Mechanisms Underlying Nerve Growth Factor Signaling Specificity: Role of membrane trafficking and selective coupling to localized Ras family members

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ABSTRACT

The major focus of this thesis has been to further understand the molecular pathways through which extracellular stimuli achieve specificity in regulating the proliferation and differentiation of neuronal cells. Hormones and growth factors are known to regulate neuronal cell growth and differentiation through their actions on the mitogen activated protein (MAP) kinase cascade. Activation of MAP kinase, also known as extracellular signal-regulated kinase (ERK), has either positive or negative effects on growth and differentiation, depending on both the cell type and the extracellular stimuli. We have previously identified a novel ERK cascade involving the small GTPase Rap1 and the MAP kinase kinase kinase (MKKK), B-Raf, which mediates the cell-type specific growth effects of cAMP-coupled hormones. The work presented here demonstrates that this novel Rap1/B-Raf pathway contributes to growth factor signaling specificity as well.

The question of growth factor specificity has arisen from the observation that related growth factors acting through similar signal transduction pathways elicit very different cellular responses. To address how growth factors achieve specificity in their actions, we exploited the well-studied model of neuronal precursors, PC12 cells. In these cells, epidermal growth factor (EGF) induces proliferation, while nerve growth factor (NGF) triggers neuronal differentiation. ERK activation is required for both of these responses. NGF induces a more prolonged activation of ERKs than does EGF and it has been proposed that this sustained ERK activation mediates NGF-induced neuronal differentiation. We show that NGF activates the small GTPases Ras and Rap1 to mediate the rapid and sustained ERK activation, respectively. The selective activation of the Rap1-dependent pathway by NGF, and not EGF, is required to induce sustained ERK activation, neuron-specific gene expression, and electrical excitability. We further show that phosphoinositide 3-kinase (PI3-K)-dependent endocytosis of the NGF receptor, TrkA, is required for activation of Rap1 initiated pathways. In contrast, Ras activation by NGF is independent of both PI3-K activity and endocytosis. This may be due to the distinct localization of Ras at the plasma membrane and Rap1 within endosomal compartments. These data suggest that different TrkA effectors (Rap vs. Ras) may be activated depending on the stage of endocytosis. Clearly, these observations support the hypothesis that a common activator may couple to distinct signaling pathways at particular subcellular locales. Furthermore, we demonstrate that these localized signals can engage distinct subsets of a convergent signaling web to differentially modulate the activation kinetics of a common effector and thereby exert opposing actions on a single cell.

CHAPTER ONE

INTRODUCTION

The field of signal transduction has blossomed from the trailblazing efforts of Krebs and co-workers beginning in the mid-twentieth century. Their pioneering studies led to the discovery that the state of phosphorylation can alter a protein's enzymatic activity, which ultimately led to the isolation of the cAMP-dependent protein kinase, PKA [1]. The following years have witnessed remarkable advances in our understanding of how extracellular stimuli induce cytoplasmic changes. For examples, the identification of heterotrimeric G-proteins and their role in regulating intracellular cAMP levels along with the identification of transmembrane receptor tyrosine kinases (RTKs) established a means by which extracellular signals could be transmitted across the plasma membrane by stimulating intracellular phosphorylation events. It quickly became clear that many receptors induce changes in gene expression upon ligand binding. Importantly, it was discovered that growth factors are able to induce a set of genes independent of new protein synthesis. Thereafter, a major focus in the field has been to understand how extracellular signals are transmitted to the nucleus to regulate transcription. Indeed, the ability to decipher a signaling pathway "from membrane to nucleus" has become the reverie of many researchers at the turn of the century. During the past several decades, a wealth of knowledge has been uncovered which aids our understanding of how extracellular signals are transmitted to the nucleus to induce changes in cell behavior. For instance, thousands of kinases and phosphatases with varying substrate specificities, signaling modules which direct phosphorylation-dependent protein interactions, heterotrimeric G-proteins, small G-proteins, new second messengers, and lipid-based signaling events have all been identified. While initial studies have categorized many of these signaling components into distinct linear pathways, it is now clear that most of these identified pathways effect one another, or "cross-talk." It is also clear that multiple stimuli can invoke the same fundamental pathways yet elicit different responses. Therefore, one of the major challenges in signal transduction studies is to determine how signaling specificity is achieved when the same pathways mediate different cellular outcomes. By examining hormonal and growth factor regulation of the extracellular signal-regulated kinase (ERK) cascade, we have gained insight into the mechanisms through which extracellular stimuli achieve signaling specificity in order to evoke precise changes in cell behaviors governing growth and development.

The ERK Cascade

ERKs, also known as mitogen-activated protein kinases (MAPKs), were identified as transcriptional modulators through the convergence of two areas of research. Specifically, the search for enzymes that effect the phosphorylation status of transcription factors and the search for downstream kinases activated by RTKs [2]. ERKs were subsequently found to regulate a diverse array of functions including cell growth and proliferation, differentiation, and survival [3-6]. The ERK cascade, like all MAP kinase modules, consists of three protein kinases that sequentially phosphorylate and activate one another in series (Figure 1.1). The upstream activators of ERKs consist of members of the mitogen and extracellular signal-regulated kinase (MEK) [7] family of MAP kinase kinases (MKKs) and members of the Raf family of MAP kinase kinase kinases (MKKKs) [8, 9]. The actions of ERKs have been best studied in the context of growth factor signaling [2, 10-12] where they have been shown to play a pivotal role in mitogenesis and differentiation [13, 14]. The classical pathway leading to ERK activation involves the binding of growth factors to RTKs in the cell membrane. These receptors then recruit adaptor molecules carrying the Ras-specific guanine-nucleotide exchange factor (GEF), SOS, to the receptor complex [15]. This recruitment places SOS in close proximity to its specific small GTPase effector, Ras, allowing the exchange of GDP for GTP on Ras [16, 17]. The GTP-loading of Ras is required for its activation of the serine/threonine kinase Raf-1 [18, 19]. Activated Raf-1 activates MEK, which then activates ERK [20]. Once activated, ERK can migrate into the nucleus where it can phosphorylate and activate transcription factors to regulate gene expression [21]. This RTK/ERK pathway is essentially conserved in species ranging from *C. elegans* to humans, with many of the components being functionally interchangeable between species.

Selective expression of Raf isoforms

Each component of the ERK cascade consists of multiple isoforms. The two ERK isoforms in this cascade, ERK1 and ERK2, and the two MEK isoforms, MEK1 and MEK2, are expressed ubiquitously in mammalian cells. For both ERK and MEK, isoforms within each family appear to have largely overlapping functions and regulatory mechanisms. Whether different MEK and ERK isoforms contribute to signaling specificity is not clear. However,

important to the work presented here, mammalian cells express three Raf isoforms termed Raf-1, A-Raf and B-Raf. In contrast to the ubiquitously expressed Raf-1, A-Raf and B-Raf display selective spatio-temporal expression patterns. A-Raf is expressed predominantly in epididymis and ovary [22]. In contrast, B-Raf is the major Raf isoform in the brain and neural-crest derived cells and is expressed to markedly higher levels during central nervous system (CNS) development [22, 23]. Although neuronal cells lack A-Raf, they also express Raf-1 (Figure 1.2). However, it is important to note that B-Raf, not Raf-1, has been identified as the major MEK activator in neuronal tissue [24, 25].

Regulation of Raf activity

Amongst members of the ERK cascade, Raf activity appears to have the most complex regulation. Consequently, the molecular mechanisms regulating Raf activities remain poorly understood. Most studies of Raf regulation have focused on the Raf-1 isoform. All Raf isoforms are highly conserved within three regions (CR1-3) [26, 27]. The catalytic domain of Raf kinases is encoded by CR3, which is most closely related to the Src family of tyrosine kinases [28]. The N-terminal domain of Raf-1 (containing CR1-2) seems to function to suppress its catalytic activity, because the deletion of the N-terminus results in constitutive activation of the kinase domain [29, 30]. For Raf-1, activity is regulated by both membrane localization and by multiple phosphorylations on serine, threonine, and tyrosine residues (Figure 1.2). Upon growth factor stimulation, Raf-1 is recruited to GTP-bound Ras (Ras-GTP) at the plasma membrane, where it becomes activated [9]. Artificially targeting Raf-1 to the membrane by expression of a fusion protein (Raf-1-CAAX) containing the membrane targeting C-terminal domain of Ras leads to constitutive activation of Raf-1-CAAX [31, 32]. In addition, recombinant Raf-1-CAAX expression is able to induce the activation of ERK2 in the absence of stimuli [31]. Several studies have suggested that the activation of wild-type Raf-1 requires its direct interaction with active GTP-bound Ras [33-38]. However, this interaction does not lead to activation of Raf-1 *in vitro* unless Ras-GTP is membrane bound and an unidentified cytosolic factor is present [20, 39-42]. These data suggest that the interaction with Ras-GTP serves to bring Raf-1 to the plasma membrane, but additional events are required for maximal Raf activation. Hormonal activation of the Ras-related small GTPase, Rap1, also induces membrane localization of Raf-1 [43-45]. This recruitment to Rap1, however, does not lead to Raf-1 activation. Although it is not clear from these studies if the Rap1/Raf-1 complex is present at the plasma membrane or another membrane compartment, this observation supports a role of additional Raf activators. Unlike Raf-1, B-Raf is recruited and activated by both Ras and Rap1 in a GTP-dependent manner. Therefore, both

Ras and Rap1 may serve as important links between RTKs and the ERK kinase cascade in neuronal cells and other tissues where B-Raf is highly expressed.

Ras and Rap1

Ras-related GTP-binding proteins constitute a large superfamily of small GTPases that are categorized into several subfamilies. The Ras family consists of Ras, Rap, and Ral, with each having various isoforms. Proteins of the Ras subfamily exist as three isoforms termed H-, N-, and K-Ras. All three Ras isoforms have been implicated in growth regulation in a wide range of human tissues [46, 47]. Like all G-proteins, the Ras family of small G-proteins function as molecular switches cycling between the GDP-bound inactive state and the GTP-bound active state. In the absence of external activation, small G proteins exist predominantly in the inactive form. Activation is triggered by the action of specific guanine-nucleotide exchange factors (GEFs) that accelerate GDP release from small G proteins. The binding of the more abundant guanine nucleotide, GTP, rapidly follows this release. By definition, once activated, small G proteins are able to interact with effector molecules. The hydrolysis of bound GTP to GDP restores the resting (inactive) state. Specific GTPase-activating proteins (GAPs) accelerate the intrinsic GTPase activity of small G proteins. Therefore, specific activating GEFs and inhibitory GAPs potentially regulate each member of the Ras superfamily [48-50].

The Ras-related small G-protein Rap1 was cloned on the basis of its sequence homology to Ras [51]. The two isoforms of Rap1, Rap1a and Rap1b, share 97% homology within their amino acid sequence, differing in only 9 out of 184 residues [52]. The functional difference between these two isoforms is unclear and in most studies no discrimination between the two has been made. Rap1a and Rap1b share about 60% homology with K-Ras. The homology between Ras and Rap1 proteins is greatest in their GTP-binding domains and their effector domains. Accordingly, several analogous mutations exist in Ras and Rap1 that function in a similar manner to alter the activation state of these G-proteins (Figure 1.3). For example, substitution of a valine for glycine at amino acid 12 in Rap1 (RapV12) results in constitutive activation by blocking the intrinsic GTPase activity of Rap1. This effect is similar to that of the constitutively active Ras mutant (RasV12). In addition, the mutation of Ala17 to Asn17 (RapN17) in the guanine nucleotide binding region, like the corresponding mutation in Ras (RasN17), blocks the binding of the activating GTP molecule. The expression of RapN17, like the corresponding RasN17, can act as an interfering mutant to prevent activation of its endogenous counterpart [53].

Furthermore, the mutation in RapN17 blocks Rap1's biological effects on Ras transformation [54].

Rap1 was originally named Krev-1 (Kirsten-Ras revertant) based on its ability to revert Ras-dependent transformation of fibroblasts [51, 54-57]. Rap1 has since been shown to antagonize Ras signaling to the ERK cascade and to the induction of mitogenesis in multiple cell types [58-62]. In contrast, activated Rap1 is unable to block signals generated from v-Raf, suggesting that Rap1 acts at a site between Ras and Raf-1 [61]. It has been suggested that Rap1 may antagonize Ras by competing for Ras effector molecules [63]. In support of this hypothesis, Rap1 was independently identified as a Raf-1 binding protein in yeast two-hybrid screens [20, 64]. In addition, activated Rap1 (RapV12) binds to other known Ras effectors including Ras-GAP, PI3-K, and Ral-GDS [65-68]. In contrast to Ras binding, however, coupling of these molecules with Rap1 generally results in an inactive complex.

Given the strong similarity between Ras and Rap1, particularly in their effector domains [68], what accounts for their different actions on downstream effectors? Subcellular localization may provide one explanation. Ras is known to associate with the plasma membrane in most cell types [69-74]. In contrast, Rap1 is located on different membrane compartments depending on both the cell type and cellular context [72, 75-78]. In neurons, for instance, Ras and Rap1 have been found in distinct membrane compartments [75, 79]. Activation of downstream effectors may require membrane-associated events subsequent to membrane recruitment by active GTPases, as predicted for Rafs. Therefore, the different subcellular localizations of Ras and Rap1 may serve to recruit these effectors to different sets of regulators that alter the magnitude and kinetics of their activation at distinct locations.

Ras and Rap1 regulation: exchangers and adaptors

As mentioned previously, Ras and Rap1 activity is regulated by specific guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). Although a role for GAPs is undeveloped, GEFs are clearly important determinants of signal specificity. In the context of growth factor signaling, the function of GEFs is generally controlled by their recruitment from the cytoplasm to multi-protein complexes at the cell membrane [80]. This process involves specific SH2-/PTB-domain containing adaptor molecules that bind to RTK-dependent phosphorylated tyrosine (pTyr) residues. Hence, a principle function of RTK activity is to provide binding sites for the SH2 domains of specific receptor targets. Additional contacts with residues in close proximity to the pTyr provides specificity, thus allowing the sequence

context of the pTyr to determine which SH2-containing proteins will bind [81]. Other conserved modules, SH3 domains, bind proline-rich peptides of about 10 amino acids [82]. SH3 domains are often found in the same polypeptides as SH2 domains allowing for the formation of multi-protein complexes. In this way, proline-rich GEFs for Ras and Rap bind to activated receptors via interactions with the SH3-domains of adaptor molecules such as Grb2 and members of the Crk family. Like SH2 domains, SH3 domains display distinct binding preferences [82, 83]. For instance, the Crk family of adaptors can bind to both the Ras-specific GEF, SOS, and the Rap-specific GEF, C3G (figure 1.4). In contrast, Grb2 selectively associates with SOS, but not C3G [84-86]. Therefore, the initiation and recruitment of specific RTK:adaptor:GEF interactions may direct growth factor signals down characteristic paths.

Aside from those mentioned above, several additional RTK-associated adaptor molecule families have been identified, including Shc, FRS2, rAPS, and SH2-B [87-90]. All of these adaptors have been implicated as upstream ERK activators. Some of these adaptor families contain multiple isoforms whose temporal and spatial regulation, particularly during development, may confer some signal specificity. Disrupting the function of these adaptors can effect not only ERK signaling, but also neuronal processes such as neurite outgrowth, axon elongation, and survival. The role of some of these adaptor families in mediating growth factor signals to ERK will be discussed in greater detail below. In addition to newly discovered adaptor molecules, recent efforts focused on the cross-talk between ERK signaling and other second-messenger systems have led to the identification of novel families of Ras- and Rap-GEFs. These GEFs appear to represent a new class of exchangers, distinct from the RTK/adaptor molecule-associated GEFs, in that direct binding to second messengers such as calcium, cAMP, and DAG activates them [91]. Interestingly, similar to many adaptors, each GEF displays a very different and restricted CNS expression pattern, suggesting they too may act as regulators of signaling specificity in neuronal cells.

ERK Signaling Specificity in Cell Growth Control

Activation of ERK has either positive or negative effects on growth and differentiation, depending on both the cell type and the extracellular stimuli. For example, several members of the ERK cascade were originally identified as oncogenes, demonstrating the importance of this pathway in cell growth regulation. Mutations or over-expression of Ras, Raf, and MEK have all been found in human cancers [92, 93]. Furthermore, ERKs are required for Ras, Raf, and MEK induced cell transformation and for growth factor induced mitogenesis [2, 94]. These studies

clearly define a role of ERKs in proliferation. However, in many cell types, ERK pathways also induce the cessation of growth [95], and the stimulation of differentiation [4, 5]. Proliferation and differentiation represent opposite ends of the spectrum for the developing cell. How then does a single enzyme generate the appropriate biological response?

Direct enzyme-substrate interactions offer a potential framework for generating signal specificity. Although much has been learned about the basic principles underlying ERK substrate specificity, relatively little is known regarding the regulation of this specificity. It is well established that ERKs are proline-directed serine/threonine kinases that preferentially phosphorylate the consensus sequence Pro-X-Ser/Thr-Pro (where X is any amino acid) [96, 97] or the minimal sequence Ser/Thr-Pro in substrate proteins [98, 99]. The three-dimensional structures of both active and inactive ERK provided by X-ray crystallography studies has clarified the basis of this preference for proline containing substrates [100]. ERK substrate specificity is determined, in part, by the geometry of the catalytic cleft. Upon activation by MEK, ERK becomes phosphorylated on adjacent tyrosine and threonine residues within a TEY motif (Thr183 and Tyr185 in ERK2). Like other kinases, ERKs have a small N-terminal domain and a large C-terminal domain. These two domains form a deep catalytic cleft into which ATP binds. ERK undergoes a conformational change as a result of phosphorylations on the TEY motif, which is located on the lip of this cleft [101, 102]. When phosphorylated, the activation lip of ERK moves to allow substrates access to the catalytic cleft by creating a surface pocket that is specific for proline. The side chain of Arg192 in ERK2 occupies this pocket when the enzyme is inactive. In addition to the restricted access of substrates, domains in ERK and its targets outside of the proline-directed interaction seem to play an important role in determining substrate specificity [100-107]. At least one of these domains in ERK does not seem to be effected by the state of ERK activation [100]. Interestingly, studies in yeast using kinase-deficient point mutants of the ERK homologs Kss1 and Fus3 show that inactive kinases can regulate signaling pathways [108-111], possibly through protein:protein interactions mediated by these additional domains. Whether these additional docking sites responsible for substrate recognition are regulated in a manner that contributes to signaling specificity remains to be determined.

Consistent with the role of ERK in a broad range of cellular responses, a variety of membrane-associated, cytoplasmic, and nuclear targets of ERK have all been identified. Figure 1.6 lists some of the ERK targets found in neuronal cells through which ERK mediates multiple actions. Whether activation of the ERK pathway leads to either positive or negative effects on cell growth may depend on the cell type-dependent expression, or cell context-dependent availability of these downstream targets [14, 112]. On the other hand, studies in some cell types have

demonstrated that the strength and duration of ERK activation determines whether the cells undergo proliferation or differentiation [94, 113-119]. Therefore, signals upstream of ERK which modulate ERK activation kinetics may also explain its ability to direct diametrically opposed cellular processes. This is best illustrated in the context of growth factor signaling.

The Question of Growth Factor Specificity

To understand how a signaling pathway may accurately mediate multiple biological responses, we have considered the question of growth factor specificity. This long-standing question originated from the observation that many growth factors can promote the opposing actions of either proliferation or differentiation. For example, nerve growth factor (NGF) plays an important role during the development of both the peripheral and central nervous systems by triggering the differentiation and survival of neuronal precursor cells; as well as survival and adaptive responses of mature neurons. However, other cell types, including hematopoetic cells and TrkA expressing fibroblasts [120], respond to NGF by proliferating. In addition, a number of growth factors can promote either the proliferation or differentiation of neuronal precursors depending upon the cellular context [121-124]. Furthermore, in many cells, different growth factors act through structurally similar receptor tyrosine kinases coupled to a similar set of intracellular targets and signal transduction pathways, yet elicit very different cellular responses [94]. These observations raise several important questions. First, what allows neuronal precursors to respond to NGF with a differentiating response rather than a mitotic response? Second, do growth factors use similar or different pathways to regulate cell growth, differentiation, and survival? Finally, how can receptor tyrosine kinases act in a single neuron to promote the opposing processes of cell differentiation and cell growth? One goal of this work was to address these questions by dissecting the molecular pathways through which extracellular stimuli regulate these processes in neuroblasts.

PC12 Cells as a Model System

Our ability to investigate growth factor specificity in neuroblasts was greatly facilitated by the isolation of the rat PC12 cell line, derived from an adrenal tumor of neural crest origin [125, 126]. PC12 cells exhibit the basic phenotype of adrenal chromaffin cells and respond to NGF by differentiating into sympathetic-like neurons. This differentiation is characterized by electrical

excitability, the induction of a set of neuron-specific genes, and neurite outgrowth [127, 128]. Importantly, not all growth factors induce differentiation of PC12 cells. For instance, EGF induces proliferation of these cells. Comparing the effects of NGF to those of proliferating agents like EGF has proved useful in determining the molecular events critical to achieving neuronal differentiation and has provided insight into the mechanisms dictating growth factor specificity. For example, the observation that EGF stimulates a transient activation of ERK, whereas NGF induces a larger and more sustained activation of ERK, led to the model suggesting that the magnitude and duration of signals through the ERK cascade dictate the physiological outcome [94]. Indeed, several laboratories have demonstrated a link between sustained activation of ERK and aspects of neuronal differentiation [14, 121, 122, 124, 129, 130]. It was proposed that this prolonged ERK activation is required to allow active ERKs to accumulate in the nucleus above a threshold level needed to activate transcription factors and gene expression driving the differentiation process. Therefore, understanding the molecular mechanisms that regulate the duration of ERK activation in these cells may be critical to our understanding the specificity of growth factor action toward neuronal differentiation.

Classical growth factor-stimulated ERK Cascade in PC12 cells

As outlined above, the classical pathway from growth factor signals to the ERK cascade involves the small G-protein Ras. The coupling of RTKs to Ras requires recruitment of the Ras-specific guanine nucleotide exchange factor, SOS, to the plasma membrane [131, 132]. In this model, growth factors initiate the ERK cascade upon binding to the extracellular domain of their RTKs. This binding induces a conformational change and dimerization of neighboring RTK proteins resulting in the activation of their cytoplasmic catalytic domains. The active RTK dimer pairs undergo a trans-autophosphorylation of specific tyrosine residues within their intracellular domains [133]. The adaptor protein Grb2 rapidly associates with specific phosphotyrosines on the activated RTKs via the interaction of Grb2's SH2-domain [134]. The Grb2 SH3-domain is thought to be constitutively associated with proline-rich sequences present in SOS. Formation of the RTK/Grb2/SOS complex within the plasma membrane places SOS in proximity to inactive Ras (Ras-GDP). SOS is then positioned to activate Ras by stimulating the exchange of GDP for GTP on Ras [16, 17]. Ras-GTP, in turn, translocates Raf-1 to the plasma membrane for subsequent activation of the ERK cascade. Additional phosphotyrosine residues within the cytoplasmic domain of the receptor provide the docking sites for SH2-domain containing proteins other than adaptor molecules. Some of these have enzymatic activities, such as phosphoinositide-specific phospholipase C; PLC, phosphoinositol-3-phosphates kinase; PI3K, Src tyrosine kinase,

and the tyrosine phosphatases SHP1 and SHP2. Generally, the recruitment of these enzymes generates intracellular signals by acting on substrates that are located to the plasma membrane. At least two of these additional enzymes (SHP2 and PLC) have been implicated in ERK activation under certain circumstances [135, 136].

Feedback control of ERK activity

Aside from the substrates listed in figure 1.6, additional targets of ERK serve to regulate ERK signaling itself. For example, in response to growth factor stimulation, ERK and other ERK-dependent kinases phosphorylate the upstream component of the ERK cascade, SOS. This modification is thought to play a negative feedback role on the Ras pathway, by causing the dissociation of SOS from the adaptor molecule, Grb2 [137-139]. Alternatively, ERK-dependent phosphorylation of SOS may uncouple RTK signals from the Ras/ERK pathway by causing the dissociation of the Grb2/SOS complex from the receptor [140-143]. In both cases, Ras-dependent ERK activation is rapidly terminated. As illustrated in figure 1.5, the classical model through which RTKs activate ERKs relies entirely on Ras-dependent pathways and is therefore insufficient to explain the sustained ERK activation following NGF stimulation.

