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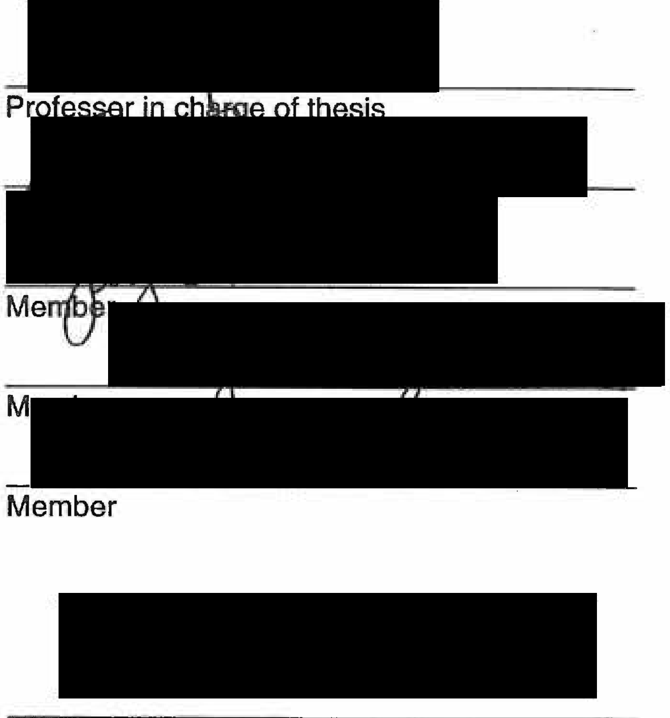

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Interaction between Hormone and Growth Factor Signaling: cyclic adenosine monophosphate activation of a novel mitogen-activated protein kinase cascade

by

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A DISSERTATION

Presented to the Department of Molecular Microbiology and
Immunology Oregon Health Sciences University
School of Medicine

in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy
February 1998

ACKNOWLEDGMENTS

Many people deserve my thanks for their support and help during my graduate study. Although it would be impossible to thank all of them here, I would like to express my special appreciation to those who made an important impact both on my research comprising this dissertation and my life.

I am grateful to my mentor, Dr. Philip J. S. Stork, who has been an outstanding advisor, from whom I have learned a tremendous number of valuable lessons that will be with me through my professional life. I thank him for his critical advice, support, encouragement, stimulating ideas, giving me freedom to perform experiments and for his efforts to ensure that I received the best training possible.

I also like to thank the members of my thesis committee, Dr. Richard Maurer, Dr. Jay Nelson, and Dr. Mary Stenzel-Poore for their precious time, helpful advice and their great efforts to help me succeed in my graduate study.

I would like to thank every current members in the Stork lab, Dr. Tara Dillon, Cindy Ellig, Chris Fenner, Savraj Grewal, Dr. Mary Minette, and Randall D. York, and former associates, Dr. Tullio Florio, Dr. Ming-Gui Pang, Dr. Anita Misra-Press, Caroline S. Rim, and Dr. Mark Vossler, who have been contributed much to this project through their work, friendship, discussion and support. I also wish to recognize the help of Savraj Grewal who carefully read my thesis and provided excellent advice. I also thank him for sharing exciting ideas with me. Special thank should be given to Dr. Mark Vossler and Randall D York for their significant contribution to this whole project.

I would like to thank the faculty, staff and fellow students in the Department of Molecular Microbiology and Immunology and in the Vollum Institute. In particular, I want to thank Dr. Scott Landfear for his supervision and encouragement throughout all years of my graduate study, Dr. Mary Stenzel-Poore for her great support and Eric Snapp for his help and scientific discussion.

I am grateful to my father Yirui Yao, who had a great influence on my life and career choices during his life; my mother Ruiying Lin, who continues to provide love and encouragement; and my brothers Jianhua Yao, Jianguo Yao, Jianzhong Yao and Jianjun Yao for their support and enthusiasm. I thank my friends, Megumi Adachi, Mei-shya Chen, Yang Chen, Changsheng Guo, Martin Kholer, Brian Little, Liming Luo, Yang Xiang, Weihong Yu and Yuan Zhang for their friendship and support in my work and my life.

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Abstract

It has been well documented that the regulation of cell growth and differentiation by growth factors can be mediated by mitogen-activated protein (MAP) kinases. The sequential activation of small G protein; Ras, the MAP kinase kinase kinase; Raf, the MAP kinase kinase; MEK, and the MAP kinase; ERK, are composed of the MAP kinase cascade initiated by growth factors. Hormones can also exert significant growth effects via the MAP kinase cascade. In particular, hormones that elevate intracellular levels of the second messenger, cyclic adenosine monophosphate (cAMP) can either stimulate or inhibit MAP kinase signals, in a cell type-specific manner. We demonstrate that cAMP can synergize with EGF to stimulate differentiation of PC12 cells and potentiate MAP kinase activation in these cells. The studies presented in this thesis will focus on the regulation of MAP kinases by cAMP. The major effector of cAMP is the cAMP-dependent protein kinase, PKA. We show that cAMP activation of MAP kinase requires PKA but is independent of small G protein Ras.

We further demonstrate that cAMP's activation of MAP kinase utilizes the small G protein Rap1. Rap1, like cAMP, activates MAP kinase in a cell type-specific manner. This cell-type specificity is dictated by the Rap1 effector B-Raf, which is expressed in cells where cAMP activates, but not in cells where cAMP inhibits, MAP kinase. These results suggest that cAMP utilizes a novel pathway to activate ERKs, this pathway is Ras-independent and requires B-Raf and Rap1. PKA/Rap1/B-Raf pathway might represent a mechanism to activate MAP kinase that is used by other agents as well. We show that PKA is required for NGF to trigger the Rap1/B-Raf pathway to MAP kinase, as well as NGF's stimulation of nuclear translocation of MAP kinase. The significance of this action of PKA in NGF signaling is that the Rap1/B-Raf pathway mediates the sustained activation of ERKs in PC12 cells. We propose that PKA/Rap1/B-Raf may provide specificity to growth factor action through the regulation of the magnitude and duration of MAP kinase.

Chapter 1

1.1 INTRODUCTION

Cell-to-cell communication is crucial for the development and subsequent maintenance of multicellular organisms. Most communication from one cell to another is achieved by signaling molecules (ligands) that are secreted from one cell and are recognized by specific receptor proteins within the membranes of the target cell. Two types of extracellular ligands are hormones (e.g. dopamine, vaso-intestinal peptide, parathyroid hormone), that circulate through the blood stream and growth factors (e.g. epidermal growth factor and fibroblast-derived growth factor) that circulate in the blood and can also be released locally near their target cells. Both ligands mediate many responses in the target cells including proliferation and differentiation (Dhanasekaran et al., 1995; Schlessinger and Ullrich, 1992; van Biesen et al., 1996). Studies on the growth of neuronal cells, endocrine cells and fibroblasts have identified two major pathways regulating proliferation and differentiation. These signals are initiated by: (a) growth factor stimulation of receptor protein tyrosine kinases; and (b) hormonal activation of G protein-coupled receptors (Cowley et al., 1994; Dhanasekaran et al., 1995; Ginell and Brown, 1996), resulting in the activation of specific intracellular pathways conveying signals from the membrane to the nucleus to trigger specific transcriptional events. While it had been thought that these two pathways are functionally and mechanistically independent, recent studies have demonstrated that they can converge on the same intracellular effector molecules and may use analogous mechanisms for the coordination of signals.

In many cell types, the regulation of cell growth and differentiation by growth factors is mediated by signals that activate the mitogen-activated protein kinase (MAP kinase). This thesis will identify points of the convergence between hormone and growth factor

signaling on proteins within the MAP kinase cascade. Since hormones and growth factors bind to functionally distinct classes of receptors, signals arising from each receptor classes (G protein-coupled receptor vs. tyrosine kinase receptor) must converge at points downstream of the receptors themselves.

An important group of hormones activate receptors that are coupled to the stimulatory G protein, Gs, that triggers the synthesis of cyclic adenosine monophosphate (cAMP) and the activation of the cAMP-dependent protein kinase A, PKA. In this thesis, I will describe the studies of the regulation of MAP kinases by extracellular signals that increase the levels of cAMP. Unlike some proliferative signals, the growth effects of hormones that increase cAMP are cell-type specific. My thesis project was carried out to address three questions: 1) Does cAMP, via PKA, interact with components of the growth factor signaling pathway in the regulation of MAP kinase activity? 2) What are the biochemical mechanisms of these interactions? 3) To what extent are these mechanisms shared by other extracellular signals?

1.2 MAP KINASE CASCADE

1.2.1 Activation of MAP kinase cascade

The MAP kinase cascade (see Fig. 1.1) refers to the cascade of phosphorylations that are downstream of the small GTP-binding protein p21Ras (Ras). MAP kinase activation is a well studied consequence of growth factor signaling. Many growth factors, such as epidermal growth factor (EGF), nerve growth factor (NGF) and fibroblast-derived growth factor (FGF), are able to elicit MAP kinase cascade by binding to and activating the specific receptor tyrosine kinase, RTK. Subsequently, the receptors recruit activators of the small GTP-binding protein Ras to the receptor complex. Ras becomes activated by increasing its GTP content which induces a

conformational change in the Ras and initiates a cascade of phosphorylation of a MAP kinase kinase kinase (MAPKKK) Raf, a MAP kinase kinase (MAPKK) MEK and finally MAP kinase itself. Activated MAP kinase either remains in the cytoplasm to activate its cytoplasmic substrates or move into the nucleus to stimulate transcription. Through this pathway, extracellular signals are transduced into the nucleus where a specific set of genes for cell growth or differentiation are induced (Cobb and Goldsmith, 1995; Cobb et al., 1994; Marshall, 1994; Marshall, 1995).

Both gain-of-function (constitutively active) and loss-of-function mutations (interfering or dominant negative) of components within or upstream of MAP kinase cascade have been made (Table 1.1a). They have been intensively used to define the role of corresponding individuals in different signals leading to MAP kinase activation and other cellular responses. Gain-of-function mutations result in an constitutive activity which is independent of external stimuli. These mutants can be used to demonstrate whether MAP kinase cascade is sufficient for certain biological events. Loss-of-function mutations produce inactive mutants which are still capable of binding endogenous substrates and effectors and therefore can compete with the corresponding endogenous wild type proteins. These kind of mutants are often used as interfering mutants to disrupt the normal signaling of the respective proteins, and can be used to examine the necessity of a particular protein in a specific biological process. Additional pharmacological inhibitors, such as PD98059 (Dudley et al., 1995), a specific MEK inhibitor, are also useful tools to demonstrate the requirement of MAP kinase and MEK in specific pathways, as well. The individual components within the MAP kinase cascade will be discussed in more detail below.

1.2.2. MAP kinase family

Mitogen-activated protein kinases (MAPKs) are protein serine/threonine kinases that have been strongly conserved through evolution suggesting their importance in intracellular signaling. There are at least three MAPK subfamilies present in mammalian cells (Cobb and Goldsmith, 1995; Sugden and Clerk, 1997). These are: (i) extracellular signal-regulated kinases (ERKs) which were initially known simply as MAPKs; (ii) the c-jun N-terminal kinases (JNKs) or stress-activated protein kinases (SAPKs); and (iii) the p38-MAPKs. The ERK cascade is intimately connected with the regulation of cell growth and differentiation and a major target of receptor tyrosine kinase signaling. The JNKs/SAPKs and p38-MAPKs are involved in the cellular responses to environmental stresses (Campbell et al., 1995; Cano and Mahadevan, 1995; Cobb and Goldsmith, 1995; Denhardt, 1996; Marshall, 1994; Marshall, 1995), such as UV light, X-ray, inflammatory cytokine, bacterial toxins and osmotic shock.

Historically, the term MAP kinase has referred to the ERK subfamily unless otherwise indicated. MAP kinase was first identified as microtubule-associated protein (MAP) kinase in 1986, in an attempt to identify the insulin-stimulated kinase which phosphorylates and inactivates the type 1 phosphatase inhibitor 2 (Sturgill and Ray, 1986). Subsequently, it was demonstrated that MAP kinase could be activated by a variety of mitogens, such as EGF in fibroblasts, insulin in adipocytes and smooth muscle cells, phorbol ester and peptide antigens in T cells, and colony-stimulating factor (CSF)-1 in macrophages and osteoblasts. The activation of MAP kinase by these mitogens provide signals that allow progression of cell cycle. Therefore, MAP kinase is now used as an acronym for mitogen-activated protein kinase, as well (Rossomando et al., 1989).

The first two MAP kinases identified, ERK1 and ERK2, show high homology at the amino acid level and have molecular weights of 44kDa and 42kDa respectively. Other

MAP kinase members have been subsequently identified and cloned based on the homology to ERKs. ERK1 and ERK2 have been purified from many different species ranging from yeast (fus3, kss1, slt2 or spk1) (Courchesne et al., 1989; Elion et al., 1990; Toda et al., 1991; Torres et al., 1991), Drosophila (rolled) (Biggs et al., 1994), sea star (p44mpk) (Sanghera et al., 1990), xenopus oocyte (Gotoh et al., 1991), murine rodent to human (Boulton et al., 1991; Ray and Sturgill, 1988; Ray and Sturgill, 1987; Sturgill and Ray, 1986). Mammalian ERK1 and ERK2 are both structurally and functionally similar. They share the same time course of activation (Meloche et al., 1992), are expressed in most cell types (Boulton et al., 1991), and are co-regulated by many mitogens (Cooper, 1989; Meloche et al., 1992). ERK1 and ERK2 are cytoplasmic proteins that, upon activation by phosphorylation, can translocate to the nucleus (Gonzalez et al., 1993; Lenormand et al., 1993).

Related MAP kinase family members, including JNK and p38, are sometimes referred to as MAP kinases. However, MAP kinase also refers to the ERK subfamily. To avoid confusion, MAP kinase will be used to refer to the ERK proteins, unless otherwise indicated. In addition, since ERK1 and ERK2 are the major mammalian MAP kinase isoforms that have been examined following activation by growth factor and hormonal signaling, the general term ERK will refer to ERK1 and ERK2 but not other ERKs in the rest of my thesis.

1.2.2.1 Regulation of ERKs

Activation of MAP kinase requires dual phosphorylation of adjacent tyrosine and threonine residues within a TEY motif (Thr190 and Tyr192 in ERK1 or Thr183 and Tyr185 in ERK2 (Boulton et al., 1991; Her et al., 1991; Marquardt and Stabel, 1992). The crystal structure of ERKs has been recently solved (Zhang et al., 1994; Zhang et al., 1995). 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. These studies show that the TEY motif is located on the lip of this cleft. Phosphorylation of Tyr and Thr induces local and global conformation changes which allow substrates access to the catalytic cleft. Substrate specificity of ERKs is determined both by the geometry of the cleft and the presence of ERK binding domains within protein substrates (Cobb and Goldsmith, 1995; Zhang et al., 1994; Zhang et al., 1995). Dephosphorylation of either threonine by phosphatase 2A or tyrosine by CD45 within the TEY motif of ERK inactivates the enzymatic activity (Anderson et al., 1990; Boulton et al., 1991). Phosphorylation and dephosphorylation of MAP kinases are mediated by dual-specific kinase MEK (see Section 1.2.3) and possibly by the dual-specific phosphatase MKP (MAP kinase phosphatase) (Sun et al., 1993; Sun et al., 1994), respectively. Therefore, MAP kinase activity can be regulated by upstream kinases as well as downstream phosphatases.

1.2.2.2 ERK substrates and the regulation of gene expression

ERK1 and 2 have a wide range of cytoplasmic, membrane-bound, and nuclear substrates consistent with its role in a variety of cellular processes. ERKs are proline-directed serine/threonine kinases that preferentially phosphorylate the consensus sequence Pro-X-Ser/Thr-Pro (where X is any amino acid) (Clark-Lewis et al., 1991; Gonzalez et al., 1991) or the minimal sequence Ser/Thr-Pro in substrate proteins (Alvarez et al., 1991). *In vitro*, ERKs show differential specificity towards a variety of conventional kinase substrates, phosphorylating its physiological substrates MAP2 and myelin basic protein (MBP) very well; in contrast, casein, histone, 40S ribosomal subunits and the peptide RRLSSLRA are not appreciably phosphorylated (Ray and Sturgill, 1988). Although ERK1 and ERK2 share the same substrates *in vitro* (Alvarez et al., 1991; Gille et al., 1992; Pulverer et al., 1991; Sturgill et al., 1988), it is likely that, in mammalian cells, they phosphorylate distinct set of substrates in addition to

similar substrates. This notion is supported by the observation that ERK1, but not ERK2, moves to the ruffling membrane surface as well as the nucleus in serum-treated NIH3T3 cells, while ERK2 only translocates into the nucleus in these cells (Gonzalez et al., 1993).

Some of the *in vivo* substrates of ERK are listed in Table 1.2. MAP kinase's ability to phosphorylate cytoplasmic substrates such as translation factor PHAS-I/eIF4E-BP (Haystead et al., 1994; Hu et al., 1994; Lin et al., 1994; Pause et al., 1994), protein kinase p90 ribosomal S6 kinase (p90RSK) (Sturgill et al., 1988), protein tyrosine phosphatase 2C (Peraldi et al., 1994), cytoplasmic phospholipase A2 (Lin et al., 1993) and tyrosine hydroxylase (Halloran and Vulliet, 1994; Haycock, 1993; Haycock et al., 1992) directly influence the corresponding translational function or enzymatic activity of these substrates. ERK1 and ERK2 also phosphorylate structural proteins (see Table 1.1) to regulate the organization of cell structure, spreading and chemotactic migration (Reszka et al., 1997). Some of the targets of ERK serve to regulate ERK signaling itself. For example, ERK phosphorylates the upstream component of the ERK cascade, SOS, in response to insulin or EGF. This modification has been thought to play a negative feedback role on the Ras pathway, by causing the dissociation of SOS from Grb2 (Cherniack et al., 1995; Corbalan-Garcia et al., 1996; Waters et al., 1995) or alternatively, dissociation of the Grb2/SOS from receptor (Klarlund et al., 1995; Langlois et al., 1995; Porfiri and McCormick, 1996; Rozakis-Adcock et al., 1995).

