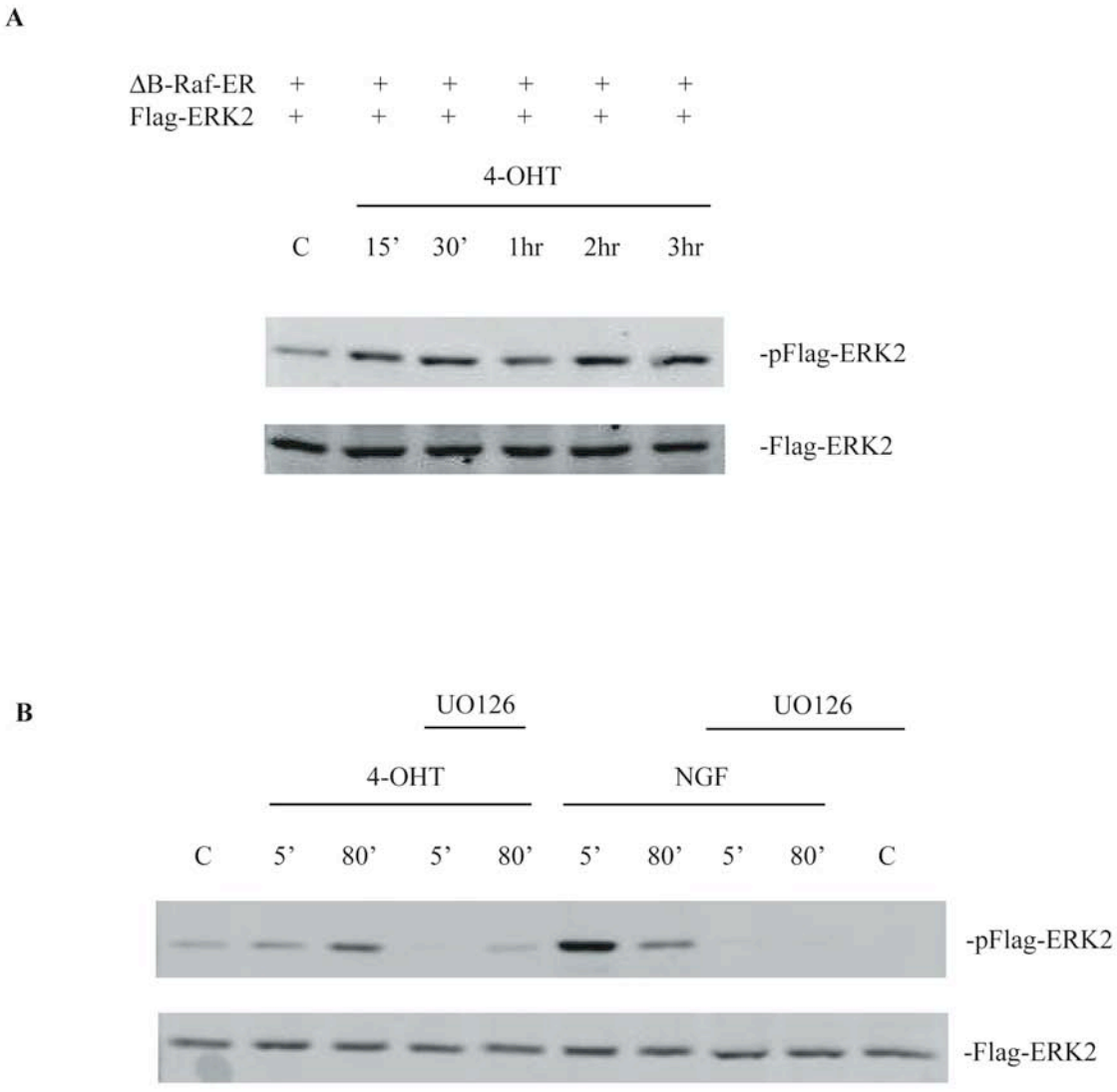
A
ΔB-Raf-ER + + + + + +
Flag-ERK2 + + + + + +
4-OHT
C 15' 30' 1hr 2hr 3hr
-pFlag-ERK2
-Flag-ERK2
B
UO126
UO126
4-OHT
NGF
C 5' 80' 5' 80' 5' 80' 5' 80' C
-pFlag-ERK2
-Flag-ERK2

Fig 3.6 Tamoxifen (4-OHT) activates ΔB-Raf-ER and a Flag-ERK2 reporter. (a) PC12 cells were transfected with ΔB-Raf-ER and a Flag-ERK2 reporter. Cells were stimulated with tamoxifen and lysed at the indicated time points. Flag-ERK2 was immunoprecipitated using anti-Flag (M2) agarose-coupled beads and SDS-PAGE was performed. Activation levels of Flag-ERK2 were measured using pERK1/2 Ab (top panel). Equal levels of Flag-ERK2 were demonstrated by measuring levels of Flag-ERK2 in lysates following SDS-PAGE using Flag (M2) antibodies (bottom panel). (b) PC12 cells were transfected with ΔB-Raf-ER and a Flag-ERK2 reporter. Cells were pretreated with U0126 for 20 min where indicated. Cells were then stimulated with either tamoxifen (4-OHT) or NGF and lysed at the indicated time points. Flag-ERK2 was immunoprecipitated using anti-Flag (M2) agarose-coupled beads and SDS-PAGE was performed. Activation levels of Flag-ERK2 were measured using pERK1/2 Ab (top panel). Equal levels of Flag-ERK2 were demonstrated by measuring levels of Flag-ERK2 in lysates following SDS-PAGE using Flag (M2) antibodies (bottom panel).

3.4f Maximal 4-OHT-dependent neurite outgrowth in cells expressing ΔB-Raf-ER requires sustained ERK1/2 signaling

Now that we can selectively regulate the ERK1/2 cascade using 4-OHT and ΔB-Raf-ER, we were interested in measuring neurite outgrowth. We compared neurite outgrowth of transfected PC12 cells with ΔB-Raf-ER and treated them with 4-OHT to cells that had been treated with NGF (Fig. 3.7). Both 4-OHT and NGF treatments caused neurite outgrowth over basal levels. Although NGF stimulated cells caused a greater percentage of neurite outgrowth than 4-OHT treated cells, 4-OHT treatment still caused a significant level of neurite outgrowth over basal levels (Fig. 3.7). 4-OHT stimulated neurite outgrowth was also completely ERK1/2-dependent. Pretreatment with U0126 blocked 4-OHT stimulated neurite outgrowth completely (Fig. 3.8). We were interested in determining whether addition of U0126 after the ERK1/2 cascade had already been activated would alter neurite outgrowth by 4-OHT. We then introduced U0126 at various time points after 4-OHT stimulation and measured neurite outgrowth (Fig 3.8). We found that the longer the ERK1/2 cascade was activated, the more neurite outgrowth we observed. In this paradigm, continuous ERK1/2 activation supports maximal neurite outgrowth in PC12 cells.

Figure 3.7

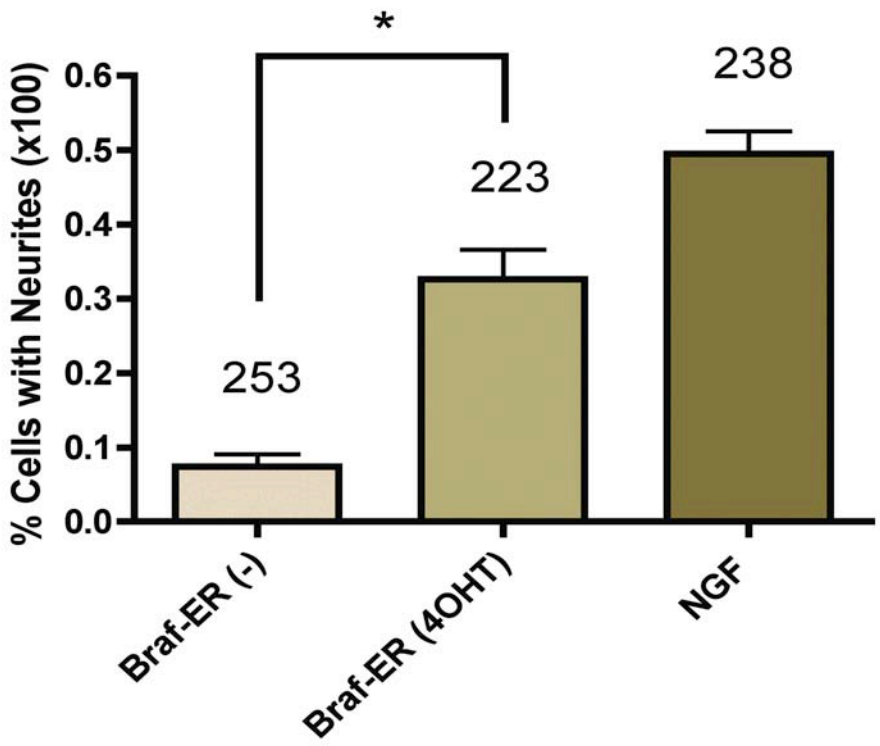

Fig 3.7 Activation of ΔB-Raf-ER with tamoxifen induces neurite outgrowth in PC12 cells. PC12 cells were transfected with GFP to better visualize neurite outgrowth as described in materials and methods. In addition, cells were transfected with ΔB-Raf-ER or pcDNA3 (vector). Cells were treated with tamoxifen or NGF as indicated and then scored for neurite outgrowth 48 hours later. The numbers above each column indicate the total number of cells scored for that condition. The data are means $\pm$ SE (n=9). An Asterisk indicates a significant difference. One asterisk (*) compares ΔB-Raf-ER (-) (1st column) versus ΔB-Raf-ER (4-OHT) (2nd column); *$p< 0.001$. The statistical significance was calculated by a one-way ANOVA with a Newman-Keuls post test.

Figure 3.8

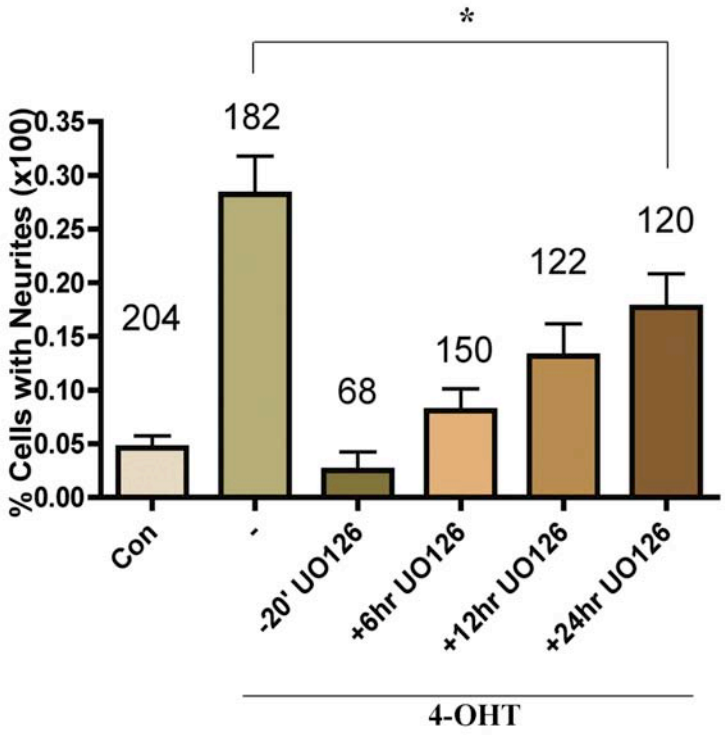

Fig 3.8 Sustained ERK1/2 activation is required for maximal neurite outgrowth by tamoxifen in PC12 cells. PC12 cells were transfected with GFP to better visualize neurite outgrowth as described in materials and methods. In addition, cells were transfected with ΔB-Raf-ER. One group was pretreated with U0126 for 20 min prior to tamoxifen stimulation (U0126 -20'). Other groups were treated with U0126 for the indicated times post-tamoxifen stimulation (+6hr, +12hr, and +24hr). Cells were scored for neurite outgrowth 48 hours after tamoxifen stimulation. The numbers above each column indicate the total number of cells scored for that condition. The data are means $\pm$ SE (n=9). An Asterisk indicates a significant difference. One asterisk (*) compares 4–OHT alone (2nd column) versus 4-OHT/+24hr UO126 (6th column); *$p < 0.05$. The statistical significance was calculated by a one-way ANOVA with a Newman-Keuls post test .

3.5 Discussion

The trademark of neuronal cells is the branching processes that emanate from the cell body of the neuron. Since 1976, PC12 cells have been used as a model for neuronal differentiation that is primarily characterized by neurite outgrowth (Greene and Tischler 1976). In this model, NGF is one agent that has been shown to initiate the genetic program for differentiation and promote extensive neurite outgrowth. As a result of this phenomenon, many studies have been directed at determining the intracellular signaling pathways that are both necessary and sufficient for this effect.

It was presented that NGF stimulated sustained activation of ERK1/2 was required for PC12 neurite outgrowth (Traverse et al. 1992). This report argued that EGF was unable to initiate neurite outgrowth because transient activation of ERK1/2 was not sufficient for translocation of ERK1/2 into the nucleus. Another study injected plasmids of constitutively active MEK mutants into naïve PC12 cells (Cowley et al. 1994). They reported that 75% of injected cells formed neurites that measured 2 times the cell body diameter. Thus, we directed out efforts at examining the ERK1/2 pathway because of evidence provided in the literature.

The MEK inhibitor PD98059 was shown to inhibit NGF-dependent neurite outgrowth in PC12 cells (Pang et al. 1995b). However, the degree of inhibition was not reported as only phase-contrast photographs were provided. We chose to use the MEK inhibitor U0126 instead of PD98059 because the latter compound, in our hands, tends to precipitate out of solution when added to culture media. The fact that we only observed a 50% block of NGF-dependent neurite outgrowth was somewhat surprising given previous reports. However, a recent report also used U0126 to inhibit NGF-dependent neurite

outgrowth in PC12 cells (Sun et al. 2006). In this report, they showed approximately 50% of NGF-treated cells showed full neurite outgrowth. Pretreatment with U0126 inhibited NGF-dependent neurite outgrowth with full outgrowth seen in approximately 18% of the NGF-treated cells. Our results do vary in the percentage of cells that are scored positive for NGF-dependent neurite outgrowth. We observe ~75% of NGF-treated cells showing neurite outgrowth. In addition, their data may have been a result of the authors also scoring intermediate neurite outgrowth (Sun et al. 2006). Another report used PC12 cells and also saw close to 50% of the cell inducing neurites (Cowley et al. 1994). They used a dominant negative MEK1 mutants and were able to see a reduction of neurite outgrowth to about 20%. Therefore, our data and that of other groups seem to suggest that the ERK1/2 pathway is required for maximal neurite outgrowth by NGF. However, there do also seem to be some ERK-independent mechanisms responsible for neurite outgrowth as well. Table 3.1 provides a list of reports that demonstrate what proteins are required for NGF-dependent neurite outgrowth in wild-type PC12 cells.