NGF: An evolving model of GF signaling in PC12 cells

In addition to the classical adaptor molecule, Grb2, other SH2 containing adaptors may associate with phosphorylated RTKs. For example, the NGF receptor, TrkA, deviates from the classical model in that it does not associate with the Grb2/SOS complex directly (Figure 1.5). Instead, Grb2/SOS binds TrkA through interactions with the Shc family of adaptor molecules [88, 144]. Members of the Shc adaptor protein family are also associated with tyrosine-phosphorylated RTKs via SH2-domain mediated interactions [145]. After binding to phosphotyrosine sequences in activated RTKs, Shc is phosphorylated at two tyrosine residues in a central Pro/Gly-rich domain. The SH2 domain of Grb2 recognizes the later tyrosine phosphorylation site. It has been assumed that, like the direct association of Grb2 with a phosphorylated RTK, the formation of the phospho-TrkA/phospho-Shc/Grb2/SOS complex recruits SOS to the membrane to convert Ras-GDP to Ras-GTP [145]. However, the requirement

of Grb2 for NGF activation of Ras has not been established. Instead, recent evidence has suggested that the Crk family of adaptor proteins is important in mediating NGF signaling to the ERK cascade. The Crk family consists of two alternatively spliced forms of Crk, CrkI and CrkII, and a closely related protein, CrkL. CrkII and CrkL contain an SH2 domain followed by two SH3 domains, while the alternatively spliced CrkI lacks the C-terminal SH3 [81]. Both CrkII and CrkL are highly expressed in PC12 cells [146], and bind via their amino terminus SH3 domains (SH3N) to SOS [84, 85, 147, 148]. Microinjection of Crk proteins induces neurite outgrowth of PC12 cells in a Ras-dependent manner [149]. Furthermore, Ras activation by NGF can be inhibited by interfering mutants of Crk [85]. However, the expression of these same Crk mutants has no effect on ERK activation by EGF [150], suggesting that the ability of Crk proteins to mediate Ras activation is specific for NGF. Unlike Grb2, Crks can recruit both SOS and C3G [84, 85, 147, 148] and therefore represent potential activators of both Ras and Rap1 pathways. Although the precise mechanisms controlling these multi-protein complexes remain unclear, it is reasonable to posit that the versatility of the interactions between adaptors and RTKs can regulate, in part, the specificity of growth factor signaling by modulating the activity of small GTPases and their downstream effectors.

We have previously described a novel pathway involving the small GTPase, Rap1, and the selectively expressed MKKK, B-Raf. This pathway was shown to mediate sustained ERK activation induced by cAMP-coupled hormones. The following chapter describes how this same pathway also contributes to growth factor specificity by regulating the kinetics of ERK activation. We demonstrate that the temporal regulation of Ras-dependent versus Rap-dependent signals to ERK dictates not only the kinetics of ERK activation, but also the specificity of growth factor effects on gene expression and cell physiology in PC12 cells. Chapter 3 reveals a potential mechanism to explain the observed kinetics of Ras, Rap1, and ERK activation and attempts to construct a spatio-temporal account of growth factor signaling in a neuronal cell. The significance of these findings and areas of important future studies are discussed in Chapter 4.

Figure Legends

Figure 1.1 The classical ERK cascade. The archetypal pathway by which growth factors activate ERKs is depicted. In this scheme, ligand binding to the appropriate receptor tyrosine kinase (RTK) induces the formation of receptor oligomers that specifically phosphorylate each other in trans on multiple tyrosine residues. In addition to regulating catalytic activity, phosphorylated tyrosine (pTyr) residues provide binding sites for signaling molecules. In the classical cascade, the adaptor protein, Grb2, is recruited to pTyr sites in the receptor via its SH2-domain and is responsible for Ras activation via its constitutive association with the guanine nucleotide exchange factor (GEF), SOS. Recruitment of this adaptor:GEF complex to the receptor brings SOS in close proximity to the plasma membrane-bound Ras, allowing for the stimulated exchange of GDP for GTP and consequent Ras activation. Activated Ras recruits the first kinase in the ERK cascade, the MAP kinase kinase kinase (MKKK) Raf. The translocation of Raf to the plasma membrane leads to its full activation by additional membrane-associated components. Active Raf is then able to phosphorylate and activate the MAP kinase kinase (MKK), MEK, which phosphorytates and activates ERKs. Activated ERKs enter the nucleus where they can phosphorylate transcription factors and thereupon influence the expression of genes that mediate cell growth and survival responses.

Figure 1.2 Structural comparison of neuronal Raf isoforms. Raf-1 and B-Raf are largely similar in three highly conserved regions (CR1-CR3), including the catalytic kinase domain (CR3) and the Ras-binding domain (RBD). Each Raf isoform can interact via their RBD with both Ras and Rap1. Interestingly, binding to Ras facilitates activation of both Raf-1 and B-Raf. In contrast, Rap1 binding leads to the selective activation of B-Raf and is associated with Raf-1 inhibition. The effector domains of Ras and Rap1 are also highly conserved (figure 1.3B), leaving the mechanisms responsible for the observed difference in Raf-1 and B-Raf activation to question. For Raf-1, full activation requires both membrane recruitment and additional phosphorylations on tyrosine, threonine, and serine residues. B-Raf shares analogous sites for some, but not all of these regulatory phosphorylations. For example, Ser259 and Ser621 are 14-3-3 binding sites which are conserved in Raf-1 and B-Raf. 14-3-3 is an essential cofactor for regulating Raf-1 activity. In contrast to the conserved 14-3-3 binding sites, Raf-1 also contains a PKA phosphorylation site at Ser43 that is not present in B-Raf. In addition, the putative Src phosphorylation sites in Raf1 (Y340/Y341) are replaced with aspartic acid (Asp) residues in B-Raf. It has been proposed that the negative charge provided by Asp residues is sufficient to constitutively mimic the phosphorylated state of the equivalent Raf-1 tyrosines (Y340/Y341) and therefore contributes to the high basal activity of B-Raf. Likewise, Ser338 in Raf-1 is not phosphorylated in the basal state but is highly responsive to Ras-mediated signals. Conversely,

the B-Raf equivalent to Ser338 is constitutively phosphorylated and this, too, may influence the basal activity of B-Raf. Finally, B-Raf also contains a unique N-terminal extension of unknown function.

Figure 1.3 Schematic of Ras family members. The Ras subfamily of the larger class of Ras-related GTP-binding proteins consists of Ras, Rap, and Ral isoforms. These proteins typically consist of approximately 180 amino acids (about 21kDa) and function as molecular switches cycling between the GDP-bound inactive state and the GTP-bound active state. Hence, they are often referred to as small G-proteins. Like the larger heterotrimeric G-proteins, Ras family members contain a guanine nucleotide binding domain (GDP/GTP) and a GTPase domain (GTPase) that is responsible for restoring the resting (GDP-bound) state by hyrolysis of GTP. Unlike some G-proteins, the intrinsic GTPase activity of these molecules is exceedingly slow and is thought to be inconsequential under physiological conditions. Instead, specific GTPase-activating proteins (GAPs) must accelerate this activity. Nonetheless, all identified regulators of Ras family GTP hydrolysis require this intrinsic GTPase domain. Therefore, mutations in this highly conserved domain result in prolonged GTP-binding and constitutive activation. For example, a mutation in which the conserved Gly12 in the GTPase domain is substituted for Val (V12) results in the constitutive activation of both Ras and Rap1 proteins. Likewise, mutating the conserved Ser17 in either Ras or Rap1 (N17) disrupts nucleotide binding by preventing interactions with the requisite magnesium ion. In this case, the mutated proteins are not only activation incompetent, but also act as interfering mutants to disrupt the activation of their endogenous counterparts (i.e. dominant negatives). B). Comparison of the effector domains of these proteins further illustrates their high degree of similarity. Ras and Rap1 are nearly identical in their core effector regions (amino acids (aa) 32-40). The structure of the effector domains for Ras and Rap1 have been determined and it was proposed that the amino acids at positions 30 and 31 are critical determinants of the different affinities of Ras and Rap1 for common effectors. Regardless of *in vitro* affinities, Ras and Rap1 appear to share common effectors *in vivo*. Interestingly, interactions with these common effectors can result in opposing actions depending on which Ras family member is active.

Figure 1.4 Adaptor coupling to Ras and Rap1. The typical adaptor protein consists of modular binding domains that provide the capacity to interact with multiple proteins simultaneously. For example, Grb2 consists of one SH2 domain that binds to tyrosine phoshporylated proteins and two SH3 domains that can interact with proline-rich sequences. It is well established that Grb2 forms a constitutive complex with the proline rich domain (PRD) of the Ras activator, SOS. By virtue of this interaction, SOS is co-recruited with Grb2 to activated receptor complexes where it

activates membrane-bound Ras. The interaction between the Grb2 SH3-domain and SOS is highly specific in that Grb2 does not interact with the PRD of other guanine nucleotide exchange factors (GEFs). The Crk-L adaptor shares a similar overall structure consisting of one SH2-domain and two SH3-domains. However, in contrast to Grb2, the more promiscuous SH3-domains of Crk proteins are capable of interacting with both SOS and the Rap1 activator, C3G. This ability to bind both SOS and C3G affords Crk-L the capacity to couple to both Ras and Rap1-dependent pathways. Truncated forms of exchange factors that contain the PRD but lack the catalytic domain (cdc25H) have been used as interfering mutants to disrupt signaling pathways. Overexpression of these mutants prevents the recruitment of active GEF complexes to effector sites by disrupting endogenous complexes. For example, in this study we have expressed a truncated form of C3G (CBR) to interfere with Crk-L function. Since both SOS and C3G bind the same N-terminal SH3-domain, over-expression of CBR is predicted to disrupt the ability of Crk-L to couple to either Rap1 or Ras. Likewise, mutations in SOS that abolish catalytic activity have the potential to block the activation of both Ras and Rap1.

Figure 1.5 Growth factor signaling specificity in PC12 cells. PC12 cells have been instrumental to the discovery of the classical ERK cascade and are particularly conducive to analysis of the mechanisms underlying specificity in growth factor responses. The preponderance of information regarding the classical ERK cascade described above originated from studies examining growth factor signaling in these cells. The resulting paradigm is depicted on the left side of this figure. In brief, upon epidermal growth factor (EGF) binding, the EGF receptor (EGFR) undergoes conformational changes that promote its dimerization and trans-phosphorylation on key tyrosines. These phosphorlated residues provide binding sites for the Grb2:SOS complex. Once recruited to the receptor complex, SOS is able to activate the small GTPase, Ras, which initiates the kinase cascade. Following EGF stimulation, this entire pathway is rapidly turned off by the inactivation of Ras resulting from a number of ERK-dependent inhibitory phosphorylations on the upstream activator, SOS. This rapid and transient activation of ERK is required for the observed proliferative response to EGF in these cells. NGF, on the other hand, induces a prolonged activation of ERK and differentiation into a sympathetic-like neuronal phenotype. Many studies have emphasized the importance of sustained ERK activation for the induction of neuronal differentiation in these cells. In spite of that, the mechanisms responsible for the different kinetics of ERK activation have remained elusive. Like EGF, NGF utilizes a Ras-dependent pathway to stimulate ERK activation and its subsequent physiological response. Importantly, although conflicting results have been published, the majority of studies observed a rapid and transient activation of Ras in response to NGF that was indistinguishable from the kinetics of EGF stimulated Ras activity. The above model, which is completely dependent on Ras

activation, is insufficient to explain the prolonged ERK activity following NGF stimulation. The NGF receptor (TrkA) recruits several adaptor complexes not present in the EGFR complex. These additional complexes have the potential to initiate Ras-independent pathways, which may account for the late phase of ERK activation.

Figure 1.6 List of potential neuronal targets of ERK phosphorylation.

Fig. 1.1 The classical ERK cascade

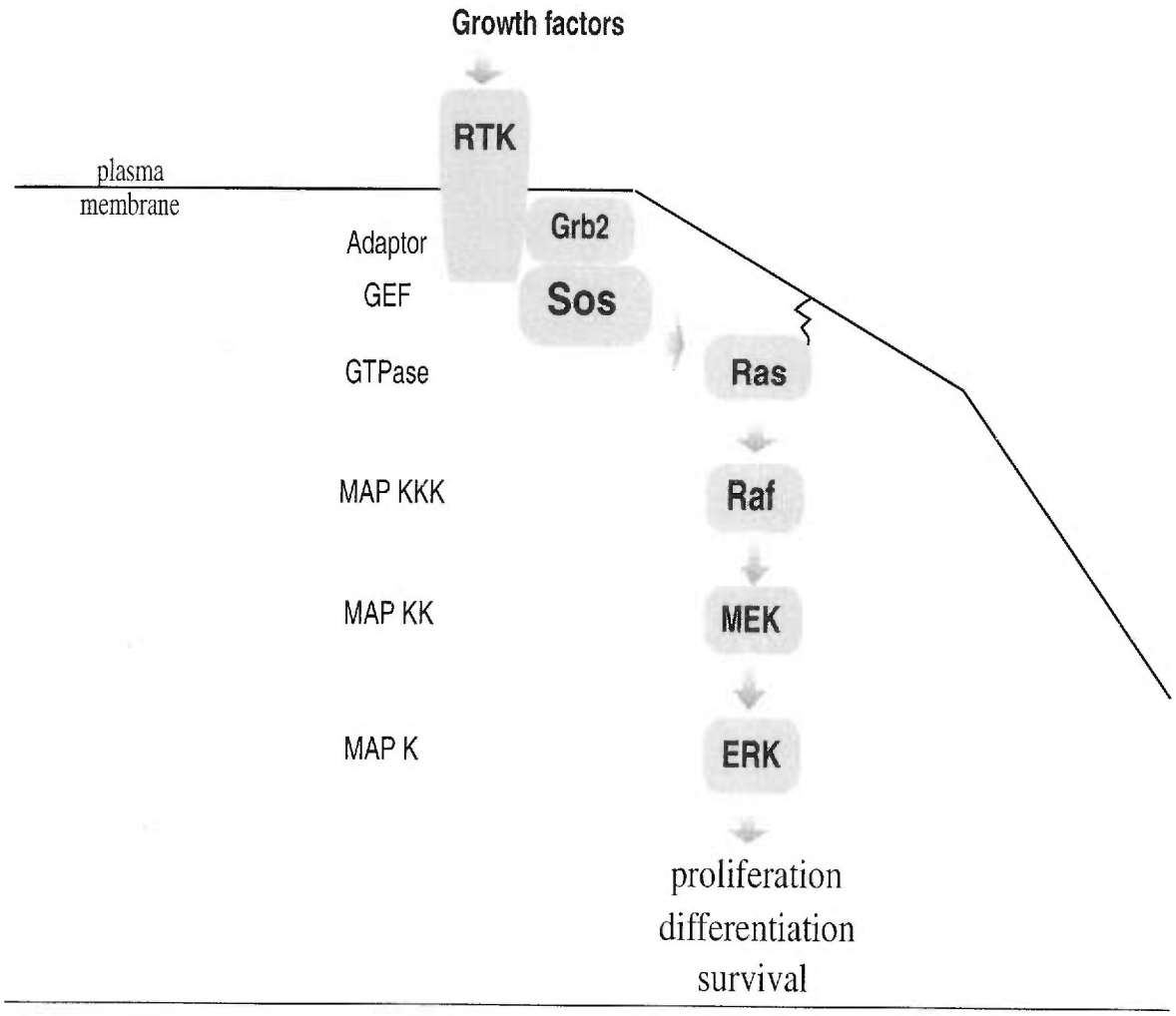


Fig. 1.2 Structural comparison of neuronal Raf isoforms

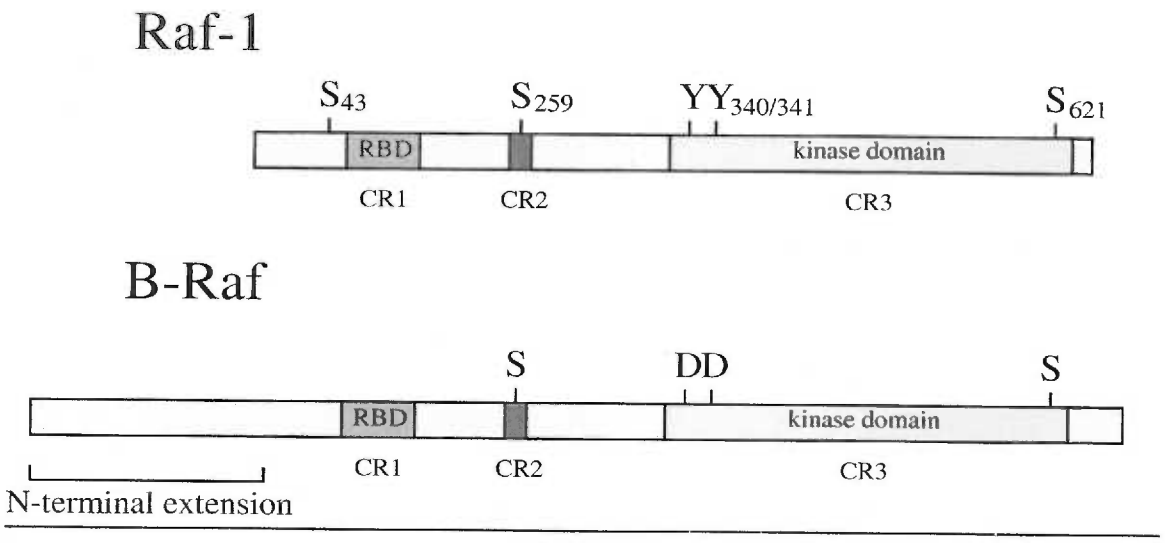
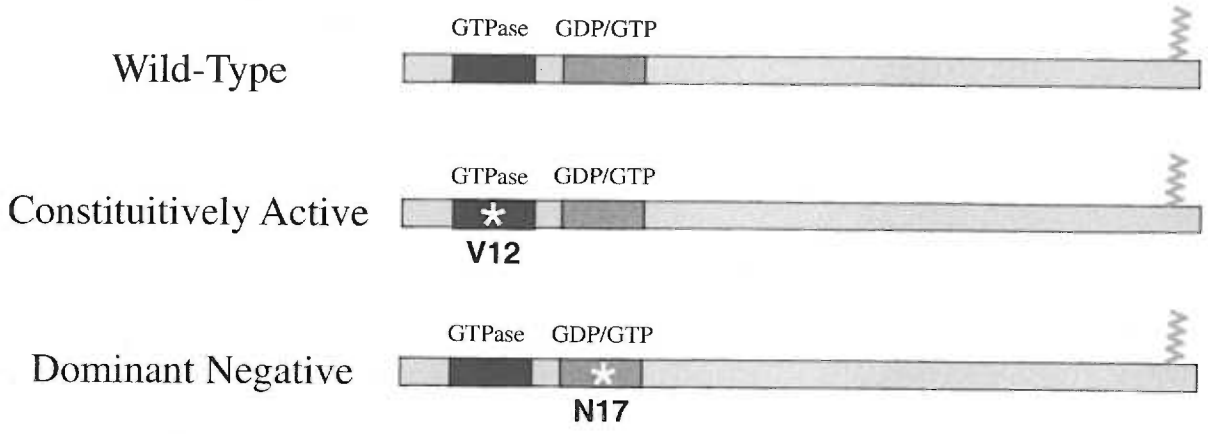


Fig. 1.3 Schematic of Ras family members

A. Conserved Ras family mutations that alter activity



B. Comparison of Ras and Rap1 Effector Domains

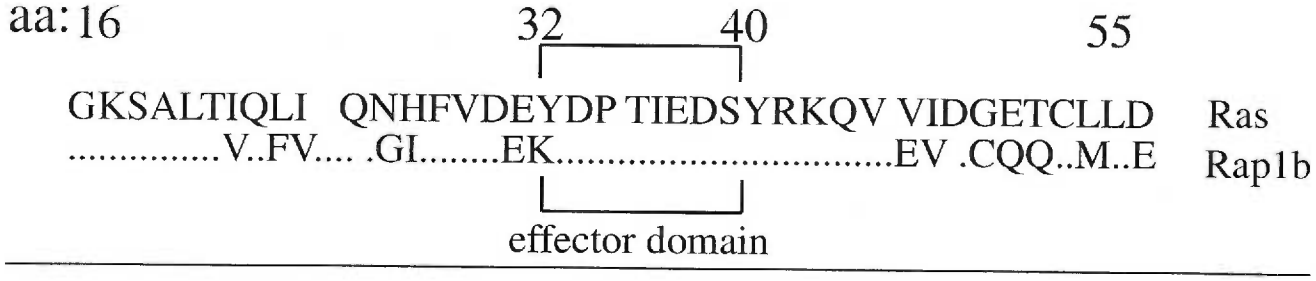


Fig. 1.4 Adaptor coupling to Ras and Rap1

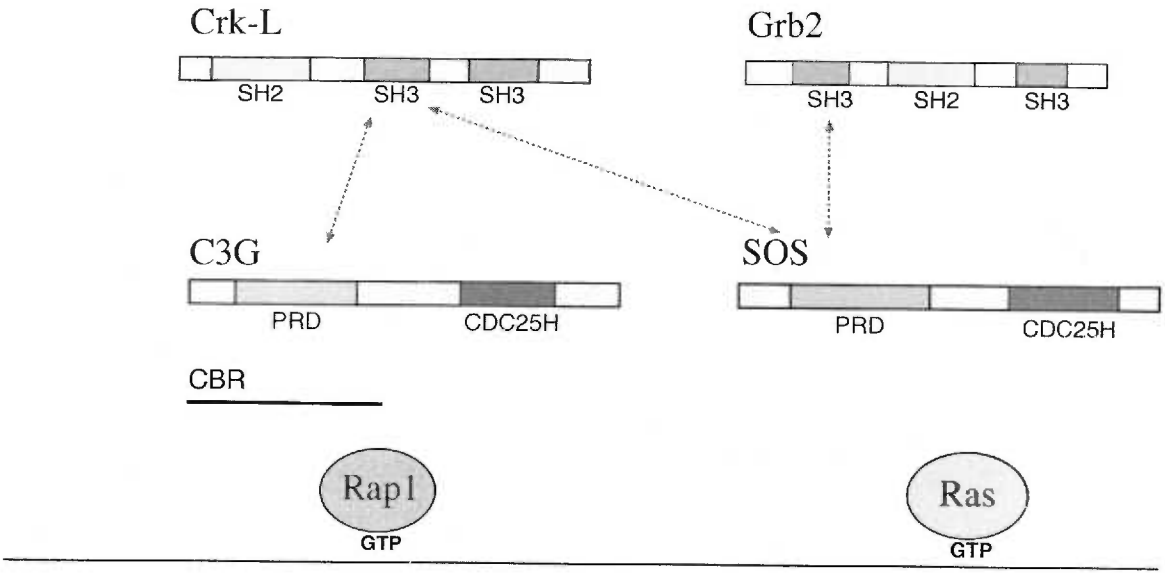


Fig. 1.5 Growth factor signaling specificity in PC12 cells

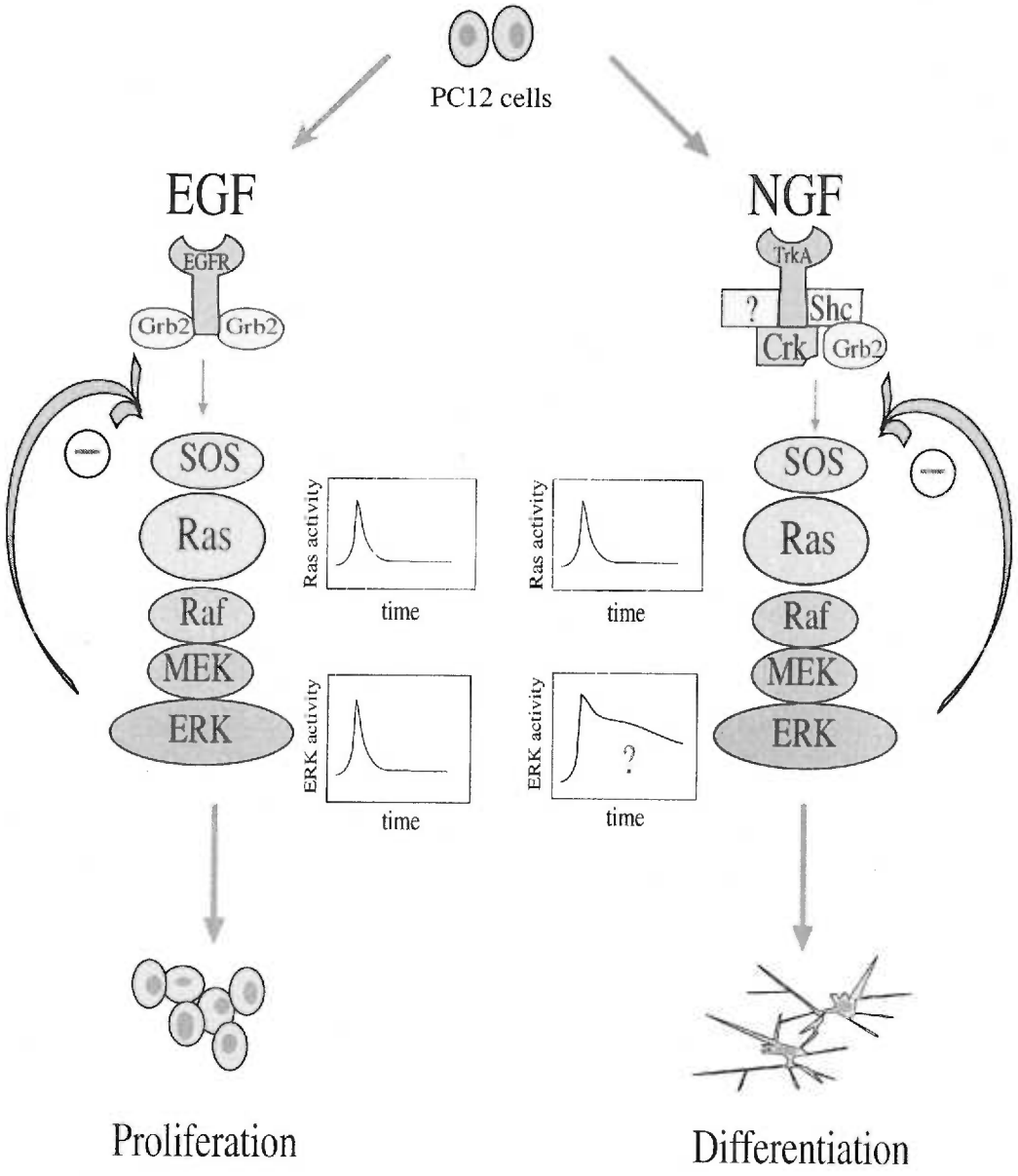


Fig. 1.6 Potential neuronal targets of ERK phosphorylation

Types of proteins — References

Nuclear proteins

ETS family transcription factors (TFs)