In addition to those substrates at the plasma membrane and cytoplasm, the ERK pathway provides a common route by which signals from different growth factor receptors activate transcription of growth factor responsive genes in the nucleus. Nuclear translocation of MAP kinase following its activation (Chen et al., 1992; Gonzalez et al., 1993; Lenormand et al., 1993) leads to the phosphorylation of

transcription factors, such as Elk-1 and Ets2, which regulate the expression of immediate-early genes such as c-fos (Hill et al., 1993; Hill and Treisman, 1995; Karin, 1994; Marais et al., 1993; McCarthy et al., 1997; McCarthy et al., 1995; Treisman, 1996). Phosphorylation of these transcription factors stimulates their transcriptional activation of the serum response element (SRE), that is located at regulatory sites within many mammalian genes. The best characterized SRE is the promoter element that regulates the immediate early gene c-fos. The major factors that activate the SRE are serum response factor (SRF) and proteins of the TCF (ternary complex factor), including the transcription factors Elk-1 and SAP-1. The activity of Elk-1 is regulated by phosphorylation of Ser or Thr within Ser/Thr-Pro motifs at its C-termini (Hill and Treisman, 1995; Treisman, 1994). SRE mutations that prevent TCF binding substantially reduce the transcription regulated by MAP kinase. Experiments with activated and interfering forms of MEK and ERK2 indicate that both MEK and ERK are necessary and sufficient for activation of Elk-1 *in vivo* (Janknecht et al., 1993; Kortenjann et al., 1994). The direct phosphorylation of Elk-1 by ERKs causes increased binding of Elk-1 to the SRE, ternary complex formation (Gille et al., 1992) involving the association of Elk-1 and SAP-1, and subsequent transcriptional activation of c-fos (Marais et al., 1993). Elk-1 can also be activated by JNKs (Cavigelli et al., 1995; Gille et al., 1995) and p38 (Raingeaud et al., 1996) via the same Ser/Thr-Pro motif in Elk-1. However, ERKs and JNKs respond differentially to different stimuli. For example, SRE-dependent gene expression is induced by JNK but not ERK in cells treated with interleukin-1 and by ERK but not JNK after treatment with phorbol ester. The Elk-1 transcription factor therefore integrates MAP kinase signaling pathways *in vivo* to coordinate biological responses to different extracellular stimuli. In addition, the activation of exogenous Elk-1/Gal4 chimera by MAP kinase to induce the reporter gene expression provides a useful tool to study MAP kinase activation (Roberson et al., 1995). Another accessory factor of SRF, SAP-1, is also a good substrate for ERKs

and the kinetics of its modification correlates well with MAP kinase activation *in vivo* (Janknecht et al., 1995; Price et al., 1995; Treisman, 1994). *In vitro* studies also demonstrated that other transcription factors, such as NF-IL6, ATF-2 and c-jun, can be phosphorylated by MAP kinase and this phosphorylation result in either increasing DNA binding ability or transactivation activity of transcription factors (Davis, 1993). However, it is not clear whether these transcription factors are ERK substrates *in vivo*. Another transcription factor that is phosphorylated and activated by ERKs in the nucleus is c-myc (Davis, 1993). In addition to the direct action on transcription by itself, nuclear ERK is also able to co-regulate gene expression with other signaling pathways including those utilizing the kinases JAK/STAT (David et al., 1995; Wen et al., 1995; Zhang et al., 1995) and estrogen receptor family (Kato et al., 1995).

1.2.3. The dual-specificity protein kinase MEK

MAP or ERK kinase (MEK) is a 45 kDa protein kinase that can phosphorylate tyrosine as well as serine/threonine residues (hence, is dual-specific) (Haystead et al., 1992). It is related to yeast mating factor-regulated protein kinases encoded by the STE7 and byr1 genes. MEK serves as an intermediate between its substrate MAP kinase and its activator Raf. MEK has been identified as the major activator of vertebrate MAP kinases. Although other MEK isoforms have been identified for JNK and p38MAPK pathways, ubiquitously expressed MEK1 and MEK2 are the specific MEK isoforms that activate ERK1 and ERK2 via simultaneous phosphorylation of adjacent tyrosine and threonine in the TEY motif of ERK (Ahn et al., 1992). Residues in the phosphorylation lip of ERK play an important role in the recognition and phosphorylation by MEK (Butch and Guan, 1996). MEK contains a nuclear export signal which keeps MEK in the cytoplasm. MEK forms a complex with inactive ERK, thereby retaining ERK in the cytoplasm. Upon phosphorylation and activation, ERK is

released from MEK and moves into the nucleus (Fukuda et al., 1996; Fukuda et al., 1997; Zheng and Guan, 1994).

MEK itself is activated by phosphorylation at two closely spaced serine residues (Ser218 and Ser222) by the Raf family of serine/threonine kinases (Zheng and Guan, 1994). Replacing these two residues with aspartic acids results in constitutively active MEK that is oncogenic (Mansour et al., 1994). The specificity or regulation of *in vivo* signaling to the mammalian MEKs (MEK1 and MEK2) was recently reported also to involve the differential phosphorylation of another region such as a proline-rich peptide located between the MEK kinase-subdomains IX and X (Robinson et al., 1996; Wang et al., 1997). It has been suggested that there are at least two types of phosphatases that can counteract MEK's actions on MAP kinases, PP2A and MKP. The Ser/Thr phosphatase PP2A can directly inactivate MEK by dephosphorylating the Raf phosphorylation sites (Sontag et al., 1993). Dual-specificity Thr/Tyr phosphatase MKP reverses MEK action by dephosphorylating MAP kinase at the MEK phosphorylation sites (Whitmarsh and Davis, 1996).

1.2.4 The Raf family of Ser/Thr kinases

1.2.4.1 Introduction

Raf was first discovered as a gain-of-function mutant with the ability to transform cells (Rapp et al., 1988). Raf proteins are protein serine/threonine kinases that are essential for growth and development in worms, flies, frogs and mammals, via their ability to function as a MEK kinase. MEK1 and MEK2 are the only known substrates of Raf although there is increasing evidence for Raf's actions in the absence of activation of MEK and ERK (Blenis, 1993; Buscher, 1993; Daum, 1994). Many other proteins, including Bcl-2 (Wang et al., 1996), cdc25 (Galaktionov et al., 1995), IκB (Finco and Jr, 1993; Li and Sedivy, 1993), KSR (Therrien et al., 1996) and Rap1B (Cook et al.,

1993; Gotoh et al., 1995), have been shown to associate with Raf-1. Activation of Raf-1 seems to be important for the functions of these interacting proteins. Receptors that regulate Raf kinases include members of the G-protein coupled receptors, receptors tyrosine kinase and cytokine receptors that regulate intracellular protein tyrosine kinases (RTKs) (Avruch et al., 1994). Raf serves as a central cytoplasmic intermediate in many of these pathways, functioning to connect upstream tyrosine kinases and Ras with downstream kinases including MEK and ERK (Avruch et al., 1994).

1.2.4.2 Tissue distribution of Raf isoforms

The Raf kinases are highly conserved in evolution and have been isolated in invertebrates such as *Drosophila* (D-Raf) and *Caenorhabditis elegans* (Ce-Raf). Genetics experiments in these organisms have identified only one functional gene. However, three such genes are present in vertebrates, A-*Raf*, B-*Raf* and C-*Raf* (also named Raf-1) (Daum, 1994). The 72kDa Raf-1 is ubiquitously expressed in almost all embryonic and adult mammalian tissues and cell types, while A-Raf and B-Raf display much more restricted tissue distribution. A-Raf encodes a 67.5kDa protein and is expressed predominantly in the epididymis and ovary (Storm et al., 1990). Its contribution to the signaling pathway has not yet been fully investigated. B-Raf is highly expressed in brain, spinal cord, ovary, and testis (Storm et al., 1990) and is readily identified in cultured neuronal and endocrine cells. Alternative splicing may contribute to the diversity of the B-Raf kinase members. B-Raf isoforms in avian, mouse and human show apparent molecular weights ranging from 65 to 70 and 95 to 105 kDa (Barnier et al., 1995; Calogeraki et al., 1993; Eychene et al., ; Moodie et al., 1994). Interestingly, B-Raf isoforms containing amino acids encoded by exon 1 and 2 which encode the unique N-terminal extension, and exon 10, which encodes a part of conserved region 2 (CR2) as well as catalytic domain (CR3), are highly expressed in neural tissues. In PC12 cells, B-Raf proteins have been reported as either a single

species at 95 kDa (Stephens et al., 1992) or as two splice variants at 67 and 95 kDa (Oshima et al., 1991), depending on the clonal isolates of PC12 cells used.

1.2.4.3 The structure of Raf isoforms

Structurally, all Raf isoforms are very similar, they share three highly conserved regions (CR1-3) embedded in variable sequences (Daum, 1994). CR1 has a composite structure consisting of a Ras-binding-domain (RBD) followed by a cysteine-rich-domain (CRD) containing zinc-finger motif of the type $C(X)_2C(X)_9C(X)_2C$, where C is cysteine and X is any amino acid). CR2 is rich in serine and threonine residues, some of which, such as S259, T268 and T269 in Raf-1, might serve as regulatory phosphorylation sites. The catalytic domain of the Raf kinase is encoded by CR3, which is more related to the Src family PTKs than to other protein kinases (Mark and Rapp, 1984). The overall structure of A-Raf is closer to that of Raf-1; but the major splice variant of B-Raf, 95kDa form, has unique N-terminus of 95 amino acids that is not present in Raf-1. The function of this 20kD extension is not known. The structural comparison of mammalian Raf-1 and B-Raf isoforms is summarized in Fig.1.2. (Daum, 1994; Morrison and R. E. Cutler, 1997).

1.2.4.4 Regulation of Raf enzymatic activity

Most studies of regulation of Raf activity have been focused on the Raf-1 isoform. The N-terminal domain of Raf-1 seems to function to suppress its catalytic activity since the deletion of or replacing single amino acid in this domain constitutively activates Raf-1 (Heidecker et al., 1990; Stanton, 1989). Therefore, in the simplest model, Raf-1 activity can be regulated by reversibly relieving the inhibitory effect of the N-terminus, and allowing the kinase domain to contact its activators and substrates. For wild type Raf-1, this is thought to be achieved by its association with Ras. It is known that Raf-1 is recruited to cytoplasmic membrane by GTP-bound Ras upon stimulation by

extracellular signals (Leevers et al., 1994; Stokoe et al., 1994; Vojtek et al., 1993). However, attempts to activate purified Raf-1 with Ras-GTP *in vitro* have generally failed and earlier experiments had indicated that Ras was insufficient to fully activate Raf kinase. These results suggest a requirement for additional membrane localized factors for its maximum activation. A potential candidate for this factor is Src or Src-like tyrosine kinase since Src phosphorylation of Raf-1 at Tyr340 and Tyr341 enhances the catalytic activity of Raf-1 *in vivo* (Fabian, 1993; Marais et al., 1995; Park, 1996; Popik, 1996; Xia, 1996). Raf-1 may also be regulated by the serine/threonine kinases PKC and PKA. PKC can activate Raf-1 activity by direct phosphorylation at Ser499 (Kolch et al., 1993; Sozeri et al., 1992; Ueda et al., 1996; Ueda and May, 1994). However, PKA activation results in the dissociation of Raf-1 from Ras to inhibit Raf-1 activation by other signals.

B-Raf isoform might be regulated in a manner distinct from Raf-1, and independent of regulation by Src phosphorylation. The amino acids homologous to Tyr340 and Tyr341 in Raf-1 that are phosphorylated by Src, are replaced with two aspartic acids at the corresponding positions in B-Raf. The negative charges at these sites may mimic Src phosphorylation and have been proposed to account for the higher basal activity of B-Raf compared to Raf-1 (Jelinek et al., 1996). The action of other kinases on B-Raf, including PKA, are not well studied.

1.2.5 Small G proteins of the Ras superfamily

The 21kDa protein Ras is a small GTP binding protein that has been implicated in the growth regulation of a wide range of human tissues (Bishop, 1987). Ras-related GTP-binding proteins constitute a superfamily of small G proteins that contain approximately 50-60 members in mammals. Based on sequence and functional homology these members can be grouped into the Rac/Rho, Rab/Ypt, Ran, Arf, Rad and the Ras

subfamilies (Wittinghofer et al., 1993). The Ras family consists of Ras, Rap, and Ral with each having various isoforms. Like trimeric G proteins, small G proteins also 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. In this inactivation form, GDP is tightly bound and the rate of dissociation of GTP is very slow. Activation is triggered by the action of specific guanine-nucleotide exchange factors (GEFs) that accelerate GDP release from small G proteins. This release is rapidly followed by the binding of GTP at the site, since GTP exists in great excess over GDP within the cells. Once activated, small G proteins are able to interact with and activate effector molecules . The restoration of small G proteins to their resting (inactive) state is accomplished by the conversion of bound GTP to GDP, which is controlled by the intrinsic GTPase activity of these G proteins. This intrinsic GTPase activity can be accelerated by specific GTPase-activating proteins (GAPs), which can act as regulatory proteins or as potential effectors. Therefore, each member of the Ras superfamily is potentially regulated by a specific activator GEF and an inhibitor GAP (Boguski and McCormick, 1993; Bourne et al., 1990; Bourne et al., 1991).

1.2.5.1 The small G protein Ras

Ras is the best studied small G protein and plays a central role in the activation of MAP kinase cascade and in many other signalings (Cobb et al., 1991). The Ras protein is plasma membrane-bound and exists as three isoforms: N-, Ha- and Ki-Ras. The accumulation of Ras-GTP can be induced by a variety of ligands including growth factors, hormones and cytokines. GTP loading of Ras is triggered by one of two distinct GEFs: SOS and RasGRF (Boguski and McCormick, 1993; Buchsbaum et al., 1996). Upon growth factor activation of RTKs, SOS is recruited to the membrane to activate Ras. In this way, SOS links Ras to growth factor actions on MAP kinase

cascade. RasGRF is stimulated by calcium (Buchsbaum et al., 1996) and may mediate MAP kinase activation by signals that increase intracellular calcium (Farnsworth et al., 1995; Rosen et al., 1994).

The best studied and perhaps the most important Ras effectors identified so far is Raf-1. The coupling of Ras and Raf-1 was first suggested by experiments where the microinjection of neutralizing anti-Ras antibodies into the cell inhibited the signaling via PDGF receptor and oncogenic v-Ras, but not oncogenic v-Raf. However, microinjection of anti-Raf antibodies inhibited v-Ras- mediated signaling (Kolch et al., 1991). Subsequent studies utilizing dominant negative mutants of Ras and Raf (see Table 1.1a), as well as co-immunoprecipitations and yeast two-hybrid studies demonstrated that Raf-1 interacts directly with Ras and that Raf-1 is the downstream effector of Ras (Koide et al., 1993; Moodie et al., 1993; van Aelst et al., 1993; Vojtek et al., 1993; Warne et al., 1993; Wittinghofer and Herrmann, 1995; Zhang et al., 1993). Targeting Raf protein by engineering the C-terminus of K-Ras which contains both the isoprenylation motif CAAX and the polybasic domain required for the plasma membrane localization of K-Ras (CAAX-Raf) (Hancock et al., 1990), can bypass the requirement of Ras for Raf-1 activation (Leevers et al., 1994; Stokoe et al., 1994). Recombinant CAAX-Raf is able to induce the activation of ERK2 and neurite outgrowth in PC12 cells and morphological transformation of NIH3T3 cells in the absence of stimuli (Leevers et al., 1994). Although the activation of wild-type Raf-1 requires its direct interaction with active GTP-bound Ras, this interaction *in vitro* does not lead to activation of Raf-1. These data suggest that Ras brings Raf-1 to the plasma membrane where other unknown proteins act for maximum Raf activation . This is in contrast to another interaction between a small GTPase, Cdc42, and a protein kinase PAK. Direct binding of the Rho family member Cdc42 to the p21-activated kinase (PAK) directly stimulates kinase activity, probably by inducing a conformational

change that promotes autophosphorylation of PAK (Manser et al., 1994). Alternatively, Raf-1 can be activated by dimeration or oligomerization after recruitment to the membrane (Luo et al., 1996). Other potential effectors of Ras are summarized in Table 1.3 (Wittinghofer and Herrmann, 1995; Marshall, 1996). It is not known whether all three isoforms of Ras bind to the same effectors with similar affinity.

1.2.5.2 The small G protein Rap1

The Ras related small G protein, Rap1, was cloned on the basis of its sequence homology to Ras (Pizon et al., 1988). Two members, Rap1 and Rap2, have been identified, each of which has two isoforms: Rap1A and 1B; Rap2A and 2B. Rap2 is a distant Ras family member and will not be discussed further. The two isoforms of Rap1 differ minimally at their C-terminus (Noda, 1993). The two human isoforms of Rap1 share 97% homology within their amino acid sequence (Noda, 1993; Pizon et al., 1990). The functional difference between the two proteins is unclear and in most studies no discrimination between the two has been made. Rap1A isoform was cloned as smg p21 after purification from bovine brain and human platelet membranes (Kawata, 1988; Ohmori et al., 1989). Rap1 shares 50% homology with Ras that is greatest in the effector domain and the GTPase domain. The substitution of a valine for glycine at amino acid 12 in Rap1 (RapV12), like the corresponding mutation in Ras (RasV12) blocks Rap1's intrinsic GTPase activity and can be suppressed by substitutions in the effector domain and GTP binding domain (Kitayama et al., 1989). Therefore, Rap1 appears to act like Ras; it is active in the GTP-bound state and is inactive in the GDP-bound state. In addition, the mutation of Ala17 to Asn17 (RapN17), like the corresponding Ras mutation (RasN17) (Feig and Cooper, 1988) also behaves as an interfering mutant, and can abolish Rap1's biological effect on Ras transformation (Kitayama et al., 1989). The striking similarity of the effector domains of Ras and Rap1 (Fig.1.3.) (Wittinghofer and Herrmann, 1995) suggest that Rap1 may

bind to the same effectors as Ras. In fact, Rap1 was independently identified as Raf-1 binding protein in a yeast two-hybrid screen (Burgering et al., 1993; Zhang et al., 1993). In addition, RapV12 binds to other known effectors of Ras including Ras-GAP, PI(3) kinase, and Ral-GDS (Aelst et al., 1994; Kikuchi et al., 1994; Spaargaren and Bischoff, 1994; Spaargaren et al., 1994; Wittinghofer and Herrmann, 1995). Lastly, like Ras, Rap1 transcripts are thought to be expressed in all cells (Kitayama et al., 1989).

Despite these similarities, Rap1 differs from Ras in many other aspects. Rap1 has been studied for many years, but no positive effectors have been identified. In sharp contrast to oncogenic Ras, Rap1 antagonizes Ras signaling to MAP kinase and to the induction of mitogenesis in multiple cell types (Campa et al., 1991; Cook et al., 1993; Kitayama et al., 1989). For example, the constitutively GTP-loaded mutant of Rap1B, RapV12, has been shown to block Ras-dependent activation of ERKs by EGF and lysophosphatidate (LPA) in Rat-1 fibroblasts (Cook et al., 1993), and to potentiate the transformation-reverting effect of Rap1 in NIH3T3 cells (Kitayama et al., 1989). In the initial cloning studies, Rap1 was characterized as the product of the *krev-1* (Ki-Ras revertant) gene, which was isolated in a genetic screen for genes that caused morphological reversion of NIH3T3 cells transformed by Ki-Ras (Gotoh et al., 1995; Kawata, 1988; Kitayama et al., 1989; Noda, 1993; Pizon et al., 1988). These observations have led to the hypothesis that Rap1 could be a possible antagonist of Ras. Additional studies also showed this antagonistic action of Rap1 on a variety of Ras-dependent responses and possibly also in the sevenless signal transduction pathway in Drosophila (Noda, 1993). For example, RapV12 can inhibit Ras-mediated induction of germinal vesicle breakdown in Xenopus oocytes (Campa et al., 1991) and the activation of c-fos promoter by v-Ras. In contrast, RapV12 is unable to block signals generated from v-Raf, suggesting that Rap1 acted at a site between Ras and

Raf-1 (Sakoda et al., 1992). Studies on chimeras of Rap1 and H-Ras have suggested that Rap1 antagonizes Ras through the competition of the effector proteins of Ras (Zhang et al., 1990). Despite 70% homology between Rap1 and Rap2, Rap2 cannot antagonize Ras (Jimenez et al., 1991) and except for an effect on mottling in Xenopus oocytes, its function is unknown.

Another major difference between Rap1 and Ras is the nature of the activating exchange factor (GEF). Rap1 is activated specifically by C3G, a GEF that is distinct from either RasGEF or SOS (Gotoh et al., 1995). The interactions between activated Rap1 and Ras effectors described above do not result in the activation of these effectors. These interactions may be inhibitory by sequestering them from Ras action. Indeed, Rap1 interferes with the activation of the major Ras effector Raf-1 by both Ras and growth factors (Cook et al., 1993; Sakoda et al., 1992). This provides evidence for a simple model for the antagonistic effect of Rap1 on Ras signaling and suggest that Rap1's ability to antagonize Ras signaling to Raf-1 might contribute to its inhibitory effects on Ras induced transformation and proliferation.