Table 3.1 Proteins required for neurite outgrowth by NGF

A. Kinases

Required Proteins	Reference
PKCs	(Hall et al. 1988)
Src, Ras	(Kremer et al. 1991)
sustained ERKs	(Traverse et al. 1992)
PKCs	(Altin et al. 1992)
MEK1	(Cowley et al. 1994)
aPKCζ	(Coleman and Wooten 1994)
PKC	(Campbell and Neet 1995)
ERKs	(Pang et al. 1995)
PI3K	(Jackson et al. 1996)
p38	(Morooka and Nishida 1998)
PKCδ	(Corbit et al. 1999)
Nuclear ERKs	(Hans et al. 2001)
PI3K, Rac1	(Cheng et al. 2002)
Mst3b	(Irwin et al. 2006)
PKCδ	(Santos et al. 2007)

B. Small G proteins

Required Proteins	Reference
Ras	(Hagag et al. 1986)
Ras	(Szeberenyi et al. 1990)
sustained Ras, sustained ERKs	(Qui and Green 1992)
Ras	(Vossler et al. 1997)
Ras	(Boglari et al. 1998)

C. Transcription factors

Required Proteins	Reference
c-jun	(Leppa et al. 1998)
Egr-1, p35, Cdk5	(Harada et al. 2001)
c-fos	(Gil et al. 2004)
CREB, ERKs, M-Ras, Rap1	(Sun et al. 2006)

D. other

Required Proteins	Reference
ZIP/p62	(Samuels et al. 2001)
ARMS	(Arevalo et al. 2004)
ARMS, ERKs	(Arevalo et al. 2006)
PLCγ, IP3	(Kiss et al. 2006)
APPL1, GIPC1	(Lin et al. 2006)
CAP	(Limpert et al. 2007)
R-RasGAP downregulation	(Iwashita et al. 2007)
ARMS, S-SCAM, Rap1, PDZ-GEF, & C3G	(Hisata et al. 2007)
RGS12	(Willard et al. 2007)

Table 3.1 Studies providing evidence of proteins required for NGF-dependent neurite outgrowth in wild-type PC12 cells. (a) Studies showing kinases (1st column) required for neurite outgrowth in PC12 cells are listed. References (2nd column) are listed in the order they were published. (b) Studies showing small G proteins (1st column) required for neurite outgrowth in PC12 cells are listed. References (2nd column) are listed in the order they were published. (c) Studies showing transcription factors (1st column) required for neurite outgrowth in PC12 cells are listed. References (2nd column) are listed in the order they were published. (d) Studies showing other types of proteins (1st column) required for neurite outgrowth are listed. References (2nd column) are listed in the order they were published.

Our experiments using constitutively active MEK mutants in PC12 cells did not result in the robust neurite outgrowth that we had anticipated (~30% positive). As discussed previously, another group injected the same mutants into PC12 cells and observed a much higher percentage of neurite outgrowth (75%) (Cowley et al. 1994). This is most likely due to the technical differences between injected plasmids and liposome-mediated transfection. Injection techniques most likely resulted in higher expression levels. This may explain the higher percentages of neurite outgrowth that Cowley *et al.* had observed. We showed that PC12 cells pretreated with U0126 completely blocked caMEK1-dependent neurite outgrowth. This demonstrates that U0126 can completely inhibit specific activation of the ERK1/2 pathway in isolation. It also lends support that there are indeed NGF-dependent ERK-independent pathways regulating neurite outgrowth because U0126 pretreatment only partially blocks neurite outgrowth in NGF treated cells.

Like NGF, Ras can activate a number of signaling pathways other than ERK1/2, including PI3K, p38, and ERK5 (English et al. 1998; Cass and Meinkoth 2000; Burry 2001). We showed that constitutively active Ras mutants (RasV12) could induce neurite outgrowth that is comparable to NGF stimulation. Like NGF-treated cells, U0126 partially inhibits RasV12-dependent neurite outgrowth. This would suggest that other Ras-dependent pathways other than ERK1/2 are involved in neurite formation. Dominant negative Ras mutants (RasN17) have been shown to inhibit NGF-dependent PC12 neurite outgrowth (Szeberenyi et al. 1990). In addition, RasV12 mutants have also been shown to induce neurite outgrowth in PC12 cells in previous reports (Sassone-Corsi

et al. 1989; Simpson et al. 1991). Thus, our results are in accordance with other studies in the literature. Molecular techniques have been used extensively to study what proteins are sufficient for neurite outgrowth in PC12 cells. Table 3.2 list studies where overexpression of either wild-type or constitutively active mutants results in neurite outgrowth in the PC12 model system. Additional treatments are also listed, if they were required with the transfected proteins to induce neurite outgrowth.

Table 3.2 Transfection models for neurite outgrowth

Transfected Protein	Treatment	Reference
N-Ras (CA)		(Guerrero et al. 1986)
Ras (CA)		(Sassone-Corsi et al. 1989)
v-Src (CA)		(Rausch et al. 1989)
K-Ras (CA), N-Ras (CA), v-Src (CA)		(Simpson et al. 1991)
v-Src (CA)		(Thomas et al. 1991)
shc		(Rozakis-Adcock et al. 1992)
Raf-1 (CA)		(Wood et al. 1993)
TrkC	NT-3	(Tsoulfas et al. 1993)
EGF receptor	EGF	(Traverse et al. 1994)
EGF receptor	EGF	(Cowley et al. 1994)
Insulin receptor	insulin	(Dikic et al. 1994)
PDGF receptor	PDGF	(Vaillancourt et al. 1995)
RasV12 (CA), RapV12 (CA)		(Vossler et al. 1997)
PI3K (CA)		(Kita et al. 1998)
c-jun (CA)		(Leppa et al. 1998)

Transfected protein	Treatment	Reference
ERK2 (CA,nuclear)		(Robinson et al. 1998)
PI3K (CA)		(Ashcroft et al. 1999)
PKCε	EGF	(Brodie et al. 1999)
Gab1		(Korhonen et al. 1999)
GTK (SFKs)		(Anneren et al. 2000)
RhoG		(Katoh et al. 2000)
shb		(Lu et al. 2000)
B-KSR	EGF	(Muller et al. 2000)
Grit (RhoGTPase)		(Nakamura et al. 2002)
Rin (CA)		(Hoshino and Nakamura 2003)
PI3K (CA)		(Sanchez et al. 2004)
M-Ras (CA), H-Ras (CA)		(Sun et al. 2006)

Table 3.2 Studies showing transfected protein overexpression that results in neurite outgrowth in wild-type PC12 cells. The table is split into a right and left section to keep it on one page. References are listed in the order that they were published (3rd and 6th column). The proteins that were overexpressed and induced neurite outgrowth are listed in the 1st and 4th columns. If additional stimulation was required after transfection, those treatments are listed in the 2nd and 4th columns. These transfected proteins require a stimulus that is normally not able to induce neurite outgrowth. CA= constitutively active mutant.

We investigated NGF-stimulated activation of PKC-dependent pathways. Studies have shown that the novel PKC isoform, PKCδ, is required for NGF-dependent neurite outgrowth in PC12 cells (Corbit et al. 1999; Santos et al. 2007). We used a general PKC inhibitor, bisindolylmaleimide II, which inhibits all of the isoforms of PKC. However, pretreatment with this inhibitor had no effect on NGF-dependent neurite outgrowth. Another group of PKC isoforms has also been reported to be required for NGF-dependent neurite outgrowth. The atypical PKC family members (aPKC) PKCζ and PKCι have been implicated in NGF-induced neurite outgrowth as well (Coleman and Wooten 1994; Samuels et al. 2001). The potency of bisindolylmaleimide II on aPKC members is the weakest compared with other isoforms. In view of this, it may be possible that our inhibitor is not having a strong effect on these isoforms and they may be still involved in NGF-dependent neurite outgrowth. Moreover, bisindolylmaleimide II did inhibit neurite outgrowth induced by the joint treatment of EGF and PMA. This would suggest that the EGF/PMA stimulation is using PKC-dependent pathways and that our inhibitor is indeed working. PMA mimics DAG and can activate all PKC isoforms except aPKCs (Brodie et al. 1999). However, long-term treatment of PMA has also been shown to downregulate PKC isoforms that it stimulates, including PKCδ (Coleman and Wooten 1994). Therefore, the exact PKC isoform utilized by the joint EGF/PMA treatment would require a more detailed study. In addition, there are many studies reporting joint stimulations, like EGF/PMA, that induce neurite outgrowth in wild-type PC12 cells (Table 3.3). Table 3.3 lists studies where treatments of PC12 cells were able to synergistically induce neurite outgrowth.

Table 3.3 Synergistic treatments causing neurite outgrowth

Synergizing Agents	Reference
K-252a & EGF	(Isono et al. 1994)
v-crk & EGF	(Hempstead et al. 1994)
EGF & cAMP (via KCl)	(Mark et al. 1995)
IL-6 & EGF (via Jak-Stats)	(Wu and Bradshaw 1996)
cAMP & EGF	(Mark and Storm 1997)
EGF & PKCε overexpression	(Brodie et al. 1999)
EGF & B-KSR overexpression	(Muller et al. 2000)
Src activity & EGF	(Yang et al. 2002)
EGF & p38 inhibition	(Yoon et al. 2004)
Epac activation & cAMP	(Kiermayer et al. 2005)
PMA & EGF	(Santos et al. 2007)

Table 3.3 Studies showing joint treatments that result in neurite outgrowth in wild-type PC12 cells. The synergizing treatments are listed in the 1st column. References are listed in the order that they were published (column 2).

The PI3K pathway has long been associated with neurotrophin-induced cell survival (Brunet et al. 2001). In our studies, we showed that inhibition of the PI3K pathway blocked NGF-dependent neurite outgrowth. Although this result was unanticipated, it is not unprecedented when the literature was examined. The PI3K inhibitor, wortmannin, has been used to pretreat PC12 cells and inhibit NGF-dependent neurite outgrowth (Jackson et al. 1996; Kobayashi et al. 1997). Interestingly, wortmannin treatment of PC12 cells with mature neurites formed after days of NGF exposure retracted and collapsed (Jackson et al. 1996). This would argue that the maintenance of PI3K signaling is required for neurite outgrowth as well. In addition, a constitutively active mutant of PI3K has been shown to induce neurite outgrowth as well (Kobayashi et al. 1997; Kita et al. 1998). Moreover, in both reports, the neurite outgrowth was also dependent on the JNK pathway. However, neurite outgrowth produced by these mutants was not identical to that produced by NGF as the authors deemed the processes "incomplete". Neurites lacked accumulation of F-actin and GAP43 (Kobayashi et al. 1997). This is consistent with another report where Trk mutants with only PI3K activity formed neurites in response to NGF in PC12nnr cells, but the cells were unable to sustain them (Ashcroft et al. 1999). The contribution of other signaling pathways, namely ERK1/2, is most likely required for fully mature neurites. This may reflect data from a study that demonstrates PI3K is required for both TrkA internalization and sustained ERK1/2 activity in PC12 cells in response to NGF(York et al. 2000).

Expression of constitutively active Src (v-Src) has been shown to induce neurites in PC12 cells (Rausch et al. 1989; Thomas et al. 1991). In our study, we showed that pretreatment with PP2, a Src family kinase (SFK) inhibitor, partially blocked neurite

outgrowth in response to NGF. Many studies have shown that Src participates in NGF signaling. Src is required for NGF-dependent EGFR down regulation and the migratory response in PC12 cells (Altun-Gultekin and A.Wagner 1996; Lazarovici et al. 1997). In fact, Src has been shown to form a complex with TrkA and is required for sustained activation of ERK1/2 in PC12 cells (Wooten et al. 2001; Obara et al. 2004). Accordingly, our results are consistent with NGF-dependent activation of Src and demonstrate that Src is also involved in neurite outgrowth in PC12 cells.

Although we discovered that ERK1/2-independent pathways were participating in neurite outgrowth in PC12 cells, we were still primarily interested in the contribution by the ERK1/2 pathway. The ΔB-Raf-ER construct was originally developed to study the role of B-Raf signaling in cellular transformation (Pritchard et al. 1995). After 5 minutes of NGF treatment, there was a much more robust activation of Flag-ERK2 compared to treatment with 4-OHT. However, our observation at the 80-minute time point show equal activation between both NGF and 4-OHT. Hence, the sustained activation of ERK1/2 by NGF is mimicked by 4-OHT using the ΔB-Raf-ER construct. More importantly, activation of the ERK1/2 pathway using ΔB-Raf-ER resulted in neurite outgrowth in PC12 cells. This effect was not as strong as NGF-induced neurite outgrowth. This can be explained by the fact that NGF stimulates various signaling pathways that contribute to neurite formation. However, sustained ERK1/2 activation without contribution from other NGF-dependent signaling pathways is sufficient in a proportion of cells to induce neurite outgrowth.

Although it is generally agreed that the transient and sustained activation of the ERK1/2 pathway by EGF and NGF, respectively, explains the difference in biological

responses to the two growth factors, the exact definition of 'sustained' has remained undefined. One unanswered question is whether there is a threshold for signaling pathway activation that makes the pathway dispensable for a certain period after stimulation. The alternative is that pathway activation must be continuous in order to maintain stable neurites. In experiments that tested the effects of U0126 treatment after NGF stimulation on neurite outgrowth, the pretreatment of PC12 cells with U0126 or with U0126 addition 1 day after treatment with NGF resulted in reduction of neurite bearing cells from ~50% to ~20% (Sun et al. 2006). This result is very different from ours where we treated ΔB-Raf-ER transfected cells with 4-OHT. We observed ~30% of the cells formed neurites in response to 4-OHT. The lower percentage of neurite outgrowth can be explained by ability of NGF to activate multiple signaling pathways that are contributing to neurite outgrowth compared to selective activation of the ERK1/2 pathway by 4-OHT. This suggests that the ERK1/2 pathway by itself is not able to induce as much neurite outgrowth as NGF. In addition, our results suggest that the length of ERK1/2 activation dictates the amount of neurite outgrowth when this pathway is activated in isolation. The addition of U0126 24 hours after cells were stimulated with 4-OHT allowed for much more neurite outgrowth than cells that were pretreated with U0126. This was not the case in the above-mentioned study (Sun et al. 2006). The percentage of cells that were inhibited by addition of U0126 was the same regardless of when U0126 was added. This suggests that the ERK1/2 pathway is indeed required for a portion of neurite outgrowth induced by NGF. However, it also suggests that other signaling pathways are able to compensate for the loss of ERK1/2 signaling and are still able to induce some neurite outgrowth. The data also seems to provide evidence that

continuous ERK1/2 activation is required for maximal neurite outgrowth in response to NGF. Consequently, if ERK1/2 signaling is terminated after NGF stimulation, the biological result is the same as if there was no ERK1/2 activation at all.

In summary, our results argue that NGF-dependent neurite outgrowth in PC12 cells utilizes more than one signaling pathway. However, we also showed that the ERK1/2 pathway by itself was sufficient to induce neurite formation as well. The ability of the cell to use multiple signaling pathways to accomplish a complex feat like neuronal differentiation seems like a logical natural design. If one pathway is deficient, other pathways are able to compensate and still elicit the correct biological response. Future experiments should focus on inhibiting combinations of these pathways in order to determine whether the pathways act in series or are parallel pathways. It will also be interesting to see if the signaling mechanisms studied here are also in primary neuronal cells.

In this chapter, the data has shown that sustained ERK1/2 signaling is critical to promote maximal NGF-dependent nerite outgrowth in PC12 cells. In the next chapter, the mechanisms underlying the role of sustained ERK1/2 activation in PC12 differentiation are analyzed. A model is proposed whereby maximal transactivation of the transcription factor c-fos and NGF-dependent gene expression are dependent on sustained ERK1/2 activation via NGF.