- Elk-1 ..... [151, 152]
- Sap-1 ..... [152]
- ETS2 ..... [153]
- ERM, ER81 ..... [154, 155]
- ERF ..... [156]
- Yan ..... [157]
- PntP2 ..... [158]

Other TFs

- c-Jun, c-Myc, c-Myb, C/EBPb (NF-IL6) ..... [151]
- Pax6 ..... [159]
- Stat3, Stat5 ..... [160, 161]
- Smad1 ..... [162]

Other nuclear proteins

- Estrogen receptor ..... [163]
- Lamins ..... [164]

Membrane-associated proteins

Plasma Membrane

- Epidermal growth factor (EGF) receptor ..... [165]
- Myristoylated alanine-richC kinase substrate (MARCKS) ..... [166]
- Connexin 43 ..... [167]
- *Aplysia* cell adhesion molecule (ApCAM) ..... [168]
- Phospholipase A2 ..... [169]
- cAMP-specific phosphodiesterase (HSPDE4D3) ..... [170]

Vesicle Membrane

- Synapsin I ..... [171]
- Caldesmon ..... [172]

Cytoskeletal proteins

- Microtubule-associated proteins (MAP2C, MAP4, tau) ..... [173]
- Neurofilaments ..... [174]
- Myelin basic protein ..... [175]

Cytoplasmic kinases

- Rsk2 ..... [104, 176]
- RskB ..... [177]
- MAPKAP kinase-2 ..... [178]
- MAPKAP kinase-3 (3pK) ..... [179]
- Mnk1/Mnk2 ..... [180, 181]
- Msk1 ..... [182]

Other cytoplasmic proteins

- PHAS-1 ..... [183]
- Tyrosine hydroxylase ..... [184]
- Hid ..... [185]

CHAPTER TWO

Rap1 Mediates the Sustained MAP Kinase Activation Induced by Nerve Growth Factor.

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SUMMARY

Activation of the mitogen-activated protein (MAP) kinase (also known as extracellular signal-regulated kinase, or ERK) by growth factors can trigger both cell growth and differentiation [94]. Therefore, the intracellular signals that couple growth factors to ERK may dictate growth factor specificity. For example, in PC12 cells, epidermal growth factor (EGF) stimulates proliferation via transient activation of ERK [94]. In contrast, Nerve growth factor (NGF) promotes differentiation of PC12 cells, in part, by inducing a sustained activation of ERK [94]. We show that NGF's activation of ERK involves two distinct pathways. The initial activation of ERK requires the small G protein Ras, whereas the sustained activation of ERK requires the small G protein Rap1. Rap1 is activated by Crk adaptor proteins and the guanine nucleotide exchange factor, C3G, and forms a stable complex with the MAP kinase kinase kinase B-Raf. Rap1 is required for at least two indices of neuronal differentiation by NGF; electrical excitability and the induction of neuron-specific genes. We propose that the activation of Rap1 by C3G represents a common mechanism to induce sustained activation of the MAP kinase cascade in B-Raf expressing cells.

INTRODUCTION AND RESULTS

PC12 cells are a well studied model of growth-factor specificity. Treatment of PC12 cells with NGF triggers differentiation into sympathetic-like neurons, characterized by electrical excitability, the induction of a set of neuron-specific genes, and neurite outgrowth [127, 186]. The ability of NGF to induce sustained activation of the ERK (extracellular signal-regulated kinase) family of MAP kinases has been implicated in these differentiating actions in PC12 cells [14, 121, 122, 127, 187]. The molecular mechanisms that maintain sustained ERK activation are not known. However, signals that limit ERK activation have been identified. In some cells, ERK directs the phosphorylation of the Ras guanine nucleotide exchange factor, Sos, to terminate Ras-dependent ERK activation [142]. Therefore, Ras-independent pathways may be required to maintain ERK activation for sustained periods of time. The small G protein Rap1 can also stimulate ERK in PC12 cells [188]. In this study, we examine the possibility that Rap1 contributes to NGF action in PC12 cells.

Using an interfering mutant of Rap1, RapN17 [188], we show that Rap1 is required for maximal activation of ERK by NGF in PC12 cells. RapN17 blocked NGF's ability to stimulate the sustained phase of ERK activation in these cells, yet had no effect on the initial rapid phase of ERK activation. RasN17, a dominant negative mutant of Ras, blocked only the initial phase of ERK activation by NGF, but did not inhibit the late phase (Figure 2.1a). Other studies have suggested that Ras is essential for NGF signaling to ERKs [4, 5]. However, these studies only examined early time points of NGF's stimulation or utilized stably transfected clonal variants of PC12 cells rather than wild type cells. Our studies suggest that Ras and Rap1 mediate the initial and the sustained phases of NGF's activation of ERK, respectively, and that these actions of both Ras and Rap1 are largely independent of each other.

One of the nuclear targets of ERK is Elk-1, a transcription factor of the Ets family [188]. NGF's activation of ERK in PC12 cells can be monitored by measuring the activation of Elk-1, using a transcription-coupled assay [188]. In PC12 cells, NGF stimulated Elk-1 to high levels, greater than those achieved by EGF (Figure 2.1b). The expression of RapN17 reduced NGF's activation of Elk-1 to those levels reached following EGF stimulation, while EGF's activation of Elk-1 was unaffected (Figure 2.1b) [188]. RasN17 blocked the activation of Elk-1 by both agents, suggesting that both Rap1 and Ras may be required for the high levels of Elk-1 activation seen following NGF stimulation.

Rap1, like other small GTP binding proteins, is active in the GTP-bound state [188]. Both the cAMP analog 8-(4-chlorophenylthio)-cyclic AMP (8-CPT) and NGF activated Rap1 for extended periods (30-45 minutes) (Figure 2.2a). Neither RapN17 nor RasN17 blocked NGF's activation of Ras and Rap1, respectively, suggesting that Ras and Rap were activated by parallel pathways (Figure 2.2a, b). Rap1 is a selective activator of B-Raf in PC12 cells [188]. Activation of Rap1 by NGF, as well as 8-CPT, recruited B-Raf to the membrane where it formed a stable complex with Rap1 (Figure 2.2c). No Rap-associated B-Raf was detected in untreated cells (data not shown) [188]. This activation of B-Raf by NGF via Rap1 may explain why B-Raf, and not Raf-1, is the major Raf isoform activated by NGF in PC12 cells [189].

Constitutive activation of Rap1 is sufficient to trigger neurite outgrowth [188], however, it is not necessary for this action. NGF's ability to induce neurites in PC12 cells was not inhibited by RapN17 at both high (100 ng/ml) [188] and low doses (10 ng/ml) of NGF (data not shown). These data, with the data from Fig. 2.1a, demonstrate that neurite outgrowth by NGF does not require sustained activation of ERK. Since, transient activation of ERK is not sufficient for neurite outgrowth, unless coupled to additional pathways [190], these data suggest that NGF activates multiple pathways leading to morphological differentiation of PC12 cells.

Sodium currents were induced by NGF in untransfected PC12 cells, as previously reported [127, 191], and in cells expressing Green Fluorescent Protein (pEGFP-C1) as a marker for transfected cells (Figure 2.3a). However, following co-transfection of RapN17, the percentage of GFP-positive cells displaying a significant sodium current was markedly reduced (Fig. 2.3a, b; right panels). The average peak sodium currents are shown in figure 2.3c. These results suggest that NGF requires Rap1, but not Ras [192, 193], to induce sodium currents in PC12 cells.

The transcriptional activation of transin (stromelysin) is a marker of neuronal differentiation of PC12 cells by NGF [193, 194]. A reporter plasmid containing the chloramphenicol acetyl transferase (CAT) gene linked to a fragment of the transin promoter (transin-CAT) has been shown to confer responsiveness to NGF, but not EGF, in PC12 cells [194]. RapN17 completely inhibited the induction of transin-CAT activity by NGF in these cells (Figure 2.3d). Therefore, transin, like Elk-1, requires both Ras [193] and Rap1 for full induction by NGF. This may explain why both EGF (a Ras activator) and cAMP (a Rap1 activator) are unable to induce transin expression alone, but are potent inducers when applied together [121]. RapN17 did not block the induction of transin by BxB-Raf, a constitutively active mutant of Raf-1, suggesting that Rap1 acts upstream or independent of Raf-1 to regulate transin expression.

Rap1 activation by NGF may involve the recently identified guanine nucleotide exchanger, C3G [84, 195]. C3G is a selective activator of Rap1 [57], and was identified by its ability to interact with members of the family of Crk adaptor proteins, including Crk-I, Crk-II, and Crk-L. *In vivo*, C3G binds specifically to the N-terminal SH3-domain of Crk proteins, but not to other SH3-containing proteins, including Grb2 [84, 86, 147, 196], suggesting that C3G is specifically activated by Crk-dependent pathways. Crk-L is the predominant Crk isoform interacting with C3G in several cell types [86, 147, 196, 197] and is highly expressed in PC12 cells (Figure 2.4a). PC12 cells also express abundant Crk-II and low, but detectable, levels of Crk-I (Figure 2.4a). NGF's activation of the ERK substrate Elk-1 can be potently augmented by the co-transfection of exogenous Crk-II and Crk-L, but only weakly by Crk-I (Figure 2.4b; left panel). In the absence of NGF, the expression of Crk isoforms activated Elk-1 minimally (data not shown). C3G dramatically increased NGF's activation of Elk-1 when co-transfected with Crk-L (Figure 2.4b), but had only a modest effect alone (data not shown).

As Crk-L/C3G also activates the c-jun N-terminal kinase, JNK [198], we used the Jun/Gal4 and Gal4/luciferase reporter system to test whether JNK contributed to the activation of Elk-1 by Crk-L/C3G in PC12 cells [199]. Crk-L/C3G expression, but not NGF stimulation, increased luciferase activity in this assay (Figure 2.4b; right panel). Furthermore, NGF blocked

the activation of JNK by Crk-L/C3G, demonstrating that the potentiation of NGF-induced activation of Elk-1 by Crk-L/C3G was not mediated by JNK. Elk-1 activation by NGF/Crk-L/C3G was blocked by interfering mutants of ERK1 and ERK2 (dnERKs) (Figure 2.4b; left panel), and by the MEK inhibitor PD98059 (Figure 2.4b; middle panel). The action of PD98059 was specific; it had no effect on the activation of Elk-1 via a constitutively-activated MEK kinase (DMEKK). These results demonstrate that NGF potentiates Crk-L/C3G signaling to Elk-1 via ERK. This potentiation was demonstrated directly using myc-ERK2 (Figure 2.4c). The expression of co-transfected Crk-L and C3G augmented NGF activation of myc-ERK2 at all time points examined. RapN17 blocked the activation of myc-ERK2 by NGF/Crk-L/C3G only at later time points (20, 30, 40, and 60 minutes) (Figure 2.4c). In contrast, RasN17 blocked this activation at early time points (5 and 10 minutes). Since SOS, but not C3G, can activate Ras [57], RasN17 may be blocking Crk-L signaling to SOS, rather than C3G, at these early time points. These data confirm that Ras is required only for the early component, and Rap1 is required only for the late component of NGF's activation of ERK.

C3G stimulated the GTP loading of Rap1 and induced the association of Rap1 with B-Raf, detected both by western blotting and kinase assays (Figure 2.4d, e). Both actions of C3G were potentiated by Crk-L (Figure 2.4d, e). In NIH3T3 cells, Rap1 activation of ERK requires exogenous B-Raf [188]. The ability of C3G to activate ERK in these cells also required exogenous B-Raf and was blocked by the expression of RapN17 (Figure 2.5a), demonstrating that C3G activates ERK in a Rap1- and B-Raf-dependent manner.

NGF may use Crk isoforms to activate Ras, as well [85, 149], since Ras activation by NGF can be inhibited by the expression of interfering mutants of Crk [85]. These same mutants of Crk have no effect on EGF-stimulated ERK activity [150], suggesting that the ability of Crk proteins to mediate ERK activation is specific for NGF. To test this possibility, we used a truncated mutant of C3G (CBR) containing the Crk-binding region that interferes with Crk function [57]. When expressed in PC12 cells, CBR inhibited NGF's activation of ERK at all time points examined, but had not effect on EGF signalling to ERK (Figure 2.5b). These results suggest that CBR interferes selectively with Crk pathways to block both C3G- and SOS-dependent signals, and confirms that EGF signalling to ERK does not require Crk [150]. The selective action of CBR on NGF signalling was reflected in the activation of Elk-1, as well (Figure 2.5c). Therefore, NGF signalling to both Ras and Rap1 may require Crk isoforms.

Crk-L and C3G are activated by multiple growth factors in many cell types, where they are thought to activate Rap1 [86, 147, 196, 197, 200]. Since Rap1 activates B-Raf, but inhibits Raf-1 [188], Rap1 may have two opposing functions; to limit ERK activation in B-Raf-negative

cells and to augment ERK activation in B-Raf-positive cells. In PC12 cells, and possibly other neuronal cells that express B-Raf, NGF's activation of Rap1 promotes sustained activation of ERK and is required for the induction of electrical excitability and a subset of neuron-specific genes. Furthermore, since B-Raf can convert Rap1 into a positive regulator of ERK, the regulation of B-Raf expression in tissues may provide a novel mechanism to modulate growth factor signalling via Rap1.

METHODS

Materials. Polyclonal anti-Rap1 and anti-B-Raf were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), and used in an unbound form for immunoblotting and bound to protein A-Sepharose for immunoprecipitations. The common Crk antibody used recognizes both Crk-I and II (anti-Crk-I/II) and was purchased from Transduction Labs (Lexington, KY). Crk-L antisera and monoclonal antibodies to Ras were purchased from Santa Cruz Biotechnology Inc. Agarose-conjugated monoclonal anti-c-myc antibody (9E10) was purchased from Santa Cruz Biotechnology Inc. and used to immunoprecipitate exogenous myc-ERK2 fusion protein. Nickel agarose (Ni-NTA-Agarose) was purchased from Qiagen Inc. (Chatswoth, CA). Radioisotopes were purchased from NEN-DuPont. PD98059 was purchased from CalBiochem (La Jolla, CA). All other reagents were from Sigma (St. Louis, MO).

Cell culture. PC12-GR5, COS-7, and NIH3T3 cells were maintained as described [188]. For immune complex assays and western blotting, cells were deprived of serum and maintained in DMEM for 8-16 hours at 37°C in 5% CO_2 prior to treatment with NGF (100 ng/ml), EGF (50 ng/ml) or 8-CPT (175 mM) unless otherwise indicated. Cell fractionation and co-precipitation studies were performed as described [188].

Plasmids and Transfection-based Assays. Cell lines were grown to approximately 50% confluence on 100 mm plates that were uncoated (NIH3T3, COS-7 cells) or coated with polylysine (PC12-GR5) prior to transfection. Transfections were performed using calcium phosphate per the manufacturer's instructions (GIBCO-BRL). In all experiments, total DNA transfected was kept constant with addition of pcDNA3 vector (Invitrogen).
For ERK activity assays, cells were transfected with myc-ERK2 (5 mg) and assayed for ERK2 activity as previously described [188]. For B-Raf assays, eluates were immunoprecipitated with B-Raf antisera and assayed *in vitro* using recombinant MEK-1 as a substrate, as described [188].
For luciferase assays assays, the plasmids encoding Elk-1/Gal4 (3 mg), or c-jun/Gal4 (3 mg),

and 5xGal4-E1b/luciferase (3 mg) were transfected into PC12 cells with additional plasmids as indicated and assays performed as described [121]. For CAT assays, the plasmid encoding transin-CAT (5 mg) was transfected into PC12 cells along with other plasmids as indicated and assays performed as described [121]. Additional cDNAs included His-Rap (15 mg); His-Ras (15 mg); RapN17 (10 mg); RasN17 (10 mg); Crk-I (10 mg); Crk-II (10 mg); Crk-L (10 mg); C3G (10 mg); B-Raf (12 mg); BxB-Raf (5 mg); dnERKs (10 mg); DMEKK (1 mg); and pEGFP-C1 (2 mg). Elk-1/Gal4, and 5xGal4-E1b/luciferase were gifts of R. Maurer, OHSU; c-jun/Gal4 was provided by R. Goodman, OHSU; cDNA encoding GFP (pEGFP-C1) was purchased from Clonetech (Palo Alto, CA.); transin-CAT was provided by G. Ciment, OHSU; RasN17 was provided by N. Nathanson, University of Washington; BxB-Raf was provided by U. Rapp, University of Wurzburg; myc-ERK2 was provided by C. Marshall, Chester Beatty Institute for Cancer Research, London; dnERKs were provided by M. Cobb, University of Texas, Southwestern; DMEKK was provided by Gary Johnson, University of Colorado Medical Center; Crk-I, Crk-II, and Crk-L were provided by B. Druker, OHSU; and C3G was provided by M. Matsuda, National Institute of Health, Tokyo.
CBR was made by PCR amplification of the cDNA encoding amino acid 279 to amino acid 660 of C3G using oligonucleotide primers containing a 5' HindIII site (sense oligo: 5'-GCGAAGCTTGAGACCATGGATAATAGTCCTCCACCA-3') and a 3' XhoI site (antisense oligo: 5'-ATTCTCGAGCTGAGCCGACTCAGAGC-3'), and subcloned into pcDNA3. cDNA encoding His-Ras was amplified by PCR from cDNA encoding wild type Ha-Ras, using specific primers that directed the fusion of the coding region (minus the start methionine) in frame with the poly-histidine amino terminus [188]. All plasmid constructions were sequenced prior to use.

Electrophysiological studies. PC12 cells were plated on collagen, transfected, and treated with NGF (10 ng/ml). Two to three days later, whole cell sodium currents were recorded with a patch clamp amplifier using 2.5 MOhm pipettes, holding potential -70 mV. The pipette solution contained 140 mM N-methyl glucamine, 10 mM EGTA, 10 mM HEPES, and was adjusted to pH 7.2 with HCl. The external solution contained 140 mM NaCl, 1 mM KCl, 1 mM $CaCl_2$, 2 mM $MnCl_2$, 10 mM HEPES, and was adjusted to pH 7.2 with NaOH. Voltage steps were made from -60 mV to +20 mV in 20 mV increments; peak sodium current was measured at 0 mV. Histograms were created from peak currents binned in 100 pA increments and expressed as percent of cells within each group. All cells used in histograms had input resistances greater than 300 MOhms, confirming intact cell membranes.

GTP loading. Cells were transfected with 15 μg of His-Rap or His-Ras and the indicated plasmids as described above. Prior to treatment, cells were serum starved in DMEM for

four hours, washed three times and incubated in phosphate free DMEM at 37°C. After one hour, 0.5 mCi/ml of [^{32}P]-orthophosphate in DMEM was added and cells were incubated for an additional one to two hours. Rap1 was precipitated with Ni-NTA Agarose and GTP loading calculated as described [188].

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FIGURE LEGENDS

Figure 2.1 Ras- and Rap-dependent components of NGF's activation of ERK. **a,** Stimulation of ERK activity by NGF and inhibition by RapN17 and RasN17. Cells were transfected with myc-ERK2 alone (top set of panels), or with RapN17 or RasN17 and treated with NGF as indicated and assayed for myc-ERK2 activity. The position of the ERK2 substrate, myelin basic protein (MBP), is shown. **b,** Requirement of Rap and Ras for NGF's activation of ERK-dependent gene transcription. PC12 cells were transfected with Elk-1/Gal4, 5xGal4-E1b/luciferase and RapN17 or RasN17 and treated with NGF or EGF and luciferase activity measured. Standard error is shown (n = 3).

Figure 2.2 Rap1 activation and association with B-Raf. **a,** Ras-independent activation of Rap1 by 8-CPT and NGF. Rap GTP loading was assayed in NGF or 8-CPT-treated PC12 cells in the presence or absence of RasN17, as indicated. The percent GTP loading is provided above each lane. **b,** RapN17 does not block Ras activation by NGF. Ras GTP loading was assayed in PC12 cells in the presence or absence of RapN17. In a and b, the levels of His-Rap and His-Ras protein within parallel eluates were determined by western blot (lower panels). **c,** NGF and 8-CPT induce the association of Rap1 with B-Raf. Membranes (M) and cytosolic (C) fractions were prepared from PC12 cells treated as indicated. Rap1 immunoprecipitates were examined by western blot using B-Raf antisera.