Unlike Ras which is a plasma membrane-associated protein (Hancock et al., 1990; Kikuchi and Williams, 1994; Willumsen et al., 1984), Rap1 is located on many different membrane compartments. Rap1 stays in Golgi complex (Beranger et al., 1991) and endosomes/lysosomes in fibroblasts (Pizon et al., 1994), phagosomes in macrophages (Pizon et al., 1994). Rap1 is also very abundant in brain where it locates at particulate fraction including mitochondria (Kim et al., 1990). Therefore, it is possible that binding of Rap1 to Raf-1 forms a nonproductive complex because other membrane proteins that are necessary for Raf-1 activation are missing in intracellular organelle membranes. Alternatively, these organelle membranes may contain enzymes that inactivate Raf-1 in some way. However, in some cells, such as platelets,

neutrophils and megakaryocytes, Rap1 was located at the inner plasma membrane and in granules (Berger et al., 1994; Quinn et al., 1992). The last difference between Ras and Rap1 is their regulation by PKA. The GTP-loading of Rap1 can be stimulated by PKA *in vivo* (Altschuler et al., 1995) and can be phosphorylated by PKA *in vitro* (Altschuler and Lapetina, 1993). No known effects of PKA on Ras have been reported.

1.3. GROWTH FACTOR SIGNALING TO RAS AND THE MAP KINASE CASCADE

Growth factors are a group of secreted molecules that regulate proliferation and differentiation in a wide range of cells. One large group of growth factors acts by binding to and activating cell surface receptors. These receptors are designated receptor tyrosine kinases (RTKs) due to their intrinsic protein tyrosine kinase (PTK) activity. The tyrosine kinase activity of RTK is critical for the physiological actions of growth factors. Growth factors and their RTKs participate in the regulation of growth, differentiation and survival of individual cell types in higher organisms in part via activation of the MAP kinase cascade (Fig.1.1) (Marshall, 1994; Marshall, 1995; van der Geer and hunter, 1994). Like components in MAP kinase cascade, some of RTKs, such as c-kit (Yarden et al., 1987), trk (Martin-Zanca et al., 1986; Martin-Zanca et al., 1989), sis (Hunter, 1997), ret (Takahashi and Cooper, 1987) and met (Park et al., 1987), as well as the receptors for growth factors steele, NGF and other neurotrophins, PDGF (platelet-derived growth factor), GDNF (glial-derived neurotrophic factor), and HGF (hepatocyte-derived growth factor) respectively, were identified as oncogenes, suggesting their important roles as a regulator of mitogenesis (Hunter, 1991; van der Geer and hunter, 1994).

Growth factors signal to MAP kinase cascade via the small G protein Ras. The coupling of RTKs to Ras requires the recruitment of the Ras-specific guanine nucleotide exchange factor, SOS to the membrane (McCormick, 1993; Schlessinger, 1993). This process involves a cascade of tyrosine phosphorylation and protein-protein interaction. Growth factors initiate MAP kinase 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 the cytoplasmic catalytic domains. The active tyrosine kinase domains undergo a trans-autophosphorylation of specific tyrosine residues within the adjacent intracellular domain of RTK dimer (Schlessinger and Ullrich, 1992). These phosphotyrosines (in the context of neighboring C-terminal residues) are recognized by specific protein binding motifs called the Src-homology 2 (SH2)-domains. The adaptor protein Grb2 rapidly associates with activated RTKs via the interaction of Grb2's SH2-domain and specific phosphotyrosines on the RTK (Lowenstein et al., 1992). Grb2 also contains another protein recognition domain called an SH3-domain which recognizes proline-rich sequences in other proteins. 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), this movement allows SOS to stimulate the exchange of GDP for GTP on Ras, thereby producing the active form of Ras (Ras-GTP) (Buday and Downward, 1993; Schlessinger, 1994). As outlined before, Ras-GTP, in turn, translocates Raf-1 to the plasma membrane for subsequent activation of the MAP kinase cascade. Some RTKs do not associate with Grb2/SOS complex directly. For example, NGF receptor TrkA interacts with Grb2/SOS through another family of adaptor molecules Shc (Obermeier et al., 1994; Stephens et al., 1994). Members of the Shc adaptor protein family are associated with tyrosine-phosphorylated RTKs via SH2 domain of Shc (Bonfini et al., 1996). After binding to phosphotyrosine sequences in

activated RTKs, Shc is phosphorylated by RTKs at a tyrosine residue in central Pro/Gly-rich domain. The later tyrosine phosphorylation site is recognized by the SH2 domain of Grb2. Like the direct association of Grb2 with a phosphorylated RTK, the formation of the phospho-RTK/phospho-Shc/Grb2/SOS complex recruits SOS to the membrane to convert Ras-GDP to Ras-GTP which recruits the Raf-1 to the membrane for the stimulation of Raf-1 activity (Bonfini et al., 1996). Other RTKs phosphorylate additional RTK-associated proteins to provide alternative docking sites for Grb2. For example, FGF receptor requires another type of adaptor molecule FRS2 (FGF receptor substrate 2) between receptor and Grb2/SOS (Kouhara et al., 1997). Similar to FRS2, insulin receptor substrate IRS1 connects insulin receptor to Grb2/SOS in addition to Shc/Grb2/SOS (Bonfini et al., 1996). All of these intermediate adaptor molecules are required for growth factor signaling to MAP kinase in selected cases and elicit a growth response via Ras. In addition to Grb2, other SH2 containing proteins can bind to the RTK to recruit additional proteins to the RTK. One of additional proteins is encoded by the Crk family of proto-oncogenes. Crk, like Grb2 contains both SH2 and SH3 domains and can recruit SOS or C3G to RTKs independently of Grb2 (Hempstead et al., 1994; Matsuda et al., 1994; Teng et al., 1995). 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 tyrosine phosphatases SHP1 and SHP2. The recruitment of each of these enzymes generates intracellular signals by acting on substrates that are located to the plasma membrane. At least two of these enzymes (SHP2 and PLC) have been reported to be involved in MAP kinase activation under certain circumstances (Stephens et al., 1994; Wright et al., 1997). It is believed that the versatility of the interaction between adaptors and the RTKs regulate, in part, the specificity of growth factor signaling.

1.4 SIGNALING TO MAP KINASE CASCADE VIA G PROTEIN-COUPLED RECEPTORS

1.4.1 G proteins and G protein-coupled receptors

An increasingly large group of hormone ligands bind to, and activate, G protein-coupled receptors. These ligands include small peptides, large proteins, glycoproteins, catecholamines, lipids, and others. These ligands circulate in the blood and do not need to enter their target cells to elicit a signal. Therefore, they can be distinguished from the other major class of hormones, the steroid family that interacts with cytoplasmic receptors. Instead, these hormones induce intracellular signals via conformational changes within specific transmembrane receptors. These receptors all share structural features including seven transmembrane domains, and cytoplasmic loops that bind intracellular proteins. The most important intracellular proteins that bind these loops are the heterotrimeric GTP-binding proteins (G proteins) (Spiegel et al., 1992). G proteins are composed of three subunits α, β and γ which are the products of three gene families (Exton, 1996). Based on sequence and/or functional homology of the α-subunit, the known G proteins can be broadly categorized into four families Gs, Gi, Gq and G_{12}. The multiplicity of gene products of α, β and γ subunits allows formation of many heterotrimeric species which may confer increased specificity or flexibility of signaling although not all interaction are favored. G protein-coupled receptors (GPCRs) have characteristic seven transmembrane domains with α helices (Neer, 1995; Spiegel et al., 1992). GPCRs display their amino terminus and three interhelical loops to the extracellular environment, while three more loops and the carboxyl terminus, are exposed to the cytoplasm and contribute to G protein binding (Probst et al., 1992; Strader et al., 1995). Binding of an agonist results in the simultaneous dissociation of the G protein heterotrimer from the receptor, exchange of GTP for GDP on the α subunit and dissociation of the α subunit from the $\beta\gamma$ dimer. Although only α subunits were originally proposed to signal to the downstream effectors and $\beta\gamma$ dimers were

originally viewed as passive partners, it is now firmly established that βγ dimers have signaling functions in their own right (Gudermann et al., 1997; Spiegel et al., 1992; Wess, 1997).

The list of known GPCRs' effectors is growing rapidly. Each of G protein subfamily has distinct properties and effectors which are summarized in Table 1.4 (Gudermann et al., 1997; Spiegel et al., 1992). They have been reviewed in detail. I will not attempt to provide a comprehensive review of G-protein-receptor coupling and G-protein-effector coupling, but will discuss the aspects of their function that are related to the activation of PKA as following.

1.4.2 Gs proteins

The expression of G proteins can be highly restricted, as in the case of G proteins involved in visual and odorant transduction, or ubiquitous as is the case for the Gs protein involved in regulation of the intracellular second messenger, cAMP. For Gs proteins, both the structure and function have been conserved (Spiegel et al., 1992). Gs stimulates cAMP formation in fruit flies as well as in mammals (Quan et al., 1991). Many receptors are capable of stimulating adenylyl cyclase through Gs. Examples are the βAR (β2-adrenergic receptor), PTH (parathyroid hormone) receptor, TSH (thyroid stimulating hormone) receptor, ACTH (adrenocorticotropic hormone) receptor and the VIP (vaso-intestinal peptide) receptor. Spontaneous mutations in the human αs-subunit gene (gsp mutations) can trigger high levels of basal adenylyl cyclase activity independent of hormonal stimulation (Landis et al., 1989). Like other G proteins, the specificity of Gs activation is determined by several intracellular receptor domains including the second and third cytoplasmic domains and the N-terminal segment of the cytoplasmic tail. Portions of these loops are important both in determining relative affinity for different G proteins, and in transmitting the signal produced by agonist

binding. For example, a mutant βAR with a seven amino acid deletion (residues 267-273) in the C-terminal region of its third cytoplasmic loop retains its ability to bind Gs but cannot transmit a stimulatory signal (Hausdorff et al., 1990). This finding suggest that binding and activating functions may be subserved discrete cytoplasmic domains.

The principal physiological function of Gsα is to stimulate the membrane-bound adenylyl cyclase and thus increase the concentration of intracellular cyclic adenosine monophosphate (cAMP). Since the major effector of Gs is adenylyl cyclase, The action of Gs can be mimicked by the addition of direct activator of adenylyl cyclase such as forskolin (Seamon and Daly, 1986), or cAMP analogues such as 8-CPT (Young et al., 1994) and dibutyryl-cAMP (db-cAMP), 8-bromo-cAMP (Br-cAMP). Another G protein that can regulate adenylyl cyclase activity is Gi. However, unlike Gs, Gi inhibits adenylyl cyclase activation (Spiegel et al., 1992).

The major effector of cAMP is the cAMP-dependent protein kinase (PKA). The Ser/Thr protein kinase PKA is a heterdimeric protein consisting of two cAMP-binding regulatory subunits (R) and two protein kinase catalytic subunits (C). cAMP activates PKA by binding to the regulatory subunit and releasing the catalytic subunit. Free catalytic subunit is capable of phosphorylating its protein substrates at sites resembling the RRXS/T consensus motif (Kemp and Pearson, 1990). There are two isoforms of the regulatory subunit, RI and RII, where the RI-C (PKA-I) complex is essentially cytosolic while the RII-C (PKA-II) complex is largely associated with particulate fractions of the cell. The compartmentalization of RII subunits via their interaction with members of A-kinase-anchoring proteins may provide another level of regulation of PKA (Houslay and Milligan, 1997). In addition, PKA can be negatively regulated by a specific protein kinase inhibitor PKI. PKI binds PKA with high affinity and can act as a PKA pseudosubstrate (Scott et al., 1986; Walsh et al., 1971; Pattern, 1992).

1.4.3 Regulation of MAP kinase cascade through G protein-coupled receptors

It has become increasingly apparent that GPCRs and G proteins, like RTKs, are involved in the regulation of cell growth, proliferation and differentiation (Dhanasekaran et al., 1995). Many of these effects are mediated by the MAP kinase cascade. Each of four subfamilies of G proteins has specific effectors, yet all of them can influence the activity of the ERK cascade. In addition, the activation of Gα can lead to stimulation or inhibition of growth factor pathways in different cell types (Graves et al., 1993; Hazlerigg et al., 1996; Parma et al., 1993). The G protein families that have been studied in most detail with respect to the regulation of MAPK cascades are Gq and Gi. Although ERKs were only partially characterized in the late 1980s and early 1990s, it was known that they could be stimulated by phorbol esters as well as by growth factors acting through RTKs. In addition, agonists for Gq-coupled receptors, which stimulate PtdInsP2 hydrolysis and activate DG-regulated PKCs, can activate ERKs in a PKC-dependent manner (Bogoyevitch et al., 1995; Bogoyevitch et al., 1994; Bogoyevitch et al., 1993; Clerk et al., 1994; Clerk et al., 1996). For example, chronic treatment with TPA (12-O-tetradecanoyl phorbol 13-acetate) which depletes cells of DG-regulated PKCs in response to agonists, inhibits ERK activation by the Gq-coupled agonist endothelin-1 (ET-1) or TPA (Bogoyevitch et al., 1995). These data are consistent with results obtained following the transfection of constitutively active α_q subunit, which activates ERKs independently of additional stimuli (Faure et al., 1994). In contrast to constitutively active α_q, transfection of constitutively active α_i into cells does not result in the activation of ERKs effectively . Instead, coexpression of α_i and $\beta\gamma$ dimers are required to activate ERK activity in these experiments, suggesting $\beta\gamma$ dimers play an important role in G_i mediated ERK activation (Faure et al., 1994). The role of $\beta\gamma$ dimers in ERK activation has recently

been confirmed. Active βγ subunits associate with and activate a form of PI-3 kinase which is required to activate the adaptor molecule, Shc, an upstream adaptor of Ras (Crespo et al., 1994; Hawes et al., 1996; Touhara et al., 1995).

Other mechanisms by which Gi and Gq activate ERK have been proposed. First, GPCRs may act through RTPKs. Activation of ERKs by either endothelin (ET-1), lysophosphatidic acid (LPA),or thrombin was decreased by a transfected dominant negative EGF receptor mutant or by a PTK inhibitor with selectively for the EGF receptor-coupled pathway (Daub et al., 1997; Daub et al., 1996). Second, the cytoplasmic tyrosine kinase PYK2 (proline rich tyrosine kinase 2) has been identified as a potential activator of Shc. PYK2 is rapidly phosphorylated on tyrosine and activated in response to the increase in calcium concentration or PKC activation to induce tyrosine-phosphorylation of Shc and subsequent recruitment of Grb2/SOS. These results suggest a role of PYK2 in the Ras-dependent activation of ERKs by Gq (Lev et al., 1995). Third, activation of the Src family tyrosine kinases by GPCR agonists might also participate in signaling to ERKs. Treatment with GPCR agonists result in the activation of Src and the ability of both Gi-coupled receptor and Gq-coupled receptor to activate the ERK cascade is reduced in cells depleted of Src family tyrosine kinases (Wan et al., 1996). The targets of GPCR-stimulated Src PTKs are unknown, but may include Shc (Ptasznik et al., 1995) and Raf-1 (Fabian, 1993; Jelinek et al., 1996; Marais et al., 1995). All of the pathways discussed above require Ras for the activation of ERKs, with the possible exception of that mediated by PKC (Kolch et al., 1993). Even for PKC, recent data suggests that it may be acting upstream of Raf-1 via a Ras-dependent mechanism (Mahmoud et al., 1997). This dependence on Ras signaling is not seen in the cAMP (Gsα) dependent pathways.

1.5 THE MAP KINASE CASCADE IN CELL GROWTH AND DIFFERENTIATION

1.5.1. MAP kinase in cell growth and differentiation

Many components in and upstream of the MAP kinase cascade have been identified as oncogenes in human tumors. Likewise, mutations in certain G-protein coupled receptors (Parma et al., 1993) or G proteins themselves (Landis et al., 1989) are oncogenic when they are constitutively activated by mutations. Such oncogenes often share the activation of MAP kinase as a common target (Waskiewicz and Cooper, 1995). Together, these findings suggest a key role for MAP kinase in the proliferative process.

In most cell types, MAP kinase acts as a mitogenic factor. Activated forms of Ras are found frequently in human tumors, and activated forms of Ras, Raf and RTK are oncogenic in a variety of cell types *in vitro* (Bos, 1989; Han et al., 1993; Köster et al., 1991; MacNicol et al., 1993; Nishida et al., 1988; Pritchard et al., 1996). Correspondingly, in these transformed cells, ERK activity is consistently elevated. Much evidence points to a positive role for MAP kinase on cell cycle progression in the normal cell in response to a variety of growth factors as well. It has been well documented that growth factors such as EGF, insulin, IGF, FGF, TPA, CSF and antigen, stimulate mitogenesis in many cell types including fibroblasts, adipocytes, muscle cells, hematopoietic cells and T lymphocytes (Rossomando et al., 1989; Herschman et al., 1989; Kumar et al., 1997; Menapace et al., 1987; Reiss et al., 1997). Again, these agents are strong activators of ERK in these cells and these mitogenic effects are believed to be mediated by the ability of ERK to regulate the cell cycle. For example, in mammalian cells, MAP kinase is sufficient to drive cells through late G1 into S phase (Pelech and Sanghera, 1992). Many studies have used constitutively activated or dominant negative MEK mutants (Cowley et al., 1994), a specific MEK

inhibitor (Dudley et al., 1995) and ectopically expressed MKP (Sun et al., 1994) to demonstrate that ERKs are required for Ras- or Raf-induced transformation or growth factor stimulated cell growth. All these observations suggest the importance of MAP kinase and MAP kinase cascade in cell growth.

It has also been demonstrated that MAP kinase can exert negative actions on cell growth. For example, in MCF-7 human breast cancer cells, TPA inhibits proliferation via the MAP kinase-dependent stimulation of the synthesis of $p21^{WAF1/CIP1}$ (Dufourny et al., 1997). The induction of the cell cycle inhibitor p21 may require a high threshold of MAP kinase activity since high levels of Raf and Ras signal leads to cell cycle arrest in mouse fibroblasts while intermediate level of Raf activity induces proliferation. High levels of Raf activity correlates with potent ERK activation; intermediate level of Raf activity correlates with weak activation of ERKs. The proliferative effects seen following intermediate levels of Raf activity are associated with upregulation of cell cycle activator cyclin D1 in the absence of induction of the cell cycle inhibitor p21 (Sewing et al., 1997; Woods et al., 1997).

In other cell types, MAP kinase activation does not lead to cell growth but rather triggers differentiation. Examples for these effects of MAP kinase are seen in neuronal cells. In neuronal and pheochromocytoma (PC12 cells), FGF and NGF are able to stop cell growth and stimulate neuronal differentiation. The interesting aspect of these growth factors is that unlike in neuronal cells, they both stimulate proliferation in fibroblasts (NIH3T3 cells) through endogenous FGF receptor and ectopically expressed NGF receptor respectively (Barbacid et al., 1991; Cordon-Cardo et al., 1991; Glass et al., 1991; Levi-Montalcini, 1987; Schlessinger and Ullrich, 1992; Wagner, 1991). The neurotrophic factor NT-3 also can differentiate and act as a mitogen in neuronal cells (Cordon-Cardo et al., 1991; Segal et al., 1992). In addition

to neuronal cells, MAP kinase also play an important role in differentiation of other cell types. It has recently been demonstrated that MAP kinase is involved in cAMP or steel/kit induced melanogenesis by activating AP-1 or Micropthalmia transcription factors which are important for the expression of tyrosinase, an critical enzyme for melanogenesis (Englaro et al., 1995; Hemesath et al., 1998). MAP kinase is also necessary and sufficient for the megakaryocytic differentiation in megakaryocyte (Melemed et al., 1997; Whalen et al., 1997) and necessary for double-negative thymocytes differentiating into double-positive thymocytes (Crompton et al., 1996). MAP kinase even has opposing effects on cell differentiation. In myocytes, MAP kinase activity inhibits myocyte differentiation and inactivation of MAP kinase by MKP1 blocks this inhibition by MAP kinase (Bennett and Tonks, 1997). Another example for this inhibitory regulation of differentiation by MAP kinase is that TNF-α's inhibition of insulin-induced adipogenesis of preadipocytic cell line 3T3-L1 is blocked by MEK inhibitor PD98059 (Mora et al., 1997).