Chapter 4

Sustained activation of extracellular signal-regulated kinase by nerve growth factor regulates c-fos protein stabilization and transactivation in PC12 cells

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4.1 Abstract

The duration of intracellular signaling is thought to be a critical component in effecting specific biological responses. This paradigm is demonstrated by growth factor activation of the extracellular signal-regulated kinase (ERK) signaling cascade in the rat pheochromocytoma cell line (PC12 cells). In this model, sustained ERK activation induced by nerve growth factor (NGF) results in differentiation whereas transient ERK activation induced by epidermal growth factor (EGF) results in proliferation in these cells. Recently, the immediate early gene product c-fos has been proposed to be a sensor for ERK signaling duration in fibroblasts. In this study, we ask whether this is true for NGF and EGF stimulation of PC12 cells. We show that NGF, but not EGF, can regulate both c-fos stability and activation in an ERK-dependent manner in PC12 cells. This is achieved through ERK-dependent phosphorylation of c-fos. Interestingly, distinct sites regulate enhanced stability and transactivation of c-fos. Phosphorylation of Thr325 and Thr331 are required for maximal NGF-dependent transactivation of c-fos. In addition, a consensus ERK binding site (DEF domain) is also required for c-fos transactivation. However, stability is controlled by ERK-dependent phosphorylation of Ser374, while phosphorylation of Ser362 can induce conformational changes in protein structure. We also provide evidence that sustained ERK activation is required for proper post-translational regulation of c-fos following NGF treatment of PC12 cells. Because these ERK-dependent phosphorylations are required for proper c-fos function, and occur sequentially, we propose that c-fos is a sensor for ERK signaling duration in the neuronal-like cell line PC12.

4.2 Introduction

NGF triggers neuronal differentiation in the PC12 cell model through the sustained activation of the MAP (mitogen-activated protein) kinase (extracellular signal-regulated kinase, or ERK). Neuronal differentiation by NGF is characterized by the induction of immediate early genes that encode transcription factors that promote the transcriptional activation of a set of NGF-responsive genes. Because of the requirement of sustained ERK activation for differentiation, NGF action on PC12 cells has served as a model for the role of duration of intracellular signals in dictating physiological responses. However, the mechanism by which transcription factors sense sustained ERK activation in PC12 cells is not known.

Other cell types have provided insight into how incremental changes in the duration of ERK activation can have profound effects on cellular responses (Weber et al. 1997; Bottazzi et al. 1999; Roovers et al. 1999; Adachi et al. 2002; Murphy et al. 2002; Koike et al. 2003; Werlen et al. 2003). For example, on the one hand, in Swiss 3T3 fibroblast cells, the sustained activation of ERKs is required for growth factor-induced proliferation by PDGF. On the other hand, transient activation of ERKs by EGF is not mitogenic in these cells. One ERK target, the transcription factor c-fos, has been proposed to mediate this action (Murphy et al. 2002). c-fos is a proto-oncogene that, unlike its oncogenic counterpart, v-fos, requires additional signals to achieve maximal proliferative potential (Chen et al. 1993; Okazaki and Sagata 1995; Chen et al. 1996; Monje et al. 2003). ERK-dependent signals stimulate c-fos at multiple levels (Monje et al. 2005), but perhaps the best studied of these actions is the stimulation of c-fos transcription (Monje et al. 2003; Tanos et al. 2005). This is achieved by phosphorylation of the transcription factor Elk-1 which

functions with other serum response factors to turn on the c-fos promoter (Gille et al. 1992; Hipskind et al. 1994).

Sustained ERK activation is not always associated with proliferation (Okazaki and Sagata 1995; York et al. 2000; Boss et al. 2001; Garcia et al. 2001). In the rat pheochromocytoma cell line (PC12 cells) (Tischler and Greene 1975; Greene and Tischler 1976; Traverse et al. 1992; Marshall 1995), sustained activation of ERKs by NGF is required for the induction of neuronal differentiation and growth arrest (Cowley et al. 1994; Marshall 1995; Kao et al. 2001). In a recent report, c-fos was also shown to be required for NGF-dependent differentiation and neurite outgrowth of PC12 cells (Gil et al. 2004). It is possible that, despite differences in the cellular response to sustained activation of ERKs, PC12 cells and Swiss 3T3 cells share targets of sustained ERK activation.

Direct phosphorylation of c-fos protein by ERKs can also enhance c-fos function at AP-1 promoters (Sutherland et al. 1992; Monje et al. 2003). This occurs via two interdependent mechanisms. First, ERK-dependent phosphorylations on Ser362 and Ser374 within the C-terminus (called "priming" phosphorylations) can stabilize c-fos, possibly by interfering with degradation signals within the c-fos protein (Okazaki and Sagata 1995; Ferrara et al. 2003). The Ser374 site has been shown to be phosphorylated by ERKs *in vivo* and *in vitro* (Chen et al. 1996). However, ERK does not phosphorylate c-fos at Ser362 *in vitro* (Monje et al. 2003), and additional kinases have been proposed for the phosphorylation of the Ser362 site (Tratner et al. 1992; Chen et al. 1993). RSK2, an ERK-dependent kinase, has been implicated in growth factor-induced phosphorylation of this site. Most previous studies examining these sites within c-fos utilized c-fos double mutants that were mutated at both sites (Chen et al. 1993; Okazaki and Sagata 1995; Chen et al. 1996; Murphy et al.

2002; Murphy et al. 2004). By examining individual mutants of these sites, we show that phosphorylations at these sites have distinct contributions to c-fos stability and priming of c-fos for further phosphorylations.

Second, these phosphorylations prime additional ERK phosphorylations within the transactivation domain (TAD) of c-fos that Gutkind and colleagues have shown potentiate AP-1-dependent transcription (Monje et al. 2003). These phosphorylations on Thr325 and Thr331 (and possibly Thr232) are thought to contribute to the retarded electrophoretic mobility shift associated with elevated c-fos phosphorylation and function (Monje et al. 2003). TAD phosphorylation by ERKs may be enhanced by directing the binding of ERKs to an ERK targeting domain, also known as a DEF domain, which has been identified near these sites (Murphy et al. 2002).

In this study, we test whether c-fos is a sensor of sustained ERK activation in PC12 cells. We show that phosphorylations of Thr325 and Thr331 are required for maximal NGF-dependent transactivation of c-fos in PC12 cells. Like in fibroblasts, c-fos requires an intact DEF domain for transactivation. c-fos stabilization and conformational changes in protein structure are also regulated by ERK-dependent sites, namely Ser374 and Ser362, respectively. We provide evidence that sustained ERK activation is required for post-translational regulation of c-fos during differentiation of PC12 cells. Thus, our data suggest that both neuronal and non-neuronal cell types utilize c-fos as a sensor for ERK signaling duration despite the fact that this transcription factor is coupled to distinct physiological outcomes, differentiation and proliferation, respectively in these cell types.

4.3 Materials and methods

Materials

PC12 cells were kindly provided by Patrick Casey (Duke University, Durham, NC). The MEK1/2 inhibitor, U0126, was purchased from Calbiochem (La Jolla, CA). Nerve growth factor was purchased from Roche (Indianapolis, IN). Epidermal growth factor and anti-Flag (M2) antibody were purchased from Sigma (St. Louis, MO). Anti-ERK2 (C-14 & D-2) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Phosphorylation-specific ERK1/2 antibodies (Thr202/Tyr204) (#9101-polyclonal) and (#9106L-monoclonal) were purchased from Cell Signaling Technology (Beverly, MA). Anti-Fos (pSer374) antibodies (#ST1029) were purchased from Calbiochem (La Jolla, CA). LipofectAMINE 2000 was purchased from Invitrogen (Carlsbad, CA). All other chemical reagents were purchased from Sigma.

Cell culture

PC12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% horse serum and 5% fetal bovine serum at 37° C in 5% CO_2 (70% confluence). Unless otherwise stated, cells were deprived of serum for 16 hours before being treated with various reagents for luciferase assays, TransAM™ assays, and western blotting.

Plasmids and transfections

PC12 cells were transiently transfected with LipofectAMINE 2000 as per the manufacturers instructions. After transfection, cells were serum starved and treated with NGF (100ng/ml), EGF (50ng/ml), and U0126 (20μM), unless otherwise stated. Mouse c-fos cDNA (clone ID/ATCC # 3439554) was purchased from the I.M.A.G.E. Consortium/ATCC (Manassas, VA). c-fos was amplified by PCR using oligonucleotide primers and cloned with specific restriction enzyme sites (EcoR1/Xba). The resulting fragment was cloned into pcDNA3 (Invitrogen) containing a Flag epitope (Flag-Fos). The c-fos mutations were engineered into Flag-Fos by PCR site directed mutagenesis. All plasmids were verified by sequencing. The CMV promoter was used to drive expression of the Flag-Fos. To generate Gal4-Fos constructs, full-length c-fos and mutants were subcloned into a pcDNA3 vector encoding the DNA binding domain of the yeast transcription factor Gal4. A Gal4 regulated luciferase reporter (Gal4-luciferase) was provided by Richard Maurer (OHSU). For Western experiments, the following amounts of DNA were used: 2μg of Flag-c-fos and 2μg Flag-GFP, with pcDNA3 (vector) to make total amounts of transfected DNA equal. For luciferase assays, the following amounts of DNA were used: 250ng of Transin-luciferase, 500ng AP-1 luciferase (Stratagene, La Jolla, CA), 20ng pRL-Null (Promega, Madison, WI), 500ng of Gal4-c-fos plasmids, and 500ng vector. For TransAM™ assays, 10cm plates were transfected with 10μg of CMV-Flag-Fos plasmids or 10μg of vector.

AP-1-luciferase & transin-luciferase reporter gene assays

PC12 cells were plated onto 24 well plates and transfected the following day as indicated. Cells were serum starved and pretreated with U0126 or dimethylsulfoxide (DMSO) for 20

minutes, then treated with growth factors for 6 hours. Cells were lysed in luciferase lysis buffer (1% triton-X, 110mM K_2HPO_4, 15mM KH_2PO_4, pH 7.8) and equal amounts of protein were assayed for luciferase enzyme activity in an Autolumat LB953 luminometer (Berthold, Bundoora, Australia). 0.15mM luciferin (Sigma) in water was used as a substrate along with 5mM ATP, 15mM $MgSO_4$, 25mM gly-gly to drive the reaction. Data are expressed as relative light units (RLUs).

Gal4-luciferase reporter gene assays

PC12 cells were plated onto 24 well plates and transfected the following day as indicated. Cells were serum starved and treated with NGF for 6 hours. Cells were lysed in Passive Lysis Buffer (Promega, Madison, WI) and the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) was used to determine luciferase activity as per the manufacturers instructions. Luciferase values were measured in a Veritas Microplate Luminometer (Turner Biosystems, Sunnyvale, CA). Equal volumes of samples were analyzed and both firefly (*photinus pyralis*) and sea pansy (*Renilla reniformis*) luciferase were measured sequentially from the same. Final values are taken as a ratio of the firefly readings (experimental) over the sea pansy readings (internal control), and expressed as arbitrary units (AUs). Unless otherwise stated, each experiment was performed 3 times with 4 replicates each and all values normalized to the values of the NGF treated wild type Gal4-c-fos (Gal4-WT).

TransAM™ DNA binding assays

To determine DNA binding of Flag-c-fos and mutants, the AP-1 Transcription factor Assay Kit (Active Motif, Carlsbad, CA) was used with minor modifications to the manufacturers protocol. PC12 cells were plated onto 10cm plates and transfected the following day as indicated. Cells were treated with NGF for 2hours and nuclear extracts were prepared using the Nuclear Extract Kit (Active Motif, Carlsbad, CA) as per the manufacturers instructions. Protein concentrations were determined using the Coomassie Plus Bradford Assay kit (Pierce, Rockford, IL). Nuclear extract (20µg) was added to the matrix containing a TPA-response element (TRE) with the 5'-TGAC/GTCA-3' sequence. The primary antibody used in this ELISA-based assay was anti-Flag (M2) antibody (Sigma) in place of the manufacturer's primary antibody. Accordingly, an anti-mouse horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, Buckinghamshire, England) was used in place of the manufacturer's secondary antibody. Absorbances were read on a Labsystems Multiskan RC Microplate Reader (Fisher Scientific, Rochester, NY) as instructed. Data are generated from an average of three individual experiments normalized to NGF treated wild type Flag-c-fos samples (FosWT) (set at unity).

Lambda protein phosphatase experiments

PC12 cells were plated onto 6 well plates and transfected as indicated. Cells were pretreated with U0126 or vehicle (DMSO) for 20 minutes. Cells were treated with NGF for 2 hours and then lysed in Low Stringency Lysis Buffer (1x PBS, 0.1% NP40, 50mM β-glycerolphosphate, 10mM NaF, 1mM sodium orthovanadate, 10µg/ml aprotinin, 10µg/ml leupeptin, 1mM phenylmethylsulfonyl fluoride). 20µl reactions were prepared with equal

volumes of lysate with lambda protein phosphatase (New England Biolabs, Beverly, MA) as per the manufacturers instructions. Briefly, reactions were incubated at 30ºC for 20 minutes and laemmli buffer was added to stop the reactions. Some samples received water in place of phosphatase (DMSO and U0126 samples). Western blotting was then performed as described and Flag (M2) antibody was used to examine changes in the mobility shifts of Flag-Fos.

Western blotting

Unless otherwise stated, cells were deprived of serum for 16 hours prior to treatment. Cells were lysed in RIPA buffer (1% triton-X 100, 1% sodium deoxycholate, 0.2% sodium dodecyl sulfate, 125mM NaCl, 50mM Tris pH 8.0, 10% glycerol, 1mM EDTA, 25mM β-glycerolphosphate, 25mM NaF, 1mM sodium orthovanadate, 10µg/ml aprotinin, 10µg/ml leupeptin, 1mM phenylmethylsulfonyl fluoride) and equal amounts of lysate were sonicated in a bath sonicator (S-3000, Misonix INC., Farmingdale, NY) at level 6 for 30 seconds 3 times. Laemmli buffer was then added to the lysates and they were boiled for 2 minutes. Proteins were resolved by SDS-PAGE and two blotting techniques were employed. In one, proteins were transferred onto polyvinylidine difluoride membranes and blocked in 5% milk diluted in PBS + 0.1%Tween-20 (PBST). Membranes were probed with primary antibodies in PBST as per the manufacturer's instructions. Horseradish peroxidase-conjugated secondary antibodies were used to detect proteins by enhanced chemiluminescence (Western Lightning, PerkinElmer Life Sciences, Boston, MA). In the second method, proteins were transferred onto nitrocellulose (Protran, Schleicher & Schuell, Keene, NH) and blocked in Odyssey™ blocking buffer (LI-COR biosciences INC, Lincoln, NE).

Primary antibodies were diluted in Odyssey blocking buffer 1:1 with PBST, as per the manufacturers instructions. The appropriate fluorescently labeled secondary antibodies were diluted in Odyssey blocking buffer 1:1 with PBST, 0.01% SDS. Membranes were scanned with the Odyssey Infrared Imaging System (LI-COR biosciences, INC.).