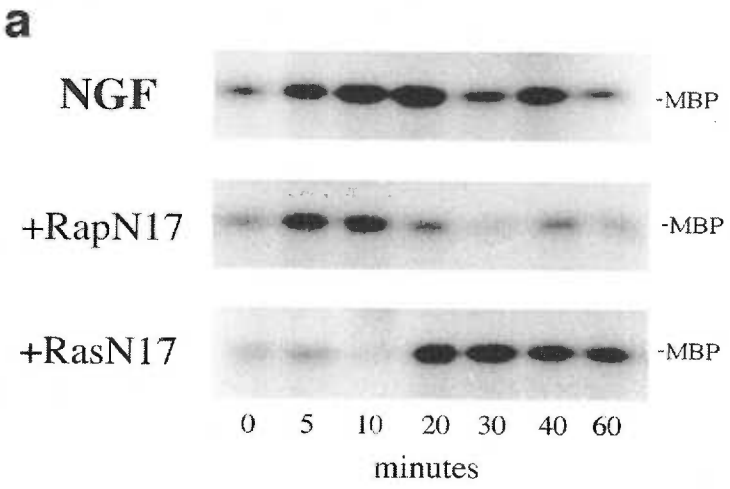
Figure 2.3 The action of RapN17 on components of neuronal differentiation. **a,** Na^+ currents from PC12 cells after 2-3 days in NGF (10 ng/ml). Currents from a representative untransfected (left panel) and RapN17/GFP-transfected cell (right panel). **b,** Histograms of peak Na^+ current amplitudes. Left Panel: Peak currents from untreated PC12 cells (black, n=17) and PC12 cells treated with NGF (gray, n=39). Right panel: NGF-treated PC12 cells transfected with GFP (2 μg) plus RapN17 (black, n=27), or GFP alone (gray, n=37). **c,** Mean current expressed in pA for each treatment group, ± standard error. **d,** Inhibition of NGF induction of transin by RapN17. PC12 cells were transfected with transin-CAT, RapN17, and BxB Raf as indicated, and treated with NGF.

Figure 2.4 Enhancement of NGF signalling by Crk-L and C3G via Rap1 and B-Raf. **a,** Expression of Crk isoforms in PC12 cells. PC12 cells were transfected with Crk-I, Crk-II, or Crk-L, as indicated, and lysates (20 μg of protein) examined by western blot. Left panel: PC12 cells and control Hela cells (H) probed with an antibody recognizing both Crk-I and Crk-II (anti-Crk-I/II). Right panel: PC12 cells and control A-431 cells (A) probed with anti-Crk-L. **b,** Elk-1 activation by NGF signalling via Crk-L/C3G. Elk-1/Gal4 (left and middle panel) and c-jun/Gal4

(right panel) activities were measured by luciferase assay in cells transfected and treated as indicated. Middle panel: cells were treated with PD98059 (PD) (20 μM) ten minutes prior to NGF treatment. **c,** ERK2 activation by NGF/Crk-L/C3G via Rap1. PC12 cells were transfected with the indicated plasmids, and treated with NGF. Myc-ERK2 activities of the transfectants are grouped by NGF treatment. **d,** Crk-L/C3G activation of Rap1. GTP loading was measured in PC12 cells expressing His-Rap with C3G and Crk-L as indicated. **e,** Crk-L and C3G induce the association of B-Raf protein and kinase activity with Rap1. COS-7 cells were transfected as indicated. Top panel: western blot of B-Raf eluting with His-Rap. Bottom panel: B-Raf immune complex kinase assays of His-Rap eluates using recombinant MEK-1.

Figure 2.5 Requirement of Crk/C3G for maximal activation of ERK. **a,** C3G activation of ERK2 requires B-Raf and Rap1. NIH3T3 cells were transfected as indicated and assayed for myc-Erk2 activity. **b,** CBR inhibits activation of ERK2 by NGF, but not EGF. PC12 cells were transfected, and treated with NGF (upper panel) or EGF (lower panel), as indicated. The expression of equal levels of myc-ERK2 protein in a and b was confirmed by western blot (lower panels). **c,** CBR inhibits Elk-1 activation by NGF, but not EGF. Transfected PC12 cells were treated with NGF or EGF as indicated and luciferase assays performed.

Fig. 2.1 Ras- and Rap1-dependent components of NGF's activation of ERKs



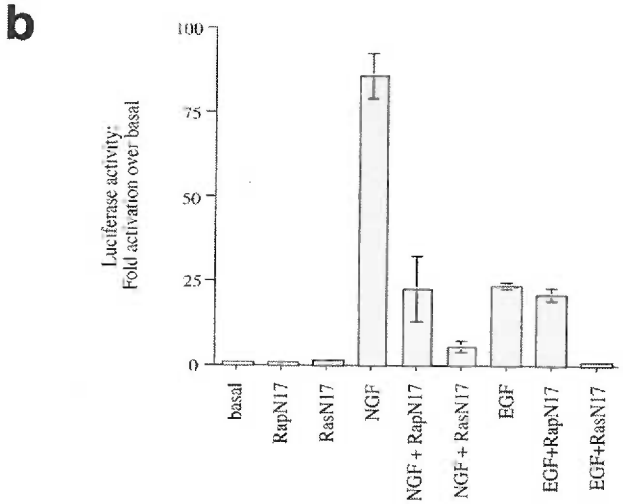


Fig. 2.2 Rap1 activation and association with B-Raf

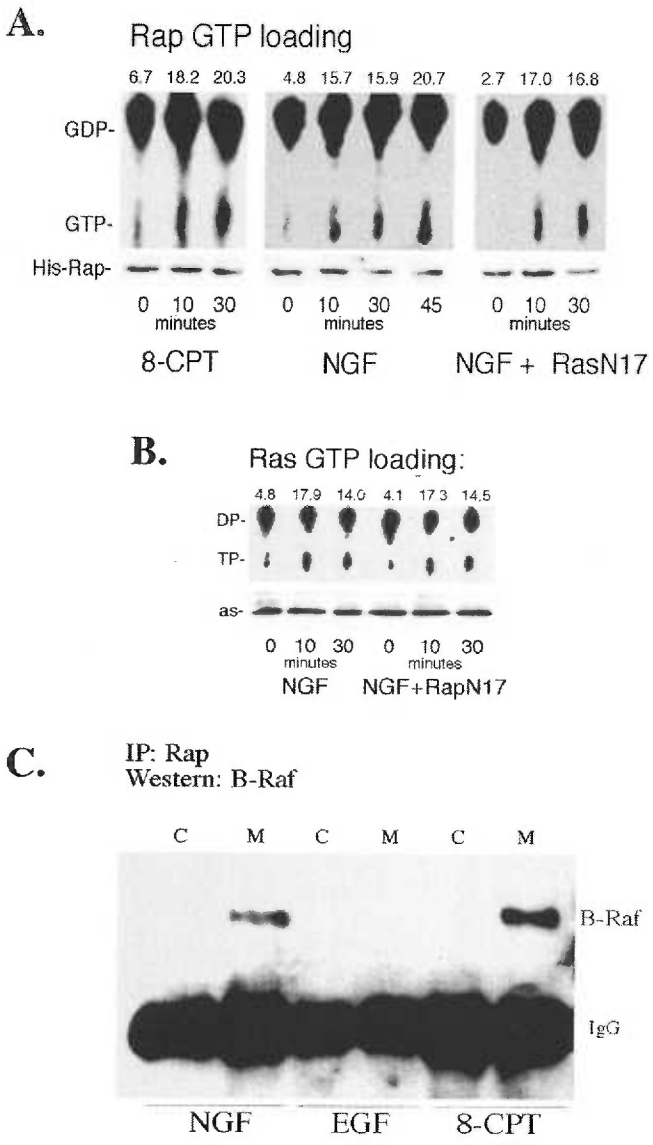


Fig. 2.3 The action of RapN17 on components of neuronal differentiation

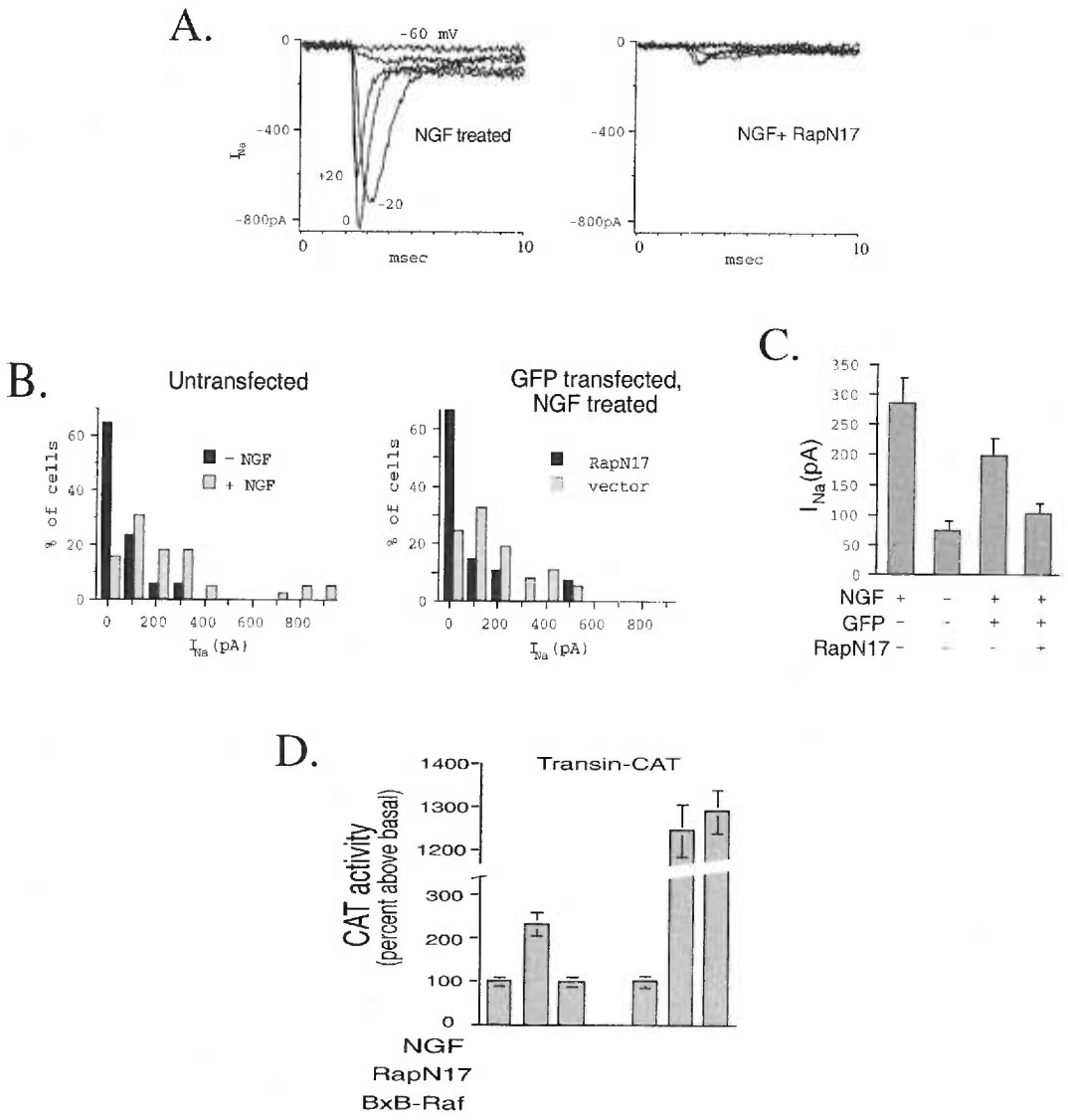


Fig. 2.4 Enhancement of NGF signaling by Crk-L and C3G via Rap1 and B-Raf

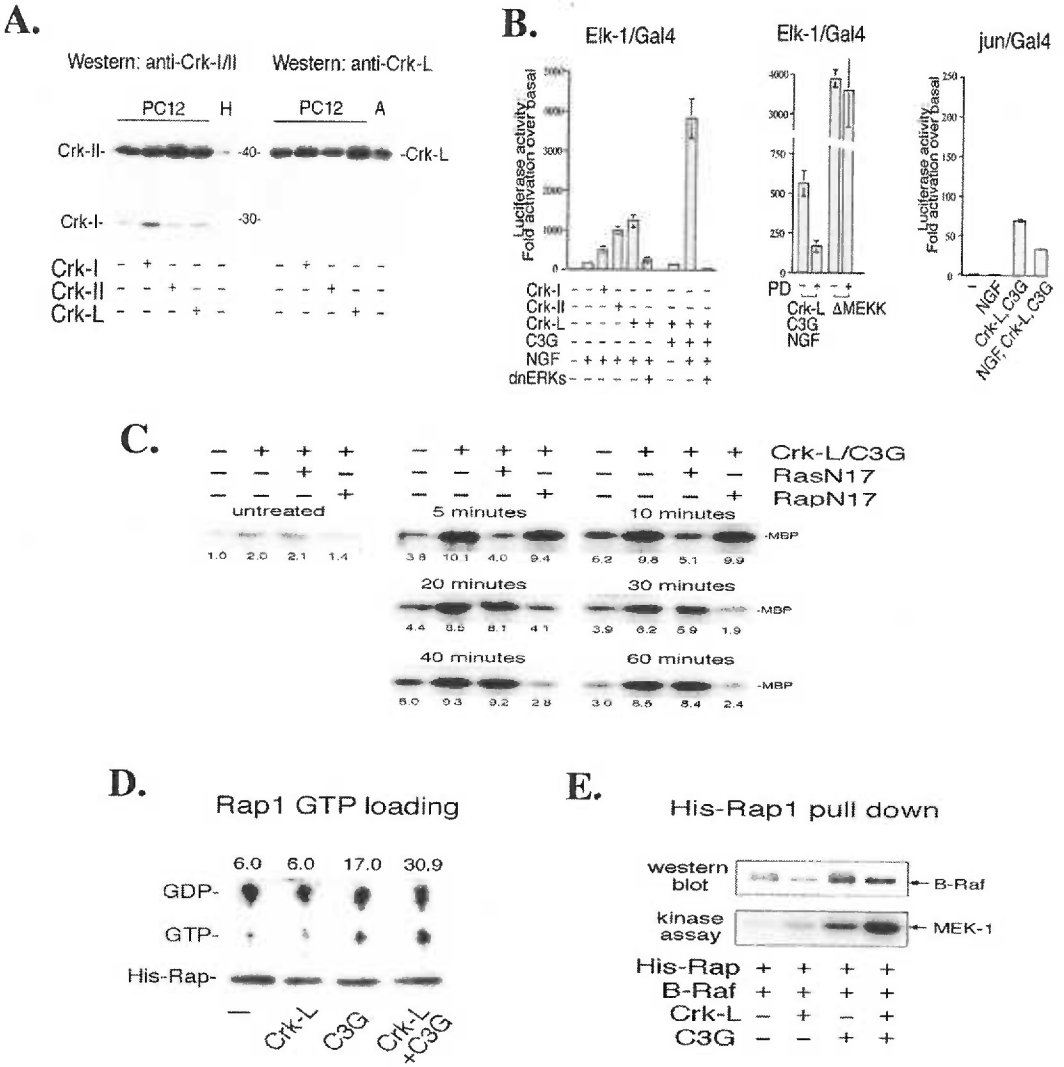
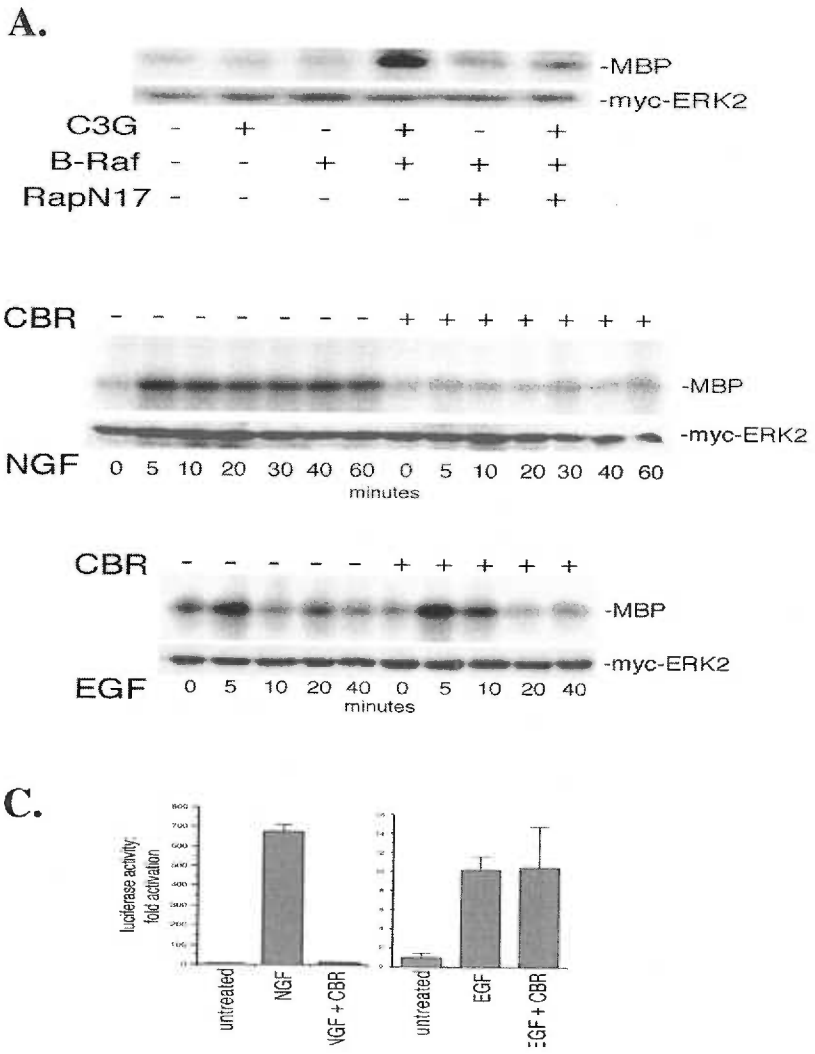


Fig. 2.5 Requirement of Crk/C3G for maximal activation of ERK



CHAPTER THREE

Role of Phosphoinositide 3-Kinase and Endocytosis in NGF Induced Extracellular Signal-Regulated Kinase Activation via Ras and Rap1.

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Running title: Rap1 activation by NGF requires PI3-K

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ABSTRACT

Neurotrophins promote multiple actions on neuronal cells including cell survival and differentiation. The best studied neurotrophin, nerve growth factor (NGF) is a major survival factor in sympathetic and sensory neurons and promotes differentiation in a well-studied model system, PC12 cells. To mediate these actions, NGF binds to the TrkA receptor to trigger intracellular signaling cascades. Two kinases whose activities mediate these processes include the mitogen-activated protein (MAP) kinase [or extracellular signal-regulated kinase (ERK)] and phosphoinositide 3-kinase (PI3-K). To examine potential interactions between the ERK and PI3-K pathways, we studied the requirement of PI3-K for NGF activation of the ERK signaling cascade in dorsal root ganglion (DRG) cells and PC12 cells. We show that PI3-K is required for TrkA internalization and participates in NGF signaling to ERKs via distinct actions on the small G proteins Ras and Rap1. In PC12 cells, NGF activates Ras and Rap1 to elicit the rapid and sustained activation of ERKs, respectively. We show here that Rap1 activation requires both TrkA internalization and PI3-K, whereas Ras activation requires neither TrkA internalization nor PI3-K. Both inhibitors of PI3-K and inhibitors of endocytosis prevent GTP loading of Rap1 and block sustained ERK activation by NGF. PI3-K and endocytosis may also regulate ERK signaling at a second site downstream of Ras since both rapid ERK activation and the Ras-dependent activation of the MAP kinase kinase kinase B-Raf are blocked by inhibition of either PI3-K or endocytosis. These studies suggest that PI3-K may be required for the signals initiated by TrkA internalization, and demonstrate that specific endocytic events may distinguish ERK signaling via Rap1 and Ras.

INTRODUCTION

Neurotrophins have long been recognized for their role in regulating neuronal survival, cell growth, differentiation, and neuronal plasticity. The archetypal neurotrophin, nerve growth factor (NGF), elicits most of these effects by binding and activating the receptor tyrosine kinase (RTK), TrkA, which leads to the activation of several well defined signaling cascades. Of these, the phosphoinositide 3-kinase (PI3-K) and extracellular signal-regulated kinase (ERK) pathways are two of the most extensively studied. PI3-Ks have been implicated in multiple biological

responses including membrane trafficking, proliferation, differentiation, and survival [201]. These kinases consist of a family of proteins which phosphorylate phosphatidylinositol (PI) at the D3 position and have been categorized into three classes based on their lipid substrate specificity in vitro [202]. The lipid products of PI3-Ks (PI(3)P, PI(3,4)P, PI(3,5)P, and PI(3,4,5)P) are known to act as second messengers and mediate most of the known functions of PI3-Ks in cells [203].

The mitogen activated protein kinase (MAPK) family members, ERK1 and ERK2, can also be activated by a wide variety of stimuli to promote a diverse array of cellular functions [3]. In addition to their established role in mitogenesis, recent advances have identified both novel mechanisms of activation and novel functions of ERKs in neurons [91]. Following growth factor stimulation of neuronal cells, ERK is phosphorylated and activated by the dual specificity kinase, MEK, which is phosphorylated and activated by members of the Raf serine/threonine kinase family. The Raf family of protein kinases consists of Raf-1, B-Raf, and A-Raf. Neurons lack A-Raf, but express the ubiquitous Raf-1 and the neuronal isoform B-Raf. Although Raf-1 is generally considered the classic upstream activator of MEKs in non-neuronal cells [204], Raf-1 is not a major MEK kinase in neuronal tissue [24]. Furthermore, in the neuronal model system, PC12 cells, Raf-1 may contribute less than five percent of the total MEK kinase activity following NGF treatment [205], whereas B-Raf has been shown to be the major Raf isoform activated by NGF in these cells [25, 189, 206]. These studies emphasize the need to examine B-Raf regulation in order to understand ERK signaling in PC12 cells and neurons.

Activation of the B-Raf/ERK cascade is linked to RTK signaling by members of the Ras superfamily of small GTPases. We have previously shown that NGF can activate B-Raf and ERKs via two distinct pathways utilizing Ras and the related Ras family member, Rap1 [146]. The significance of these two pathways is that the engagement of Rap1-dependent signaling by NGF, but not EGF, affords specificity to growth factor signaling. Ras-dependent signaling to ERKs is transient whereas Rap1 dependent signaling to ERKs is sustained [146]. The sustained activation of ERKs via Rap1 has been proposed to participate in NGF-dependent PC12 cell differentiation and a role for Rap1 in the induction of electrical excitability and NGF-dependent gene expression has been shown [146, 207]. Like all small GTPases, Ras and Rap1 are activated by specific guanine nucleotide exchange factors (GEFs) which stimulate the exchange of bound GTP for GDP. The association of B-Raf with either Ras-GTP or Rap1-GTP is an essential step in B-Raf activation. Binding to its upstream small GTPase activator alone, however, is not sufficient for full B-Raf activation, suggesting that other factors are required [208-210].

While PI3-K and ERKs are well-studied downstream targets of NGF, prevailing models have them existing as two distinct pathways with PI3-K and its target kinase Akt controlling cell survival and ERK signaling controlling cell growth/differentiation [94, 211]. As such, no one has looked extensively at the cross-talk between these two cascades in NGF signaling. Indeed, the role of PI3-K in ERK signaling in general is not completely clear. For example, overexpression of a constitutively active p110-α, a PI3-K isoform, has been shown to activate the Ras/ERK pathway in at least one system [212], but not in others [213-216]. In addition, many studies have demonstrated a requirement of PI3-K activity for ERK activation by multiple diverse stimuli [216-225], while other reports failed to show a sensitivity of some of these same stimuli to PI3-K inhibitors [226-229]. One explanation for these apparent discrepancies may be that the ability of PI3-K inhibitors to block ERK activation is dependent on the cell-type, type of stimuli and strength of the signal [219, 230]. However, the mechanisms through which PI3-K facilitates ERK activation are not known, nor has a role of PI3-K in regulating B-Raf activity been established.

The major goal of this study was to examine the role of PI3-K in NGF signaling to ERKs. We show here that PI3-K inhibitors block the activation of B-Raf and ERK by NGF in primary sensory neurons and PC12 cells. We demonstrate that PI3-K inhibitors block the activation of Rap1, but not Ras, and suggest that this may be due to the ability of these inhibitors to block TrkA internalization. In addition to these actions upstream of Rap1, we identify a requirement of PI3-K downstream of Ras. Both actions contribute to NGF-dependent signaling to ERKs in PC12 cells.