1.5.2 The specificity of signaling pathways

As we have seen above, MAP kinase has both stimulatory and inhibitory effects on cell growth and differentiation. Therefore, the study of MAP kinase raises two important questions concerning how cells achieve specificity in signaling pathways: A) How does an extracellular signal have opposing growth effects in different cell types via similar signal transduction pathways? B) How does the activation of a specific signaling cascade induce different cellular responses within the same cells in response to different stimuli? As a model for the first question, I will examine cAMP's cell-type specific effects on growth via its cell-type specific regulation of the MAP kinase cascade. As a model for the second question, I will test the ability of MAP kinase to mediate proliferation by EGF and differentiation by NGF in the neuro-endocrine cell line, PC12 cells.

1.5.2.1 PC12 cells: a model system

To study the specificity of MAP kinase signaling and its role in differentiation, I chose PC12 cells as a model system. PC12 cells were derived from rat adrenal tumor of adrenergic neural crest origin (Tischler and Greene, 1975). They express multiple tyrosine kinase receptors for growth factors and G-protein coupled receptors for hormones. Examples of receptors for growth factors in these cells include EGFR, NGFR, FGFR and IGFR. Hormone receptors expressed on the cells mediate the biological functions of B adrenergic receptor (βAR) and receptors for VIP, prostaglandin (PGE), bradykinin and adenosine as well as others. The PC12 cell line is one of few neuronal systems in which differentiation can be induced *in vitro* by activation of a tyrosine kinase growth factor receptor. In response to some growth factors, such as NGF, FGF, they stop growing and differentiate into neuron-like cells (Greene and Tischler, 1976; Togari et al., 1985; Wagner, 1991). Differentiated PC12 cells develop characteristics of sympathetic neurons (Greene, 1978; Wagner and D'Amore, 1986). This neuronal differentiation phenotype includes morphological changes (i.e. flattening and extension of neurite processes referred to as neurite outgrowth), expression of neuronal markers such as transin, GAP43, neurofilaments, formation of synaptic-like vesicles and display of action potentials that reflects sodium channel expression (Eves and al., 1994; Eves and al., 1992; Greene and Tischler, 1976). However, in response to other factors such as EGF, these cells do not differentiate; instead, they proliferate (Cowley et al., 1994; Huff et al., 1981). Proliferation stimulated by EGF is accompanied by increase in the levels of several cell cycle progression factors including cdk2, cdk4, and cyclin B1 (Mark and Storm, 1997). The MAP kinase pathway is activated by both proliferative and differentiative growth factors in these cells. Because of the ability of PC12 cells to respond, in distinct ways, to growth factors that all activate MAP kinase, this cell line has been widely used for the studies of signal transduction pathways in growth factors' actions.

Unlike PC12 cells, primary neural progenitor cells are heterogeneous. Therefore, it is informative to test the role of MAP kinase in the differentiation of neuronal cells by using this clonal cell line.

1.5.2.2 cAMP regulation of MAP kinase

The PC12 cell line responds to cAMP to induce differentiation (Heidemann et al., 1985; Togari et al., 1985; Young et al., 1994). cAMP's actions in these cells serve as a model for cAMP's activation of MAP kinase in many cell types including pituitary, thyroid, melanocytes and others. Our studies of these actions of cAMP in these cells have provided us some insights into the mechanism by which cAMP regulates cell growth in a cell-type specific manner.

1.5.2.2.1 cAMP's cell-type specific growth effects

One interesting aspect of hormonal control of cell growth and differentiation is the cell-type specificity of the action of hormones elevating intracellular level of cAMP. These types of hormones can both stimulate and inhibit cell growth in different cell types. One example for this opposing effects of hormone is PTH which stimulates proliferation in choroid cells (Verheijen and Defize, 1997) and inhibits cell growth in osteosarcoma cells (Verheijen and Defize, 1995). Directly applying cAMP analogues or adenylyl cyclase activator to the cell cultures also has similar opposing effects on cell growth in different cell types. In fibroblasts, adipocytes, smooth muscle cells and cortical astrocytes, T cells and macrophages, cAMP inhibits cell growth and antagonizes mitogenesis induced by growth factors (Burgering et al., 1993; Dumont et al., 1989; Faure and Bourne, 1995; Graves et al., 1993; Hordijk et al., 1994; Kurino et al., 1996; Sevetson et al., 1993; Wu et al., 1993). In contrast, cAMP promotes the growth of certain endocrine cells, such as thyroid and pituitary somatotrophs, human and mouse primary melanocytes and potentiates growth factor action in Swiss 3T3 cells

(Dumont et al., 1989; Faure and Bourne, 1995; Miyazaki et al., 1992; Rosengurt, 1986; Zachary et al., 1990). In follicular thyroid cells, cell growth requires the combined action of insulin-like growth factor and thyroid stimulating hormone, a known activator of adenylyl cyclase (Dumont et al., 1992).

Evidence for cAMP's role in endocrine cell growth also comes from studies of pituitary and thyroid tumors. Mutant forms of Gsα that constitutively activate adenylyl cyclase have been identified in human GH-secreting pituitary adenomas (Climenti et al., 1990; Landis et al., 1989; Lyons et al., 1990; Vallar et al., 1987) and thyroid toxic adenomas (Lyons et al., 1990; O'Sullivan et al., 1991). Greater than 40% of all human GH-secreting pituitary adenomas and a small fraction of thyroid adenomas harbor an oncogene (*gsp*) along the cAMP signaling pathway, specifically within the Gsα protein that activates adenylyl cyclase (Climenti et al., 1990; Vallar et al., 1987). These Gsp mutants constitutively activate adenylyl cyclase in pituitary adenomas and thyroid adenomas (Goretzki et al., 1992; Landis et al., 1989).

1.5.2.2.2 cAMP and MAP kinase

The reasons why cAMP stimulates cell growth in some cells but inhibits it in others are not clear. However, it is known that cAMP can inhibit cell growth by antagonizing Ras-dependent activation of MAP kinase. A number of laboratories have demonstrated that cAMP inhibited ERKs in some cells, such as adipocytes, NIH3T3 and Rat1 fibroblasts (Burgering et al., 1993; Cook and McCormick, 1993; Faure and Bourne, 1995; Graves et al., 1993; Hordijk et al., 1994; Sevetson et al., 1993; Wu et al., 1993), where it also inhibits cell growth and proliferation. Since MAP kinase is critical for cell proliferation, we sought out to examine whether increased cAMP level and PKA activation can also activate MAP kinase in some other cells.

Recent work by our laboratory (manuscripts in Chapter 2 and 3) and others demonstrates that cAMP can activate MAP kinase in multiple neuronal and endocrine cells including the adrenal medullary cell line PC12 cells (Frodin et al., 1994), cerebellar granule neurons (Villalba et al., 1997) and rat somatotrophic tumor cell line GH_4C_1 cells (Tashjian et al., 1996; Vossler et al., 1998). cAMP, like NGF, is associated with neuronal differentiation, but not proliferation in PC12 cells [Chapter 3; (Huang et al., 1996; Young et al., 1994)]. As we will see in the following chapters, the ability of MAP kinase to stimulate differentiation is a property of PC12 cells. Others have shown that cAMP also stimulates MAP kinase activation and growth in melanocytes (Englaro et al., 1995), choroid cells (Verheijen and Defize, 1997), pituitary cells (Hazlerigg et al., 1996) and in the cell line Swiss 3T3 cells (Faure and Bourne, 1995). Since MAP kinase plays a critical role in growth factor-mediated growth response, cAMP's cell-type specific action on cell growth and proliferation may be related to its cell type specific ability to regulate MAP kinase. Therefore, how cAMP controls cell growth in cell-type specific manner can be answered by addressing how cAMP regulates MAP kinase. The major goal of my thesis is to identify the molecular basis for the cell type specificity of cAMP's regulation of MAP kinase.

1.5.2.2.3 Rationale

PKA's inhibitory effects on MAP kinase have been reported by several groups (Cook and McCormick, 1993; Graves et al., 1993; Sevetson et al., 1993; Wu et al., 1993). It has been shown that PKA inhibits MAP kinase by blocking Raf-1 activation. It has been proposed that PKA directly phosphorylates Raf-1 on its Ras-binding domain and subsequently dissociating Raf-1 from Ras. This has been proposed to account for the inhibition of MAP kinase by PKA (Cook and McCormick, 1993; Wu et al., 1993). However, the mechanism by which PKA activates MAP kinase is not known. Based on the following observations, we propose a model that cAMP/PKA activates MAP

kinase in a cell type-specific manner via two proteins: a ubiquitously expressed small G protein Rap1 and a tissue specific Raf isoform B-Raf.

--The expression pattern of B-Raf correlates with cAMP's actions.

When cAMP activation of MAP kinase was examined by using a number of interfering mutants (Table 1.1a) within MAP kinase cascade, we demonstrated that cAMP stimulates MAP kinase in a Ras-independent but Raf- and MEK- dependent manner (Chapter 3). The requirement of Raf was surprising since Raf-1 is known to be inhibited by cAMP. We proposed that other Raf isoforms might be involved in cAMP's actions. One of the candidates we examined was B-Raf, since the expression pattern of B-Raf is correlated with cAMP's actions on MAP kinase and cell growth. The 95kDa form of B-Raf is highly abundant in the endocrine cells PC12 cells (Chapter 3) and GH_4C_1 (Vossler et al., 1998) where cAMP activates MAP kinase. It is not detectable in Rat1 (Erhardt et al., 1995) or NIH3T3 fibroblasts where cAMP antagonizes Ras signaling to MAP kinase. Swiss 3T3 cells which have properties of endocrine cells despite a fibroblast-like appearance, express abundant B-Raf (Faure and Bourne, 1995). cAMP potently activates MAP kinase in these cells as well. These observations suggest to us that B-Raf expression might be important for cAMP to activate MAP kinase.

Structural features of Raf-1 and B-Raf also suggest that they may regulated in distinct ways. B-Raf does not have PKA consensus site around the amino acid corresponding to Ser43 in Raf-1 which has been proposed to be the site of PKA's inhibition of Raf-1 activation. In addition, B-Raf has 95 amino acids at N-terminus which are not present in Raf-1 (Daum, 1994; Morrison and R. E. Cutler, 1997). Therefore, we proposed the hypothesis that the expression of B-Raf dictates cAMP's ability to activate MAP kinase. To test this hypothesis, we chose PC12 cells and NIH3T3 cells as model systems for

cAMP's positive and negative actions on MAP kinase, respectively, in part because they differ in the expression of B-Raf.

--Rap1 is potential target for PKA

The Ras independence of PKA's activation of MAP kinase and the inability of PKA to activate B-Raf *in vitro* (Peraldi et al., 1995) suggest that other components are required for PKA to activate B-Raf. Two lines of evidence suggest that the small G protein Rap1 might mediate cAMP's activation of B-Raf and MAP kinase. First, the GTP loading of Rap1 can be stimulated by cAMP *in vivo*.. PKA phosphorylates Rap1 on a single site near the carboxyl-terminus (serine 179) *in vitro* and *in vivo* (Altschuler and Lapetina, 1993; Farrell et al., 1992; Kawata et al., 1989; Lapetina et al., 1989; Lerosey et al., 1991; Siess et al., 1990). This phosphorylation increases its GTP loading *in vivo* (Altschuler et al., 1995) and potentiated the ability of a putative Rap exchanger Smgp21 GDS to activate Rap1 *in vitro* (Hata et al., 1991). Second, a high degree of correlation exists between cAMP and Rap1 actions on cell growth; Rap1 antagonizes the ability of Ras to activate both MAP kinase and cell growth in the same cell type where cAMP also inhibit Ras signals (Cook et al., 1993) and induction of cell growth (Kitayama et al., 1989; Yoshida et al., 1992). Activated Rap1 stimulated cell growth in only one cell type of the many examined (Yoshida et al., 1992), Swiss 3T3 cells, cells shown by others to be stimulated by cAMP as well.

1.5.2.3 Specificity of growth factor signaling

The second major question that I will address is how two extracellular signals can activate a similar set of intracellular pathways yet induce distinct cellular responses. The model system I will use to examine this question of specificity is the role of MAP kinase in the actions of EGF and NGF in PC12 cells. Although EGF and NGF have dramatically different actions on cell growth and differentiation in PC12 cells, both

trigger the activation of MAP kinases and a similar set of early response including Egr/NGFI-A/Krox24/zif268, fos and jun family members, NGFI-B/nur77, β actin, and ornithine decarboxylase (Chao, 1992), neither of which is wholly specific for EGF or NGF. In addition, studies using constitutively active and interfering mutants of proteins within the MAP kinase cascade demonstrate that MAP kinase is necessary for differentiation by NGF as well as proliferation by EGF (Cowley et al., 1994; Huff et al., 1981; Wood et al., 1992). The only differences in MAP kinase activation by both agents is the duration of the MAP kinase activation. ERKs are activated rapidly (within 5 minutes) in response to EGF, but this activity returns to basal levels by 15-20 minutes. In contrast, NGF rapidly and persistently activates ERKs. This sustained activation can last at least two hours (Lenormand et al., 1993). The difference in the kinetics of ERK activation has led others to propose that duration of ERK activation dictates the cellular responses (Cowley et al., 1994; Marshall, 1995; Traverse et al., 1994). One important consequence of sustained activation is the translocation of the majority of cytoplasmic ERKs into the nucleus. Whether nuclear ERKs dictate differentiation is not known. However, nuclear translocation of ERKs is important for distinct set of gene expression required for differentiation. Proponents of this model have proposed that sustained activation of MAP kinase is necessary and sufficient for PC12 differentiation while transient activation of MAP kinase is required for proliferation. Support for this model comes from the studies using PC12 cells in which EGF receptor is overexpressed. In contrast to wild type cells, cells that overexpress EGF receptor allow EGF to induce a sustained activation and nuclear localization of ERK. These alterations also correlate with the occurrence of neuronal differentiation (Traverse et al., 1994). Overexpression of the adapter molecule Crk also alters EGF signaling, resulting in sustained activation of MAP kinase and differentiation (Hempstead et al., 1994; Teng et al., 1995). To further test the role of sustained

activation of MAP kinase in inducing NGF-like differentiating effects, we examined the interaction between cAMP and growth factor signaling to MAP kinase cascade.

1.5.2.4 Rationale

To test whether sustained activation of MAP kinase is sufficient for differentiation of PC12 cells, we can try to increase the duration of MAP kinase activation pharmacologically. One simple way to do this is to identify agents that by themselves activate MAP kinase modestly or transiently, that when applied to cells together can induce a more robust sustained activation. Using this approach, we examined MAP kinase activation by different reagents that activate PKA. We demonstrated that forskolin, on its own, only modestly stimulates MAP kinase in PC12 cells and does not induce differentiation in PC12 cells. However, forskolin synergistically acts with growth factors such as EGF and NGF to activate MAP kinase. Both the magnitude and duration of MAP kinase activation by the costimulation of forskolin and EGF is comparable to NGF alone. Using this treatment, we examine whether sustained activation correlates with neuronal differentiation and nuclear localization of ERKs, in multiple situations.

Another approach examine whether sustained activation of MAP kinase is necessary for differentiation is to convert the sustained activation of MAP kinase into the transient activation by inhibiting sustained phase of activation and examining the consequence of this inhibition on neuronal differentiation. However, this approach requires that 1) the sustained portion of MAP kinase activation be regulated by distinct pathways, and 2) that these pathways can be selectively inhibited by pharmacological or molecular means. Therefore, my goal for this part of the thesis was to identify the molecular mechanisms of sustained activation of MAP kinase in PC12 cells. The ability of PKA to stimulate sustained activation of MAP kinase provided us agents to test whether

PKA's activation of MAP kinase contributed to the ability of NGF to induce sustained activation of MAP kinase. Previous evidence suggests that PKA might be activated by NGF. PKA inhibitors block NGF induction of specific genes (Ginty et al., 1991; Hawley et al., 1992) and block the expression of sodium currents (Kalman et al., 1990). If PKA is activated by NGF, it might provide an addition pathway to MAP kinase and therefore might contribute to the sustained activation of MAP kinase by NGF. The demonstration that cAMP/PKA utilizes Rap1/B-Raf to stimulate MAP kinase led us to examine the possibility that sustained activation of MAP kinase utilizes pathways that were distinct from the classical Ras-MAP kinase cascade. The rationale for this study is that NGF, and any other agent that induces a sustained activation of MAP kinase, must activate MAP kinase independently of Ras in order to avoid the feedback loops that terminate Ras signals (Porfiri and McCormick, 1996). PKA which avoids these feedback loops by activating Rap1, instead of Ras, might provide NGF a possible mechanism to sustain MAP kinase activation. Therefore, we can test the hypothesis that PKA participates in NGF signaling to MAP kinase by using pharmacological and molecular inhibitors of the cAMP/PKA pathway. If we are able to prove that PKA is required for the sustained activation of MAP kinase by NGF in PC12 cells, these inhibitors will also allow us to examine the necessity of sustained activation of MAP kinase for neuronal differentiation.

Three set of experiments have been performed to address the questions outlined above. They are described in the following manuscripts. In chapter 2, experiments were carried out to test whether forskolin can convert EGF into a differentiating agent in PC12 cells; if it does, whether this change correlates with sustained activation of MAP kinase. In chapter 3, we studied the mechanism of the interaction between cAMP and growth factor signaling by answering how cAMP/PKA activates MAP kinase in PC12 cells. In chapter 4, we examined to what extent cAMP/PKA pathway is shared by

NGF. By utilizing inhibitors and interfering mutants along the cAMP/PKA pathway to convert sustained activation of MAP kinase by NGF into a transient one, we tested whether sustained activation of MAP kinase is required for differentiation in PC12 cells.

Fig. 1.1. The MAP kinase Cascade

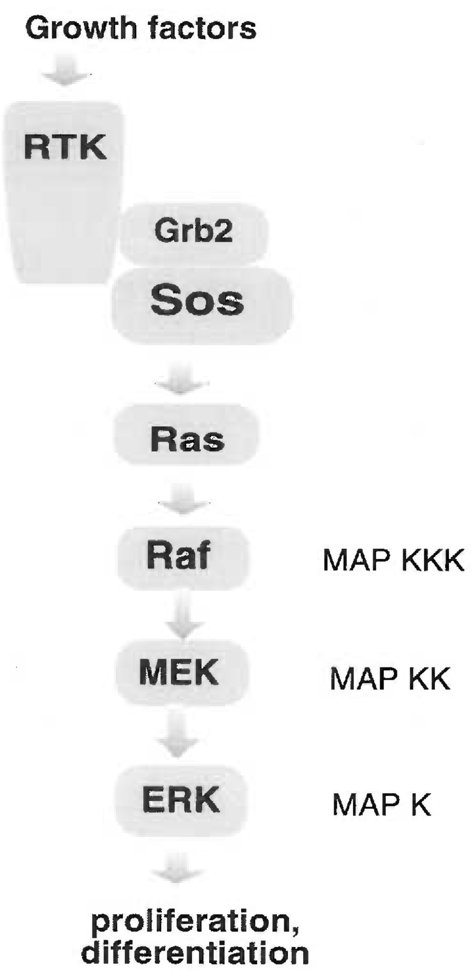

Fig. 1.1. The MAP kinase cascade is dipicted above. Growth factors activate receptor tyrosine kinases (RTK) that recruit the Grb2/SOS complex to the receptor. SOS then activates the GTP binding protein Ras which triggers activation of the MAP kinase cascade itself. The MAP kinase cascade is composed of three kinases: the MAP kinase kinase kinase (MAP KKK) Raf, MEK (MAP KK), and ERK (MAP K).