4.4 Results

4.4a ERKs are required for AP-1-dependent gene expression by NGF

NGF and EGF are well known activators of ERKs in PC12 cells (Vaudry et al. 2002; Horgan and Stork 2003). However, these growth factors activate ERKs with very different kinetics. For comparison purposes, we chose concentrations of each growth factor that gave maximal ERK activation at 5minutes, a time point of activation shared by both NGF and EGF, and used those concentrations for the duration of this study (Fig. 4.1). One target of ERKs is the transcription factor c-fos, which participates in the activation of AP-1 promoter elements. Using AP-1 luciferase as a reporter of AP-1-dependent gene expression, we show that NGF, when compared with EGF, caused a more robust activation of AP-1-luciferase in PC12 cells. Although both growth factors had a statistically significant increase in AP-1 dependent gene expression, the effect elicited by NGF was greater than twice that of EGF. Both effects were dependent on activation of the ERK cascade as they were blocked by U0126, an inhibitor of the ERK kinase MEK (Fig. 4.2a). The ERK dependence of growth factor's actions on AP-1 is reflected in the induction of c-fos-response genes. One well-studied gene encodes the metalloprotease transin, which contains well-defined AP-1 sites within its promoter (Machida et al. 1991). Both NGF and EGF stimulated expression of the

transin promoter linked to luciferase. Although both effects were statistically significant, NGF activation of the transin promoter was roughly 5 times greater than that of EGF. Activation of the transin promoter by both NGF and EGF was blocked by U0126 (Fig. 4.2b). These data suggest that the differences between NGF and EGF may reflect differences in their ability to activate ERKs.

Figure 4.1

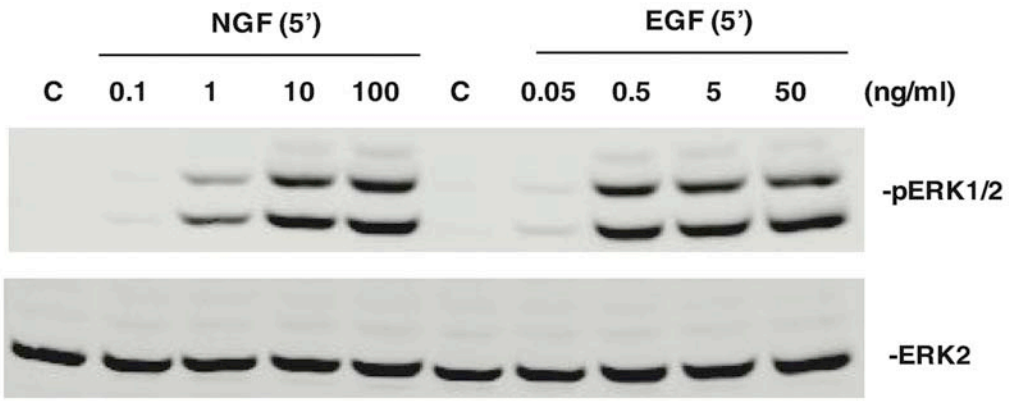
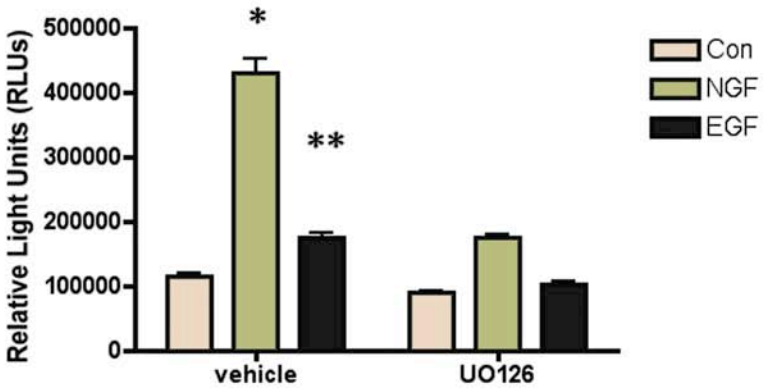


Figure 4.1 Dose dependent activation of ERK1/2 by growth factors. PC12 cells were treated with NGF and EGF at the indicated concentrations (ng/ml) for 5' and lysates were resolved by SDS-PAGE. Activation of ERK1/2 was measured using pERK Ab as shown in the top panel. Equal loading of protein was demonstrated by measuring levels of ERK2 (bottom panel). Maximal activation of ERK1/2 at 5' was maximal at 10ng/ml (NGF) and 0.5ng/ml (EGF).

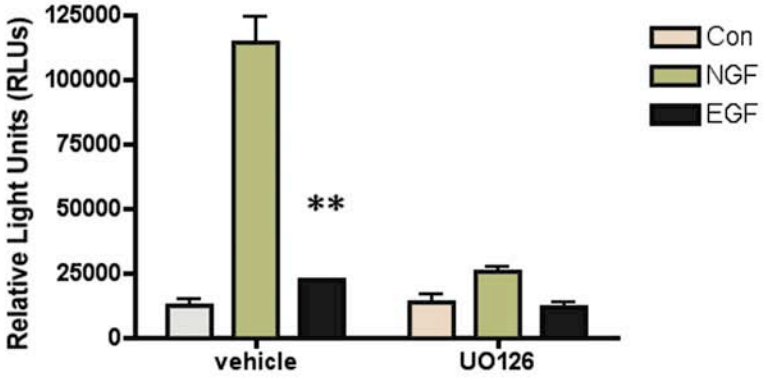
Figure 4.2

(a)



AP-1 luciferase

(b)



Transin luciferase

Fig. 4.2 NGF specific activation of AP-1-dependent gene expression requires ERKs in PC12 cells. (a) PC12 cells were transfected with an AP-1-luciferase plasmid (Stratagene). Cells were pretreated with U0126 or vehicle (DMSO) for 20 minutes prior to simulation with NGF or EGF (6 hours). Lysates were harvested for luciferase assay as described in materials and methods. Luciferase activity is shown as relative light units (RLUs). The data are means $\pm$ SE (n=4). Asterisks indicate significant differences compared to vehicle control conditions. The statistical significance was calculated by a one-tailed unpaired Student's t-test. Single asterisks (*) indicate p=0.0000045. Double asterisks (**) indicate p=0.00026. (b) PC12 cells were transfected with a Transin-luciferase plasmid. Cells were pretreated with U0126 or vehicle (DMSO) for 20 minutes prior to simulation with NGF or EGF (6 hours). Lysates were harvested for luciferase assay as described in materials and methods. The data are means $\pm$ SE (n=4). Asterisks indicate significant differences compared to vehicle control conditions. The statistical significance was calculated by a one-tailed unpaired Student's t-test. Single asterisks (*) equal p=0.00024. Double asterisks (**) indicate p=0.00529.

4.4b NGF induction of c-fos protein requires sustained activation of ERKs

One of the hallmarks of c-fos induction in fibroblast cells is the absence of c-fos protein in unstimulated cells (Muller et al. 1984). The more robust induction of AP-1 and the transin promoter by NGF, versus EGF, in PC12 cells may reflect differences in the levels of c-fos protein induced by these growth factors. We examined whether treatment with either NGF or EGF could result in the accumulation of c-fos protein in PC12 cells. NGF triggered a sustained activation of ERKs that remained readily detectable for 120 minutes (Fig. 4.3a, middle panel), and was still detectable at 6 hr (Fig. 4.4). c-fos protein was undetectable in resting PC12 cells and was induced within 60 min of NGF treatment and remained detectable at 120 minutes (Fig. 4.3a, top panel). U0126 dramatically reduced the level of c-fos protein induced by NGF (Fig. 4.3b, top panel). In contrast to NGF, EGF activation of ERKs in PC12 cells was robust but transient, returning close to baseline levels after 10 minutes (Fig. 4.3a, middle panel), and remained at basal levels for at least 6 hr (Fig. 4.4). This transient activation was much less efficient at inducing detectable levels of c-fos protein (Fig. 4.3a, top panel).

Figure 4.3

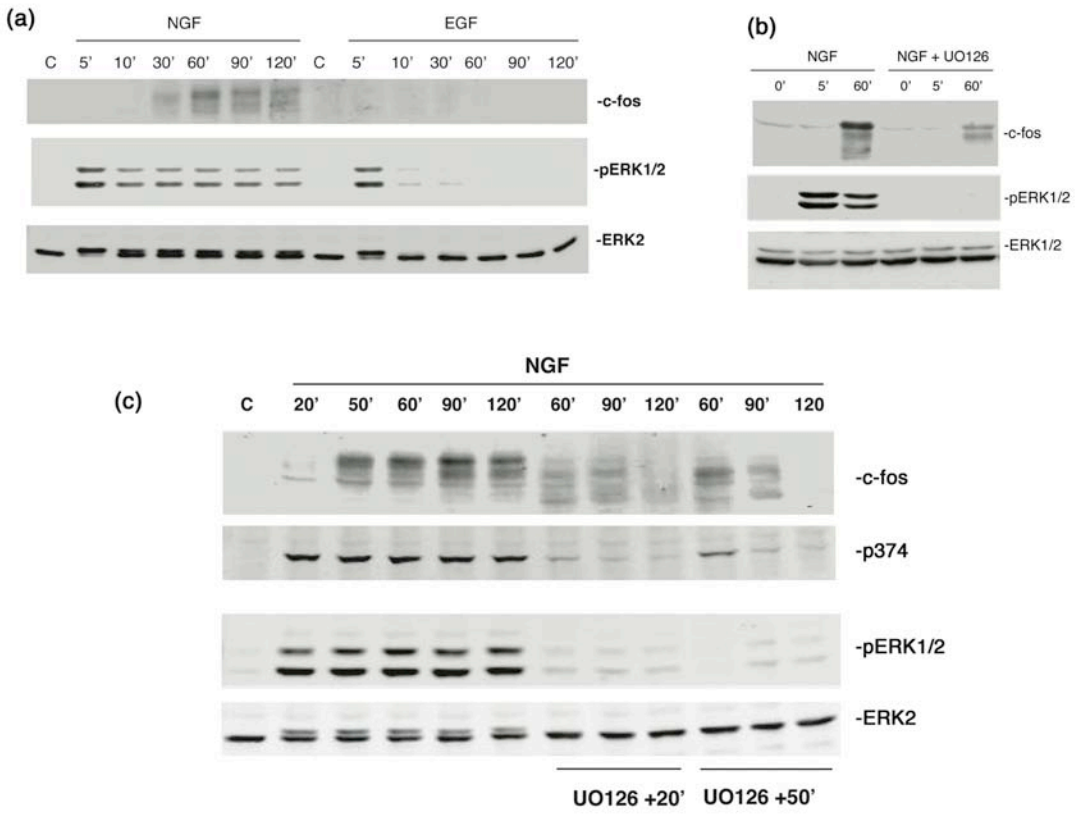

(a)
NGF
EGF
C 5' 10' 30' 60' 90' 120' C 5' 10' 30' 60' 90' 120'
-c-fos
-pERK1/2
-ERK2
(b)
NGF
NGF + UO126
0' 5' 60' 0' 5' 60'
-c-fos
-pERK1/2
-ERK1/2
(c)
NGF
C 20' 50' 60' 90' 120' 60' 90' 120' 60' 90' 120
-c-fos
-p374
-pERK1/2
-ERK2
UO126 +20'
UO126 +50'

Fig. 4.3 NGF stimulation of c-fos protein levels requires MEK. (a) PC12 cells were treated with NGF and EGF and the lysates were resolved by SDS-PAGE. Induction of c-fos protein was detected by SDS-PAGE at the indicated time points (top panel). ERK1/2 activation, measured by phospho-ERK1/2 antibody (pERK Ab), is shown in the middle panel. Equal loading of protein was demonstrated by measuring levels of ERK2 (bottom panel). (b) Cells were pretreated with U0126 or vehicle (DMSO) for 20 minutes prior to stimulation with NGF. Cells were harvested at the indicated times and c-fos protein levels are shown in the top panel. Activation of ERK1/2 was measured using pERK Ab (middle panel). Equal loading of protein was demonstrated by measuring levels of ERK1/2 (bottom panel). (c) PC12 cells were treated with U0126 before NGF application (-10′) and after NGF application (+20′ and +50′). c-fos protein levels were measured using c-fos antibodies (first panel). Phosphorylation of c-fos on residue Ser374 is measured using Anti-Fos (pSer374) antibodies (second panel). Activation of ERK1/2 was measured using pERK Ab (third panel). Equal loading of protein was demonstrated by measuring levels of ERK2 (fourth panel).

Figure 4.4

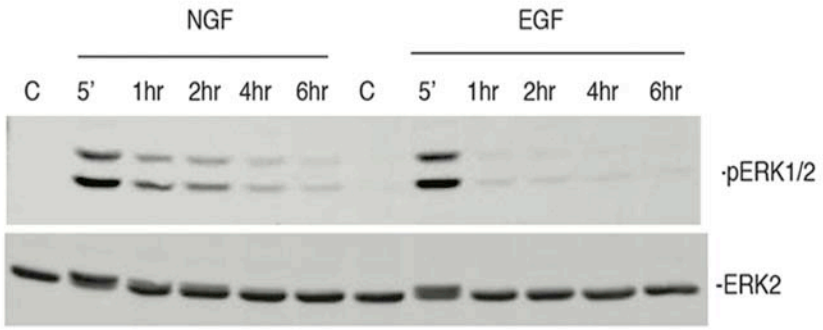


Figure 4.4 Extended timecourse of ERK1/2 activation by growth factors. PC12 cells were treated with NGF (100ng/ml) and EGF (50ng/ml) for the indicated time points. Lysates were resolved by SDS-PAGE and ERK1/2 activation was measured using pERK Ab as shown in the top panel. Equal loading of protein was demonstrated by measuring levels of ERK2 (bottom panel).

Sustained activation of ERKs has recently been proposed to mediate c-fos protein stabilization (Murphy et al. 2002). To determine whether sustained activation of ERKs is required for NGF's ability to accumulate detectable levels of the c-fos protein, we incubated cells with U0126 at various time points after NGF treatment. Pretreatment of cells with U0126 (-10') inhibited NGF-dependent increases in c-fos protein levels (Fig. 4.3b, top panel). Interestingly, inhibition of ERKs at times (+20', +50') subsequent to NGF treatment also causes a marked inhibition of c-fos protein levels (Fig 4.3c, first panel). In fibroblasts, Ser374 phosphorylation has been shown to be ERK-dependent and involved in c-fos protein stabilization (Chen et al. 1996). U0126 application causes inhibition of NGF-dependent Ser374 phosphorylation in PC12 cells (Fig. 4.3c, second panel). These data suggest that sustained ERK signaling enables NGF to stimulate and maintain c-fos protein levels and phosphorylation.