MATERIALS AND METHODS

Materials. PC12-GR5 cells were kindly provided by R. Nishi, Oregon Health Sciences University, Portland, Oregon. Forskolin, PD98059 and LY294002 were purchased from Cal Biochem (Riverside, CA). Monodansylcadaverine (MDC), wortmannin, and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma (St. Louis, MO). NGF and EGF were from Boehringer Mannheim (Indianapolis, IN). Phosphorylation-specific mouse monoclonal antibodies (mAb) which recognize phosphorylated ERK1 (pERK1) and ERK2 (pERK2) at residues threonine 183 and tyrosine 185, as well as anti-ERK1/2 polyclonal antibodies which recognize ERK1 and ERK2 independent of phosphorylation on residues 183 and 185, were purchased from New England Biolabs (Beverly, MA). Phosphorylation-specific antibodies which recognize TrkA phosphorylated on tyrosines 674/675 (pTrkA) and Akt phosphorylated on serine 308 (pAkt) were also purchased from New England Biolabs (Beverly, MA), as were

phosphorylation state-independent Akt antibodies. Polyclonal antibodies to ERK2(C14-AC), B-Raf(C19), Rap1/Krev-1 and TrkA(C14) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-Flag (M2) antibody was purchased from Sigma (St. Louis, MO.). Anti-Ras mAb and anti-PI3-K(p85) rabbit polyclonal antibodies were from Upstate Biotechnology, Inc. (Lake Placid, N.Y.). Anti-myc mAb (9E10) was kindly provided by Andrey Shaw, Washington University, St. Louis, MO. Nickel agarose (Ni-NTA-Agarose) was purchased from Qiagen Inc. (Chatswoth, CA.) and Radioisotopes were from NEN-DuPont (Boston, MA). All other reagents were from Sigma (St. Louis, MO).

Cell Culture. PC12 cells were maintained in DMEM (Dulbecco-Modified Eagle Medium) plus 10% horse serum and 5% fetal calf serum on 100 mm plates to 60-70% confluence at 37°C in 5% CO_2 prior to harvesting. For immune complex assays and western blotting, PC12 cells were maintained in DMEM containing 0.1% horse serum and 0.5% fetal calf serum for 16 hours at 37°C in 5% CO_2 prior to treatment with various reagents. Adult DRG neurons were cultured as previously described [231]. Briefly, ganglia were dissected from 250-300g Sprague-Dawley rats, dissociated enzymatically and plated on poly-lysine and laminin-coated chamber slides for immunocytochemistry or 35 mm plates for B-Raf assays. Cells were maintained in F12 media with 10% fetal calf serum. DRG cultures were serum starved in F12 media for 4-6 hours prior to treatment. The following drug concentrations were used to treat both DRG cultures and PC12 cells, unless otherwise stated: NGF (50ng/ml), EGF (50ng/ml), Forskolin (10 μM), IBMX (100 μM), LY294002 (20 μM), wortmannin (200 nM), MDC (100 μM) and PD98059 (40 μM). All inhibitors were added 10 minutes prior to treatment.

Transfections. Sixty percent-confluent PC12 cells were co-transfected with the indicated cDNAs using Superfect from Qiagen Inc. (Chatswoth, CA) according to the manufacturer's instructions. The vector pcDNA3 (Invitrogen Corp.) was added to each set of transfections to ensure that each plate received the same amount of DNA. Following 24 hours of recovery, cells were starved overnight in DMEM containing 0.1% horse serum and 0.5% fetal calf serum before treatment and harvest.

Western blotting. Cell lysates were prepared as described [188]. Protein concentrations were assessed using Bradford protein assay. Equal protein amounts of cell lysate per treatment condition were resolved by SDS-PAGE followed by transfer onto polyvinyldine diflouride (PVDF) membranes. Membranes were blocked in 5% milk and probed with primary antibodies per manufacturers instructions followed by the appropriate HRP-conjugated anti-rabbit

or anti-mouse monoclonal secondary antibody (Amersham). Proteins were detected by enhanced chemiluminescence.

Immune complex assays. For ERK and PI3-K assays, treated and untreated cells were lysed in a buffer containing 1% NP-40, 10% Sucrose, 20mM Tris-HCl (pH 8.0), 137mM NaCl, 10% glycerol, 2mM EDTA, 1mM PMSF, 1mg/ml Leupeptin, 1mM sodium orthovanadate, and 10mM sodium fluoride. The lysates were spun at low speed to remove nuclei and the supernatant was assayed for kinase activity. ERK activity was assayed as described [188] using myelin basic protein (MBP) and [^{32}P]-gATP as substrates with equal protein amounts per treatment condition. For PI3-K assays, supernatants containing 0.5 mg of total protein were incubated with anti-PI3-K (p85) antibodies overnight at 4°C. A 50% slurry of protein-G-Sepharose beads (20ml) was then added for 45 minutes. Beads containing immunoprecipitates were washed 1X with lysis buffer, 2X with 1% NP40 in Phosphate Buffered Saline (PBS), and 1X with distilled water. PI3-K activity was measured using phosphatidylinositol (PI) as a substrate. PI (10mg/sample) was dried under nitrogen stream, resuspended in 10ml of 20mM Hepes, and sonicated for 3 minutes. Washed samples were preincubated with sonicated lipid substrate for 10 minutes on ice prior to the addition of 40ml/sample of a kinase reaction mixture containing 20mM Hepes, 30mM $MgCl_2$, 20mM ATP, and 10mCi [^{32}P]-gATP. After 15 minutes of incubation at room temperature, the reactions were terminated by the addition of 80ml 1M HCl. Lipids were extracted with a 1:1 chloroform:methanol solution and separated by TLC on Silica gel 60 plates in a solvent containing chloroform/methanol/water/ammonium hydroxide (45:35:2.6:7.4, v/v). The incorporation of ^{32}P into PI was visualized using a PhosphorImager (Molecular Dynamics). To determine the location of phosphorylated PI, unlabeled PIP was run in parallel and visualized using an I_2 vapor chamber.

For B-Raf assays, untreated and treated cells were lysed in 1% NP-40 buffer containing 10mM Tris (pH 7.4), 5mM EDTA, 50mM NaCl, and 1mM PMSF. Immune complex kinase assays were performed as described [188] using MEK-1 and [^{32}P]-gATP as substrates with equal protein amounts per treatment condition. The reaction products of all kinase assays were resolved by SDS-PAGE and analyzed with a PhosphorImager (Molecular Dynamics).

Luciferase reporter gene assays. Following transfection of GAL4-CREB [232], and 5XGal-E1b-TATA-luciferase (gal-luciferase) [233], PC12 cells were treated with the appropriate stimuli for four to five hours. Cells were then lysed and equal protein amounts of lysate per condition were assayed for luciferase activity as previously described [188]. All experiments were performed with at least three independently treated plates per condition.

***In vivo* Rap1 and Ras activation assays**. Activated Rap1 was isolated from cell lysates using a protocol adapted from Franke et. al. [234]. Treated cells from three 70% confluent 100 mm plates were lysed in 400ml ice-cold Rap1 lysis buffer (10% glycerol, 1% NP-40, 50mM Tris-HCl (pH 8.0), 200mM NaCl, 5mM $MgCl_2$, 1mM PMSF, 1mM leupeptin, 10mg/ml soybean trypsin inhibitor, 10mM NaF, 0.5mM aprotinin, and 1mM Na_3VO_4). Lysates were clarified by low-speed centrifugation and supernatants containing 2mg of total protein were incubated with 40mg of GST-RalGDS fusion protein (gift of Dr. J.L. Bos, Utrecht University, The Netherlands) coupled to glutathione agarose beads for 1 hr at 4°C. Beads were pelleted and rinsed three times with lysis buffer, and protein was eluted from the beads with Laemmli buffer. Activated Ras was isolated from stimulated cell lysates using agarose coupled GST-Raf-1-RBD provided in the Ras Activation Assay Kit (Upstate Biotechnology, Inc., Lake Placid, NY) following manufacturer's recommended protocol. The amount of Rap1 or Ras bound to beads was detected by western blot.

Nickel affinity chromatography. For studies examining polyhistidine-tagged Ras (His-Ras) or polyhistidine-tagged Rap1 (His-Rap1), transfections were performed using Superfect. Cells were lysed in a buffer containing 1% NP40, 10% glycerol, 10mM Tris (pH 8.0), 20mM NaCl, 30mM MgCl2, 1mM PMSF, 1mg/ml Leupeptin, 0.5mM aprotinin and 1mM Na_3VO_4 and supernatants prepared. Transfected His-tagged proteins were precipitated from supernatants containing equal amounts of protein using Ni-NTA Agarose and washed with 10mM imidazole in lysis buffer and eluted with 500mM imidazole, 5mM EDTA in PBS. Eluates containing His-tagged proteins were separated on SDS-PAGE and B-Raf protein was detected by western blotting. Equal amounts of each eluate were immunoprecipitated with B-Raf antisera and B-Raf activity measured by immune complex assay. Equal amounts of His-Ras or His-Rap1 in the eluates was confirmed by western blotting.

Cell surface biotinylation. PC12 cells were grown in 35 mm 6-well plates to 60-70% confluence prior to treatment. Treated cells were quickly washed on ice with ice-cold PBS. 1.5 mg/ml NHS-biotin (Pierce) was added to cells followed by a 30 minute incubation with gentle motion at 0-4°C. Cells were rinsed 3 times with 0.1M glycine in PBS to quench unreacted biotin prior to lysis in 1% NP-40, 20mM Tris-HCl (pH 8.0), 137mM NaCl, 10% glycerol, 2mM EDTA, 1mM PMSF, 1mg/ml Leupeptin, 1mM sodium orthovanadate and 10mM sodium fluoride. Cell lysates were incubated with 100µl of a 50% suspension of UltraLink Immobilized NeutrAvidin (Pierce) for 2-4 hours at 4°C. The beads were pelleted and washed 2 times with a high salt wash buffer [0.1% Triton X-100, 500mM NaCl, 5mM EDTA and 50mM Tris (pH 7.5)]

and once with 50mM Tris (pH 7.5). Biotinylated proteins were eluted from the beads by incubating at 80°C for 5 minutes in 2X Laemmli buffer. The amount of TrkA bound to the beads was detected by western blotting.

Immunocytochemistry. Cultures were fixed for 10 minutes in 3% paraformaldehyde, 15% picric acid in PBS, followed by 10 minutes in cold 100% methanol. They were then rinsed in PBS and incubated for 30 minutes in blocking buffer consisting of 1.5% normal goat serum, 1% porcine gelatin and 0.2% Triton X-100 in PBS. Primary and secondary antibodies were diluted in the same solution. Cells were incubated in primary antibody overnight, washed in PBS and placed in secondary antibody incubation for 30 minutes (CY3-conjugated donkey anti-rabbit or CY2-conjugated goat anti-mouse diluted 1:200; Jackson Immunoresearch Laboratories, West Grove, PA). As a negative control, cells were processed without a primary antibody. Primary antibodies were used at the following dilutions: pERK, 1:1000; Rap1/Krev-1, 1:500; B-Raf, 1:500; Ras, 1:500. Cells were examined by epifluorescence and confocal microscopy. For pERK1/2 immuno-staining in DRG cultures, the signal intensity in neuron cell bodies was quantitated from at least 100 cells per treatment condition using NIH image.

Electron Microscopy. PC12 cells were fixed in 4% paraformaldehyde and 0.05% glutaraldehyde in 100mM phosphate buffer (pH 7.2) for 1 hour at ambient temperature. Cells were rinsed in 100mM phosphate buffer (pH 7.2), scraped off tissue culture dishes with a rubber policeman and microfuged into a pellet. The fixed cell pellets were infused with polyvinylpyrrolidone and sucrose [235] and prepared for cryosectioning [236]. Cryosections were immunolabeled as described [237]. Diluted antibodies were microfuged prior to use. Following incubation with anti-Rap1/Krev-1 primary antibody (1:50-1:100 dilution), the sections were rinsed and incubated with protein A-gold (1:10) (Amersham Life Sciences, Inc.). Controls included substitution of primary antibody with purified rabbit IgG or irrelevant antibodies.

RESULTS

PI3-K inhibitors block B-Raf and ERK activation by NGF in DRG sensory neurons. Neurotrophins are known to promote the survival and differentiation of sensory neurons of the dorsal root ganglia (DRG) during development. In adult sensory neurons, NGF acts as a potent survival factor following injury and is required for maintenance of the differentiated phenotype. Here, we examined the ability of NGF to regulate ERKs in cultures of adult rat DRG neurons. ERK activation was monitored by immunocytochemistry with a widely used phospho-specific ERK antibody which recognizes the phosphorylation of the two sites known to be responsible for ERK activation. NGF treatment of DRG cultures resulted in

increased phospho-ERK (pERK) staining in nearly half of the sensory neurons present in the DRG culture, consistent with previous studies showing TrkA expression in 40 to 50 percent of neurons in the ganglia [238, 239]. No pERK staining was observed in glial cells, which do not express TrkA [240]. The increase in ERK activation by NGF was completely abolished in the presence of the PI3-K inhibitor, LY294002 (LY; 20 mM) (Figure 3.1A, left panels). Wortmannin (200 nM), a fungal metabolite which inhibits PI3-K activity through an independent mechanism [241], also blocked NGF induced pERK staining in these cells (data not shown). ERK activation by cAMP dependent signals was elicited by treatment with Forskolin, an activator of adenylyl cyclases. LY had no effect on Forskolin stimulation of ERKs (Figure 3.1A, right panels). Interestingly, Forskolin stimulated ERK activation in all of the sensory neurons in DRG cultures, but did not stimulate ERKs in glia. In cultures of DRG cells, B-Raf, an upstream activator of ERKs, was expressed exclusively in neurons (Figure 3.1B). Together, these results are consistent with previous reports [242, 243], and support a model in which B-Raf expression accounts for the cell type specific actions of cAMP on ERK activation [43, 188, 244].

Biochemical examination of ERKs in cultured DRG neurons was complicated by the large number of glial cells which also express ERKs. However, the selective expression of B-Raf in DRGs (Figure 3.1B) allowed us to directly evaluate B-Raf activity in sensory neurons by immune-complex kinase assay in mixed cell cultures. Using this assay, we observed an increase in B-Raf kinase activity following NGF treatment which was inhibited by the presence of LY (Figure 3.1C, D).

PI3-K inhibitors block ERK activation by NGF in PC12 cells. To understand the mechanism through which PI3-K inhibitors block ERK activation by NGF, we examined this pathway in the neuronal model system, PC12 cells. In these cells, NGF activation of ERKs at both 5 and 40 minutes was blocked by LY (20 mM), as assessed by both western blot with pERK antibodies and by immune-complex kinase assay (Figure 3.2A, B). Raising intracellular cAMP levels by treatment with Forskolin and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) stimulated ERK activation, as expected [188]. This activation by Forskolin/IBMX was not blocked by LY (Figure 3.2A, B), at concentrations that blocked NGF-stimulated PI3-K activity (Figure 3.2C). We further examined the role of PI3-K in NGF signaling by monitoring a physiological downstream target of ERKs. Previous studies have shown that NGF-induced gene expression mediated by the transcription factor, CREB, occurs via ERK-dependent mechanisms [182, 245-247]. To determine whether PI3-K played a role in NGF's regulation of physiological targets of ERKs in PC12 cells, we examined CREB-dependent transcription using a GAL4-CREB/gal-luciferase reporter system. NGF treatment of PC12 cells stimulated CREB-dependent transcription which was blocked by LY (Figure 3.2D, left panel).

The magnitude of inhibition by LY was similar to that seen with the MEK inhibitor, PD98059 (Figure 3.2D, right panel). These studies suggest that the PI3-K-dependent regulation of ERKs in PC12 cells is important in controlling the activity of downstream ERK targets as well.

To confirm the specificity of the PI3-K inhibitors, we compared the actions of LY and wortmannin on ERK with their actions on PI3-K. Both LY and wortmannin blocked NGF-induced ERK2 activation in a dose-dependent manner(Fig. 3A). The ability of LY to inhibit NGF's activation of PI3-K (Figure 3.3B), as well as the inhibition of the PI3-K-dependent phosphorylation of Akt (Figure 3.3C), displayed a similar dose dependence as did ERK inhibition (Figure 3.3A, B). NGF-induced PI3-K and ERK signals also displayed a similar dose response to wortmannin (data not shown). We independently confirmed the role of PI3-K signals in NGF activation of ERKs by attenuating the action of PI3-K activity via the expression of lipid phosphatases which remove the phosphate from the D3 position. Transfection of cDNA encoding PTEN, a PI3-K antagonizing PI(3,4,5)P phosphatase [248], abolished the ability of NGF to activate co-transfected myc-tagged ERK2 (myc-ERK2) (Figure 3.3D), suggesting that the lipid products of PI3-K were required for ERK activation. This was confirmed in experiments expressing a mutant PTEN cDNA (PTEN-G129E) encoding a single amino acid substitution which specifically ablates its lipid phosphatase activity [249, 250]. PTEN-G129E had no effect on NGF induced myc-ERK2 activation (Figure 3.3D). Together, these studies suggest that the ability of LY and wortmannin to inhibit ERK activation by NGF was a function of their specific inhibition of the lipid kinase activity of PI3-K.

PI3-K inhibitors block activation of Rap1, but not Ras, by NGF. To determine if PI3-K was required for the activation of upstream activators of ERKs in PC12 cells, we analyzed endogenous B-Raf activity by immune-complex kinase assay following NGF treatment. Inhibition of PI3-K activity by LY completely blocked B-Raf activation at both early (5 minutes) and late (40 minutes) time points (Figure 3.4A). Our lab has previously shown that NGF activates B-Raf and ERKs via Ras and Rap1 [146]. Figure 3.4B shows the relative expression of Ras and Rap1 in both PC12 cells and DRGs. To determine the effect of PI3-K inhibition on Ras and Rap1, we monitored their activation by widely used affinity purification protocols. Rap1 activation was examined using Gst-RalGDS and Ras activation was examined using Gst-Raf-1-RBD (Ras binding domain). Interestingly, pretreatment with LY completely blocked the ability of NGF to activate Rap1, but had no effect on NGF's activation of Ras (Figure 3.4C, D). Similar effects were observed with wortmannin (data not shown). In these same cell lysates, the PI3-K-dependent phosphorylation of Akt by NGF was completely inhibited in the presence of LY (data not shown), demonstrating that LY was inhibiting PI3-K in these experiments. As with ERK activation, Forskolin-stimulated Rap1 activity was not blocked by LY

pretreatment (Figure 3.4C). The ability of LY to inhibit NGF activation of Rap1, but not Ras, was also observed using GTP loading assays (data not shown).

Active Ras also requires PI3-K to couple to B-Raf and ERK activation. Previous results have suggested that ERK activation 5 minutes after NGF treatment is independent of Rap1 [146]. To confirm this, we have measured ERK activation by NGF in the absence or presence of the specific enzymatic inhibitor of Rap1, Rap1GAP1. As shown is Figure 3.5A, the expression of Rap1GAP1 completely inhibited the late phase of ERK activation by NGF (>20 minutes) with no effect on the early phase (5 minutes). At 10 minutes, we observed a partial inhibition of ERK which is consistent with the contribution of both Ras- and Rap1-dependent signals to ERK activation at this time [146]. PI3-K inhibitors blocked B-Raf and ERK activation following 5 minutes of NGF treatment, without effecting Ras activity. This finding suggests that PI3-K activity might also be required at a step between Ras and B-Raf activation. To test this, we examined the requirement of PI3-K for Ras-GTP to activate ERK2. Transfection of cDNA encoding constitutively active Ras (RasV12) induced Flag-ERK2 activation, as assessed by anti-Flag immunoprecipitation followed by pERK western blot. This activation was blocked by either co-transfection of PTEN or treatment with LY, while neither PTEN nor LY blocked ERK activation by constitutively active Raf (BXBRaf) or constitutively active MEK (caMEK) (Figure 3.5B). These data demonstrate that PI3-K was acting downstream of Ras and upstream of B-Raf. To directly examine the ability of Ras to couple to B-Raf, we monitored the recruitment of B-Raf to Ras upon NGF treatment. NGF stimulation markedly increased the association of B-Raf with Ras. This association was blocked by LY (Figure 3.5C). In addition, both LY and PTEN blocked the ability of NGF to stimulate Ras associated B-Raf kinase activity (Figure 3.5D). LY also blocked the ability of B-Raf to associate with NGF stimulated His-Rap1 (data not shown), as expected, since LY blocked Rap1 activation, as shown in Figure 3.4C.

PI3-K inhibition blocks TrkA internalization. In sensory neurons, PI3-K has been shown to be required for the retrograde transport of [I^{125}]-NGF from nerve terminals [251], but whether this action involved endocytosis or subsequent transport steps was not established. As shown in Figure 3.6A and B, the cell surface expression of TrkA in PC12 cells was reduced by NGF, presumably via endocytosis into vesicles containing activated TrkA [252, 253]. This effect was largely inhibited in the presence of LY. We detected a reduction in TrkA cell surface expression as early as 5 minutes of NGF treatment with a peak at 10 minutes. LY blocked this effect at all time points examined (data not shown). LY completely blocked phosphorylation of Akt, but not TrkA (Figure 3.6C, D), demonstrating that LY's actions were specific for targets downstream, but not upstream, of PI3-K. This suggests that PI3-K facilitates the endocytosis of TrkA, similarly to its action on the PDGF receptor [254].

Rap1 is associated with submembraneous vesicles in PC12 cells. While both Ras and Rap1 are tightly associated with membranes [73, 74, 255], several studies have shown that they are localized to different subcellular regions. In neuronal cells, Ras and Rap1 display distinct subcellular distributions [75]. In other cell types, Ras proteins are known to localize to the plasma membrane [72, 74], while Rap1 expression has been detected in intracellular compartments including the Golgi complex [72, 77] and late endosomes [78]. Here, we examined the subcellular localization of Ras and Rap1 using immunofluorescent techniques. In both DRG neurons and PC12 cells, Ras displayed a plasma membrane distribution while Rap1 was localized to intracellular structures (Figure 3.7A). To further characterize these Rap1-containing structures, we examined the subcellular localization of Rap1 in PC12 cells by immuno-gold electron microscopy. Rap1 expression was not detected in the plasma membrane but was found associated with structures which resemble endocytic vesicles (Figure 3.7B). This observation, combined with the ability of PI3-K inhibitors to block TrkA internalization, suggests that one mechanism through which PI3-K inhibitors block NGF induced Rap1/ERK activation might be through the inhibition of receptor-mediated endocytosis.

Blocking endocytosis mimics the effects of PI3-K inhibition on NGF induced Ras, Rap1 and ERK activation. Clathrin-mediated endocytosis is required for ERK activation by EGF [256-258] and IGF [259]. TrkA has also been shown to undergo endocytosis via a clathrin-mediated process [252]. Once internalized into endocytic vesicles, TrkA remains phosphorylated and continues to signal to downstream effectors [253, 255, 260-263], suggesting that internalization may be important for TrkA signaling as well. We employed the primary amine monodansylcadaverine (MDC), which is known to block clathrin-mediated endocytosis [264-266], to examine Ras and Rap1 activation in the absence of TrkA internalization. As expected, MDC pretreatment blocked NGF-mediated endocytosis of TrkA (Figure 3.8A, B). At 10 minutes following NGF treatment of PC12 cells, when both Ras and Rap1 are active, pretreatment with MDC (100 μM) largely inhibited Rap1 activation by NGF but did not inhibit Ras activation (Figure 3.8C, D). As seen with LY (Figure 3.4B), activation of Rap1 by Forskolin plus IBMX was not blocked by MDC (Figure 3.8C).

Ras recruitment and activation of B-Raf was also blocked by MDC (Figure 3.8E). This suggests that endocytosis may be required for ERK activation at two sites; one site upstream of Rap1 activation and a second site downstream of Ras activation. Indeed, examination of ERKs revealed that MDC completely blocked ERK activation at 10 minutes (Figure 3.9A), presumably by blocking both sites. As seen with MDC's inhibition of Rap1, MDC blocked phosphorylation of ERKs by NGF, but not Forskolin/IBMX (Figure 3.9A). This inability of LY or MDC to block

activation of Rap1 and ERK by Forskolin/IBMX may be due to the nature of the intracellular signal, cAMP, that is generated by Forskolin. Unlike NGF, cAMP appears to be able to activate intracellular pathways upstream of Rap1 independently of endocytosis. NGF's activation of ERKs at 10 minutes was completely abolished following expression of an interfering mutant of dynamin, (K44E), known to block clathrin-mediated endocytosis [267-269] (Figure 3.9B, C). This result is consistent with recent evidence demonstrating that dynamin is required for TrkA internalization in PC12 cells [263]. As seen in PC12 cells, blocking endocytosis with MDC also blocked ERK activation in DRG neurons, as measured by pERK immunofluorescence (Figure 3.9D).