Figure 1.2. Structural comparison of Raf-1 and B-Raf

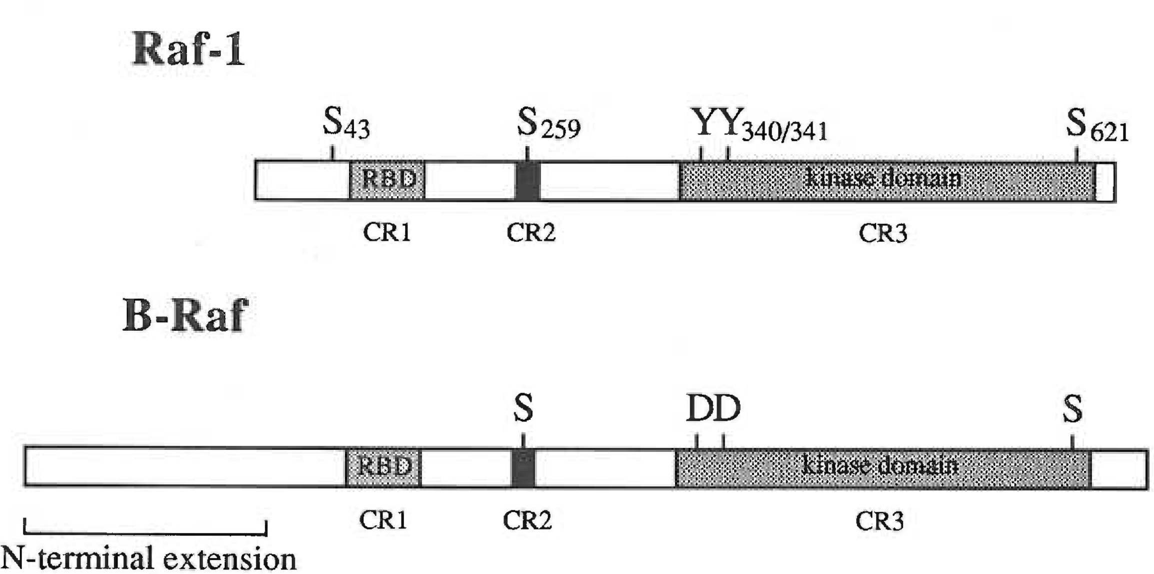

Fig. 1.2 Structral comparison between Raf-1 and B-Raf. Raf-1 contains a consensus PKA phosphorylation site at Ser43 that is not present in B-Raf. Ser259 and Ser621 are 14-3-3 binding sites which are conserved in Raf-1 and B-Raf. Both Raf isoforms have homologous cysteine-rich (CR) domains. Ras binding domain (RBD) and kinase domains are highly homologous between these two Raf isoforms. The putative Src phosphorylation sites in Raf1 (Y340/Y341) are not present in B-Raf and are replaced with Asp. B-Raf also contains a unique N-terminal extension.

Fig. 1.3. Comparison of the effector domains of Ras and Rap1.

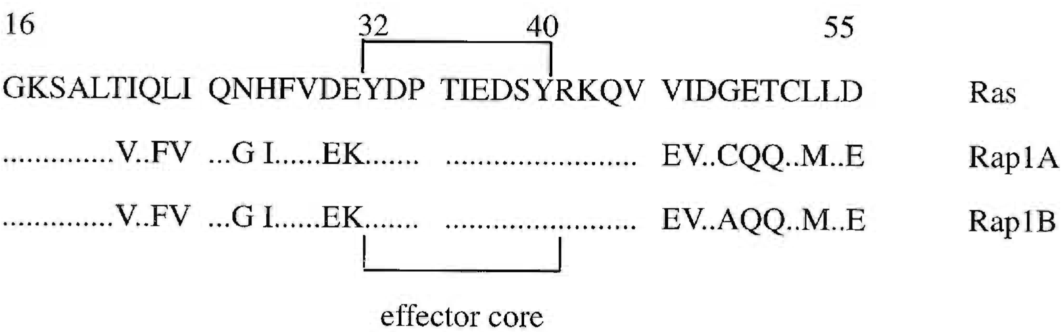

Fig. 1.3. Schematic presentation of the effector domains of Ras and Rap1 small G protein.
Ras and Rap1 are highly homologous in their effector core (amino acids 32 to 40). The different amino acids at 30 and 31 have been proposed to be important for the different binding affinities of Ras and Rap1 for both Raf-1 and RalGDS.

Table 1.1a. Constitutively active and dominant negative mutants of components in MAP kinase cascade.

Mutant	Action	Mutation	Reference
RasV12	Constitutively active	Val12 for Gly12 substitution in GTPase domain	Bar-Sagi & Feramisco, 1985
RasN17	Dominant negative	Asn17 for Ser17 substitution in the Mg^{2+} binding site	Stacy et al., 1991
Raf301	Dominant negative	Met375 for Lys375 substitution in the ATP binding domain.	Bruder et al., 1992
BXB-Raf	Constitutively active	In frame deletion of amino acid 26-302 in the regulatory domain.	Bruder et al., 1992
MEK(K97R)	Dominant negative	Arg97 for Lys97 in the catalytic domain.	Cowley et al., 1994
MEK E217/221	Constitutively active	Glu for Ser substitutions at Raf phosphorylation sites.	Mansour et al., 1994
dnERK1 and dnERK2	Dominant negative	Arg for Lys substitution in the ATP binding domain.	Robbins et al., 1993

Table 1.1b. Rap1 mutants used in this study.

Mutant	Action	Mutation
RapV12	Constitutively active	Val12 for Gly12 substitution in GTPase domain.
RapN17	Dominant negative	Asn17 for Ser17 substitution in the Mg^{2+} binding site.
RapAA	Unknown	Ala for Ser substisutions at the PKA phosphorylation sites.

Table 1.2. Substrates of MAP kinase ERKs

Cell surface proteins		EGF receptor, SOS,
Cytoplasmic proteins	translational factor	eIF4E-BP
	protein kinase	p90 ribosomal S6 kinase, Raf, MEK, MAPKAP kinase-2.
	protein phosphatase	phosphatase 2C
	other signaling molecule	phospholipase A2
	other enzyme	tyrosine hydroxylase
	structural proteins	MBP, MAP2 and MAP4, tau, caldesmon.
Nuclear proteins	transcription factor	Elk1, c-jun, c-fos, c-myc, TAL1, ATF-2, NFIL6.
	RNA polymerase	RNA polymerase II

Table 1.3. Effectors of mammalian Ras protein.

Effector	Function
p120^{GAP}	GAP for p21Ras.
Neurofibromin	GAP for p21Ras.
p110^{PI3K}	3' phosphoinositol-lipid kinase.
Raf1, A-Raf, B-Raf	MAPKKKs in ERK-dependent signalling from receptor tyrosine kinase
Ral-GDS	Guanine nucleotide exchange factor for p21Ral
Rin	Unknown
Rsbs 1, 2 and 4	Unknown
PKC-ζ	Protein kinase C family member
MEKK-1	MAPKKK in Jun kinase pathway

Table 1.4. Properties of G proteins.

α-Subunit	Toxin sensitivity	Effector
G_s		
α_s	CTX	↑ Adenylyl cyclase, Ca^{2+} channel
α_{olf}	CTX	↑ Adenylyl cyclase
G_i		
α_{I1}	PTX	↓ Adenynyl cyclase, secretion?
α_{i2}	PTX	↓ Adenynyl cyclase, tyrosine kinase?, phosphatase?
α_{i3}	PTX	↓ Adenynyl cyclase, Golgi vesicular transport?
$\alpha_{o1,2}$	PTX	↓Ca^{2+} channels (L, N type)
$\alpha_{t\ rod}$	PTX/ CTX	↑ cGMP phosphodiesterase
$\alpha_{t\ cone}$	PTX/ CTX	↑ cGMP phosphodiesterase
α_{gust}		Taste signal transduction
α_z		Adenylyl cyclase
G_q		
α_q		↑ Phospholipase C_β
α_{11}		↑ Phospholipase C_β
α_{14}		↑ Phospholipase C_β
$\alpha_{15,16}$		↑ Phospholipase C_β
G_{12}		
α_{12}		Na^+, H^+ antiporter?, growth reg.?
α_{13}		Na^+, H^+ antiporter?, growth reg.?, egr-1
β-subunits		
β1, β2, β3, β4......		K^+ ir channel, phospholipase Cβ2 and 3, PI-3 kinase, Ras exchange factors?
γ-subunits		
γ1, γ2, γ3, γ4, γ5.....		

Chapter 2

Reversal of growth factor signaling by cyclic adenosine monophosphate in neuronal cells.

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Published in J. Biol. Chem. Vol. 270, 20748-20753, (1995)

ABSTRACT

The rat pheochromocytoma (PC12) cell line is a model for studying the mechanism of growth factor action. Both epidermal growth factor and nerve growth factor stimulate mitogen-activated protein (MAP) kinase in these cells. Recent data suggest that the transient activation of MAP kinase may trigger proliferation, whereas sustained activation triggers differentiation in these cells. We have tested this model by asking whether agents that stimulate MAP kinase without inducing differentiation can act additively to trigger differentiation. Neither forskolin nor epidermal growth factor can stimulate differentiation, yet both activate MAP kinase in these cells. Together, their actions on MAP kinase are synergistic. Cells treated with both agents differentiate, measured morphologically and by the induction of neurite-specific genes. We propose that cellular responses to growth factor action are dependent not only on the activation of growth factor receptors by specific growth factors but on synchronous signals that may elevate MAP kinase activity within the same cells.

INTRODUCTION

In many cells, both proliferation and differentiation are triggered by specific growth factors (Aaronson, 1991). Despite differences in their physiological actions, many growth factors engage similar intracellular signaling pathways, initiated by the auto-phosphorylation of specific transmembrane receptors. This phosphorylation recruits multiple signaling molecules into a membrane-associated complex that includes the GTPase p21 Ras. Activation of Ras initiates a cascade of phosphorylations and activation of protein kinases, including the activation of MAP kinase (Blenis, 1993). Growth factor activation of MAP kinase is required for proliferation in many cell types (Mansour et al., 1994).

The rat pheochromocytoma PC12 cells (Greene and Tischler, 1976) have provided researchers with the best studied example of dual regulation within a single cell through the distinctive actions of nerve growth factor (NGF) and epidermal growth factor (EGF) (Chao, 1992). Both signals are mediated through a family of receptors with intrinsic tyrosine kinase activity yet display dramatically different effects on neural phenotype. NGF-induced differentiation of PC12 cells is characterized by the prolongation of neurites, the induction of neural markers, including transin (stromelysin), that are involved in maintaining neuronal phenotypes (D'Arcangelo and Halegoua, 1993a; Greene and Tischler, 1982), and the cessation of cell division. In contrast, EGF stimulates PC12 cell proliferation. Interestingly, both EGF and NGF activate a receptor tyrosine kinase to phosphorylate and activate similar intracellular substrates including Ras and MAP kinase. However EGF and NGF treatments stimulate distinct physiological responses, resulting in proliferation and differentiation, respectively (Chao, 1992). Though Ras activation is necessary and sufficient for this differentiation (Guerrero et al., 1986; Thomas et al., 1992), the requirement of MAP and ERK kinase (MEK) activation has only recently been established (Cowley et al., 1994).

Despite the contrasting actions of EGF and NGF, the points of divergence accounting for the expression of such different cellular phenotypes have not been determined. Recent data suggest that the duration of MAP kinase activation may dictate proliferative and differentiative responses in PC12 cells (Traverse et al., 1994). In addition, it has been shown that sustained activation of MAP kinase (ERK, extracellular signal-regulated kinase) by NGF in these cells allows for the nuclear translocation of ERKs (Nguyen et al., 1993). This may initiate a program of differentiation and growth arrest, presumably through the action of nuclear substrates of MAP kinase or associated kinases.

We have tested this model by asking whether agents that stimulate MAP kinase without inducing differentiation can act additively to trigger differentiation. Modest increase in cAMP levels has been shown to activate MAP kinase in PC12 cells without differentiating these cells (Frodin et al., 1994; Connolly et al., 1984; Ho et al., 1992). Here, we show that stimulation of cAMP levels by forskolin, an activator of adenylyl cyclase, induces a transient stimulation of MAP kinase activity and a persistent localization of MAP kinase (ERK1) within the cytoplasm. The application of forskolin and EGF together to these cells altered the physiological action of both agents by inducing differentiation as judged by changes in morphology and gene expression. This differentiation was associated with a sustained activation and nuclear localization of MAP kinase. The morphological changes induced by EGF and forskolin were blocked by specific inhibition of MAP kinase activation by MAP kinase phosphatase-1 (MKP1), demonstrating that the synergistic effects of EGF and cAMP are mediated by the activation of MAP kinase. We propose that cellular responses to growth factor's action are dependent not only on the activation of growth factor receptors by specific growth factors but on synchronous signals that elevate intracellular signals like cAMP that can activate MAP kinase within the same cells. Because cAMP is regulated by hormonal stimulation, these data suggest that the specificity of

growth factor action depends not only on signals generated by growth factor receptors but also on the hormonal milieu in which growth factors act.

Examination of the magnitude and time course of MAP kinase activation in PC12 cells by EGF and NGF suggests how one intracellular pathway can stimulate both differentiation and proliferation in the same cell. Proliferative agents like EGF activate MAP kinase rapidly and transiently. During this brief activation, MAP kinase remains in the cytoplasm in PC12 cells (Blenis, 1993; Nguyen et al., 1993). In contrast, differentiation by NGF is characterized by a prolonged activation and translocation of MAP kinase (Nguyen et al., 1993; Traverse et al., 1992), where it may regulate differentiation-specific genes through the phosphorylation of nuclear transcription factors (Blenis, 1993).

Forskolin induces an activation of MAP kinase that is lower in magnitude than that induced by NGF (Connolly et al., 1984; Frodin et al., 1994; Ho and Raw, 1992) and does not stimulate differentiation (Frodin et al., 1994). Still, cAMP is synergistic with NGF (and fibroblast growth factor) to potentiate differentiation, measured morphologically (Gunning et al., 1981) and by the induction of specific genes (Heidemann et al., 1985; Richter-Landsberg and Jarstoff, 1986). Therefore, it is possible that MAP kinase activation must reach a threshold of sufficient magnitude and duration to trigger differentiation in these cells.

The possibility that a threshold of MAP kinase activation exists, above which a cell is committed to a pathway of differentiation, could have profound implications for the physiological regulation of growth and differentiation in normal cells. Signals that activate MAP kinase to levels that are not sufficient to trigger differentiation when acting alone might be sufficient to trigger differentiation when acting together. To test this hypothesis,

we examined the physiological response of PC12 cells to EGF and agents that stimulate cAMP.

MATERIALS AND METHODS

Cell culture

PC12-GR5 cells (kindly provided by Rae Nishi, Oregon Health Sciences University) were maintained at 37° C, in a 5% CO2 humidified atmosphere on collagen-coated plates in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated horse serum and 5% fetal calf serum (GIBCO, BRL). Sixteen hours prior to treatments, cells were switched to serum-free medium. 50 ng/ml NGF (Boeringer Mannheim), 100 ng/ml EGF (Boeringer Mannheim), 10 µM H89 (Calbiochem) and 10 µM forskolin (Cal. Biochem) were added as indicated, and the cells incubated for 48 hours. The extension of neurites was assayed as previously described (Greene and Tischler, 1976).

Plasmids and Transient Transfections

For all transfections, the calcium-phosphate precipitation method was used (GIBCO, BRL). Transfections were performed as manufacturer's instruction described (GIBCO, BRL). One day prior to transfection, cells were plated at a density of 2×10^7 cells/plate as described. The following day they were transfected with combination of the following plasmids: 5 µg of RSV-ß-galactosidase, 30 µg of pMKP-1, 5 µg of pEXV3 MAPKK1, 15µg of dn ERK1, 15µg of dnERK2, 30 µg of pCaN420 and 5µg of pTRCAT. Four hours following the transfection the cells were washed in hypotonic medium (osmotic shock) to facilitate DNA uptake (deSouza et al., 1995). The cells were maintained in complete media for an additional 24 hours and fed with supplemented serum-free medium

(N2) containing 5 μg /ml insulin, 100 μg/ml transferrin, 30 μM sodium selenite, 100 μM putrescine, and 20 μM progesterone (deSouza et al., 1995).

CAT assays

CAT assays were performed as previously described (deSouza et al., 1995). Briefly, CAT activity was measured using ^{3}H-Acetyl-CoA as a substrate. Radioactivity was counted by scintillation counting.

MAP Kinase assays

Treated and untreated PC12 cells (1 x 10^7 cells) were lysed in 1% Nonidet P-40 lysis buffer (10% Sucrose, 1% NP-40, 25 μM Tris-HCl (pH 7.4), 150 μM NaCl, 10% glycerol, 1 μM EDTA, 1.5 μM $MgCl_2$, 1 μM EGTA, 1 μM PMSF, 10 μg/ml leupeptin, 2 μM sodium vanadate, and 10 μM sodium fluoride). The lysates were centrifuged at 10,000g for 10 min at 4° C. The supernatants were immunoprecipitated with agarose-conjugated anti-ERK1 (Santa Cruz Technology, Santa Cruz, CA) for 2 hr at 4°C. The immunoprecipitates were assayed for kinase activity by incubating with 25 μg MBP and 50 μM [^{32}P]-γ-ATP (10 μCi) in 50 μl of buffer containing 40 μM Hepes, pH 7.4, 40 μM $MgCl_2$, and 0.2 μM ATP for 30 min at 30°C. The reactions were terminated by adding 50 μl of 2x Laemmli buffer and resolved in a 10% SDS-polyacrylamide gel and then analyzed by autoradiography. The bands were quantitated using a PhosphorImager (Molecular Dynamics.

RNA analysis

Total RNA from PC12 cells treated with NGF, EGF, forskolin, NGF plus forskolin, and EGF plus forskolin for 24 hours were prepared using guanidium salts, separated by electrophoresis and transferred to Nylon filters. The filters were hybridized at 65° C with one million cpm of antisense cRNA (1 μg) generated from a riboprobe vector (Bluescript)

containing the full length transin cDNA (kindly provided by Gary Ciment) (Machida et al., 1991). The filters were analyzed by autoradiography.

Immunohistochemical localization of ERK1

Ten cm plates of PC12 cells grown to 50% confluency were treated with NGF, EGF, forskolin and EGF plus forskolin for 90 minutes. The cells were washed, fixed in 4% formaldehyde (at 4° C for 5 minutes) and scraped into 1 ml of phosphate buffered saline (PBS). The suspension of whole cells was centrifuged at 2000 x g for ten min at 4° C, and the pellet dehydrated in graded ethanols. The cell block was perfused in paraffin and embedded for microtomy. Five micron sections were mounted on glass slides, pretreated with trypsin for 10 minutes and used for immunohistochemical staining with antisera against ERK1 (Santa Cruz Technology, Santa Cruz, CA; 2 μg/μl, representing dilution of 1:50). The reaction was carried out in an automated immunohistochemistry instrument, the Ventana 320/ES (Ventana Medical Systems, Tuscon AZ). Antigen-antibody reactions were revealed with standardized development times by the instrument utilizing the avidin biotin complex (ABC) method using diaminobenzidine (DAB) as substrate.