4.4c NGF stabilization of c-fos requires ERKs

ERK stimulates the induction of c-fos protein by both transcriptional and post-translational mechanisms. To specifically examine these post-transcriptional effects, independent of transcriptional effects, we examined the expression of an epitope tagged c-fos construct (Flag-Fos) under the control of the CMV promoter. Initial experiments using Flag-GFP under control of the CMV promoter demonstrated that NGF did not regulate this promoter as shown by equal levels of Flag-GFP protein (Fig. 4.5a, top panel). In addition, U0126 had no effect on basal Flag-GFP levels (data not shown). In PC12 cells, low levels of Flag-Fos were detectable in the absence of stimulation due to basal activation of the CMV promoter. NGF treatment caused an increase in Flag-Fos protein levels that was evident at 2 hours

(Fig. 4.5b, top panel). In addition, NGF induced mobility shifts previously associated with hyperphosphorylation of c-fos (Monje et al. 2003). The ERK-dependence of these mobility shifts was confirmed by pretreating cells with U0126, which resulted in the loss of all but the lowest (fastest) migrating band (Fig. 4.5c, top panel). The phosphorylation-dependence of these shifts was confirmed by their absence upon treatment of lysates with lambda phosphatase (Fig. 4.5c, top panel). In contrast, EGF stimulation had a minor, albeit significant effect, on stabilization and mobility shift of Flag-Fos (Fig. 4.5b, top panel). Flag-GFP expression levels were constant and served as an internal control (Fig. 4.5b, bottom panel). Thus, we focused on NGF's regulation of c-fos in the remainder of the study.

Figure 4.5

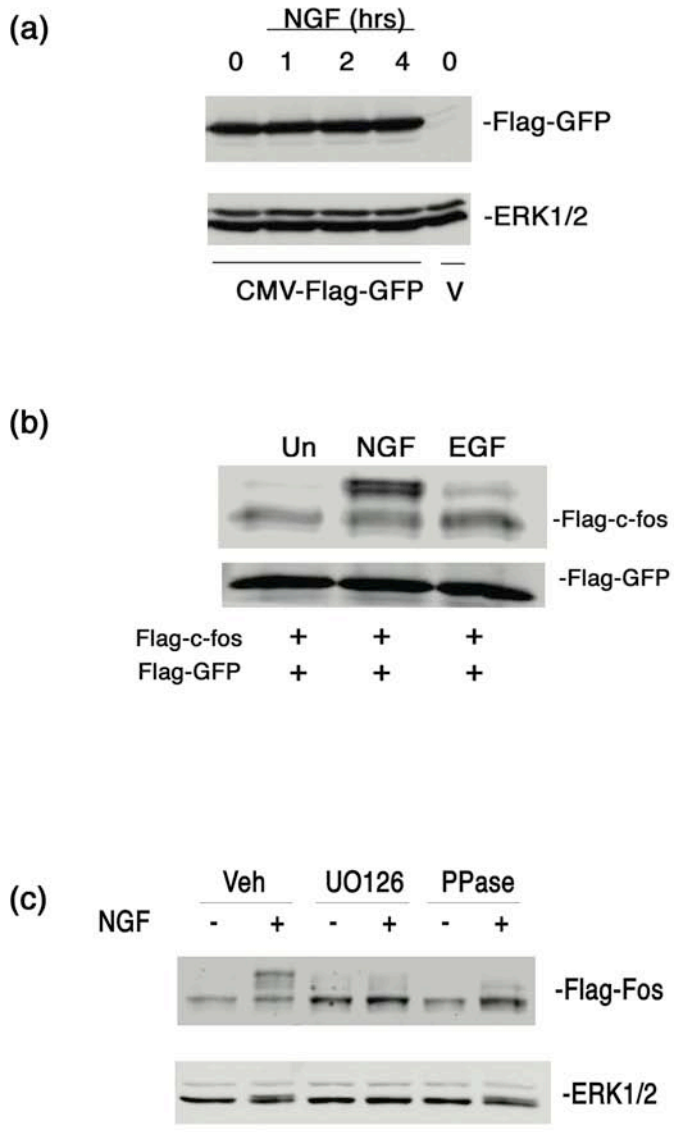

(a)
NGF (hrs)
0 1 2 4 0
-Flag-GFP
-ERK1/2
CMV-Flag-GFP V
(b)
Un NGF EGF
-Flag-c-fos
-Flag-GFP
Flag-c-fos + + +
Flag-GFP + + +
(c)
Veh UO126 PPase
NGF - + - + - +
-Flag-Fos
-ERK1/2

Fig. 4.5 Sustained ERK1/2 activation regulates Flag-c-fos phosphorylation and protein levels. (a) PC12 cells were transfected with equal amounts of Flag-GFP plasmids under the control of the CMV promoter. Cells were treated with NGF for 4 hours and lysed at the indicated time points. Vector (V) DNA was used as a negative control. Levels of Flag-GFP were determined using anti-Flag antibodies (top panel). Levels of ERK1/2 are provided to demonstrate equal loading of total protein (bottom panel). (b) PC12 cells were transfected with both Flag-c-fos (Flag-Fos) and Flag-GFP and stimulated with NGF or EGF for 2 hours. Western blotting was performed with anti-Flag antibodies to detect levels of both Flag-Fos (top panel) and Flag-GFP (bottom panel). (c) PC12 cells were transfected with Flag-Fos and pretreated with U0126 or vehicle for 20 minutes. Cells were treated with NGF for 2 hours. Lambda phosphatase reactions were then performed on lysates as described in materials and methods. Flag-Fos protein levels and mobility shifts were measured using Flag antibodies (top panel). Levels of ERK1/2 are provided to demonstrate equal loading of total protein (bottom panel).

4.4d Thr325 and Thr331 are required for c-fos activation by NGF

In fibroblasts, Thr325 and Thr331 have been shown to be important for c-fos function (Murphy et al. 2002). We examined whether phosphorylation of these residues was responsible for any of the mobility shifts of Flag-Fos induced by NGF. We mutated both residues to alanine, both together and as single mutations, to inhibit potential phosphorylation at these sites (Flag-Fos-T325A/T331A, Flag-Fos-T331A, and Flag-Fos-T325A referred herein as T325A/T331A, T331A, and T325A, respectively) and compared mobility shifts of wild type Flag-Fos (WT) with this panel of mutants in response to stimulation by NGF. The band with the highest (slowest) mobility was lost in the T325A/T331A mutant demonstrating that phosphorylation of these residues are required for the conformational change in the protein associated with this mobility (Fig. 3.6a, first panel). However, NGF did increase the protein levels of the mutant. Moreover, it also induced an intermediate shift. This suggests that other sites can contribute to the stability of c-fos and produce a protein of intermediate mobility. Both T331A and T325A had similar mobility shifts and protein levels as WT demonstrating that phosphorylation of either Thr325 or Thr331 are sufficient for migration at the slowest mobility. Flag-GFP levels are shown as an internal control (Fig. 4.6a, second panel). Activation of ERK1/2 is shown (Fig. 4.6a, third panel) as well as total levels of ERK2 (Fig. 4.6a, fourth panel).

We then examined whether these phosphorylations were responsible for c-fos activation. Gal4-Fos fusion proteins are established tools to examine transactivation of c-fos (Monje et al. 2003). We constructed Gal4-Fos fusion proteins encoding full-length wild-type c-fos (Gal4-WT) full length T331A (Gal4-T331A), full length T325A (Gal4-

T325A), and full length T325A/T331A (Gal4-T325A/T331A) and measured transactivation of a Gal4-luciferase reporter construct as reported (Monje et al. 2003). In this manner, luciferase activity will only reflect activation by transfected c-fos constructs, with minimal interference from endogenous c-fos. In this assay, NGF induced strong transactivation of Gal4-WT but was unable to activate the threonine double mutant (Gal4-T325A/T331A) (Fig. 4.6b). Gal4-T331A has about 40% of the activity of Gal4-WT in response to NGF while Gal4-T325A activation is barely over basal levels (Fig. 4.6b). The data suggest that both Thr325 and Thr331 are required for full activation of c-fos by NGF in PC12 cells, although Thr325 seems to play a greater role.

Figure 4.6

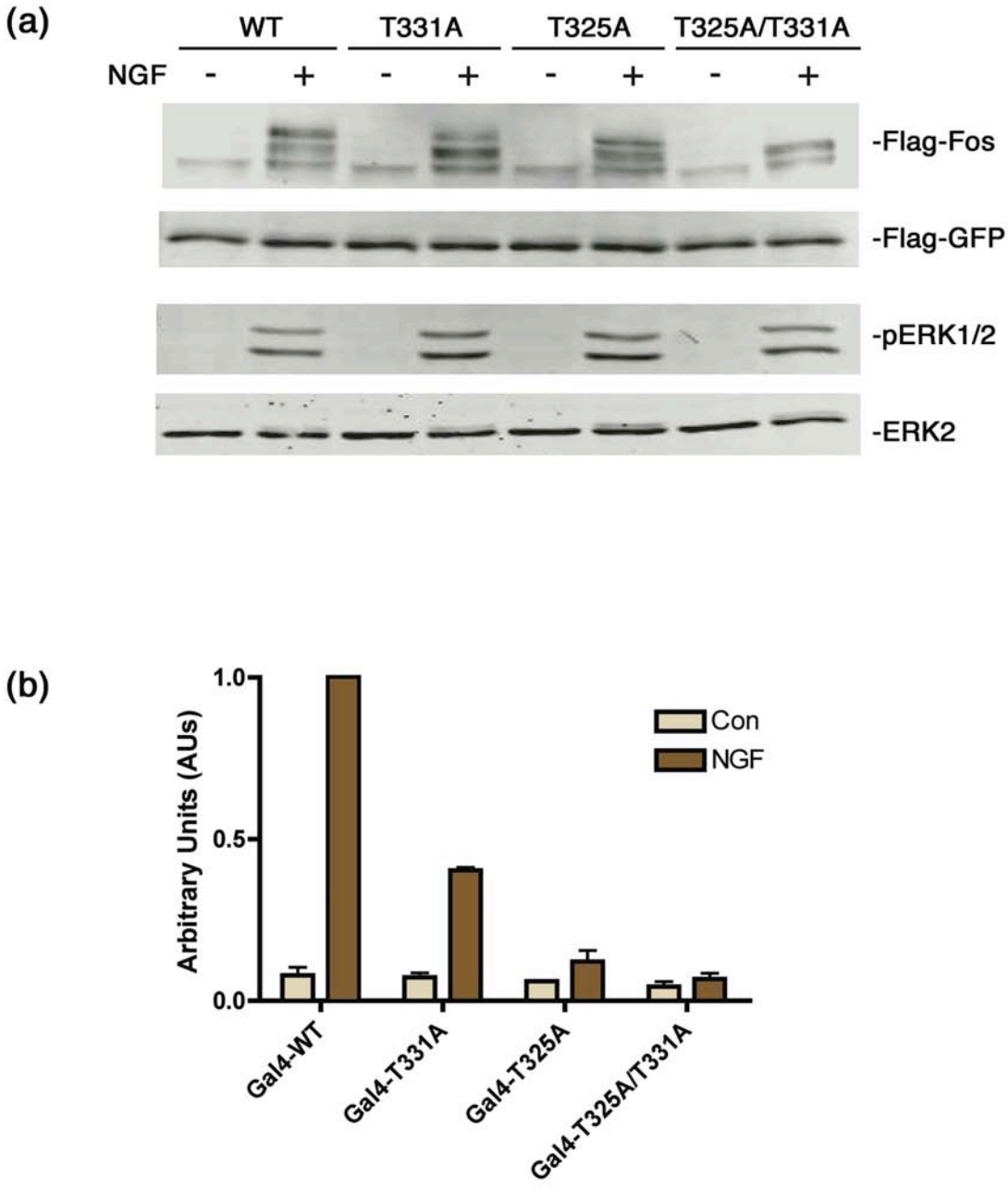

(a)
WT
T331A
T325A
T325A/T331A
NGF - + - + - + - +
-Flag-Fos
-Flag-GFP
-pERK1/2
-ERK2
(b)
Arbitrary Units (AUs)
1.0
0.5
0.0
Con
NGF
Gal4-WT
Gal4-T331A
Gal4-T325A
Gal4-T325A/T331A

Fig. 4.6 Thr325 and Thr331 are required for c-fos transactivation by NGF. (a) PC12 cells were transfected with Flag-c-fos wild type (WT), Flag-c-fos T331A (T331A), Flag-c-fos T325A (T325A), Flag-c-fos T325A/T331A (T325A/T331A), and Flag-GFP. Cells were stimulated with NGF for 2 hours and western blotting was performed with anti-Flag antibodies to detect levels of Flag-Fos (first panel) and Flag-GFP (second panel). Activation of ERK1/2 was measured using pERK Ab (third panel). Equal loading of protein was demonstrated by measuring levels of ERK2 (fourth panel). (b) PC12 cells were transfected with Gal4-luciferase, pRL-Null (*Renilla* control), Gal4-WT, Gal4-T331A, Gal4-T325A, and Gal4-T325A/T331A. Cells were treated with NGF for 6 hours, harvested, and luciferase activity was measured as described in materials and methods. The data presented are an average of three independent experiments with four replicates each. The data are means $\pm$ SE. All values were then normalized to NGF treated Gal4-WT (set at unity). Luciferase activity is measured as arbitrary units (AUs) representing the ratio of firefly light units/*Renilla* light units.

c-fos contains an ERK binding site known as a DEF domain (docking site for ERK, F/Y-X-F/Y-P) (Dimitri et al. 2005). Previous reports in fibroblasts have suggested that binding of ERK to the DEF domain is required for subsequent phosphorylation of Thr325 residue and c-fos transactivation (Murphy et al. 2002). ERK binding can be inhibited by mutating the functional DEF domain ($F^{343}TYP$) to $A^{343}TAP$. Using this mutant (FosDEF), we compared its mobility with that of FosWT. In response to NGF, FosDEF was shifted to high mobilities, although the highest mobility seen with FosWT was not apparent (Fig. 4.7a). This is consistent with the reported loss of Thr325 phosphorylation in DEF mutants (Murphy et al. 2002). We then examined if DEF-dependent phosphorylations were required for c-fos activation using the Gal4-luciferase reporter gene assay. Interestingly, like T325A, activation of the DEF mutant (Gal4-DEF) was drastically reduced in response to NGF when compared with wild type (Gal4-WT) (Fig. 4.7b). Therefore, the DEF domain is required for full activation of c-fos by NGF in PC12 cells through its participation in phosphorylation of downstream sites.