DISCUSSION

In this study we show that NGF activation of B-Raf and ERK signaling in both DRG cultures and PC12 cells is blocked by inhibition of PI3-K. Multiple methods of inhibiting PI3-K, using both pharmacological and molecular agents, all had the same effect. Furthermore, this requirement of PI3-K was specific for NGF; the ability of cAMP to activate ERKs in both DRGs and PC12 cells did not require PI3-K.

We have previously demonstrated that both Ras and Rap1 pathways contribute to the activation of ERKs by NGF. Indeed, the co-ordinated signaling via these two pathways accounts for the ability of NGF to induce both sustained activation of ERKs and neuronal differentiation [146]. Interestingly, in the present study, we demonstrate that the requirement for PI3-K in ERK activation by NGF may reflect distinct actions on signaling via Ras and Rap1. Thus, PI3-K inhibition blocked activation of Rap1 but not Ras. However, PI3-K did block the ability of activated Ras to couple to B-Raf and ERKs. Both actions of PI3-K prevent NGF from utilizing either Ras-dependent or Rap1-dependent signals to activate ERKs.

Our data demonstrating the requirement for PI3-K in the activation of Rap1 may reflect a role in the endocytosis of activated TrkA receptors. We found that inhibition of PI3-K activity blocked TrkA internalization after NGF stimulation. Other blockers of endocytosis had the same effect as PI3-K inhibitors, preventing activation of Rap1 but not Ras. Taken together, we propose a model in which TrkA activation can stimulate Ras at the plasma membrane but requires PI3-K-dependent internalization to activate Rap1 (See Figure 3.10).

A role for endocytosis in NGF signaling is consistent with the emerging view that internalization of active TrkA receptors into signaling vesicles is required for the downstream

actions of neurotrophins [63, 263]. For example, the ability of both NGF and TrkA to be transported in a retrograde fashion from the nerve terminal to the cell body has been demonstrated by many groups [255, 261, 270, 271]. Recent studies demonstrate that this retrograde transport process delivers active TrkA to the cell body which may be required for the stimulation of gene expression by NGF [255, 261, 262]. These conclusions are supported by studies in which signaling competent vesicles containing active NGF:TrkA complexes were isolated from PC12 cells following clathrin-mediated endocytosis of TrkA [252, 253, 272]. Furthermore, these data are consistent with studies examining other growth factor receptors, as well as G-protein coupled receptors, where clathrin-mediated endocytosis has been implicated in ERK activation [256-259, 273]. Therefore, receptor trafficking may serve an important signaling function in addition to simply mediating receptor down-regulation via lysosomal degradation. It is possible that the sorting determinants and mechanisms for targeting proteins to signaling vesicles versus lysosomes may be differentially regulated for different ligand:receptor complexes [274-277]. Whether such sorting events impart specificity in the ability of growth factors to activate Rap1-dependent pathways remains to be determined.

A proposed role for endocytosis is to bring activated receptors to the location of downstream signaling molecules [278]. Accordingly, the localization of Ras and Rap1 to distinct membrane compartments may account for the differential roles of PI3-K and endocytosis in Ras and Rap1 activation. Our data showing that Rap1 resides with vesicular membranes are consistent with the localization of Rap1 to endosomal compartments. In contrast, Ras is at the plasma membrane itself, and can be activated by TrkA without additional endocytic events. Membrane targeting of Ras-like molecules is determined by postranslational modifications of their C-termini. Differences in the C-terminal motifs found in Rap1 and Ras may account for their different membrane distribution [47, 279, 280]. Importantly, these differences in membrane distribution of Ras family members influence downstream signaling actions [47]. Our data suggest that their location may also determine how these small G proteins become activated.

PI3-K-dependent TrkA internalization may control differential activation of Ras vs. Rap1 signaling to dictate the specificity of NGF action. Our lab has previously shown in PC12 cells that Rap1 is activated by NGF [146], but not EGF [188]. Furthermore, we have shown that Rap1-dependent signals contribute to growth factor specificity by mediating both the sustained phase of ERK activation by NGF and aspects of neuronal differentiation [146]. Differences in the kinetics of receptor internalization have also been proposed to impart specificity to NGF versus EGF signaling in PC12 cells [272]. In this study, Huang et. al. have shown that TrkA remains associated with caveolae-like membranes following NGF treatment, whereas EGF treatment results in the rapid depletion of the EGF receptor (EGFR) from this membrane population.

Consistent with these results, we have observed a more rapid internalization of the EGFR compared to TrkA following ligand stimulation (data not shown).

The slower internalization of TrkA following NGF treatment may allow for the assembly of signaling complexes which subsequently lead to Rap1 activation within the mature signaling endosome. It has been shown that the phosphorylation of the adaptor molecule FRS2 recruits additional adaptor molecules that contribute to the sustained ERK activation seen following treatment with NGF [281]. One of these adaptor molecules which binds phosphorylated FRS2 is Crk [282]. Crk has been shown to contribute to Rap1 activation via its association with C3G, the Rap1-specific exchanger [146, 283, 284]. Accelerating TrkA internalization by incubating PC12 cells with an NGF-antibody complex results in a transient activation of ERKs similar to that seen with EGF treatment [285]. Interestingly, acceleration of TrkA internalization resulted in a TrkA signaling complex that lacked critical phosphorylations, including phosphorylation of FRS2, that are required for sustained activation of ERKs. Here, we propose that the contribution of Rap1 signaling and endocytosis to sustained ERK activation are intimately connected; Rap1 participates in the late phase of ERK activation by NGF because Rap1 activation by NGF requires endocytic events.

One finding of this study was that the ability of Ras to couple to its downstream effector, B-Raf, was also blocked by inhibitors of PI3-K. This action explains the contribution of PI3-K to the early (Ras-dependent) activation of ERKs. This inhibition of Ras-dependent signaling downstream of Ras activation may also reflect a requirement of PI3-K for endocytic events. B-Raf is localized to vesicles within DRG neurons and PC12 cells and this localization did not appear to change following NGF treatment (data not shown). Interestingly, Grimes and co-workers [252] have shown that NGF treatment leads to the redistribution of TrkA immunostaining to a pattern similar to that seen for B-Raf. Therefore, it is possible that endocytosis and subsequent vesicular fusion is required to bring Ras in contact with B-Raf, consistent with the ability of MDC to disrupt Ras/B-Raf complexes. This model is consistent with recent evidence suggesting that Ras is internalized into enodcytic vesicles following growth factor stimulation [286]. The requirement of PI3-K in Ras-dependent activation of B-Raf may also depend on a second action of PI3-K. For example, recent studies suggest that phosphorylation of Raf-1 by kinases downstream of PI3-K may stimulate Raf kinase activity [287, 288]. In contrast, the direct phosphorylation of Raf-1 by the PI3-K target, Akt, has been shown to be inhibitory in some cells [289, 290]. However, this ability of Akt to inhibit Raf-1 appears to depend on the cell-type and cellular context [291]. Similar studies examining B-Raf have also been reported [292], although no studies have been carried out in neuronal cells. In any

case, the data presented here demonstrate that PI3-K has indirect actions that enhance signaling through B-Raf.

The major significance of these studies is that we show PI3-K is required for NGF activation of ERKs through its specific action in regulating TrkA internalization. Both PI3-K and ERKs are well studied targets downstream of NGF. A prevailing view is that they represent the end-points of distinct linear pathways and trigger distinct physiological actions. However, recent reports suggest that these pathways may be interconnected [293-295]. Here, we provide a biochemical basis for a model that these two pathways share overlapping functions within neurons and suggest that the regulation of endocytosis by PI3-K may provide an important mechanism to modulate ERK signaling cascades.

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FIGURE LEGENDS

FIGURE 3.1 Role of PI3-K in neuronal ERK activation. (A) ERK activity was assessed by immuno-staining fixed cells with a phospho-specific ERK1/2 (pERK1/2) antibody. Adult rat dorsal root ganglia cultures were treated for 20 minutes with either 50ng/ml NGF (left) or 10 μM Forskolin (right) in the presence or absence of LY294002 (LY; 20 μM), a specific PI3-K inhibitor. Left panels; light field. Right panels; fluorescent micrograph showing pERK1/2 immunoreactivity. The signal intensity in neurons was quantitated for each treatment condition using NIH image. Histograms represent the distribution of fluorescent intensities as a function of frequency (n = 100 cells). LY completely abolished ERK activation by NGF but had no effect on Forskolin stimulated ERKs. (B) Immunofluorescent localization of B-Raf expression in adult rat dorsal root ganglia cultures. Note that expression is only detected in neurons. (C) Biochemical examination of B-Raf in cultured DRG neurons. B-Raf activity was measured by immune complex kinase assay using anti-B-Raf antisera and recombinant MEK-1 as a substrate. A representative gel is shown (n=3). (D) Average of three experiments as in C, presented as fold activation with standard error (s.e.).

FIGURE 3.2 Requirement of PI3-K for ERK activation by NGF in PC12 cells. PC12 cells were treated with Forskolin plus IBMX (F/I) for 20 minutes or NGF for either 5 minutes or 40 minutes, as indicated. In (A), phosphorylation of ERKs was used as a measure of ERK activation in cell lysates monitored by western blot using pERK1/2 antibodies. In (B), ERK activation was measured in cell lysates by immune-complex kinase assay using an anti-ERK2 antibody and myelin basic protein (MBP) as a substrate. In both assays, LY blocked ERK activation by NGF but not by Forskolin. (C) PI3-K assay showing blockade of NGF-induced PI3-K activity by LY (20 mM); left panel. Average of three assays is presented as fold activation with s.e. in the right panel. (D) LY inhibition of the transcription factor CREB, an ERK-dependent target of NGF. Following transfection with GAL4-CREB (2mg) and 5XGal-E1b-TATA-luciferase (gal-luciferase, 2mg), cells were left untreated or treated with NGF, LY or the MEK inhibitor PD98059 (PD, 40 mM) as indicated. CREB-dependent transcription was monitored by luciferase assay and reported as fold increase in luciferase activity. CREB activation by NGF was sensitive to LY, as well as PD.

FIGURE 3.3 PI3-K inhibitors block NGF activation of PI3-K and ERK. (A) Dose-dependent action of LY and wortmannin on ERK activation by NGF (15′). Cells were treated with NGF and either LY (0.05, 0.5, 5.0, and 50 mM) or wortmannin (0.01, 0.05, 0.1, 0.5 and 1 mM), as

indicated. ERK activation was measured in cell lysates by immune-complex kinase assay using an anti-ERK2 antibody and MBP as a substrate. A representative gel is shown (upper panels) along with the average of three experiments, with standard error (bar graphs). ERK activity is presented as fold activation over basal levels. (B) Dose dependency of LY's inhibition of PI3-K activity. Cells were treated with NGF and LY (0.05, 0.5, 5.0, and 50 mM) and PI3-K activity was measured as described in Methods (upper panel). Fold increase in PI3-K activity is shown numerically. Lower panels show the action of LY on NGF-induced phosphorylation of ERK in the same cell lysates, as measured by western blot using pERK antibodies (middle panel). The expression of total ERKs is shown as a loading control (bottom panel). (C) Dose dependency of LY inhibition of phosphorylatated Akt (pAkt). Cells were treated with NGF and LY (0.05, 0.5, 5.0, and 50 mM), lysates prepared, and the levels of pAkt were visualized by western blot (upper panel). The expression of total Akt is shown (bottom panel). (D) Inhibition of NGF activation of ERK by PTEN. PC12 cells were co-transfected with myc-tagged ERK2 (myc-ERK2) and wild type PTEN or a mutated PTEN (PTENG129E) which lacks lipid phosphatase activity. myc-ERK2 activation was measured in cell lysates by immune-complex kinase assay using an anti-myc antibody (9E10) and MBP as a substrate. A representative gel is shown (upper panel) along with the average of three experiments, with standard error (bar graph). myc-ERK2 activity is presented as fold over basal.

FIGURE 3.4 LY inhibition of Rap1/B-Raf activation. (A) LY inhibition of NGF-induced B-Raf activation. PC12 cells were treated with NGF and LY as indicated and B-Raf activity was measured by immune complex kinase assay using anti-B-Raf antisera and recombinant MEK-1 as a substrate. (B) Western blot showing expression of Rap1 (left panel) and Ras (right panel) in PC12 cells and DRG cells, as indicated. (C) LY inhibition of NGF-induced Rap1 activation. PC12 cells were either left untreated or treated with NGF, Forskolin plus IBMX (Forsk/IBMX) and LY as indicated. Lysates were incubated with Gst-RalGDS and precipitated with glutathione beads. The amount of active Rap1 bound to the beads was measured by SDS-PAGE followed by western blots using anti-Rap1 antibodies. (D) Lack of LY inhibition of NGF-induced Ras activation. Lysates were either left untreated (Untr.) or treated as indicated. Lysates were prepared and incubated with Gst-Raf-1-RBD and precipitated with glutathione beads. The amount of active Ras bound to the beads was measured by SDS-PAGE followed by western blots using anti-Ras antibodies.

FIGURE 3.5 The requirement of PI3-K for Ras coupling to downstream kinases. (A) Independence of Rap1 for early activation of ERK by NGF. PC12 cells were transfected with myc-tagged ERK2 (myc-ERK2) in the absence or presence of co-transfected Rap1GAP1 (RapGAP), and treated with NGF for the indicated times. myc-ERK2 activation was measured in

cell lysates by immune-complex kinase assay using an anti-myc antibody and MBP as a substrate. The amount of myc-ERK2 within each lysate is shown in the lower panels. A representative gel is shown (n=3). (B) PC12 cells were co-transfected with cDNA encoding constitutively active Ras (RasV12), constitutively active Raf (BXBRaf), or constitutively active MEK (caMEK), along with Flag-ERK2, and treated with LY or co-transfected with PTEN, as indicated. Flag-ERK2 activity was assessed by anti-Flag immunoprecipitation followed by phospho-ERK (pERK) western blot. The amount of total ERK is shown in the lower panel. (C) The recruitment of B-Raf to Ras upon NGF treatment. PC12 cells were transfected with polyhistidine-tagged Ras (His-Ras) and treated with NGF in the absence or presence of LY as indicated. His-Ras and associated proteins were precipitated from lysates using Nickel-NTA agarose and associated proteins resolved by SDS-PAGE and B-Raf detected by western blot. The position of B-Raf (95 kD) is shown. (D) NGF stimulation of Ras-associated B-Raf kinase activity. PC12 cells were transfected with His-Ras and/or PTEN and treated with NGF or LY as indicated. B-Raf activity within His-Ras eluates was measured by immune-complex kinase assay using MEK-1 (MEK) as a substrate.

FIGURE 3.6 Facilitation of NGF-induced TrkA internalization by PI3-K. (A) PC12 cells were left untreated or treated with NGF in the presence or absence of LY, as indicated. Cell surface proteins were biotinylated as described in Methods. Biotinylated proteins were recovered using UltraLink Immobilized NeurtrAvidin (Pierce) and the amount of TrkA recovered was assessed by western blot. A representative blot with the position of TrkA is shown. (B) Bar graph showing the average of three biotinylation experiments as in A. The data is presented as percent of maximal stimulation, with s.e. (C) Phosphorylation of Akt is completely blocked by LY. Parallel plates of PC12 cells were treated as in A and examined for pAkt expression by Western blot. The position of pAkt (upper panel) is shown, along with total Akt (lower panel) as a loading control. (D) Phosphorylation of TrkA is not effected by LY. Cells were treated with LY and NGF as indicated and lysates examined for phospho-TrkA (pTrkA) expression by Western blot. A representative blot is shown and the position of pTrkA is indicated (n=3).

FIGURE 3.7 Subcellular localization of Ras and Rap1 in PC12 cells. (A) Immunofluorescent detection of Ras and Rap1. DRG cells (upper panels) or PC12 cells (lower panels) were fixed and incubated with monoclonal antibodies to Ras and polyclonal antibodies to Rap1. Anti-Ras (left panels) and Anti-Rap1 (right panels) were visualized by confocal microscopy. (B) Immuno-Electron Microscopy of Rap1. Using immuno-gold electron microscopy and anti-Rap1 antibodies, immunogold particles were detected on sections of fixed PC12 cells. Two adjacent PC12 cells are shown with their plasma membranes indicated. The nucleus of one cell can be

seen. Gold particles are clustered within vesicular structures. Magnification = 48,000 X. Insert: vesicular structures with gold particles are shown.

FIGURE 3.8 MDC inhibition of NGF signaling to ERK. (A) Monodansylcadaverine (MDC) blocks TrkA internalization. PC12 cells were left untreated or treated with NGF in the presence or absence of a 10′ pretreatment with MDC as indicated. Cell surface proteins were biotinylated and precipitated as in Figure 3.6 and the amount of TrkA recovered was assessed by western blot. A representative blot with the position of TrkA is shown. (B) Bar graph showing the average of three independent experiments as in A, with s.e. (C) Inhibition of NGF-induced Rap1 activation by MDC. PC12 cells were treated with NGF or Forskolin plus IBMX (Forsk/IBMX) for 10′ in the presence or absence of MDC as indicated. Rap1 activation was measured using Gst-RalGDS "pull-down" as in Figure 3.4. The position of Rap1 is shown. (D) Lack of Inhibition of NGF-induced Ras activation by MDC. Cells were treated with NGF and MDC as indicated and Ras activation was measured using Gst-Raf-1-RBD "pull-down" as in Figure 3.4. The position of Ras in PC12 lysates is shown. (E) Inhibition of Ras-dependent recruitment and activation of B-Raf by MDC. Cells were transfected with His-Ras and treated with NGF in the presence or absence of MDC as indicated. His-Ras and associated proteins were precipitated from lysates using Nickel-NTA agarose and associated proteins were eluted from His-Ras as described in Methods. Eluates were split and assayed for associated B-Raf protein by western blot (upper panel) or for associated B-Raf kinase activity (lower panel), as measured by immune-complex assay using MEK1 as a substrate (n=3). The position of MEK1 is shown. The position of B-Raf within PC12 lysates is shown in the upper panel (lysate).

FIGURE 3.9 Requirement of endocytosis for ERK activation by NGF in PC12 and DRG cells. (A) PC12 cells were treated with NGF or Forskolin plus IBMX (Forsk/IBMX) for 10′ in the presence or absence of MDC, as indicated. Activation of ERK was measured in cell lysates by western blot using pERK1/2 antibodies. The positions of pERKs (pErk1, pErk2) are shown. The amount of total ERKs in each lysate is shown in the lower panel. (B) PC12 cells were transfected with myc-ERK2 in the presence or absence of either wild type dynamin (Dyn-wt) or a mutated dynamin (Dyn-K44E) and cells treated with NGF (N), EGF (E), or left untreated (U). myc-ERK2 activation was measured in cell lysates by immune-complex kinase assay using an anti-myc antibody and MBP as a substrate. A representative gel is shown (upper panel) and the amount of myc-ERK2 within each lysate is shown below (lower panel). (C) Data from three independent experiments as performed in B are represented as fold activation, with s.e. (D) Adult rat dorsal root ganglia cultures were treated for 20′ with NGF in the presence or absence of MDC.

Upper panels; fluorescent micrograph showing pERK1/2 immunoreactivity. Lower panels; light field.

FIGURE 3.10 Model of NGF signaling to ERKs. In PC12 cells, NGF activates Ras and Rap1 to mediate the rapid and sustained activation of ERKs respectively. Both TrkA internalization and Rap1 activation require PI3-K. Clathrin-mediated endocytosis is also required for Rap1 activation and the sustained activation of ERK. In contrast, Ras activation by NGF is independent of both PI3-K activity and endocytosis. This may reflect the distinct localizations of Ras at the plasma membrane and Rap1 within endosomal compartments. PI3-K-dependent endocytosis may regulate ERK signaling at a second site downstream of Ras since both rapid ERK activation by NGF and Ras activation of B-Raf are blocked by both PI3-K inhibitors and inhibitors of endocytosis.