RESULTS

Forskolin, an agent that stimulates adenylyl cyclase in these cells (Balbi and Allen, 1994), did not stimulate neurite formation (Fig. 2.1), as previously reported (Frodin et al., 1994; Pollock et al., 1990). Simultaneous treatment with EGF and forskolin (EGF/forskolin) produced morphological changes including an outgrowth of neurites indistinguishable from those induced by NGF alone (Fig. 2.1). The cAMP analog 8-AHA (8-aminohexylamino-cAMP) (Skålhegg et al., 1992), but not IBMX (3-isobutyl-1-methylxanthine), gave similar results (data not shown). Neurites were first observed in greater than 50 percent of cells

following 24 hours of treatment, and they increased in density and length by 48 hours. These changes, but not those induced by NGF, could be blocked by the PKA inhibitor H89 (Chijiwa et al., 1990), confirming that NGF's actions on neurite outgrowth do not require PKA, as previously proposed (Damon et al., 1990) (Fig. 2.1). Similar results were seen with another PC12 cell line, P19 (kindly provided by Louis Reichardt; data not shown).

The requirement for MAP kinase activation was investigated by using three cDNAs whose expression inhibits MAP kinase activity. One cDNA encodes the MAP kinase phosphatase-1 (MKP-1). MKP-1 is a dual-specificity phosphatase that inactivates MAP kinase by specifically dephosphorylating both MAP kinase isoforms ERK1 and ERK2 (Noguchi et al., 1993). Transient expression of MKP-1 selectively inactivates MAP kinase in many cell types (Roberson et al., 1995; Sun et al., 1993; Sun et al., 1994). We have shown that, in PC12 cells, MKP-1 inactivates MAP kinase and blocks MAP kinase-dependent transcription (Misra-Press et al., 1995). The other two cDNAs used in these studies encode interfering mutants of ERK1 and ERK2 that block endogenous MAP kinase activity by acting as dominant negative mutants (referred to here as dn ERKs) (An et al., 1982) (kindly provided by Melanie Cobb).

To examine whether neurite outgrowth can be inhibited by MKP-1, cells were transfected with either a plasmid pcDNA3 (Invitrogen) containing the full-length mouse MKP-1 (kindly provided by Nicholas Tonks) (pMKP-1) under the control of a cytomegalovirus (CMV) immediate early promoter or vector alone (pcDNA3). Cells were also transfected with a plasmid directing the expression of the *lacZ* gene product ß-galactosidase under the control of an RSV promoter (RSV-ß-galactosidase) (An et al., 1982) as a marker for transfected cells. After 24 hours, cells were serum-starved for an additional 16 hours and treated with NGF, or EGF/forskolin as described (deSouza et al., 1995). After incubation

for an additional 48 hours, the cells were stained for ß-galactosidase using histochemical methods (Robbins et al., 1993). Using this technique, the expression of ß-galactosidase identified transfected cells. The expression of MKP-1 blocked the morphological changes induced by NGF and EGF/forskolin (Figs. 2.2A and 2.2B, and 2.3) . This inhibition reduced the fraction of neurite-containing blue cells to less than 20% without affecting the differentiation of cells that did not express ß-galactosidase (Fig. 2.3). This result demonstrates that EGF and agents that stimulate cAMP levels require MAP kinase to induce morphological changes. Similar results were seen in cells co-transfected with plasmids expressing interfering mutants of ERK1 and ERK2 (dn ERKs) (Fig. 2.3).

As a control for the previous experiments, cells were transfected with pEXV3 MAPKK1 (kindly provided by Christopher Marshall). pEXV3 MAPKK1 encodes a constitutively active mutant of MAP kinase and ERK kinase, MEK, that differentiates PC12 cells when introduced into PC12 cells via microinjection (Cowley et al., 1994). Likewise, transient transfection of this plasmid also stimulated neural differentiation (Fig. 2.2C). Cells were co-transfected with RSV-ß-galactosidase and pEXV3 MAPKK1, and the expression of ß-galactosidase was detected by the presence of blue cells, as described above. Greater than 80% of blue cells displayed neuritic processes (Fig. 2.2C and 2.3). In contrast, less than 20% of non-blue cells displayed morphological changes, demonstrating that cells expressing RSV-ß-galactosidase also expressed the co-transfected pEXV3 MAPKK1. The differentiation seen following treatment with NGF or EGF/forskolin was blocked in co-transfections with cDNAs encoding dn ERK1 and dn ERK2 or MKP-1 (Figs. 2.2A, 2.2B, and 2.3).

Expression of RSV-ß-galactosidase alone did not stimulate neurites, nor did it interfere with the extension of neurites (Fig. 2.3). These results complement those of Cowley et al. (Cowley et al., 1994) that microinjection of PEXV3 MAPKK1 is sufficient for neuronal

differentiation in PC12 cells. They also reported that injection of inactivating mutants of MEK could block NGF-induced morphological changes in PC12 cells (Cowley et al., 1994). These studies and those presented here strongly suggest that the activation of MAP kinase, as well as MEK, is required for neuronal differentiation.

Neuronal differentiation of PC12 cells by NGF is associated with the induction of both immediate early genes including Egr-1 (Sukhatme et al., 1988) and fos (Greenberg et al., 1985) and late genes including SCG10 (Stein et al., 1988) the neural adhesion marker L1 (McGuire et al., 1978) and the metalloprotease transin (stromelysin) (D'Arcangelo and Halegoua, 1993a). Transin encodes a metalloprotease that is expressed in neuronal and non-neuronal cells. Its expression in the developing nervous system has suggested a role in growth cone guidance and axonal path finding (Pittman and Williams, 1988) and correlates with neuronal differentiation in PC12 cells (Fillmore et al., 1992; Machida et al., 1991). To examine whether treatment with EGF/forskolin is able to induce transin expression, RNA was isolated from treated PC12 cells and subjected to Northern blotting using an antisense RNA probe corresponding to the transin cDNA (Machida et al., 1991). As shown in Fig. 2.4A, EGF/forskolin, as well as NGF, induced the expression of a 1.9 kb band corresponding to the rat transin mRNA (Machida et al., 1991). In contrast, neither forskolin nor EGF alone induced transin expression, as previously described (Machida et al., 1991). Surprisingly, the level of transin expression appeared to be significantly higher with EGF/forskolin (28-fold over basal) than with NGF (8-fold over basal) (Fig. 2.4B). Forskolin also potentiates NGF's induction of transin expression.

The induction of transin expression by NGF is dependent on upstream activators of MAP kinase including Ras (D'Arcangelo and Halegoua, 1993a) and may be a marker for neuronal differentiation through the MAP kinase cascade. To demonstrate a requirement of MAP kinase for the induction of transin, we examined the induction of a chloramphenicol

acetyl transferase (CAT) gene under the control of 750 bp of the transin promoter (pTRCAT) (kindly provided by Gary Ciment). The expression of pTRCAT in PC12 cells qualitatively reflects NGF's effects on transin expression through *cis*--acting promoter elements contained on the plasmid (deSouza et al., 1995). As shown in Fig. 2.4B, NGF treatment for 24 hours induced a 5-fold stimulation of CAT activity, and transfection of pEXV3 MAPKK1 induced a 16-fold stimulation of CAT activity. Both activities were blocked by co-transfection of MKP-1. Treatment with EGF/forskolin stimulated a 6-fold increase in CAT activity that was blocked by co-transfection with pCMV-MKP-1 but not by pCaN420, encoding a constitutively active mutant of calcineurin (Perrino et al., 1995), a serine/threonine phosphatase with no known activity against MAP kinase (provided by Thomas Soderling, Vollum Institute). These data suggest that MAP kinase activation participates in transin induction by these agents.

To examine the kinetics of MAP kinase activation by EGF/forskolin, PC12 cells were treated with both agents and lysed at the indicated times. Cells lysates were subjected to immunoprecipitation with ERK1 antisera and MAP kinase activity was measured within the immune complexes. EGF stimulation produced a rapid, transient activation that reached a maximum within 5 minutes and returned to low levels after 30 minutes. Forskolin treatment produced a slow rise in MAP kinase activity, reaching a maximum at 20 minutes. At all time points of forskolin treatment, MAP kinase activity was lower than the corresponding level induced by NGF and like EGF, returned to low levels for the subsequent time points examined (Fig. 2.5). In contrast, treatment of PC12 cells with EGF/forskolin produced a substantial and sustained activation of ERK1, which paralleled that was seen with NGF (Fig. 2.5). Nuclear localization of ERK1 was correlated with sustained activation (Fig. 2.6). Only those cells treated with either NGF or EGF/forskolin (but not cells treated with EGF or forskolin alone) showed detectable nuclear staining.

DISCUSSION

Using morphological, molecular, and biochemical criteria, we have established that forskolin can convert the physiological response of EGF from one of proliferation to one of differentiation. Using these same criteria, neither EGF nor forskolin alone produced a differentiated response. In all assays, co-stimulation with EGF and forskolin produced responses similar to those of NGF. In addition, we have shown that both treatments (EGF/forskolin and NGF) require MAP kinase for their induction of neurite outgrowth. It has been proposed that differentiation of PC12 cells by growth factors requires a threshold of MAP kinase activity (Nguyen et al., 1993; Traverse et al., 1992). The notion of a threshold for differentiation is supported by studies of PC12 cells that have been genetically altered to express high levels of the EGF receptor (Traverse et al., 1994) or the adapter protein Crk that couples this receptor to Ras activation (Hempstead et al., 1994). Both alterations result in cell lines displaying enhanced MAP kinase activation and neuronal differentiation in response to EGF. In addition, in contrast to forskolin, the long acting non-hydrolyzable cAMP analog 8-(4-chlorophenylthio)-cAMP (8-CPT) can stimulate sustained activation of MAP kinase to levels similar in magnitude and duration to that of NGF and can induce PC12 differentiation (Young et al., 1994). These studies are consistent with this notion that the level of MAP kinase activity may dictate the physiological responses to MAP kinase activation. In addition, these studies demonstrate that nuclear translocation of MAP kinase is associated with prolonged activation of MAP kinase, suggesting that the translocation of MAP kinase and/or associated kinases (Blenis, 1993), activates a program of differentiation and growth arrest.

The mechanism by which cAMP stimulates MAP kinase in PC12 cells is not completely understood, although this stimulation has been reported to involve the activation of MEK (Frodin et al., 1994). Although we have shown that cAMP-mediated activation of MAP kinase can augment EGF signaling, cAMP's activation of other pathways may be important

as well. For example, forskolin significantly increases the stimulation of transin mRNA levels by both NGF (data not shown) and EGF (Figure 3.4A) to a degree that far exceeds the cooperativity of forskolin with these factors in the stimulation of MAP kinase (Fig. 3.5). PKA is not required for NGF's induction of neurite outgrowth (Fig. 2.1D, inset). Similar data have been used to suggest that cAMP-dependent gene transcription is not important in mediating NGF's actions (Damon et al., 1990). cAMP-dependent gene transcription involves the phosphorylation of the transcription factor CREB (cAMP responsive element binding protein). However, it has recently been shown that CREB can be activated by NGF in PC12 cells through a protein kinase A-independent pathway downstream of Ras (Ginty et al., 1994) and possibly of MAP kinase as well. Therefore, although sustained activation of MAP kinase may be sufficient for differentiation of PC12 cells (Marshall, 1995), the involvement of CREB-dependent gene transcription cannot be ruled out.

The studies presented here demonstrate that co-stimulation by physiological agents can produce cooperative effects on MAP kinase that are sufficient to induce novel biological responses. The ability of cAMP to augment EGF's activation of MAP kinase to trigger differentiation has profound implications in the hormonal regulation of growth and differentiation. Proliferation and differentiation appear to be mediated, in part, by a common pathway. The physiological response of this pathway may be dramatically influenced by additional hormonal signals. For example, EGF receptors are expressed in cells throughout the developing brain where they are thought to exert proliferative effects during development (Plata-Salamán, 1991). These cells also express multiple receptors for hormones that are coupled to adenylyl cyclase activation, therefore their physiological response to EGF may be dictated by the synchronous contributions of hormonal signals. cAMP may regulate growth factor action in non-neuronal cells, as well. For example, cAMP's activation of MAP kinase in cardiac myocytes (Lazou et al., 1994) and in pituitary

cells (Stork, unpublished observations) may be part of a common mechanism by which cAMP mediates hypertrophic responses in the heart (Milano et al., 1994) and/or regulates differentiation and cell growth in the pituitary.

Acknowledgments--We are grateful to Gary Ciment and Sunita de Sousa for providing transin probes and technical assistance, to Christopher Marshall for providing DNA encoding constitutively active MEK, Nicholas Tonks for providing cDNA encoding MKP-1, Anita Misra-Press and Andrey Shaw for helpful discussions, and Gary Ciment and Michael Forte for critical reading of the manuscript and Sheri Medford for secretarial assistance.

FIGURE LEGENDS

Figure 2.1 EGF plus forskolin induce morphological changes in PC12 cells PC12 cells were grown to 10% confluency on collagen-coated slides and then left untreated (A) or incubated with forskolin (B), EGF (C), NGF (D), NGF plus H89 (D, insert), EGF and forskolin (E), or EGF/forskolin plus H89 (E, insert). Phase contrast micrographs were taken with a Leitz Dialux 22EB microscope. The bar represents 20 μm.

Figure 2.2 Photomicrograph of transfected PC12 cells stained for ß-galactosidase activity. PC12 cells were transfected with RSV-ß-gal (5 μg) and either 30 μg of pMKP-1 (A and B), or 5 μg of PEXV3 MAPKK1 (C), and either treated with NGF (A) or EGF/forskolin (B) or left untreated (C). Note that treatments induced neurites in non-transfected cells surrounding the blue cells in (A) and (B), and in contrast, the non-transfected cells surrounding the blue cells in (C) have not differentiated. This experiment was performed three times with similar results as shown in Fig. 2.3.

Figure 2.3 The percentage of neurites in PC12 cells transfected with cDNAs that modulate MAP kinase activation. Cells were transfected with 5 μg of RSV-ß-gal and either 30μg of MKP-1, 15 μg of both dn ERK1 and dnERK2 or 30 μg of vector pcDNA3 with or without 5 μg of PEXV3 MAPKK1. Subsequently, cells were treated either with NGF, EGF plus forskolin or left untreated as indicated. The percentage of cells with neurites is shown. White bars represent RSV-ß-gal-negative cells and shaded bars represent RSV-ß-gal-positive cells. Note that the extension of neurites in untransfected cells (white bars) is unaffected by the conditions of the transfection in all assays. Each bar represents the total of at least 100 cells.

Figure 2.4 NGF and EGF/forskolin stimulate transcription from a transin promoter. (A) NGF and EGF/forskolin stimulate transin mRNA levels. Autoradiograph of a Northern blot detecting transin mRNA demonstrating transin mRNA induction by EGF plus cAMP in PC12 cells is shown. Lane 1, untreated cells (U); lane 2, EGF (E); lane 3, forskolin (F); lane 4, EGF plus forskolin (E/F); lane 5, NGF (N). The expected size of the transin band is indicated (1.9 kb). (B) Induction of CAT activity expressed from a transin promoter. Cells were transfected with 5 μg of the plasmid pTRCAT, and either vector (pcDNA3) alone (30 μg), pEXV3 MAPKK1 (5 μg), pCMV-MKP-1 (pMKP1) (30 μg) or pCaN420 (30 μg) and stimulated with NGF or EGF/forskolin or left untreated as indicated. Basal represents the CAT activity seen in unstimulated cells transfected with pTRCAT and vector. Each value represents the average with standard error of at least three experiments and is presented as fold increase over basal levels.

Figure 2.5 Time course of MAP kinase activation by forskolin, EGF, NGF and EGF/forskolin. Cells were treated with agents for the indicated times and ERK1 activity was assayed and quantitated as described in Materials and Methods. Basal activity represents the ERK1 activity in unstimulated cells. Activity is expressed as fold increase over basal (time 0) to allow direct comparison between the following treatments: forskolin (open squares), EGF (closed diamonds), NGF (closed squares) and EGF/forskolin (open diamonds).

Figure 2.6 Photomicrograph of 10-μm sections of paraffin-embedded cell blocks demonstrating immunohistochemical detection of ERK1. PC12 cells were treated with: A) NGF; B) EGF; C) forskolin; and D) EGF plus forskolin for 90 minutes. Nuclei are counterstained with hematoxylin (purple) and cytoplasmic regions are counterstained with eosin (pink). The reddish orange histological reaction product represents localization of ERK1.

Fig. 2.1. EGF/forskolin induced morphological changes in PC12 cells

Fig. 2.2. Photomicrograph of transfected PC12 cells stained for beta-galactosidase activity.

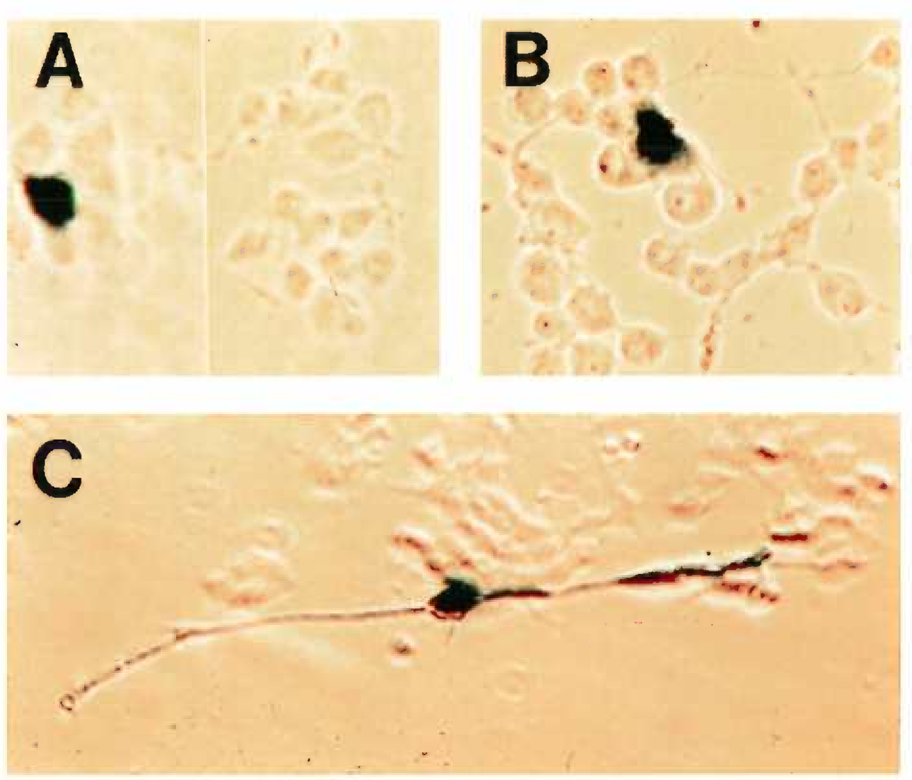


Fig. 2.3. The percentage of neurites in PC12 cells transfected with cDNAs that modulate MAP kinase activation