Figure 4.7

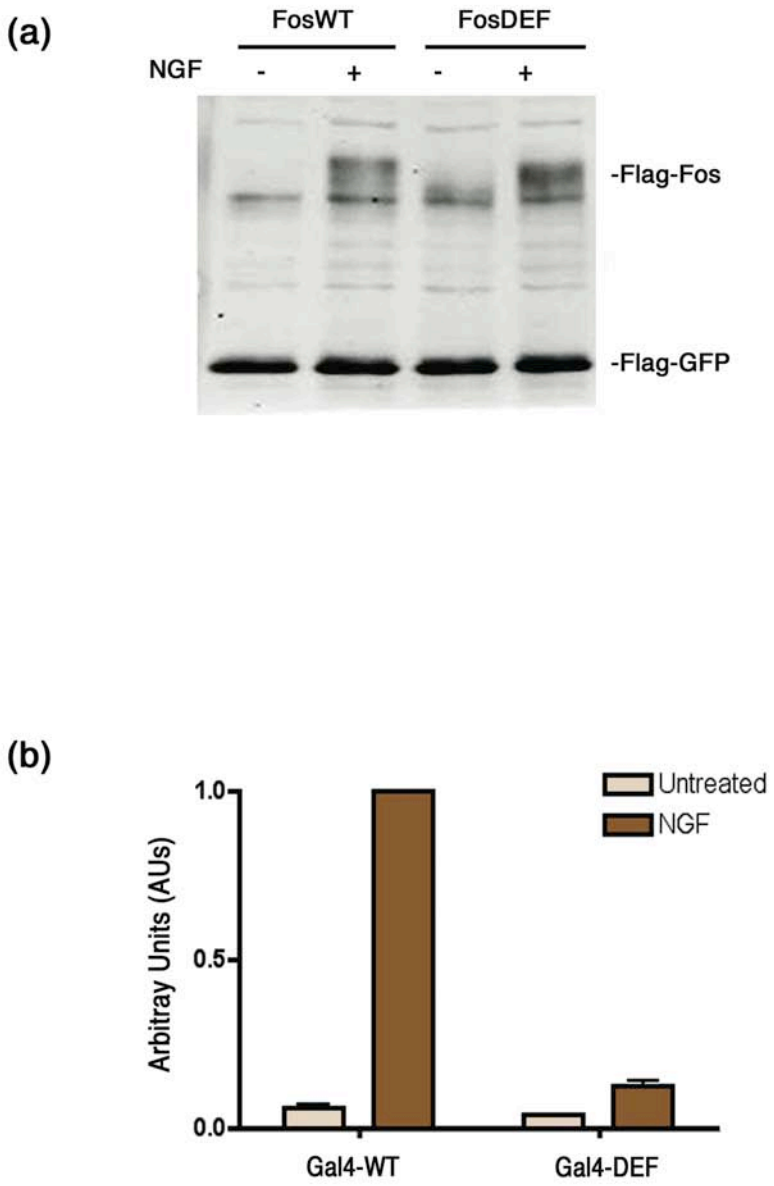

(a)
FosWT
FosDEF
NGF
-
+
-
+
-Flag-Fos
-Flag-GFP
(b)
Untreated
NGF
Arbitray Units (AUs)
1.0
0.5
0.0
Gal4-WT
Gal4-DEF

Fig. 4.7 The DEF domain of c-fos is required for maximal phosphorylation and transactivation by NGF. (a) PC12 cells were transfected with Flag-Fos-WT, Flag-FosDEF, and Flag-GFP as indicated. Cells were stimulated with NGF for 2 hours and western blotting was performed. Anti-Flag antibody was used to detect levels of FosWT and FosDEF and Flag-GFP. (b) PC12 cells were transfected with Gal4-luciferase, pRL-Null, Gal4-WT, and Gal4-DEF. Cells were treated with NGF for 6 hours, harvested, and luciferase activity was measured as described in materials and methods. The data presented are an average of three independent experiments with 4 replicates each. The data are means $\pm$ SE. All values were then normalized to NGF treated Gal4-WT (set at unity). Luciferase activity is measured as arbitrary units (AUs) representing the ratio of firefly light units/*Renilla* light units.

4.4e Priming sites mediate ERK-dependent stability

To further examine which phosphorylations are required for the intermediate shift we treated cells transfected with T325A/T331A with U0126. Treatment with U0126 inhibited the intermediate mobility of T325A/T331A induced by NGF (Fig. 4.8a). This suggests that additional ERK-dependent phosphorylations are also induced by NGF in PC12 cells. Two ERK-dependent phosphorylation sites, Ser362 and Ser374, have been shown to be important for stabilization and c-fos-dependent transformation in fibroblasts (Okazaki and Sagata 1995; Chen et al. 1996; Murphy et al. 2002). These sites do not require ERK docking to the DEF domain and have been termed "priming sites" for subsequent phosphorylations on Thr325 and Thr331 (Murphy et al. 2002). We mutated these serine residues to alanine (FosAA) and compared mobility shifts and stabilization with FosWT. FosAA was unable to undergo any mobility shift and protein levels were poorly stabilized by NGF treatment (Fig. 4.8b, first panel). Activation and total levels of ERK1/2 are shown (Fig. 4.8b, second and third panels, respectively). In order to compare DNA binding of FosAA to FosWT, we used a modified ELISA-based assay (TransAM ™) that measures Flag-Fos binding to an AP-1 consensus sequence. As expected, FosAA bound to an AP-1 target sequence poorly compared to FosWT upon NGF stimulation consistent with the modest effect of NGF on protein stabilization of this mutant (Fig. 4.8c).

Figure 4.8

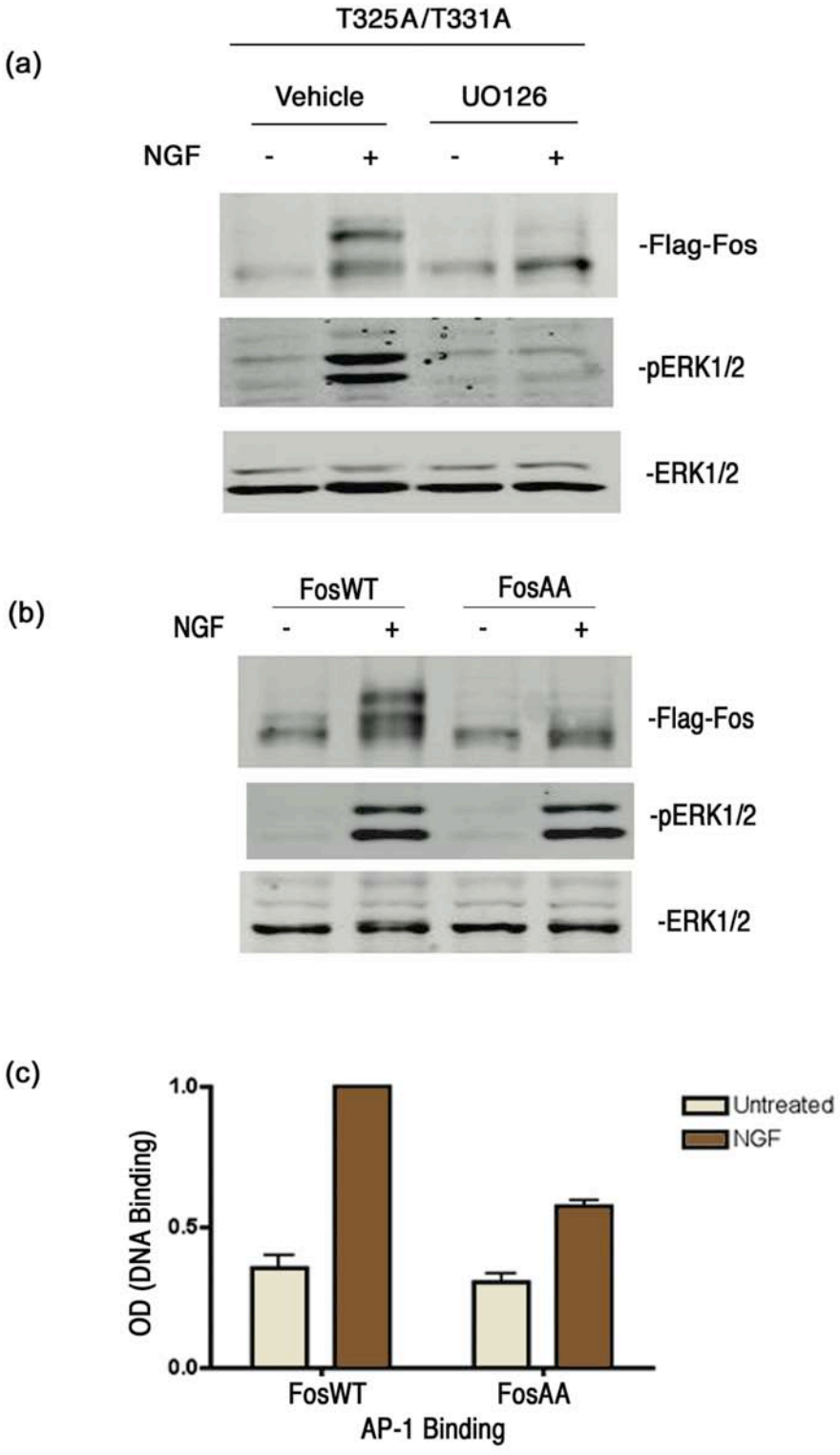

(a)
T325A/T331A
Vehicle
UO126
NGF
-
+
-
+
-Flag-Fos
-pERK1/2
-ERK1/2
(b)
FosWT
FosAA
NGF
-
+
-
+
-Flag-Fos
-pERK1/2
-ERK1/2
(c)
1.0
0.5
0.0
OD (DNA Binding)
Untreated
NGF
FosWT
FosAA
AP-1 Binding

Fig. 4.8 Ser362 and Ser374 are required for c-fos stabilization by NGF. (a) PC12 cells were transfected with T325A/T331A as indicated. Cells were pretreated with vehicle (DMSO) or U0126 for 20 minutes prior to stimulation with NGF for 2 hours. Western blotting was performed and Flag-Fos protein levels and mobility shifts were measured using anti-Flag antibodies (top panel). ERK1/2 activation and inhibition, measured by pERK Ab, is shown in the middle panel. Levels of ERK1/2 are provided to demonstrate equal loading of total protein (bottom panel). (b) As indicated, PC12 cells were transfected with FosWT and Flag-c-fos-S362A/S374A (FosAA). Cells were either left untreated or stimulated with NGF for 2 hours. Western blotting was performed using anti-Flag antibodies to detect levels of Flag-Fos (top panel). Activation of ERK1/2 was measured using pERK Ab (middle panel). Equal loading of protein was demonstrated by measuring levels of ERK1/2 (bottom panel). (c) As indicated, PC12 cells were transfected with FosWT or FosAA. Cells were treated with NGF for 2 hours and nuclear extracts were isolated as described in materials and methods. DNA binding was measured using the TransAM™ AP-1 Transcription factor Assay Kit (Active Motif, Carlsbad, CA) as described in materials and methods. The data presented are an average of three independent experiments. The data are means $\pm$ SE. All values were then normalized to NGF treated FosWT (set at unity).

In addition, we mutated both Ser362 and Ser374 to aspartic acids (FosDD) to mimic phosphorylation at these sites, and the effect on Flag-c-fos protein levels was examined. FosDD was stable basally and migrated at an intermediate mobility (Fig. 4.9a, top panel). Stimulation with NGF caused FosDD to migrate at the highest mobility, similar to FosWT. FosDD, in cells treated with U0126, did not show this highest mobility, but displayed intermediate mobilities seen in untreated cells. This demonstrates that these additional phosphorylations are indeed ERK-dependent (Fig. 4.9a, top panel). Taken together, the data suggest that mimicking phosphorylation of the two priming sites is sufficient to mimic stabilization of c-fos by NGF. Moreover, the intermediate mobility seen basally in FosDD resembles the intermediate mobility seen after NGF treatment of the T325A/T331A mutant, suggesting that phosphorylation of these priming sites represents an intermediate state of c-fos phosphorylation prior to full activation achieved by subsequent phosphorylation of Thr325 and Thr331.

We then examined the relative contribution of each priming site to both stabilization and to migration at the intermediate mobility. Table 4.1 lists the mutants at Ser362 and Ser374 that were analyzed. To determine whether mimicking phosphorylation of Ser374 provided a stabilizing signal that was independent of the status of Ser362, we examined the mutants FosSD and FosAD (Fig. 4.9b, top panel). Both mutants were basally stable; demonstrating that by mimicking phosphorylation of Ser374 stabilized c-fos even in the absence of phosphorylation of Ser362. NGF induced a shift of the majority of FosSD, but only a small fraction of FosAD, consistent with the participation of phosphorylation of Ser362 in the shift. In order to directly test whether Ser374 is required for stabilization of c-fos by NGF, we mutated Ser374 to alanine to inhibit phosphorylation at this site (FosSA).

In response to NGF, FosSA is much less stable than FosWT, although its mobility shifts are unchanged (Fig. 4.9c, first panel). Taken together, these data support a model where phosphorylation of Ser374 appears to be primarily responsible for the stability. Phosphorylation of Ser362 contributes significantly to the intermediate mobility shift.

Table 4.1

Flag-c-fos construct	residue 362	residue 374
FosWT	S	S
FosAA	A	A
FosAS	A	S
FosSA	S	A
FosDD	D	D
FosDS	D	S
FosSD	S	D
FosAD	A	D
FosDA	D	A

Table 4.1 Analysis of Ser362 and Ser374 Flag-c-fos mutants. This table provides a key to determine the identity of Flag-c-fos mutants corresponding to Ser362 and Ser374. FosWT (wild type), FosAA, (S362A, S374A), FosSA (S374A), FosDD (S362D, S374D), FosDS (S362D), FosSD (S374D), FosAD (S362A, S374D), and FosDA (S362D, S374A). S=serine, D=aspartate, and A=alanine.

Figure 4.9

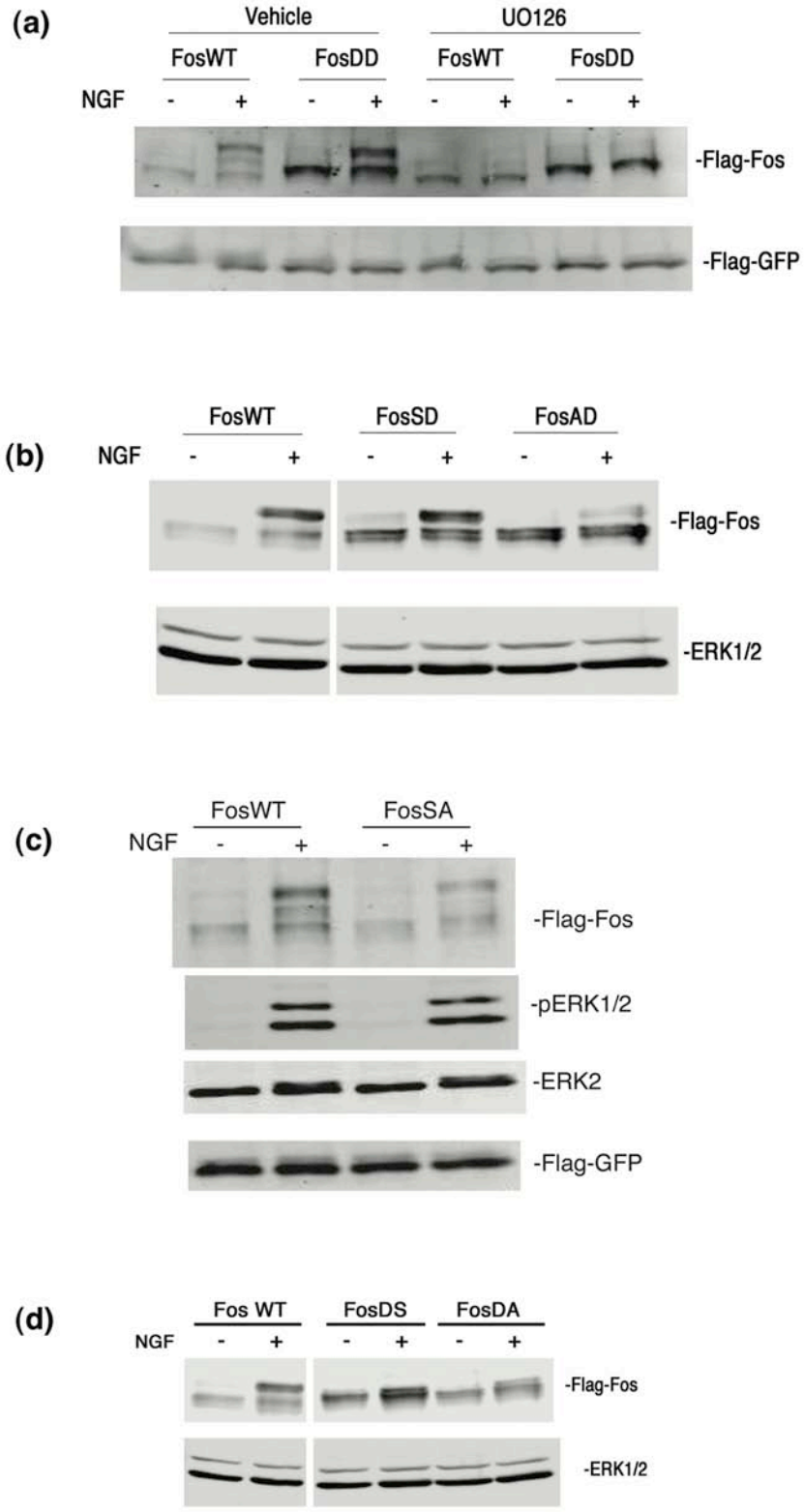

(a)
Vehicle
UO126
FosWT
FosDD
FosWT
FosDD
NGF
- + - + - + - +
-Flag-Fos
-Flag-GFP
(b)
FosWT
FosSD
FosAD
NGF
- + - + - +
-Flag-Fos
-ERK1/2
(c)
FosWT
FosSA
NGF
- + - +
-Flag-Fos
-pERK1/2
-ERK2
-Flag-GFP
(d)
Fos WT
FosDS
FosDA
NGF
- + - + - +
-Flag-Fos
-ERK1/2