Fig. 3.1 Role of PI3-K in neuronal ERK activation

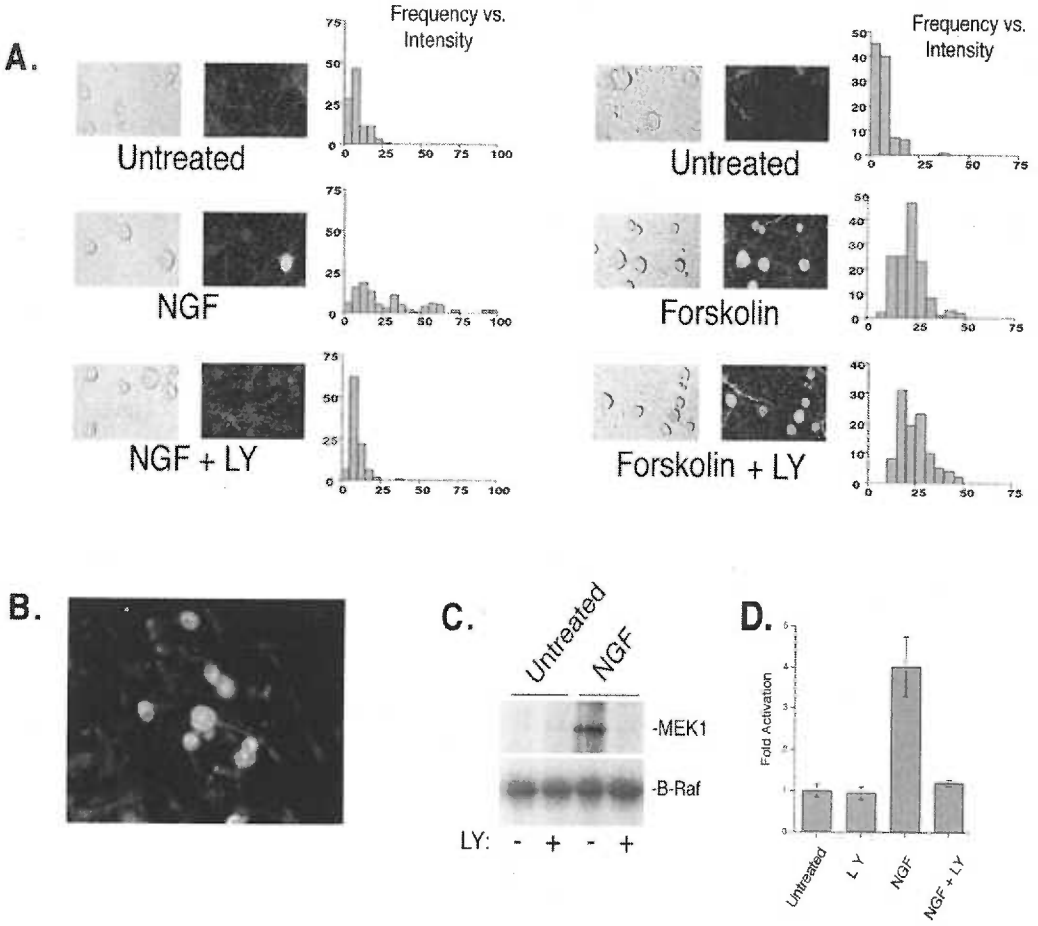


Fig. 3.2 Requirement of PI3-K for ERK activation by NGF in PC12 cells

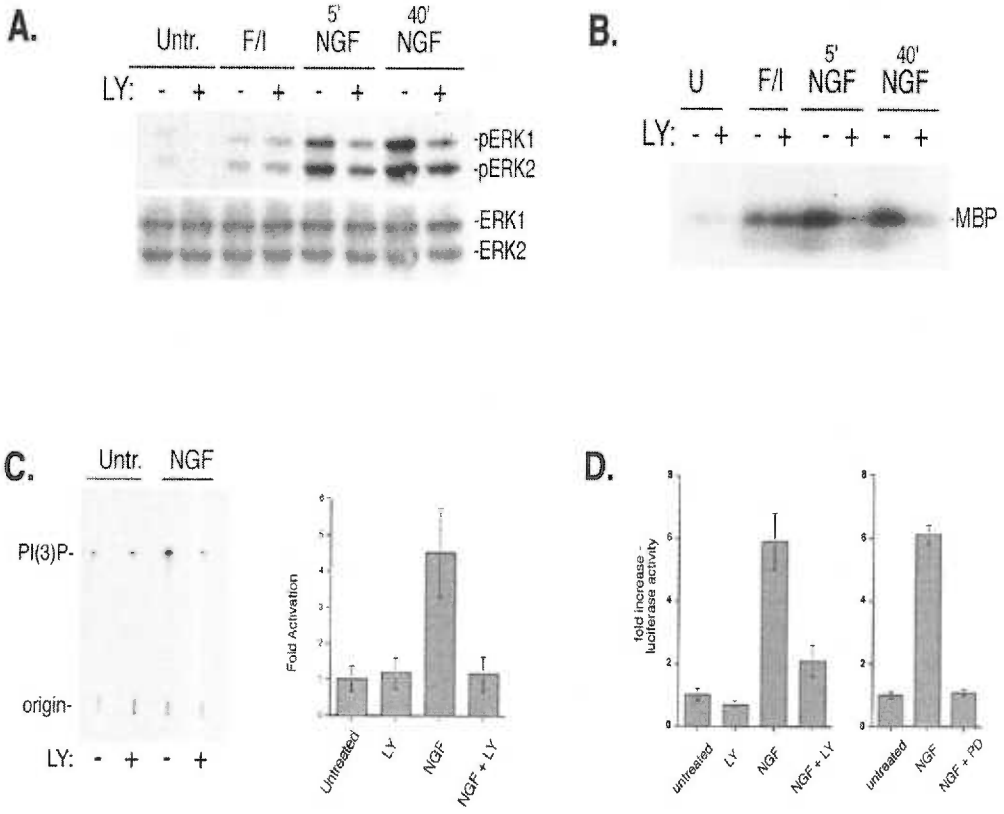


Fig. 3.3 PI3-K inhibitors block NGF activation of PI3-K and ERK

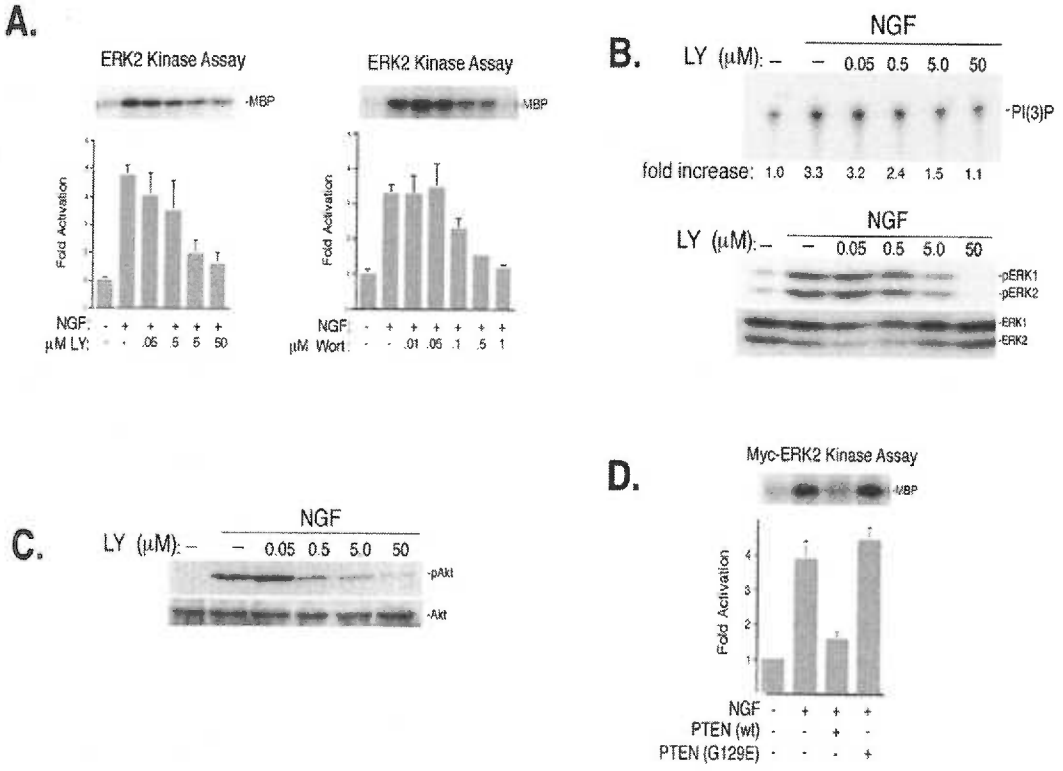


Fig. 3.4 LY inhibition of Rap1/B-Raf activation

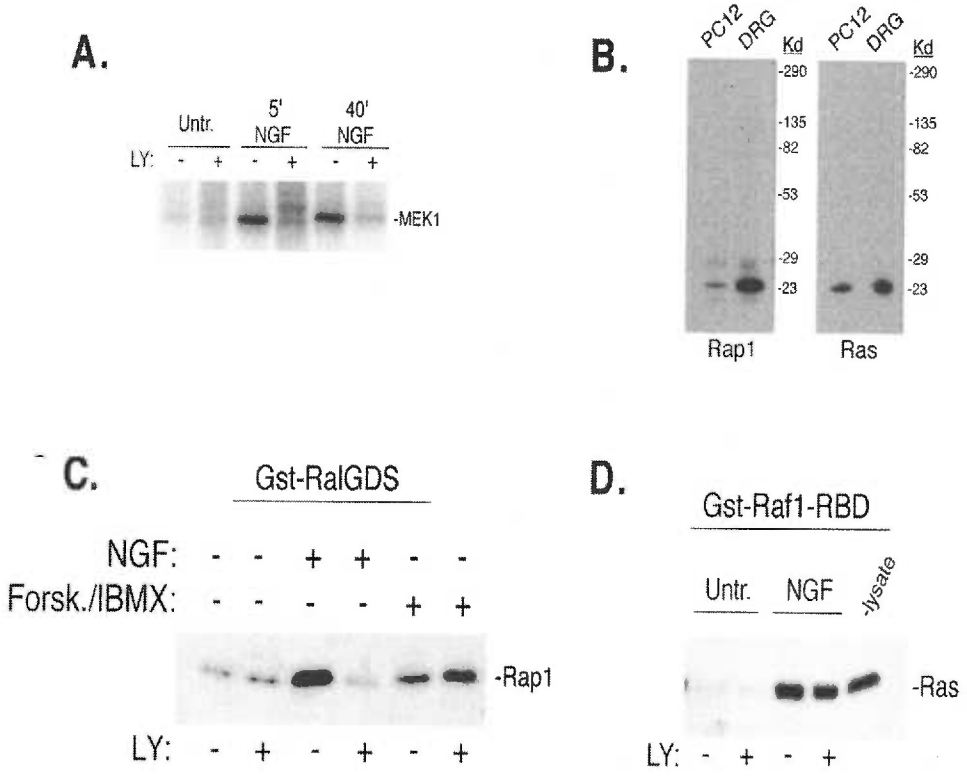


Fig. 3.5 The requirement of PI3-K for Ras coupling to downstream kinases

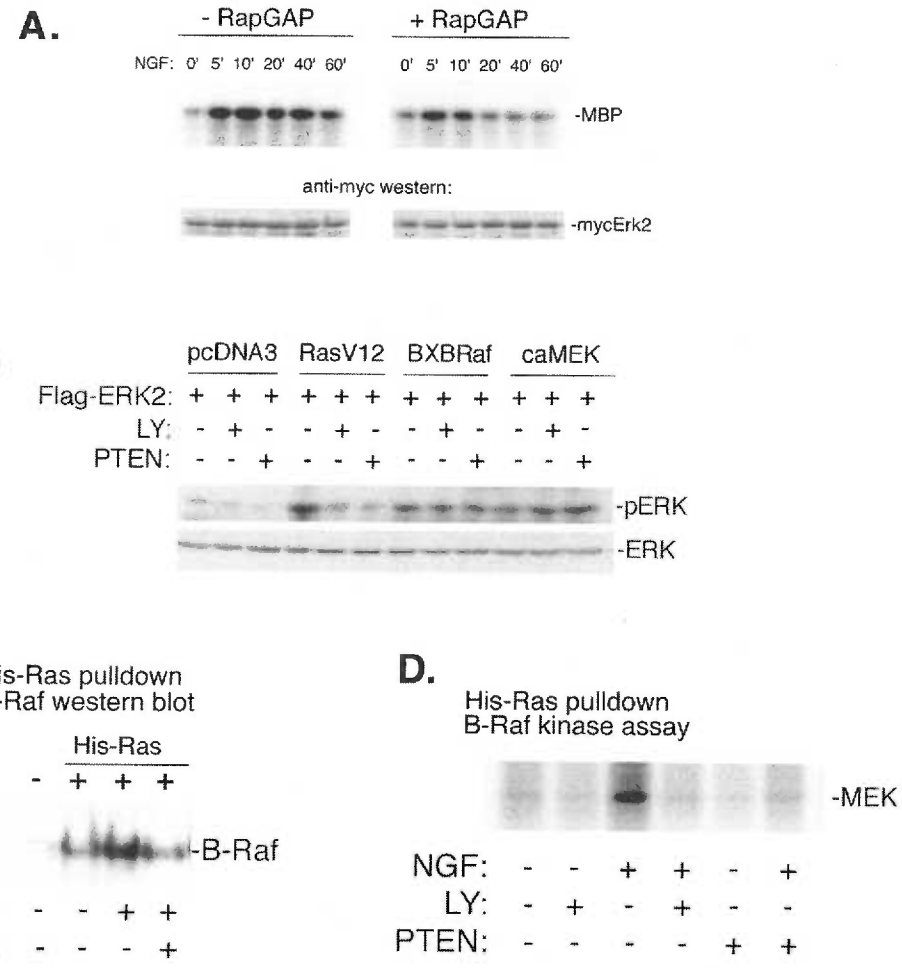


Fig. 3.6 Facilitation of NGF-induced TrkA internalization by PI3-K

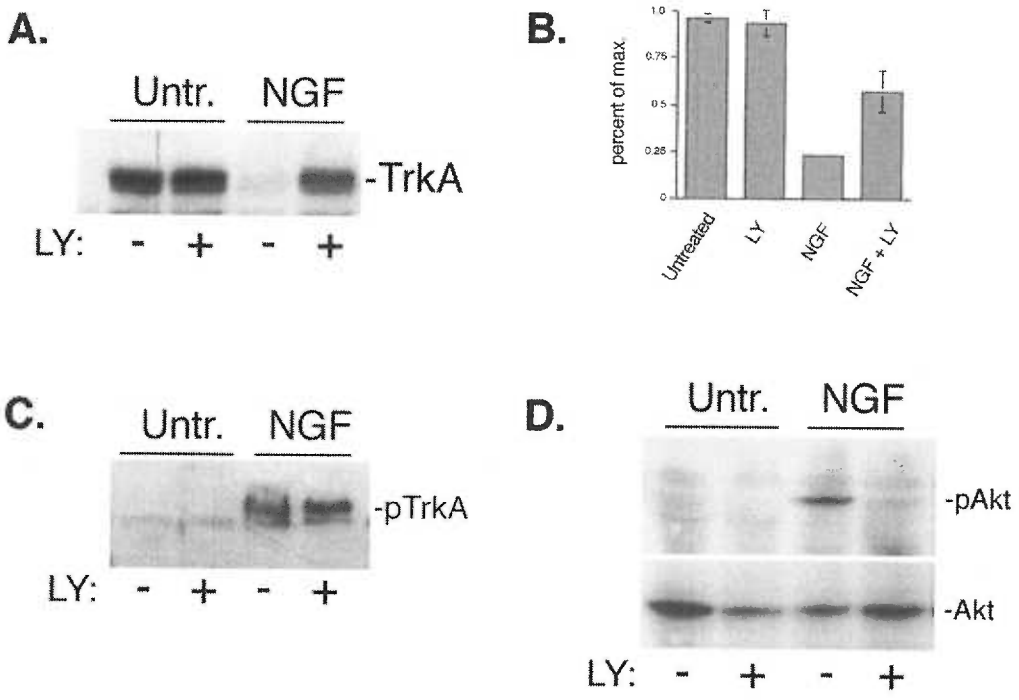


Fig. 3.7 Subcellular localization of Ras and Rap1 in PC12 cells

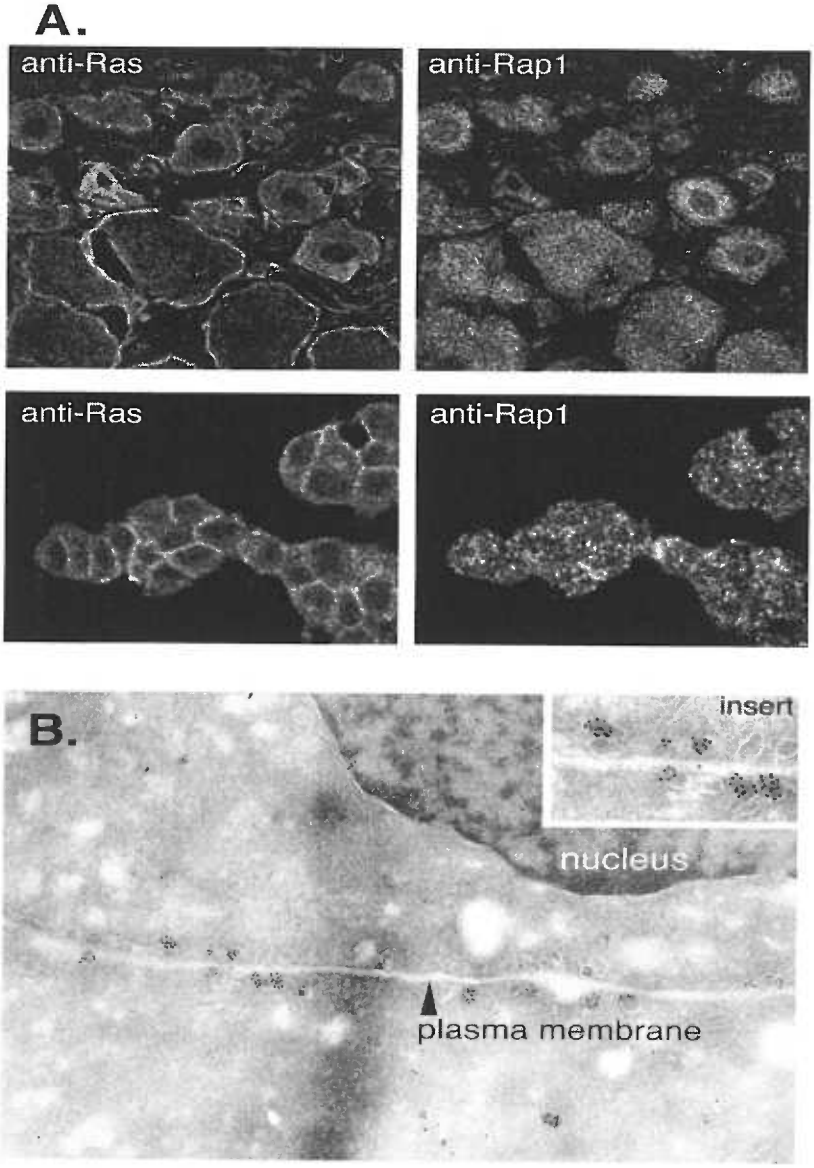


Fig. 3.8 MDC inhibition of NGF signaling to ERK

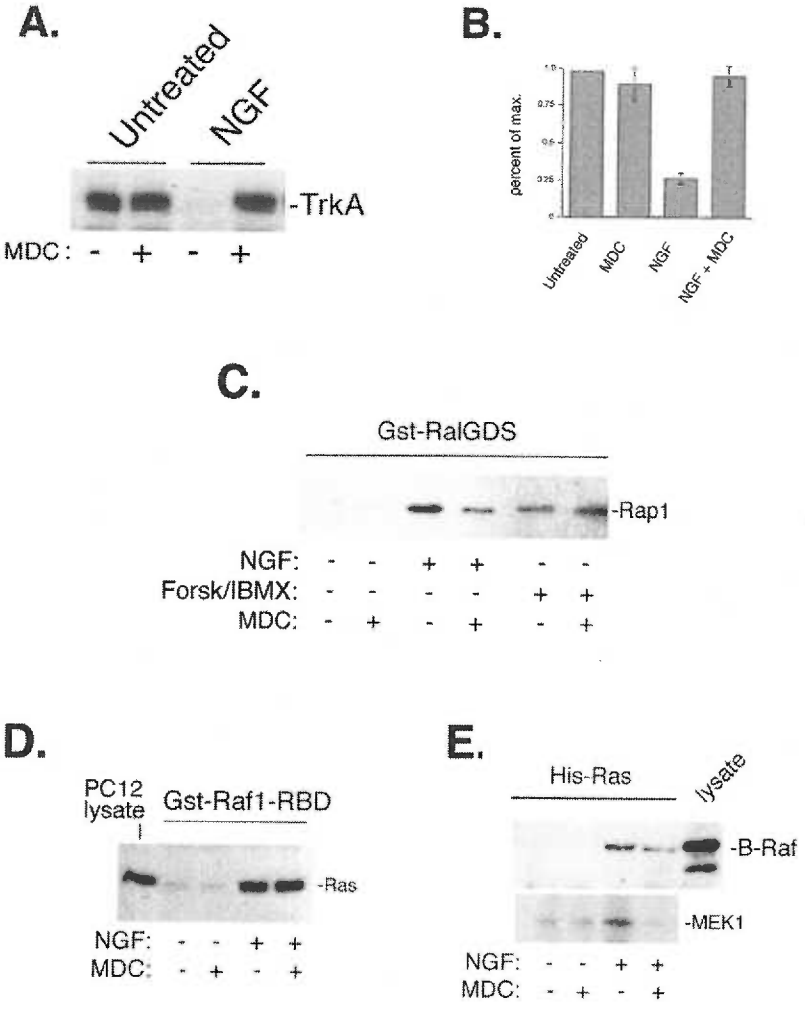


Fig. 3.9 Requirement of endocytosis for ERK activation by NGF in PC12 and DRG cells

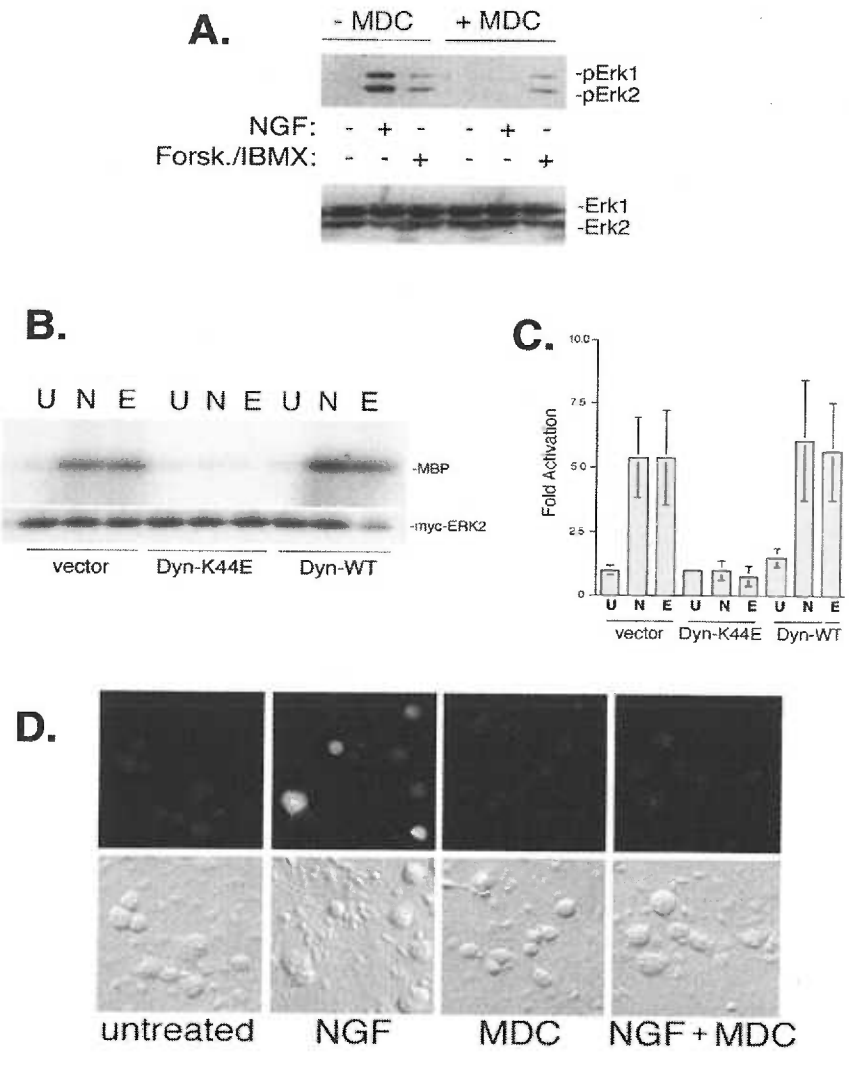
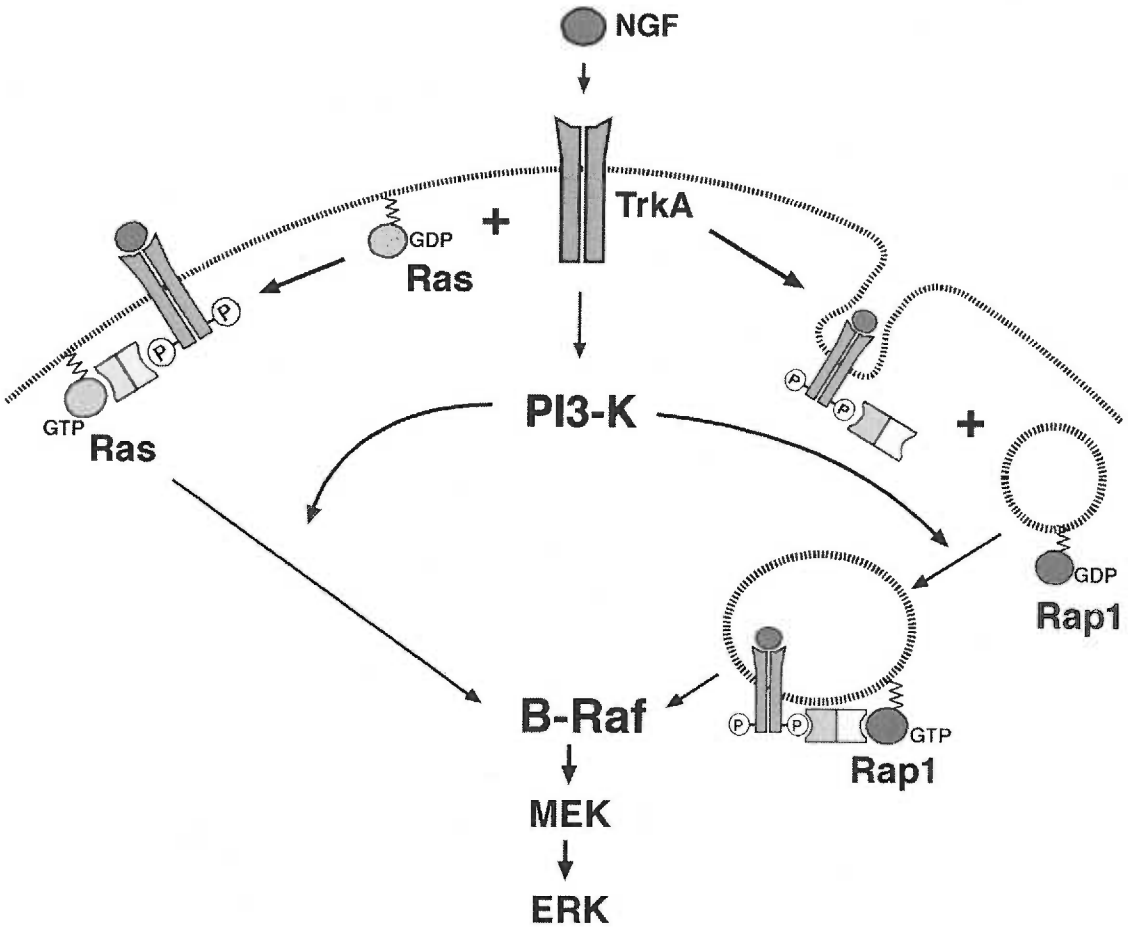


Fig. 3.10 Model of NGF signaling to ERKs



CHAPTER FOUR

DISCUSSION

Knowledge of the molecular mechanisms controlling the proliferation and differentiation of neuroblasts is essential for the construction of therapeutic strategies to combat both developmental disorders and cancers of the nervous system. My graduate work constitutes an incremental advance toward this ultimate goal by providing a framework for determining how extracellular stimuli achieve specificity in regulating these critical cell fate decisions.