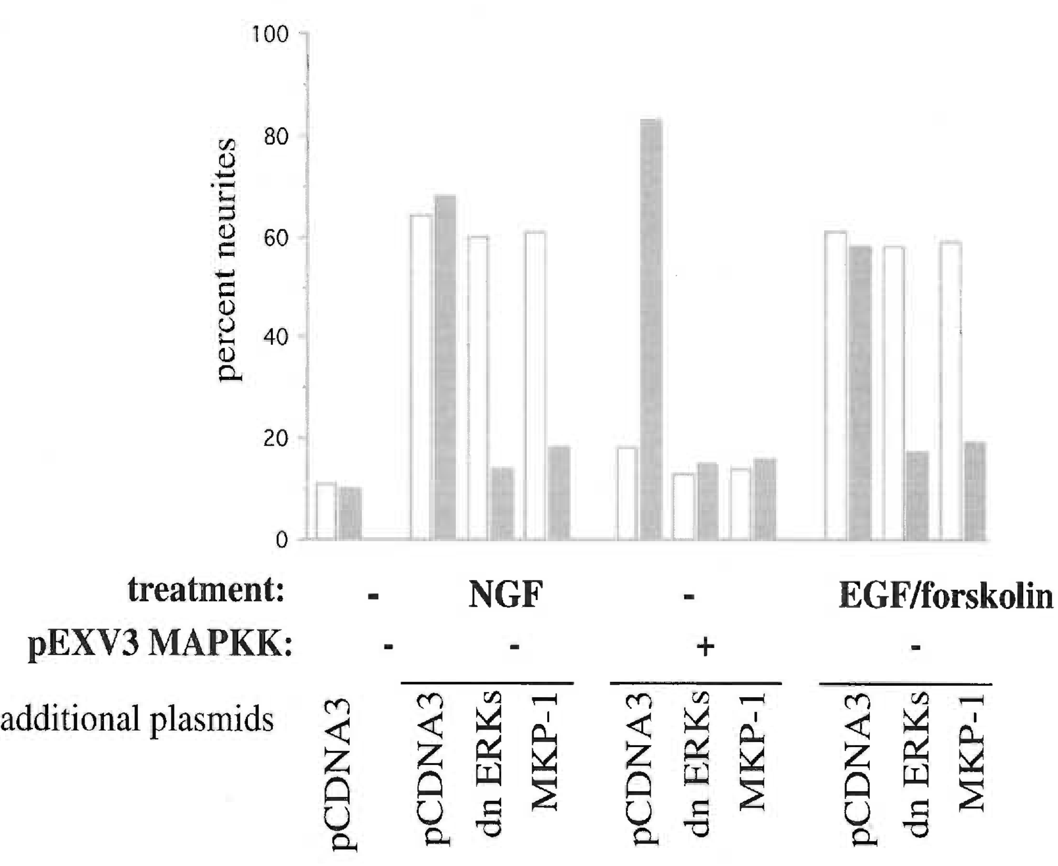
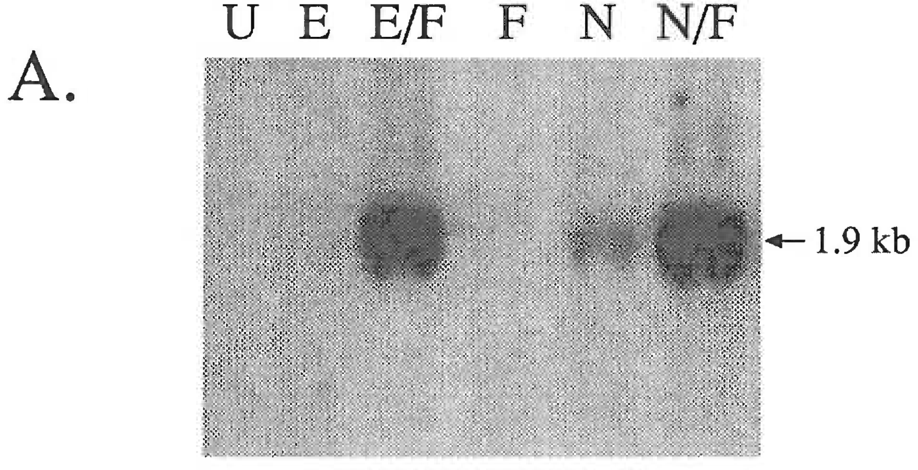


Fig. 2.4. NGF and EGF/forskolin stimulate transcription from the transin promoter

A.



B.

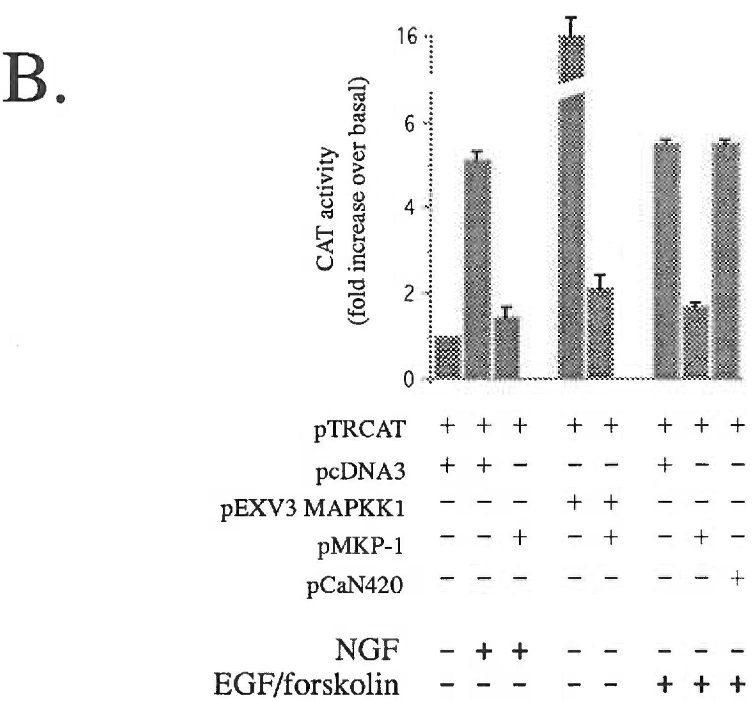


Fig. 2.5. Time course of MAP kinase activation by forskolin, EGF, NGF, and EGF/forskolin

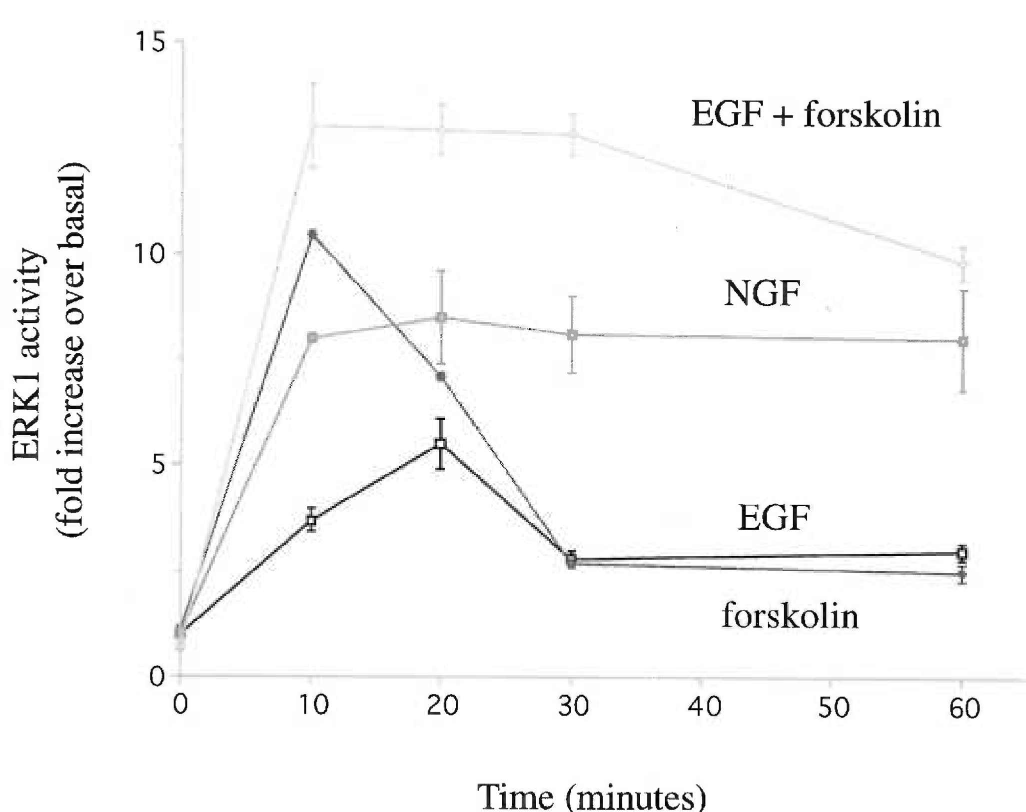
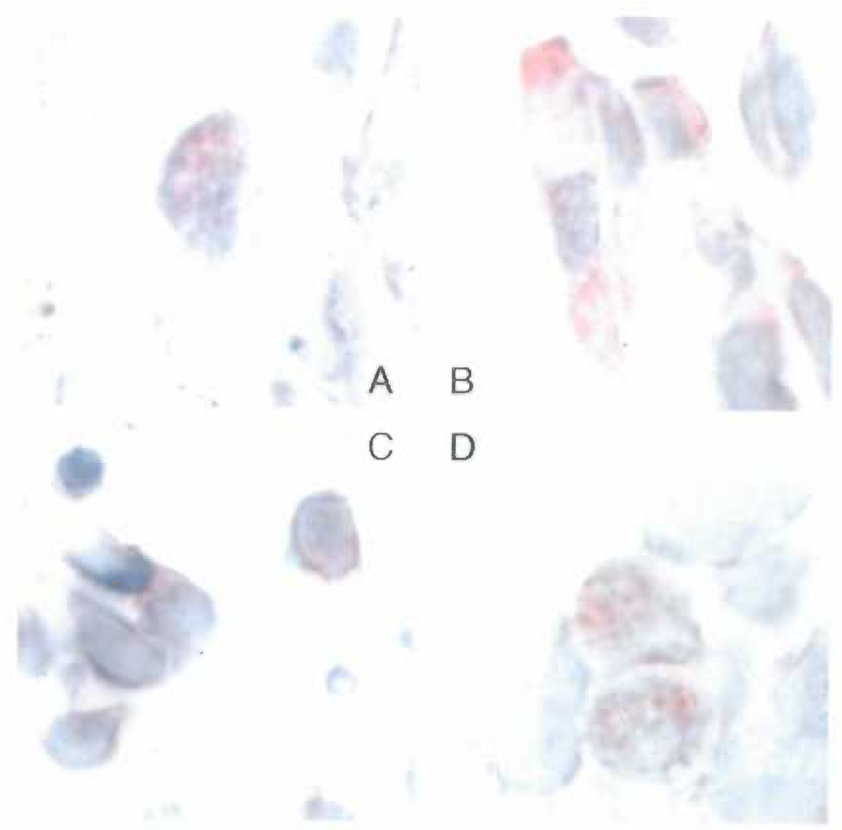


Fig. 2.6. Photomicrograph of PC12 cells showing immunohistochemical detection of ERK1



Chapter 3

cAMP Activates MAP Kinase and Elk-1 through B-Raf and Rap1-dependent Pathway.

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Published in Cell, Vol.89, 73-82 (1997)

Summary

Cyclic adenosine monophosphate (cAMP) has tissue-specific effects on growth, differentiation and gene expression. We show here that cAMP can activate the transcription factor, Elk-1, and induce neuronal differentiation of PC12 cells via its activation of the MAP kinase cascade. The cell-type specific actions of cAMP require the expression of the serine/threonine kinase B-Raf and activation of the small G protein Rap1. Rap1, activated by mutation or by the cAMP dependent protein kinase PKA, is a selective activator of B-Raf and inhibitor of Raf-1. Therefore, in B-Raf-expressing cells, the activation of Rap1 provides a mechanism for cAMP's tissue-specific regulation of cell growth and differentiation via MAP kinase.

Running Title: PKA activates ERKs via Rap1 and B-Raf

Introduction

Elevation of intracellular cAMP produces growth effects in a variety of cell types including inhibition of growth factor-stimulated cell growth in fibroblasts (Rat-1 and NIH3T3 cells), smooth muscle cells, and adipocytes. The ability of growth factors to stimulate cell proliferation requires the small G protein Ras and MAP kinase or ERK (extracellular signal-regulated kinases) (Marshall, 1995). Ras activates the serine/threonine kinases Raf-1 and B-Raf (Lange-Carter et al., 1993; Vaillancourt et al., 1994), which, in turn, activate the MAP kinase kinase, MEK, which then activates ERKs (Marshall, 1995). It has been proposed that the inhibitory effects of cAMP on cell growth reflect cAMP's inhibition of growth factor activation of ERKs (Cook and McCormick, 1993; Wu et al., 1993). In other cell types, including PC12 cells, cAMP does not antagonize the activation of ERKs by growth factors, but activates ERKs (Frodin et al., 1994; Pan et al., 1995; Young et al., 1994) and potentiates the effects of growth factors on differentiation and gene expression (Heidemann et al., 1985; Yao et al., 1995).

PC12 cells are derived from rat adrenal medullary cells and differentiate into sympathetic neurons upon treatment with nerve growth factor (NGF) (Marshall, 1995). This action of NGF in these cells requires activation of ERKs and induction of gene expression. ERKs regulate gene expression, in part, through the phosphorylation and activation of transcription factors of the Ets family, including Elk-1 (Marais et al., 1993). In PC12 cells, PKA can induce morphological changes consistent with neuronal differentiation (Young et al., 1994), can potentiate NGF's morphological effects during differentiation, and can synergize with NGF in the induction of specific genes (Heidemann et al., 1985; Yao et al., 1995). It is not known whether the differentiating actions of PKA require ERKs, as has been reported for NGF (Cowley et al., 1994).

Potential mediators of cAMP's activation of ERKs include kinases that lie upstream along the MAP kinase cascade (Frodin et al., 1994; Young et al., 1994). B-Raf is abundantly expressed in the brain (Barnier et al., 1995) and is activated in PC12 cells by Ras, following stimulation by NGF (Oshima et al., 1991) and epidermal growth factor (EGF) (Vaillancourt et al., 1994). Like Raf-1, both the activation of B-Raf by growth factors, as well as its association with Ras, are inhibited by PKA (Moodie et al., 1994; Vaillancourt et al., 1994; Wu et al., 1993). However, differences in the regulation of B-Raf and Raf-1 by cAMP have been proposed (Erhardt et al., 1995). Although Ras-dependent activation of B-Raf by growth factors can be blocked by cAMP, the ability of cAMP to regulate B-Raf via Ras-independent pathways has not been characterized in detail. In this study, we sought to examine the mechanisms by which cAMP activates ERKs and Elk-1, and to ask whether this pathway depends on Ras or other small GTP-binding proteins.

B-Raf can be activated by the small G protein Rap1 *in vitro* (Ohtsuka et al., 1996). Rap1 is a member of the Ras superfamily of GTP binding proteins that shares 50% amino acid sequence identity with Ras (Burgering and Bos, 1995; Kitayama et al., 1990), and, like Ras, binds Raf isoforms *in vitro* and *in vivo* (Nassar et al., 1996). The GTP loading of Rap1 is stimulated by cAMP *in vivo* (Altschuler et al., 1995). Although it is likely that GTP loading activates Rap1, *in vivo* effectors of Rap1 have not been defined. Instead, almost all the actions of Rap identified, to date, involve the inhibition of Ras-dependent signaling (Cook et al., 1993; Kitayama et al., 1990). We show here that both PKA and Rap1 are selective activators of B-Raf *in vivo* and can activate selected transcription factors and stimulate neuronal differentiation via an ERK-dependent, but Ras-independent, pathway.

Materials and Methods

Materials

Agarose-conjugated ERK1, ERK2, and JNK1 antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA.). Polyclonal anti-Rap1 [Krev-1(121)], anti-Raf-1 (C-12), anti-B-Raf (C-19), Ras monoclonal antibodies (Y13-259 and Y13-238), Gst-c-jun and MEK-1 protein were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA.). Anti-myc antibody 9E10 (provided by A. Shaw, Washington University, St. Louis, MO) was used to immunoprecipitate exogenous Myc-ERK2 fusion protein. MKK 8E and MKK 36A were provided by N. Ahn (University of Colorado, Boulder, CO). Nickel agarose (Ni-NTA-Agarose) was purchased from Qiagen Inc. (Chatsworth, CA.). Radioisotopes were from NEN-DuPont. All other reagents were from Sigma (St. Louis, MO).

Cell culture

PC12-GR5 cells were provided by R. Nishi (Oregon Health Sciences University, Portland, Oregon) and 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 prior to harvesting. COS cells and NIH 3T3 cells were maintained in DMEM plus 10% fetal calf serum. For immune complex assays and western blotting, the cells were deprived of serum and maintained in DMEM for 16 hr at 37°C in 5% CO2 prior to treatment with forskolin (10μM), 8-CPT (175μM), NGF (100ng/ml), EGF (50ng/ml), VIP (10μM), or isoproterenol (10μM) unless otherwise indicated.

Immune complex assays

Cells were grown to 50% confluence, serum-starved for 16 hours, and treated with agents as indicated. ERK assays were performed using myelin basic protein (MBP) and [^{32}P]-γATP as substrates as described (Pan et al., 1995). JNK assays were performed using c-

jun and [^{32}P]P-γATP as substrates as described (Dérijard et al., 1994). For Raf assays, untreated and treated cells were lysed in 1% NP-40 buffer containing 10 μM Tris (pH 7.4), 5 μM EDTA, 50 μM NaCl, and 1 μM PMSF. Immune complex kinase assays were performed as described using MEK-1 and ^{32}P-γATP as substrates (Vaillancourt et al., 1994). The reaction products of all kinase assays were resolved by 10% SDS-polyacrylamide gel and analyzed with a PhosphorImager (Molecular Dynamics). Transfection of B-Raf into COS-7 cells was required to detect B-Raf activity, consistent with the low levels of endogenous B-Raf expressed in these cells (Faure and Bourne, 1995).

Construction of expression vectors

Wild type Rap1b was cloned by PCR from a bovine brain cDNA library using specific primers to the published Rap1b sequence: (sense oligo: GGCAAGCTTGAGACCATGCG-TGAGTATAAGCTAGT and antisense oligo: GGCGAATTCTATTAAAGCAGTGAC-ATGATGACT) and subcloned into pcDNA3 (Invitrogen). RapV12 was constructed following PCR amplification of the full length clone using a mutant 5' oligonucleotide primer: (sense oligo: GGCGGATCCATGCGTGAGTATAAGCTATCGTTCTT-GGCTCTGTAGGCCTT) resulting in a valine for glycine substitution at amino acid 12, and subcloned into pcDNA3. RapD179 was constructed following PCR amplification of the full length clone using a mutant 3' oligonucleotide primer: (antisense oligo: GGCGAATTCTATTAAAGCAGCTGACATGAGTCCTT-TTTGCGGGCCTTC) resulting in an aspartic acid for serine substitution at amino acid 179.

Rap N17 was constructed following PCR amplification of the full length clone using a mutant sense oligonucleotide primer: GGCGGATCCGAGACCATGCGTGAGT-ATAAGCTAGTCGTTCTTGGCTCTGGAGGCGTTGGAAAAAATGCTCTGACTGTA. His-Rap (wild type) and His-RapV12 were constructed by amplifying the corresponding

Rap sequences with a 5'oligonucleotide encoding a Kozak recognition site, start codon and poly-Histidine sequence in frame with the second amino acid of the Rap coding region (sense oligo: ACCGAGCTCGGATCCATGGGACATCACCACCACCACCACAGCG-GGAATTCTCGTGAGTATAAG). The resultant amplification products were subcloned into pcDNA3 (Invitrogen) and sequenced.

Plasmids and Transfection

All cell lines were grown to 50% confluence prior to transfection. Unless otherwise indicated, 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. For luciferase assays, 5xGal4-E1b/luciferase and a plasmid encoding the Gal4-Elk-1 or Gal4-c-jun transactivation domain were used in combination with other plasmids as indicated. MEK K97R (gift of K-L. Guan, University of Michigan) was subcloned into pcDNA3. RasV12, Elk-1/Gal-4, 5xGal4-E1b/luciferase, cPKA and PKI were gifts of R. Maurer, Oregon Health Sciences University; RasN17 (Stacy et al., 1991) was provided by N. Nathanson, University of Washington; MEKKΔ was provided by G. Johnson, National Jewish Hospital; Gal4-c-jun, was provided by R. Goodman, Vollum Institute. cDNA encoding Raf-1 was provided by A. Shaw, Washington University; dominant negative (dn) mutants of ERK1 and ERK2 were provided by M. Cobb, University of Texas, Southwestern; dnJNK was provided by R. Davis, University of Massachusetts; myc-ERK2 and pEXV3 MAPKK1 (Cowley et al., 1994) were provided by C. Marshall, Chester Beatty Institute for Cancer Research, London and C3G was provided by M. Matsuda, National Institute of Health, Tokyo. CMV-CAT was transfected as an internal control for gene expression. Cells were transfected as described, and treated with forskolin, 8-CPT or growth factors. After 6 hours of treatment, cell lysates were prepared and luciferase activity was assayed as

described (Misra-Press et al., 1995). Luciferase activity was normalized to CAT activity and results reported as fold activation above basal levels.