Fig. 4.9 Mimicking phosphorylation on Ser374 produces mutant proteins that are basally stable. The designations for the mutants are listed in Table 4.1. (a) PC12 cells were transfected with FosWT, FosDD, and Flag-GFP as indicated. Cells were pretreated with vehicle (DMSO) or U0126 for 20 minutes prior to stimulation with NGF (+) for 2 hours. Western blotting was performed using anti-Flag antibodies to detect levels of Flag-Fos (top panel) and Flag-GFP (bottom panel). (b) PC12 cells were transfected with FosWT, FosSD, and FosAD. Cells were left untreated (-) or treated with NGF for 2 hr. (+). Total levels of transfected proteins were examined using anti-Flag antibodies (top panel). ERK 1/2 levels were measured to confirm equal protein loading (bottom panel). (c) PC12 were transfected with FosWT or FosSA, and cells were left untreated (-) or treated with NGF for 2 hr (+). Western blotting was performed using anti-Flag antibodies to detect levels of Flag-Fos (first panel) and Flag-GFP (fourth panel). Activation of ERK1/2 was measured using pERK Ab (second panel). Equal loading of protein was demonstrated by measuring levels of ERK2 (third panel). (d) PC12 cells were transfected with FosWT, FosDS, and FosDA, and cells were left untreated (-) or treated with NGF for 2 hr. (+). Total levels of transfected proteins were examined using anti-Flag antibodies (top panel). ERK 1/2 levels were measured to confirm equal protein loading (bottom panel).

To determine whether mimicking phosphorylation of Ser362 could induce a mobility shift independently of Ser374, we compared the mutants FosDS and FosDA (Fig. 4.9d, top panel). As expected, NGF increased the stability of only FosDS, but not FosDA. In both mutants, the presence of an aspartate at position Ser362 produced a c-fos protein displaying an intermediate mobility in the absence of stimulation. Upon NGF stimulation, the migration of both mutants was further retarded to the highest shifted forms. Together these data strongly support the role of pSer374 in NGF induced c-fos stability, and suggest that priming of additional phosphorylations can occur in the absence of pSer374. We propose a model whereby c-fos is induced at the transcriptional level by growth factor stimulation (Fig. 4.10). Newly translated c-fos proteins undergo a series of phosphorylations that allows c-fos to activate AP-1-dependent genes. Firstly, phosphorylation of Ser374 stabilizes c-fos allowing protein levels to accumulate in the cell. Ser362, another ERK-dependent site, is phosphorylated which changes the conformational structure of the protein, seen as the intermediate mobility shift. Presumably, these 2 'priming' phosphorylations allow for binding of ERKs to the DEF domain of c-fos. Finally, phosphorylation of Thr325 and Thr331, and possibly other sites (Thr232), induce c-fos transactivation. This sequence of phosphorylations provides a mechanism of how sustained activation of ERKs by NGF is required for maximal activity of c-fos.

Figure 4.10

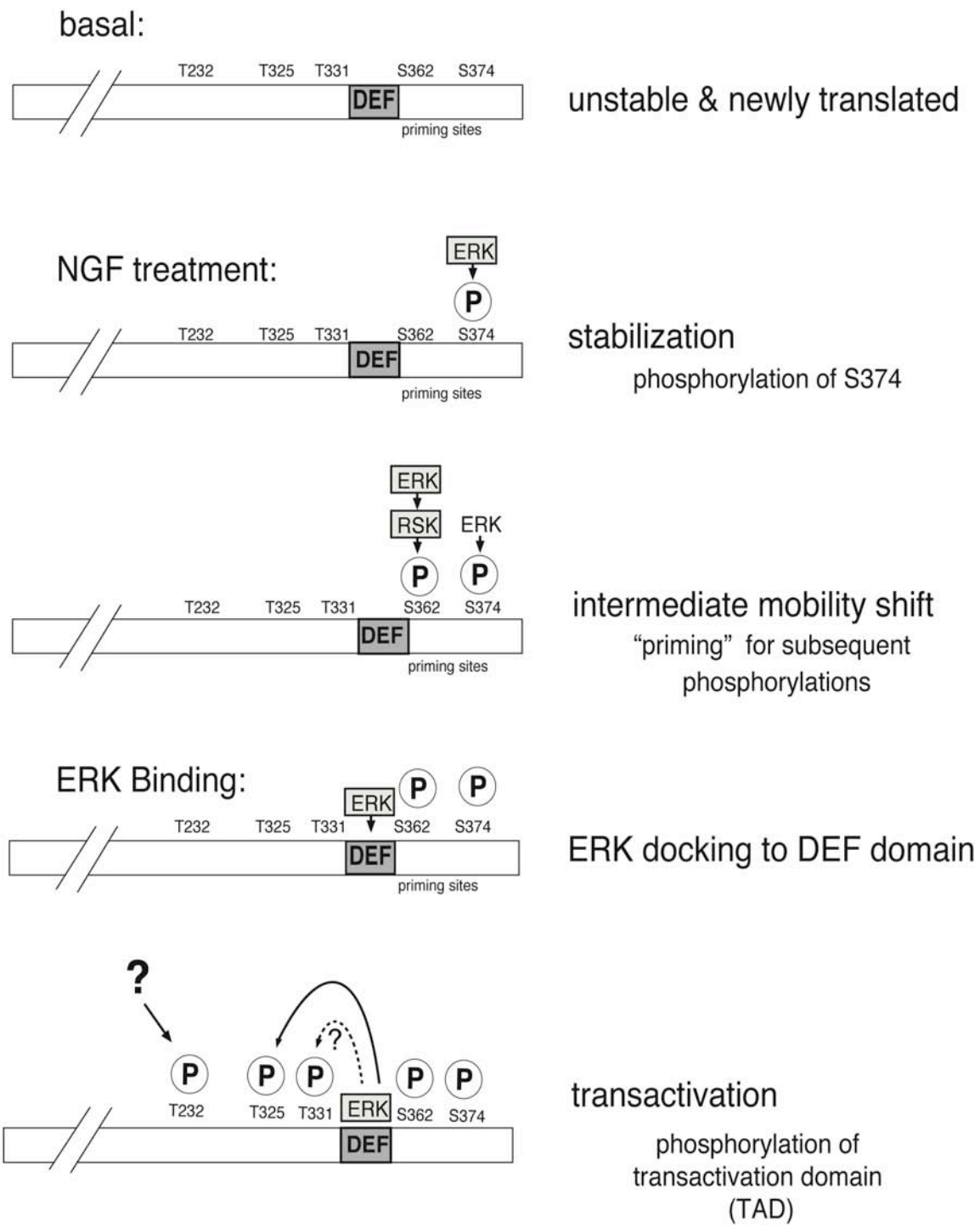

basal:
T232
T325
T331
S362
S374
DEF
priming sites
unstable & newly translated
NGF treatment:
ERK
P
stabilization
phosphorylation of S374
RSK
intermediate mobility shift
"priming" for subsequent phosphorylations
ERK Binding:
ERK docking to DEF domain
?
transactivation
phosphorylation of transactivation domain (TAD)

Fig. 4.10 Proposed model of NGF-dependent c-fos transactivation. c-fos is basally maintained in an unstable state. Maximal stabilization by NGF is achieved by the ERK-dependent phosphorylation of Ser374, which stabilizes c-fos (Chen et al. 1993), possibly by blocking PEST sequences within this region (Ferrara et al. 2003). The phosphorylation of Ser362, which is thought to occur via the ERK-dependent kinase RSK, contributes to the intermediate mobility shift of c-fos. ERK binding to the DEF domain is required for priming of additional phosphorylations including Thr325 and possibly Thr331 (Murphy et al. 2002). Thr232, another site involved in c-fos transactivation, is phosphorylated by an unknown kinase and recent evidence suggests that it may not be ERK2 (Monje et al. 2003).

4.4f c-fos requires constant ERK activity to maintain a hyperphosphorylated state

We propose that sustained activation of ERKs is required to induce the sequence of phosphorylations that are required for maximal activation of c-fos protein. We speculated that EGF might be able to fully activate FosDD since it's already primed and stabilized. Indeed, transient ERK activation by EGF induced a transient mobility shift in both FosWT and FosDD. However, although FosDD was more stable than FosWT at all time points, the shift rapidly returned to basal levels (Fig. 4.11a, top panel) as ERK levels returned to baseline (bottom panel). This transient effect was insufficient to activate c-fos as seen by EGF's inability to activate Gal4-DD (Fig. 4.11b). As a result, although FosDD is basally stable, sustained phosphorylation is still required for maximal c-fos function.

Figure 4.11

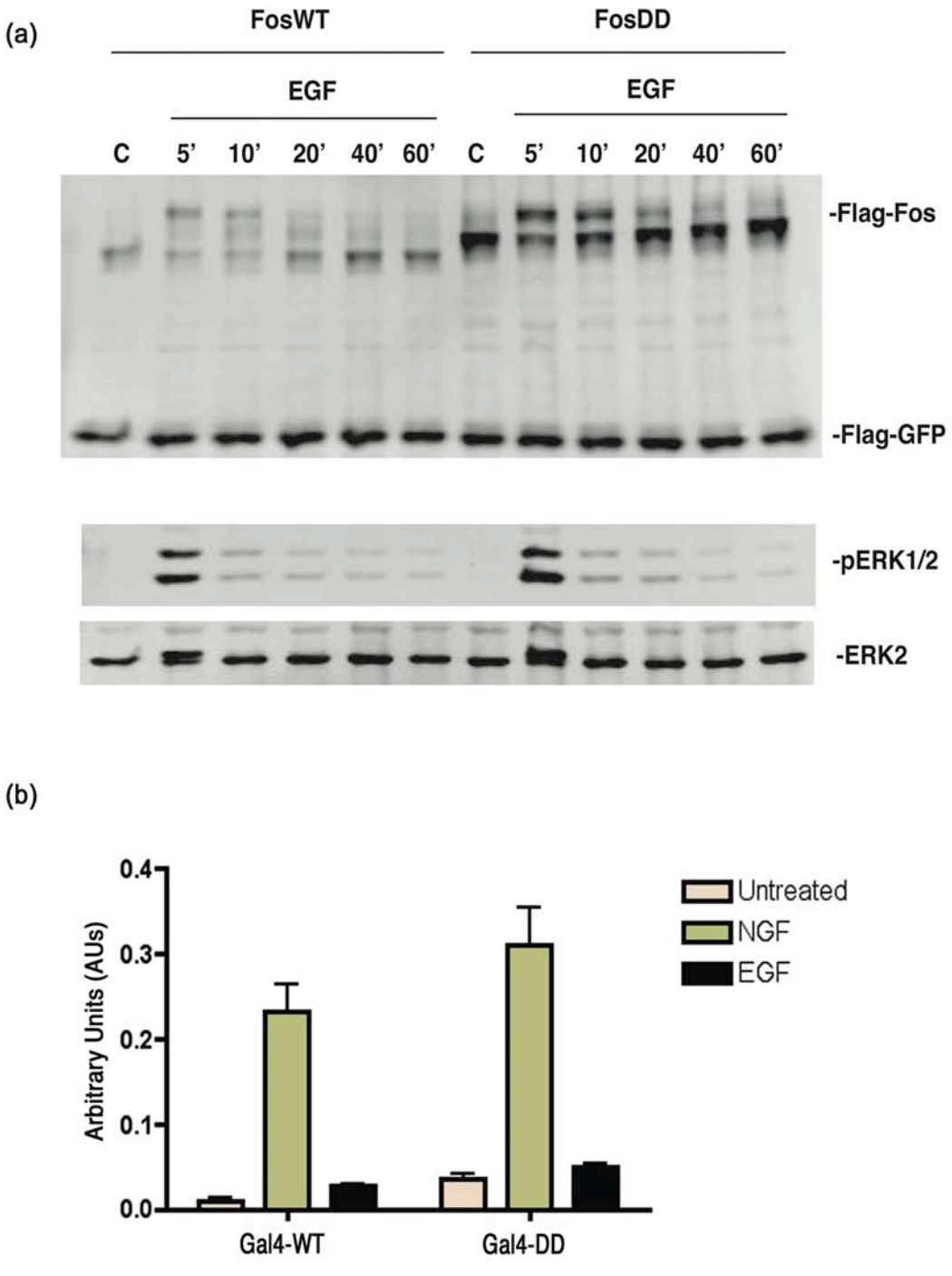

(a)
FosWT
FosDD
EGF
EGF
C
5'
10'
20'
40'
60'
C
5'
10'
20'
40'
60'
-Flag-Fos
-Flag-GFP
-pERK1/2
-ERK2
(b)
Arbitrary Units (AUs)
0.4
0.3
0.2
0.1
0.0
Untreated
NGF
EGF
Gal4-WT
Gal4-DD

Fig. 4.11 Maximal phosphorylation of c-fos is dependent on sustained ERK activation. (a) PC12 cells were transfected with FosWT, FosDD, and Flag-GFP. Cells were treated with EGF for the indicated time points and Western blotting was performed on the lysates. Total levels of Flag-c-fos proteins and Flag-GFP were examined using anti-Flag antibodies (top panel). pERK Abs show activation of ERK1/2 (middle panel). ERK2 levels were measured to confirm equal protein loading (bottom panel). (b) PC12 cells were transfected with Gal4-luciferase, pRL-Null, Gal4-WT, and Gal4-c-fos-S362D/S374D (Gal4-DD). Cells were treated with NGF for 6 hours, harvested, and luciferase activity was measured as described in materials and methods. The data presented are from a single representative experiment. The data are means $\pm$ SE (n=4). Luciferase activity is measured as arbitrary units (AUs), which equals the ratio of firefly light units/*Renilla* light units.