One important question regarding specificity has been to determine how activation of the same second messenger can produce cell-type specific growth effects. For example, hormones that elevate intracellular cyclic adenosine monophosphate (cAMP) levels exert growth effects via the ERK cascade in a cell-type specific manner. Until recently, the mechanism through which cAMP activates ERKs was unknown and the only known function of Rap1 was to antagonize the Ras-dependent activation of Raf-1. Our previous work demonstrated that increased intracellular cAMP levels activate Rap1 and that, contrary to its inhibitory action on Raf-1, Rap1 is an activator of B-Raf. Consequently, the selective expression of B-Raf dictates the action of Rap1 and cAMP on effectors downstream of Rafs, including ERKs. Therefore, we proposed that the Ras-independent activation of the selectively expressed B-Raf isoform contributes to the cell-type specific growth effects of cAMP-coupled hormones [188]. This model provides one mechanism by which extracellular signals can have opposing growth effects in different cell types via a very similar transduction pathway. The ability of B-Raf to convert a Rap1-dependent signal from an inhibitor to a stimulator of ERKs has since been demonstrated in other systems, including hormonal stimulation of astrocytes [242] and following integrin signals in fibroblasts [296]. Together, these findings demonstrate that the regulated expression of a single signaling component can reverse the growth effects of hormones and provide exciting possibilities in designing therapies for growth related pathologies.

Here, we have shown that Rap1-dependent pathways contribute to growth factor specificity, as well. Based on studies using interfering mutants of Ras and Rap1 in PC12 cells, we have proposed that NGF signaling to ERKs is mediated by two distinct pathways: a rapid and transient activation that is Ras-dependent and a sustained phase that requires Rap1. In contrast, EGF induces only a transient (Ras-dependent) activation of ERK and is unable to activate Rap1. Initially, this model raised some controversy due to the inability of one lab to detect Rap1 activation following NGF treatment in their cells [297]. Indeed, our initial studies suffered from

several potential limitations. For instance, an important criticism of our study was that technical limitations at that time had forced us to examine the activation of expressed exogenous Rap1. A second concern was that inhibition of the Rap1-dependent pathway relied upon the expression of interfering mutants. As for all interfering mutants, one can never be certain that their action is entirely specific for the molecules of interest. More recently, the development of new affinity-based assays for endogenous Ras and Rap1 activation and use of the endogenous Rap1 inhibitor, RapGAP, have allowed us to address these issues directly. Using these improved reagents, we have confirmed our initial finding that both NGF and EGF activate Ras transiently and that sustained Rap1 activation is selectively induced by NGF, but not EGF [53, 298]. This important finding has since been independently confirmed by several laboratories [299-301]. Noteworthy, Kao and colleagues found that EGF also induced a rapid and transient activation of Rap1 in their system. This EGF induced Rap1 activation occurred at very early time points and may have been missed in our experiments. Nonetheless, these studies document the central importance of the Rap1/B-Raf pathway in mediating a sustained ERK activation following NGF treatment in PC12 cells that is independent of Ras. In a similar manner, integrin signals also require the Rap1/B-Raf pathway to mediate sustained ERK activation [296]. However, the role of Rap1 pathways in regulating the kinetics of ERK activation and downstream physiology is surely dependent on a combination of the cell type, cell context, and the particular stimuli being considered.

Adaptor Molecules Contribute to Specificity

The mechanism through which NGF activates Rap1 is not fully understood. However, it is generally recognized that a growing family of adaptor molecules mediates the coupling of activated RTKs to the activation of small GTPases. These adaptor molecules consist almost entirely of conserved modular binding domains that promote protein-protein interactions. While these modular domains often contain a common core, they also maintain the ability to recognize specific sequences within their binding partners. Therefore, adaptor proteins can regulate signal transduction pathways by acting as molecular linkers to form specific multi-protein complexes. The adaptor molecule, Grb2, has been proposed to mediate the activation of Ras by both NGF and EGF. However, my work suggests that Crk adaptors, rather than Grb2, may mediate NGF's activation of both Ras and Rap1. Specifically, we have shown that NGF's activation of ERK is blocked by the expression of the truncated mutant of C3G (CBR) [146] derived from the Crk-binding region [84]. Expression of this peptide, which presumably interferes with Crk function by displacing the endogenous SH3-binding proteins, has no effect on EGF-stimulated ERK activation. These results confirm the findings of Tanaka *et al.* that EGF signaling to ERKs does not require Crk proteins [149, 150]. Furthermore, CBR abolished both the early (Ras-dependent)

and late (Rap1-dependent) phases of ERK activation by NGF, suggesting that Crk proteins may be required for ERK activation by NGF via both SOS/Ras and C3G/Rap1. More recently, we used the RalGDS and RafRBD affinity assays to demonstrate that CBR inhibits NGF's activation of both Rap1 and Ras [53]. This result is supported by those of Landreth and colleagues who showed that NGF induces the stable formation of a Crk:C3G complex that was proposed to support prolonged Rap1 activation [299]. In marked contrast, EGF treatment of PC12 cells leads to the dissociation of the Crk:C3G complex [299]. The dissociation of Crk:C3G has previously been shown to correlate with Rap1 inactivation following insulin treatment, as well [302]. Together, these data support our hypothesis that the differential use of the Crk:C3G adaptor complex by growth factors contributes to their specific actions on small GTPases and the downstream kinetics of ERK activation. We therefore propose that the differential recruitment of active adaptor:GEF complexes is important in determining the physiological outcome of extracellular stimuli. It would be of interest to further test this model with additional interfering mutants of Crk and Grb2. Two types of mutations in these adaptors have been shown to produce interfering mutants [303, 304]. The first type consists of mutations in the conserved arginine of the FLVRES sequence in the SH2 domains and the other involves SH3 domains in which the first tryptophan of the characteristic tryptophan doublet is mutated to a lysine. The use of interfering mutants, along with the expression of chimeric proteins containing the SH2 domain of one adaptor and the SH3 domain of another, would allow for a systematic evaluation of this model.

Admittedly, this model represents a simplified view of the complex multi-protein interactions known to contribute to growth factor activation of ERKs. Other adaptor molecules certainly play a role in determining growth factor specificity. For example, both the FRS2 adaptor and the SH2 domain-containing phosphatase, SHP-2, have been implicated in sustained ERK activation [45, 89, 136]. FRS2 was originally identified based on its ability to form a complex with Grb2 following stimulation with fibroblast growth factor (FGF) [89]. It is now known that FRS2 is also recruited to the TrkA receptor following NGF treatment, where it is subsequently phosphorylated on multiple tyrosine residues [89, 282, 305, 306]. We and others have shown that, upon NGF treatment of PC12 cells, FRS2 forms a stable complex with Crk and the adaptor/tyrosine-phosphatase, SHP-2 [45, 299]. Furthermore, expression of a mutant FRS2 with impaired SHP-2 binding disrupts recruitment and activation of the NGF stimulated Crk:C3G:Rap1 pathway [299]. The degree to which these complexes regulate Ras activation via a Crk:SOS association is not known. However, consistent with our hypothesis that Grb2 is dispensable for NGF induced ERK activation, Schlessinger and colleagues have suggested that the recruitment of SHP-2 to a FRS2 complex has a greater influence on ERK activation and neurite outgrowth than does Grb2 recruitment [89, 281]. Moreover, it is possible that Grb2 actually plays an inhibitory role in NGF-mediated ERK activation similar to that following PC12

cell stimulation with the NGF-related neurotrophin, NT-3 [307]. In this case, NT-3 was reported to induce the prolonged association of Grb2 with the growth inhibitory molecule Dab2 (Doc/Dab2). Interestingly, the N-terminus of Dab2 interacts with a RasGAP and the formation of a Grb2/Dab2 complex displaces the Ras activator, SOS. Therefore, similar to SOS phosphorylation, Grb2/Dab2 association may provide a means of rapidly terminating Ras-dependent signals. Interestingly, Dab2 was able to block the transient activation of ERK following neurotrophin stimulation in PC12 cells, but had no effect on the late phase of ERK activation [307]. It is tempting to speculate that the specific actions of Dab2 on TrkB-mediated ERK activation reflect specific actions on Ras-dependent versus Rap1-dependent signals. Although less understood, additional adaptor molecules recruited to Trk receptors, such as rAPS and SH2-B, may also play a role in determining growth factor specificity [90]. In future studies, the evaluation of endogenous complex formations in combination with the use of interfering and chimeric mutants should provide insight into the intricacies associated with the selective recruitment of multi-protein complexes and their role in dictating growth factor actions.

Membrane Trafficking

It has long been recognized that the internalization and subsequent trafficking of growth factor and hormone receptors through the endosome/lysosome-mediated degradation pathway is an important means of downregulating receptor signals. It is also becoming clear that endocytosis of growth factor receptors is intimately coupled to signaling events through both post-translational modifications and induced protein complexes [308, 309]. This regulation is bi-directional. That is, not only do signaling pathways regulate the endocytic process, but receptor trafficking may also regulate signaling cascades. Previous studies hinted that the NGF receptor, TrkA, must be internalized and shuttled appropriately for the normal array of NGF-induced signals to be activated [255, 260-262, 270, 310]. Although these findings led to much speculation, little is known about the selective utilization of signaling components during receptor transit. Furthermore, the mechanism of receptor internalization and the nature of the internalized bodies have remained uncertain. Here, we provide evidence that clathrin-mediated endocytosis of TrkA is essential to activation of the Rap1 signaling pathway that is necessary for sustained ERK activation in PC12 cells. This is consistent with recent reports showing internalization of NGF:TrkA complexes into clathrin coated vesicles [263, 301]. The first of these studies took advantage of a conditional mutant of dynamin, a GTP-binding protein required for the "pinching " off of clathrin-coated vesicles, to acutely regulate the location of TrkA following NGF stimulation [263]. In support of the notion that subcellular location of active receptors is critical to the biological response, the authors demonstrated that blocking TrkA endocytosis in PC12 cells prevented NGF-induced differentiation while enhancing NGF-induced survival signals.

Furthermore, this study also implicated TrkA internalization as a regulator of NGF-induced ERK activation.

From our studies, it appears that the requirement for TrkA trafficking to maintain ERK activation is due to the distinct subcellular localization of signaling molecules in the Rap1 pathway. We found that C3G, Rap1, and B-Raf are all localized to small intracellular vesicles in both PC12 cells and sensory neurons [[298, 311]. In contrast, Ras and Raf-1 appear to be localized primarily to the cell surface. This is consistent with the ability of NGF to activate Ras, but not Rap1, in the absence of TrkA internalization and trafficking [298]. More recently, using confocal microscopy in combination with biochemical fractionation techniques, Wu and Mobley provide strong evidence that Rap1 signaling complexes form on early endosomal vesicles [301]. Following NGF stimulation, these endosomal complexes contain active TrkA, C3G, Rap1, B-Raf, and ERK. Since Rap1 co-localized with endosomal markers in both control and NGF treated cells, it is likely that internalized TrkA undergoes a membrane fusion event with resident Rap1-containing vesicles in the endocytic pathway. Alternatively, signals dependent on TrkA trafficking could lead to the relocation of components of the Rap1 pathway to a distinct subset of endosomes containing TrkA.

The study by Wu et al. represents the first attempt to directly examine the subcellular distribution of Ras and Rap1 activity. However, these time-consuming experiments are limited by the purity of the fractions, their reliance on correlative markers comparing fractionation and microscopy studies, and the need to determine activation states with biochemical assays. The recent development of new tools to examine Ras and Rap1 activation kinetics in living cells represents a significant advance in this field. This elegant technique, based on fluorescent resonance energy transfer (FRET), allows one to monitor Ras and Rap1 activation in real time and space at the near-molecular level. Using these newly developed tools, Mochizuki and colleagues have confirmed our suggestion that Ras activation occurs at the plasma membrane and Rap1 activation occurs at intracellular membrane compartments [300]. In addition, this study further supported our previously controversial finding that the majority of Ras activation following NGF treatment of PC12 cells was rapid and transient while Rap1 activation was prolonged. Remarkably, the increased sensitivity of their assay allowed for the detection of a subset of active Ras molecules that persisted for up to 24 hours after NGF treatment. This relatively small pool of active Ras existed only at the extended neurites of differentiating cells and not at the cell bodies. It seems that this potentially important population of active Ras would not have been detected with conventional Ras activation assays. In contrast to Ras, FRET detected Rap1 activation only at the cell body, and never at neurites. This is consistent with our finding that Rap1 activation requires TrkA internalization and trafficking. This is also consistent with the finding of Wu et al. that disruption of membrane trafficking with brefeldin A (BFA) inhibited TrkA and Rap1 co-

localization. Moreover, BFA was found to specifically block Rap1 and sustained ERK activation without effecting Ras or early ERK activity. Together, these results lend further support to our view that TrkA trafficking is a critical determinant of downstream signal specificity.

Previous studies had shown that neuronal TrkA remains in an active state and maintains a stable complex with signaling molecules once internalized into vesicles [252, 253, 261, 312]. Based on our findings, we have extended the "signaling vesicle" hypothesis by proposing that TrkA-containing vesicles are transported to a location where they can contact effectors in the C3G/Rap1/B-Raf pathway. Although many of these studies have been performed in PC12 cells, the effect of TrkA endocytosis and transport is particularly relevant to neuronal responses during development, when neurons are dependent on target-derived NGF for survival and differentiation [313]. In concert with others, we suggest that signaling vesicles may provide the machinery for the transport of NGF signals from axon tips to neuronal cell bodies where changes in gene expression determine changes in cell behavior. In support of the signaling vesicle hypothesis, we have shown that TrkA requires endocytosis to activate ERKs in primary sensory neurons from the adult dorsal root ganglion (DRG). Since TrkA receptors are found throughout these neurons, our studies did not distinguish between signals initiated at the cell body from those at the nerve terminals. Interestingly, a very recent report has broadened our appreciation for the role of localized neurotrophin signals by examining embryonic DRG neurons grown in compartmentalized chambers. This system, which approximates the *in vivo* separation between axon tips and cell bodies, allows for the dissection of signaling components following selective stimulation of particular regions of the neuron[314]. Using this system, Watson and colleagues found that primary sensory neurons could distinguish neurotrophin stimulation at the cell soma from stimulation of distal axons by the differential activation of specific MAP kinase pathways. In particular, neurotrophin stimulation of cell bodies led to concurrent activation of both the ERK cascade (ERK1/2) and the big MAP kinase (BMK1, or ERK5) cascade. Likewise, stimulation of distal axons also led to a local activation of both ERK1/2 and BMK1. However, only BMK1 activity was extended to the cell body where it translocated to the nucleus to facilitate CREB phosphorylation and neuronal survival. In contrast, ERK1/2 activity was not detected in the cell body, suggesting that this pathway may not be used for transcriptional responses to target-derived neurotrophins. Like the activation of ERK1/2, BMK1 activation also required dynamin-dependent endocytosis. Together, these studies suggest that endocytosis of Trk receptors is generally required for signaling to MAP kinase cascades and that additional factors regulating the subcellular distribution of internalized vesicles and signaling components determine the specificity of coupling to individual pathways. The differential activation of individual pathways may provide important information regarding the location of particular stimuli in order to instruct the appropriate cellular response. The ability of neurons to decipher the location of a particular

growth factor stimulus may be especially critical to the organization of pointed responses under physiological conditions. As an illustrative example, assuming a similar response occurs *in vivo*, it is possible that the concurrent activation of ERK1/2 and BMK1 may be important in directing the repair response following nerve injury when there is a marked increase in the amount of growth factors available for direct stimulation to the proximal axons and cell bodies. In contrast, BMK1 activity alone may be sufficient for target-derived survival signals and the retrograde signals directing differentiation and cell fate determination. Undoubtedly, the exact patterns of activation that occur *in vivo* will be dependent on multiple criteria including cell-type, type of stimulus, developmental stage, and context of both the intracellular and extracellular environments. For that reason, the next phase of investigations should include the analysis of signaling specificity in primary neurons from various stages of development and, wherever possible, *in vivo* analysis. The construction of adenovirus vectors that allow for the expression of tagged-signaling components in primary neurons would be useful for co-monitoring the subcellular location and specificity of signaling pathways. In addition, the use of FRET technologies in primary neurons would be informative.

Finally, our studies suggest that PI3-K may be required for the signals initiated by TrkA internalization. Specifically, our data indicate that functional coupling of TrkA to the ERK cascade requires PI3-K activity in both PC12 cells and DRG neurons. Likewise, in PC12 cells, both TrkA internalization and Rap1 activation require PI3-K activity. This requirement for PI3-K likely reflects its role in endocytosis, similar to the well-known role of PI3-K activity in regulating yeast endocytic pathways [315]. This interpretation is supported by a similar requirement of PI3-K in early membrane trafficking following NGF treatment in sympathetic neurons [261, 316]. It is possible that PI3-K regulates ERK signaling at a second site downstream of Ras since both rapid ERK activation by NGF and Ras activation of B-Raf are blocked by PI3-K inhibitors. However, given the vesicular distribution of B-Raf and its predominant role in neuronal ERK activation, this effect of PI3-K may also be due to impaired endocytosis. This would be consistent with the observed trafficking of Ras and active ERK into distinct clathrin-coated vesicles following NGF treatment [79]. Although global ERK activation is predominantly mediated by B-Raf, the relative contribution of Raf-1 and B-Raf to ERK activation in Ras-containing vesicles remains to be determined. However, in other systems, receptor endocytosis is required for functional coupling to ERK activation downstream of Raf-1, as well [273, 317]. In summary, the effects of blocking PI3-K activity and inhibiting endocytosis were indistinguishable with respect to Ras, Rap1, and ERK activation. Disruption of endocytosis therefore seems sufficient to explain the PI3-K result. Still, PI3-K is known to intersect with multiple signaling pathways and other targets can not be ruled out.

Rap1 Activation via NGF and PKA: A Common Mechanism?

Importantly, the ability of NGF to activate Rap1 and the sustained phase of ERK activation can also be inhibited by interfering with the function of PKA, a known Rap1 activator [116]. Together with the noted synergy between PKA and NGF signals, these results suggest that NGF and PKA may be activating the Rap1/B-Raf pathway via a common mechanism [116]. In support of this hypothesis, the CBR interfering mutant prevents ERK activation by both NGF and PKA, but not EGF [45, 146]. Furthermore, activation of PKA-dependent pathways leads to the incorporation of Crk adaptors into FRS2 complexes, similar to the effect of NGF stimulation [45]. More recent studies in the Stork lab have shown that PKA's activation of Rap1 utilizes a Crk:C3G pathway that is dependent on the tyrosine kinase, Src [318]. This finding is consistent with a recent report demonstrating a Src-dependent activation of the Crk/C3G/Rap1 pathway that involves two additional proteins, Sin and p130-Cas [319]. Likewise, activation of the Src-related kinase, GTK, has been proposed to induce a p130-Cas/Crk/C3G/ Rap1 pathway [320]. Similar to PKA, NGF may utilize a Src-dependent signal to initiate the Crk/C3G pathway since the Src kinase inhibitor, PP1, blocks NGF induced tyrosine phosphorylation of Sin [299]. Together, these data support the view that both NGF and PKA utilize a Src-dependent mechanism to initiate the formation of a Crk:C3G-containing complex that activates a Rap1/B-Raf/ERK pathway. This hypothesis could be tested easily with the available molecular and pharmacological reagents. If confirmed, the challenge of this model will be to define how either NGF or PKA activate Src-pathways. In addition, given our realization that trafficking and signaling are integrated at the mechanistic level, it would be interesting to determine the effects of Src activation/inactivation on the subcellular location of signaling complexes. For example, does the requirement of Src-dependent pathways to activate Rap1 reflect a role in clathrin redistribution and receptor internalization, similar to that shown following EGF stimulation [321]? In any event, the essential nature of Rap1 for sustained activation of ERK by PKA [116] implicates this GTPase as a potential mediator of undefined neuronal signaling pathways initiated by PKA, including those leading to neuronal survival and LTP. Both of these processes are influenced by a combination of growth factor and activity-dependent neuronal signals.

Rap1 as a Mediator of Activity-Dependent Neuronal Processes

Research during the past several years has uncovered the potential of PKA and ERK signaling cascades to regulate diverse neuronal processes including cell fate determination and synaptic plasticity. In order to control these processes, neurons must decipher patterns of electrical impulses resulting from multiple activity-dependent stimuli while simultaneously

responding to complex signals in the external milieu. This multitude of information must then be translated into appropriate intracellular signaling outputs and physiological responses. The ability of Ras and Rap1 to integrate multiple stimuli and to alter the kinetics of downstream signals may represent one mechanism of achieving specific responses to complex influences. The ability of Ras and Rap1 to act as a point of convergence between hormonal and growth factor signals has already been discussed. Recently, a central role for Ras and Rap1 as integrators of neuronal function has been further suggested by the discovery of GEFs and GAPs that can be regulated directly by diverse second messengers such as calcium, DAG, and cAMP [91]. Given the selected neuronal distribution and synaptic localization of at least a subset of these GEFs and GAPs, it is likely that activation of Ras- and Rap-dependent pathways may also have important implications in the control of activity-dependent neuronal function. Indeed, several reports have implicated Ras as an important component regulating activity-dependent changes [322-325]. Activity-dependent neuronal processes are predominantly mediated by calcium initiated signals. Similar to that observed following growth factor stimulation, we have recently identified a PKA-dependent activation of the Rap1/B-Raf pathway downstream of depolarization-induced calcium signals [326]. This pathway likely involves increased intracellular cAMP levels via calcium/calmodulin-dependent activation of adenylate cyclases [326]. Importantly, this study established that the route of calcium entry dictated whether Ras (PKA-independent) or Rap1 (PKA-dependent) pathways were responsible for ERK activation. This is particularly interesting in conjunction with previous studies demonstrating that the mode of calcium influx can determine the profile of expressed genes [327-330]. Together, these findings suggest that both Ras and Rap1-dependent signals mediate calcium actions in neurons and raise the exciting possibility that the relative contribution of each pathway may be important in dictating the specificity of calcium-mediated signaling.

Summary and conclusion

It is now becoming apparent that both Ras and Rap1 represent potential integrators of multiple signaling systems leading to ERK activation. Both can be regulated by growth factor signaling via RTKs. Moreover, classic second messengers such as cAMP, PKA, DAG and calcium can modulate their activity. These second messengers may have direct effects on Ras and Rap1 regulators or they may act via a combination of elaborate multi-protein adaptor complexes and enzymatic pathways. Regulation of these small GTPase/ERK pathways may have profound effects on neuronal processes including cell growth, differentiation, survival, and synaptic plasticity. Dissecting the role of GEFs, GAPs, and adaptors in coupling different stimuli to distinct intracellular signaling pathways will be important to our understanding the mechanisms by

which neurons interpret complex stimuli. With regard to growth factor signaling specificity, our work urges an appreciation for the role of endocytosis, membrane trafficking, and subcellular localization of signaling components. The next challenge will be to elucidate the spatio-temporal regulation of these processes connected with receptor signaling. Are the receptors trafficked from one location to another, changing effector partners along the way? Are there distinct pools of receptors that are targeted to different locations where they activate a subset of effectors? What are the roles of cell adhesion, cytoskeletal organization, cell cycle, co-stimulation, and cross-talk with other pathways? Answers to these questions will certainly influence our ever-changing models of growth factor signaling and may lead to a greater appreciation of the fundamental processes by which complex biochemical networks govern the passage of life.

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