4.5 Discussion

The distinct actions of NGF and EGF on PC12 cells have provided a paradigm for the role of the duration of ERK activation in distinguishing the actions of these growth factors (Chao et al. 1992; Traverse et al. 1992; Cowley et al. 1994; Traverse et al. 1994; Marshall 1995; Vician et al. 1997; Kao et al. 2001; Vaudry et al. 2002). The ability of NGF to induce sustained activation of ERKs in these cells is required for NGF to differentiate these cells (Cowley et al. 1994; Marshall 1995). Sustained ERK activation by NGF permits nuclear localization of ERKs to promote a program of NGF-dependent transcription (Vician et al. 1997) through the induction of 'immediate-early genes' including the transcription factor c-fos (Kruijer et al. 1985; Milbrandt 1986; Kujubu et al. 1987; D'Arcangelo and Halegoua 1993; Groot et al. 2000). That we don't observe complete inhibition of c-fos stabilization by U0126 pretreatment may represent an ERK-independent effect. Indeed, other kinases have been implicated in phosphorylation of c-fos (Tratner et al. 1992; Tanos et al. 2005). Following NGF stimulation, c-fos and other immediate-early genes are rapidly induced to initiate a second wave of gene expression that contributes to this differentiated phenotype (Kalman et al. 1990; Ginty et al. 1992; deSouza et al. 1995; Nordstrom et al. 1995; Vician et al. 1997). For one of these immediate early genes, Egr-1, a direct role in neuronal differentiation by NGF has been established (Harada et al. 2001). A recent report has demonstrated that c-fos is also required to initiate the transcriptional program for differentiation in PC12 cells (Gil et al. 2004). It has also been reported that c-fos may also have a role in neurite outgrowth through its action on membrane biosynthesis (Borioli et al. 2004, 2005).

We show in PC12 cells that NGF induces the stability and transactivation of c-fos. We show that this phenomenon is dependent on the sustained activation of ERKs and is blocked by pharmacological inhibitors of ERK activation. This establishes that c-fos is a sensor for sustained ERK activation in PC12 cells. This is similar to the model proposed for c-fos activation by PDGF in fibroblasts (Murphy et al. 2002). Importantly, PDGF is a proliferative agent in these cells. Therefore, our study demonstrates that the requirement of sustained ERK activation for c-fos function is independent of the physiological outcome of c-fos activation. Recently, studies in fibroblasts have demonstrated that c-myc, Egr-1, Fra-1, Fra-2, and c-jun are also targets for activation by ERKs (Murphy et al. 2004). Indeed, a recent report demonstrates that sustained ERK1/2 activation is required for an AP-1-dependent mitogenic signal by growth factors in fibroblasts (Yamamoto et al. 2006). It is likely that some of these proteins also respond to sustained activation of ERKs by NGF to promote the induction of NGF-responsive genes in neuronal cells (Pap and Szeberenyi 1998; Cosgaya and Aranda 1999; Riccio et al. 1999; Groot et al. 2000; Boss et al. 2001).

We were able to observe a slight, but significant, increase in c-fos transactivation and protein stability by EGF. This effect of EGF can partially be attributed to increased basal levels of transfected Flag-Fos under control of the CMV promoter, which allows transient ERK1/2 activation to immediately act on premade c-fos protein. However, EGF's small increases in c-fos transactivation are unable to reach the threshold for induction of differentiation, demonstrating the requirement for sustained ERK activation to initiate this paradigm.

Several reports have examined residues in c-fos that are important for transactivation in fibroblasts (Murphy et al. 2002; Monje et al. 2003; Tanos et al. 2005). In this study, we

show that both Thr325 and Thr331 are required for maximal NGF-dependent activation of c-fos in PC12 cells. Our evidence suggests that both residues contribute to c-fos transactivation, although inhibition of Thr325 phosphorylation reduces transactivation more strongly. However, phosphorylation of these residues has a synergistic effect. This may explain why both residues are required for migration of the slowest mobility (top band). Blenis and colleagues showed a robust phosphorylation of Thr325 upon PDGF treatment in non-neuronal cells. This phosphorylation was also dependent on sustained ERK activation and an intact DEF domain. However, when they mutated these same residues, they saw a very modest effect on basal AP-1 activity. Although this difference in results could be cell-type specific, it is more likely due to differences in basal versus stimulated c-fos activity. Our data confirm that of Blenis and co-workers that demonstrated the importance of the DEF domain in promoting ERK-dependent phosphorylations critical for c-fos transactivation. At this time, we cannot rule out the possibility that this domain directs the ERK-dependent phosphorylation of additional target proteins.

c-fos is much less potent than its viral counterpart v-fos in inducing cellular transformation, despite sharing extensive homology within the DNA binding and transactivation domains (Cohen and Curran 1989; Piechaczyk and Blanchard 1994; Jotte and Holt 1996). One difference is the presence of C-terminal sequences unique to c-fos (Ofir et al. 1990). c-fos requires growth factor stimulation to become fully activated, in part because growth factors stimulate ERK-dependent phosphorylations within the C-terminal domain (Barber et al. 1987; Chen et al. 1993). ERK-dependent phosphorylations at Ser362 and Ser374 have been implicated in transformation (Okazaki and Sagata 1995; Chen et al. 1996), and transactivation (Murphy et al. 2002; Monje et al. 2003), in part by regulating

stability (Chen et al. 1996; Ferrara et al. 2003). Moreover, phosphorylations at these sites are thought to exert a priming function that directs the hyperphosphorylation of c-fos by ERK and other kinases (i.e. Thr325 & Thr331) (Murphy et al. 2002). Mutations preventing phosphorylation at both sites (FosAA) block all of these effects (Okazaki and Sagata 1995; Chen et al. 1996; Monje et al. 2003). On the other hand, phosphomimetic mutations introduced at both sites (FosDD) enhance stability and transformation (Chen et al. 1996). We show here that NGF also utilizes these sites to regulate c-fos stability in PC12 cells.

It has been assumed that phosphorylation at both sites functions similarly to enhance c-fos stability and transformation, largely because previous studies have examined c-fos mutated simultaneously at both sites. By examining a more complete series of mutants, we demonstrate that phosphorylations at these two serine sites contribute to the function of c-fos in distinct ways. Phosphorylation of Ser374 is required for the stabilizing effects of NGF stimulation, as evidenced by the finding that inhibition of Ser374 phosphorylation by U0126 correlates with decreased stability of c-fos protein. This can be shown by the absence of NGF-induced stabilization in Fos mutants lacking this serine (FosSA, FosDA and FosAA). Phosphorylation at this site appears to be sufficient for stabilization as phosphomimetic mutations at residue Ser374 produce c-fos mutants (FosSD and Fos AD) that are stable even under resting conditions. Phosphorylation of Ser362 appears to contribute significantly to the intermediate mobility shift, even in the absence of phosphorylation of Ser374 (FosDA). This likely reflects the contribution of Ser362 in priming additional phosphorylations. The exact mechanism by which phosphorylation of Ser362 promotes subsequent phosphorylations is not known. It is possible that this is achieved in part by promoting ERK binding to the DEF domain in c-fos that has been

proposed to contribute to the hyperphosphorylation and shift. This model is illustrated in Fig. 4.10.

The requirement for sustained ERK activation was seen not only in the activation of wild type c-fos proteins but also in c-fos mutants in which priming sites were replaced with aspartates (FosDD). FosDD is basally stable and no longer requires ERK-dependent phosphorylations at the "priming" sites. Even in this mutant, transient ERK activation is not sufficient to induce c-fos activation. The inability of EGF to activate the stable mutant FosDD suggests that sustained activation of ERKs is required to maintain the phosphorylation status of the activating threonines (Thr325 and Thr331). It is likely that these sites are tightly regulated by active phosphatases. This ensures that c-fos requires sustained ERK activation at multiple levels of its regulation

In summary, the ability of NGF to stimulate c-fos function requires sustained ERK activation. This requirement is due to two ERK-dependent phosphorylation sites within the C-terminus (Ser362 and Ser374) that have been proposed to confer stability on nascent c-fos protein and also serve as priming sites for subsequent phosphorylations within the TAD region. We show that phosphorylation of Ser374 is required for stabilization. As previously suggested, phosphorylation of this site may interfere with the well-characterized degradation sequence surrounding this site (Okazaki and Sagata 1995; Ferrara et al. 2003). Phosphorylation of Ser362 is critical in inducing the intermediate mobility shift, and with some contribution of phosphorylation of Ser374, enables c-fos to be targeted for additional ERK-dependent phosphorylations. These additional phosphorylations, specifically Thr325 and Thr331, are required for c-fos transactivation in this model. Moreover, an intact ERK binding site is also needed for stimulated c-fos activity, presumably for these 'activating'

phosphorylations. Because c-fos requires multiple ERK-dependent phosphorylations to be stabilized, shifted, and hyperphosphorylated, c-fos is strictly dependent on sustained ERK activation for stability and function. This requirement for sustained ERK activation serves as a paradigm for the distinct actions of NGF versus EGF on c-fos-dependent PC12 gene expression.

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Chapter 5

Summary and future directions

5.1 Summary and Conclusions

This dissertation has analyzed the signaling events associated with NGF-dependent differentiation in the PC12 model system. As discussed previously, differentiation of PC12 cells is characterized by exiting the cell cycle, the induction of electrical excitability, NGF specific gene expression, the ability to make synapses, and neurite formation. The work in chapter 3 used a pharmacological approach to show that ERK-independent pathways are also involved in NGF-dependent neurite outgrowth. In addition, we developed a system to study the ERK1/2 pathway in isolation and showed that sustained ERK1/2 activation is required for maximal neurite outgrowth in PC12 cells. The work in chapter 4 was focused on how a specific signaling pathway (ERKs) might be able to regulate NGF-specific gene expression. We demonstrated how sustained activation of ERKs was critical for the stabilization of the transcription factor c-fos. In addition, we also implicated ERK signaling in activation of c-fos-dependent gene expression. Furthermore, we identified distinct residues required for both c-fos stabilization and maximal transcriptional activation.

Analysis of NGF-dependent PC12 neurite outgrowth was the main focus of the work in chapter 3. This aspect of PC12 cell differentiation has been measured in a variety of ways since PC12 cells were first reported. The most common method, and the method chosen for the work in this dissertation, is to measure the percentage of cells that have neurites measuring greater than 2 cell body lengths 48 hours after NGF treatment. Thus, the final data is a percentage of neurite bearing cells. However, there are many other groups that use both similar and different criteria to study the very same effect.

Some groups use the same system, but measure neurite after 72 hours or 1 week (Boglari and Szeberenyi 2001; Sun et al. 2006). Some studies score a cell positive for neurite outgrowth if a neurite is equal to 1.5 times the cell body length (Morooka and Nishida 1998). Other studies measured total neurite length or the number of times neurites branched (Xiao and Liu 2003; Gil et al. 2004). In some reports, only pictures of cells are provided as evidence (Pang et al. 1995a). Therefore, the literature studying NGF-dependent neurite outgrowth uses different methods with no universal protocol. Indeed, the wide use of different criteria can have great effects on results and the general consensus in this field. Although there are commercially available automated systems for analyzing neurite outgrowth, such as ImageXpress, they can be expensive and only available to the well funded (www.moleculardevices.com/pages/instruments/imagexpress_micro.html). Recently, a computer program has been developed to provide automated measurements for neurite outgrowth (Pool et al. 2008). This paper describes a plugin for the free image-processing program, ImageJ (Abramoff et al. 2004). The plugin is called NeuriteTracer and is an automated method for measuring neurite outgrowth. NeuriteTracer is a freely available method that could be used to provide a better and more universal way to measure neurite outgrowth in all neuronal cell types. In the future, automated methods that are widely available should allow labs to better compare and understand each other's data.

The work in chapter 4 provides a model explaining why the kinetics of ERK1/2 activation is important in transmitting the proper signal to the cell. We demonstrated that transient ERK1/2 activity is not sufficient to properly regulate AP-1-dependent gene transcription. Sustained ERK1/2 signaling must be sustained in order to transcribe,

stabilize, and activate the c-fos protein. This may help explain why a transient ERK1/2 activator like EGF does not result in neuronal differentiation whereas a sustained ERK1/2 activator like NGF does. This feature of utilizing ERK1/2 signaling kinetics to dictate the cell's response to a given stimulus has also been shown in CCl39 fibroblasts (Chalmers et al. 2007). This report shows that c-fos activation and AP-1-dependent transcriptional activity are both reliant on sustained ERK1/2 activity. However, in this model cell cycle re-entry is the qualitative response to stimulation. Thus, the importance of ERK1/2 signal duration can be seen as a general mechanism used by different cell types to accomplish varied biological fates.

Kinases must bind the correct targeting sequence as well as recognize a specific phosphorylation motif in order to properly activate their substrates. Proper regulation of c-fos activation, and presumably NGF-specific gene transcription, is dependent on correct targeting by ERK1/2 to the DEF domain of c-fos. Indeed, other transcription factors such as Elk-1 and Egr-1 also contain DEF domains that are required for proper phosphorylation by ERK1/2 (Dimitri et al. 2005). Mutation of the DEF domain in Bim (EL) abolishes ERK1/2 phosphorylation and leads to cytotoxicity in HEK293 cells (Ley et al. 2005). This is due to loss of ERK1/2-dependent phosphorylation that directs Bim (EL) protein degradation. Moreover, the DEF domain has been shown to be directly involved in promoting sustained ERK1/2 signaling. MKP-1 contains a DEF domain and ERK1/2 phosphorylation promotes MKP-1 protein degradation. Down regulation of this phosphatase allows for sustained ERK1/2 signaling (Lin and Yang 2006). The D domain is another ERK1/2 binding site that is present in ERK1/2 substrates, such as RSK. The D domain in RSK seems to be required for phosphorylation by both ERK1/2

and ERK5 (Ranganathan et al. 2006). The D domain also targets JNK to the AP-1 family member c-jun (Vinciguerra et al. 2004). This report goes on to show that another related transcription factor, JunD, contains both a D and a DEF domain. As expected, these domains mediate phosphorylation by both JNKs and ERKs. Therefore, these targeting domains provide specificity for MAPK family signaling pathways. This information can be used to inhibit ERK1/2 effectors to better understand their role in biology without blocking the entire ERK1/2 pathway using pharmacological inhibitors. Specifically, cancers that implicate ERK-dependent pathways may be studied and attacked in a more specific manner by mutating targeting domains (Boucher et al. 2000; Davies et al. 2002; Dimitri et al. 2005; Gebhardt et al. 2005).

Much of the work provided in this thesis has been directed at trying to develop a better understanding of the ERK1/2 cascade and its role in regulating neurite outgrowth. It is well known that spinal cord injuries are very difficult to treat due to the incapability of axons to regenerate. In a fascinating report, viral delivery of a constitutively active MEK1 induced axon regeneration in a completely transected spinal cord of a rat (Miura et al. 2000). These regenerated neurons became electroconductive and rats regained hind limb function within 2 weeks of infection. Another recent report demonstrates that injection of the MEK inhibitor U0126 into the sciatic nerve concomitantly with a conditioning crush injury results in reduced neuronal regeneration (Perlson et al. 2005). In addition, activated ERK1/2 has been shown to be retrogradely transported after nerve injury (Perlson et al. 2006). These reports providence evidence that understanding how the ERK signaling cascade can regulate axonal regeneration may have very important clinical applications. The work presented in this dissertation adds to

our overall understanding of how ERK1/2 activation and other signaling pathways can regulate neurite outgrowth in the PC12 model system.

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