Phosphorylation and GTP loading studies of Rap1

PC12 cells were grown to 50% confluence on 100mm polylysine-coated plates and washed 3x with phosphate free DMEM. 0.5 mCi/ml of [^{32}P]-orthophosphate in DMEM was added and cells incubated for four hours at 37°C. For phosphorylation studies, cells were lysed in 1% NP40 lysis buffer, the insoluble material removed by low speed centrifugation and lysates precleared with non-immune rabbit serum and protein A sepharose. The remaining supernatant was immunoprecipitated with anti-Rap1, resolved on a 12% SDS-polyacrylamide gel and analyzed by autoradiography and PhosphorImager (Molecular Dynamics). For GTP loading studies, PC12 cells were transfected with 30 μg of His-Rap using calcium phosphate (GIBCO/BRL) and labeled as described above. Rap1 was precipitated with Ni-NTA Agarose and GTP loading was assayed as described (Urano et al., 1996) with the addition of a preclearing step using activated charcoal and agarose. 5 μl of each nucleotide sample was spotted on a PEI cellulose chromatography plate along with GTP and GDP standards (Sigma), resolved in 10mM KH_2PO_4, pH 3.4 at room temperature, and analyzed using a PhosphoImager (Molecular Dynamics). The GTP fraction was calculated as follows: (cpm GTP)/(cpm GTP + cpm GDP).

Coprecipitation studies

Cells were serum starved overnight and treated with 8-CPT for ten minutes or left untreated. After treatment, cells were immediately washed twice with ice-cold PBS and lysed in 1ml 0.1X PBS. Cell lysates were homogenized, using Dounce Homogenizer (Wheaton). Nuclei and cytoskeleton were pelleted by centrifugation at 5,000 rpm for 10 minutes. The supernatant was collected and loaded on the top of 30% sucrose and spun at 100,000 g for one hour. The pellet was designated the membrane fraction. The membrane

was resuspended in buffer containing 1% NP-40, 10µM Tris pH 7.4 and protease inhibitors. The top 0.75 ml of the gradient was collected as cytosolic proteins and was diluted with an equal amount of 2X membrane solubilization buffer. Equivalent amounts of protein from treated and untreated cells were immunoprecipitated with antisera against Rap1 as described above, resolved by 12% SDS-PAGE and immunoblotted with antisera against B-Raf. Antibody Y13-238 was used to immunoprecipitate Ras from fractionated cells, as described above, and from unfractionated cells, as described (Warne et al., 1993). B-Raf and Ras western blots were carried out as described.

For studies in COS-7 cells, transfections were performed using calcium phosphate. Cells were lysed in a buffer containing 1% NP40, 10µMTris, pH 8.0, 20 µM NaCl, 30 µM $MgCl_2$, 1µM PMSF, and 0.5µg/ml aprotinin and supernatants prepared. Transfected His-Rap proteins were precipitated from supernatants containing equal amounts of protein using Ni-NTA Agarose and washed with 20µM imidazole in lysis buffer and eluted with 500µM imidazole, 5µM EDTA in Phosphate Buffered Saline. 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 immunoprecipiatated with B-Raf antisera and B-Raf activity measured by immune complex assay. Equal amounts of Rap1 in each eluate was confirmed by western blotting.

β-Galactosidase expression

PC12 cells were maintained in DMEM with 10% horse serum and 5% fetal calf serum on primaria (Falcon) plates. Plasmids encoding ß-galactosidase (3µg/plate) and indicated plasmids were transfected using lipofectamine (GIBCO/BRL) in serum free media, stained for ß-galactosidase activity as described (Yao et al., 1995), and examined by light microscopy. The ratio of blue cells with and without neurites after transfection was compared to that in cells that received vector and ß-galactosidase alone, as described.

Processes greater than one cell body in length were counted as neurites. Greater than 200 blue cells were counted per condition, in three independent experiments.

Results

cAMP induces neuronal differentiation via ERKs

cAMP's ability to differentiate PC12 cells was reproduced by transfection of the catalytic subunit of PKA (cPKA) or treatment with the non-hydrolyzable cAMP analog 8-(4-chlorphenylthio)-cyclic AMP (8-CPT) (Figure 3.1). Cells transfected with cPKA were identified by co-transfection with a cDNA encoding β-galactosidase (β-gal). Cells transfected with ß-gal and treated with 8-CPT or NGF developed neurites in 63% and 75% of ß-gal-positive cells, respectively (data not shown). These percentages were not affected by co-transfection of plasmid vector along with β-gal (data not shown). However, they were reduced by the expression of dominant-negative mutants of the ERKs (dnERK1 and dnERK2; dnERKs) (Figure 3.1B), and MKP-2 (Figure 3.1C), a MAP kinase phosphatase that is endogenously expressed in PC12 cells (Misra-Press et al., 1995). The expression of the dominant negative mutant of Ras (RasN17) also blocked the ability of NGF (Figure 3.1E) to induce neurites but did not block neurite outgrowth induced by 8-CPT (Figure 3.1D).

cAMP's activation of ERKs is independent of Ras

Forskolin activated both ERK1 and ERK2 in PC12 cells (Figure 3.2A). This action was selective; neither forskolin nor 8-CPT activated the related c-Jun N-terminal kinases (JNKs) (Figure 3.2B). ERK2 activity was also stimulated by hormones and hormone analogs that are known activators of adenylyl cyclase, including vasoactive-intestinal polypeptide (VIP) and isoproterenol (Figure 3.2C). ERK activation by these agents was inhibited by H-89 (N-[2-(p-bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide), an inhibitor of PKA (Yao et al., 1995), suggesting that PKA was required for these hormonal

actions, as well. Epitope-tagged ERK (myc-ERK2) was used to examine the effect of transfected mutants on ERK activity. 8-CPT's activation of transfected myc-ERK2 was not blocked by expression of Ras N17, while EGF's activation was blocked (Figure 3.2D). Basal ERK activation was also blocked by RasN17 to a similar degree as EGF-stimulated ERK activity, suggesting that some constitutive activation of upstream signaling cascades occurs under basal conditions. Like signals generated by EGF, these signals appear to activate ERKs via Ras-dependent mechanisms. Stimulation of myc-ERK2 by both EGF and 8-CPT was completely blocked by expression of dominant negative MEK-1 (MEK-K97R) (Figure 3.2D). Therefore, like neuronal differentiation, cAMP's activation of MAP kinases requires MEK but is independent of Ras.

cAMP activates Elk-1 via an ERK-dependent, Ras-independent mechanism

Elk-1, a member of the ets family of transcription factors, is an important physiological substrate of ERKs and mediates serum-induced expression of immediate early genes (Marais et al., 1993). The activation of ERKs was also assayed indirectly by measuring the transactivation of the 5x Gal4-E1b/luciferase reporter gene by an Elk-1/Gal4 chimera (Misra-Press et al., 1995). cAMP-dependent Elk-1 activation was completely blocked by dnERKs, but not by interfering mutants of JNKs (Figure 3.2E), despite the ability of these mutants to block activation of c-jun (data not shown). Therefore, Elk-1 activation is a useful measure of ERK activation by cAMP. Forskolin's activation of Elk-1 was blocked by the PKA inhibitor PKI, demonstrating the requirement for PKA (Figure 3.2E). Forskolin's activation of Elk-1 was also blocked significantly by the expression of MEK-K97R but minimally by RasN17 (Figure 3.2E). As a control, we show that RasN17 can block activation of Elk-1 by NGF (Figure 3.2E, right panel). These data suggest that forskolin activates Elk-1, as well as ERKs, at a level above MEK and downstream of, or parallel to, Ras.

cAMP activates the protein kinase B-Raf

Upstream activators of MEK include Raf-1 and B-Raf (Moodie et al., 1994). In PC12 cells, 8-CPT activated B-Raf (Figure 3.3A), but, like forskolin, inhibited Raf-1 activity (data not shown) (Vaillancourt et al., 1994). cAMP's regulation of Raf isoforms resulted in changes in Elk-1 activation. Transfection of B-Raf into PC12 cells increased forskolin's activation of Elk-1. In contrast, forskolin's activation of Elk-1 was reduced following transfection of Raf-1 (Figure 3.3B). The actions of transfected B-Raf on cAMP signaling were more apparent in B-Raf-deficient cells. B-Raf is expressed primarily in neuronal cells (Barnier et al., 1995), and its expression was not detectable by immunoblot in NIH3T3 fibroblasts (Figure 3.3C). B-Raf activity has been detected in NIH3T3 cells by other techniques, suggesting that it is expressed, but at a much lower level than Raf-1 (Reuter et al., 1995). To test the hypothesis that the expression of B-Raf dictates cAMP's activation of MAP kinase, we introduced B-Raf into these cells. In the absence of B-Raf, PKA was unable to activate Elk-1, however, transfection of B-Raf converted PKA into a potent activator of Elk-1 (Figure 3.3D). Forskolin's ability to activate Elk-1 also required transfection of B-Raf into these cells, and this ability increased with increasing doses of B-Raf. In contrast, forskolin inhibited the activation of Elk-1 by Raf-1, at all doses examined (Figure 3.3E).

Rap1 activates B-Raf and ERKs *in vivo*

The mechanism by which cAMP induces Ras-independent activation of B-Raf is not known. The small G protein, Rap1, is activated by cAMP *in vivo* (Altschuler et al., 1995) and has been shown to activate B-Raf *in vitro* (Ohtsuka et al., 1996). To ask whether B-Raf is a positive effector of Rap1 *in vivo*, we examined the actions of RapV12, a constitutively activated mutant of Rap1 in which glycine at amino acid residue 12 is replaced with valine within the GTPase domain and, like the analogous Ras mutant, results in increased GTP loading and a prolonged activation state (Kitayama et al., 1990). In

contrast to its actions in other cell types, where it blocks Ras-dependent activation of MAP kinase (Cook and McCormick, 1993), RapV12 stimulates ERKs and Elk-1 in PC12 cells (Figure 3.4A and 3.4B). These effects were not blocked by RasN17 but were blocked by MEK-K97R (Figure 3.4A and 3.4B). Therefore, Rap1, like cAMP, activates the ERK cascade via a pathway that is independent of Ras, but requires MEK. This action of RapV12 was selective for ERKs; it did not stimulate basal JNK activity or potentiate JNK activity stimulated by other agents (data not shown). These data confirm that Elk-1 activation by RapV12, like that of cAMP, is mediated by the ERK cascade.

Rap1 is phosphorylated by PKA at a single site, serine 179, within its carboxy-terminus (Altschuler and Lapetina, 1993). A role of PKA in Rap1's activation of ERKs was suggested by the ability of RapD179 to activate ERKs in this assay (Figure 3.4A). RapD179 encodes a mutated Rap1 in which serine 179 was replaced with an aspartic acid residue to mimic phosphorylation at that position. Wild type Rap1 had no effect in this assay (data not shown).

The requirement for Rap1 in cAMP's activation of Elk-1 was confirmed using RapN17, a dominant negative mutant corresponding to RasN17 (Stacy et al., 1991). RapN17 blocked forskolin's activation of Elk-1 in PC12 cells. This inhibition was dose-dependent and specific; activation by EGF was not blocked at any dose (Figure 3.4C). These data demonstrate that endogenous Rap1 is necessary for forskolin's effects on Elk-1. Expression of exogenous Rap1 could potentiate forskolin's stimulation of Elk-1. In the absence of forskolin, wild type Rap1 was unable to activate Elk-1, but in the presence of B-Raf and low doses of forskolin (1μM), Rap1 activated Elk-1 to levels greater than forskolin and B-Raf alone (Figure 3.4D, right panel). In addition, transfection of B-Raf greatly increased the activation of Elk-1 by RapV12 in untreated PC12 cells (Figure 3.4D,

left panel). These results, and the results of Figure 3.3., demonstrate that cAMP's activation of Elk-1 utilizes Rap1 and B-Raf.

Rap1 is a selective activator of B-Raf and is required for PKA-dependent B-Raf activation

PKA and forskolin could also stimulate the activity of transfected B-Raf, when expressed at high levels in COS-7 cells. Basal, forskolin- and PKA-stimulated B-Raf activities were augmented following the co-transfection of wild type Rap1 (Figure 3.5A). We confirmed that B-Raf activated by cAMP phosphorylates the sites in MEK-1 (serines 218 and 222) that have previously been shown to be required for MEK activation by Raf-1(Alessi et al., 1994). MKK8E, a kinase inactive MEK that retains serines 218 and 222 (Resing et al., 1995) is phosphorylated by B-Raf co-expressed with PKA while MKK36A, a MEK mutant in which these serines are mutated to alanine is not phosphorylated (Figure 3.5A, right panel). Therefore, the results of the B-Raf kinase assays suggest that PKA stimulates both the phosphorylation and activation of MEK via B-Raf.

To determine whether Rap1 was required for PKA's activation of B-Raf, we examined the action of RapN17 on B-Raf activity in COS-7 cells. RapN17 inhibited PKA's activation of B-Raf, while B-Raf activity stimulated by EGF was unaffected (Figure 3.5B). These studies demonstrate that Rap1 is required for PKA's activation of B-Raf. The strongest activator of B-Raf in COS-7 cells was RapV12 (Figure 3.5C). This activation was selective; Raf-1 was not stimulated by RapV12. Furthermore, both EGF- and RasV12-stimulated Raf-1 kinase activity were inhibited by RapV12 (Figure 3.5C). Therefore, Rap1 is a selective activator of B-Raf and inhibitor of Raf-1.

B-Raf can convert Rap1 into an activator of Elk-1

To test the hypothesis that the expression of B-Raf permits RapV12, as well as PKA, to activate the MAP kinase cascade, we transfected NIH3T3 cells with either B-Raf or Raf-1 and examined Elk-1 activation by RapV12. RapV12 activation of Elk-1 was greatly potentiated by co-transfecting B-Raf. The expression of B-Raf alone, in the absence of RapV12, had no effect on Elk-1. In contrast, Raf-1 activation of Elk-1 was completely blocked by RapV12 (Figure 3.5D). Therefore, like PKA, RapV12 selectively activates B-Raf in multiple cells. The small amount of activation of Elk-1 following transfection of RapV12 alone may reflect low endogenous levels of B-Raf in these cells (Reuter et al., 1995), or the presence of additional unidentified targets of Rap1.

cAMP stimulates GTP-loading of Rap1 in PC12 cells

PC12 cells express abundant Rap1 protein that was phosphorylated in response to forskolin treatment (Figure 3.6A). This phosphorylation is associated with increased GTP-loading of Rap1 in these cells (Figure 3.6B) as has been shown in other cell types (Altschuler et al., 1995; Hata et al., 1991). In contrast, EGF had no effect on GTP loading of Rap1 in these cells (Figure 3.6B).

PKA induces the association of B-Raf and Rap1 in PC12 membranes.

In PC12 cells, Rap1 and B-Raf are localized to the cell membrane and cytosol, respectively (data not shown). 8-CPT stimulated the association of B-Raf with Rap1 within membranes (Figure 3.6C). This action was specific for both cAMP and Rap1; no association of B-Raf with Rap1 was detected within membranes following treatment with EGF or in untreated cells (Figure 3.6C), nor was B-Raf detected in immunopreciptates using Ras antibody Y13-238 (data not shown). Parallel western blots were performed to confirm the ability to immunopreciptate both Ras and Rap1 (data not shown).

The GTP-dependence of this interaction was examined in COS-7 cells transfected with B-Raf and 6x histidine tagged Rap1b (His-Rap) or His-RapV12. Rap and Rap-associated proteins were purified using Nickel affinity. B-Raf and its kinase activity were detected in eluates (Figure 3.6D). The small amount of B-Raf associating with His-Rap1 was increased in cells co-transfected with PKA. The highest level of B-Raf was detected in cells co-transfected with His-RapV12 (Figure 3.6D, lower panel). Only eluates from B-Raf transfected cells contained B-Raf activity, as measured by immune complex assay using B-Raf antisera. B-Raf activity associated with His-Rap1 was greatly stimulated by PKA, to a level similar to that associating with His-RapV12 (Figure 3.6D, upper panel). The expression of equal amounts of His-Rap was confirmed by immunoblotting with Rap1 antisera (data not shown). These data suggest that the association of activated B-Raf protein with Rap1 is increased upon GTP-loading, stimulated by PKA or by a V12 mutation.

Constitutively active Rap stimulates neuronal differentiation in PC12 cells

Transfection of RapV12, but not vector alone, induced neurite outgrowth in PC12 cells (Figure 3.7A, 3.7B and 3.7C). The percentage of the RapV12 transfected cells developing neurites was similar to that in cells transfected with RasV12. This action of RapV12 was completely blocked by MEK-K97R but was only modestly blocked by RasN17 (Figure 3.7D, 3.7E, and 3.7F). Co-transfection of these mutants did not alter the level of expression of transfected Rap protein as confirmed by western blotting (data not shown). RapN17 (Figure 3.7G and 3.7H), but not vector control (data not shown), interfered with the ability of 8-CPT, but not NGF, to differentiate these cells. These data demonstrate that Rap1 is required for cAMP-dependent differentiation of PC12 cells.

Discussion

We demonstrate that B-Raf can convert cAMP into an activator of ERKs and Elk-1. The action of cAMP on B-Raf is the opposite of its action on Raf-1 (Burgering and Bos, 1995; Wu et al., 1993). Unlike cAMP's inhibition of growth factor activation of B-Raf, where cAMP blocks Ras-dependent pathways (Vaillancourt et al., 1994), cAMP's activation of B-Raf utilizes the small G protein Rap1. Rap1, like cAMP, activates B-Raf and inhibits Raf-1. Therefore, in cells that express B-Raf and Raf-1, cAMP's actions on ERKs may represent a balance between its effects on each Raf isoform.

cAMP activates B-Raf in PC12 cells and transfected COS-7 cells

Although cAMP can antagonize Ras-dependent activation of B-Raf, cAMP's regulation of B-Raf activity has not been examined independently of Ras activation (Erhardt et al., 1995; Vaillancourt et al., 1994). We show that cAMP can activate B-Raf in PC12 cells in the absence of factors that activate Ras and that both cAMP and PKA can stimulate B-Raf following transfection in COS-7 cells. This MEK kinase activity of B-Raf is limited to serine 218 and 222, sites responsible for the activation of MEK by Raf-1 (Alessi et al., 1994; Resing et al., 1995). Therefore, PKA stimulates both the phosphorylation and activation of MEK via B-Raf.

cAMP's activation of B-Raf and ERKs may depend on the cell lines examined. For example, one previous study was unable to detect activation of B-Raf or ERKs by cAMP (Vaillancourt et al., 1994). The cells used in that study expressed two forms of B-Raf, 95kD and 68kD. The majority of B-Raf expressed in those cells was a 68kD form, while only the minor 95kD form was shown to be activated by growth factors (Lange-Carter et al., 1993; Vaillancourt et al., 1994). In contrast, the PC12 cells used in this study express predominantly the 95kD form (Figure 3.3C). The inability of 68kD forms to be activated by growth factors may also account for the finding that cAMP's ability to activate ERKs

does not correlate with the expression of the lower molecular weight forms of B-Raf (Faure and Bourne, 1995).

cAMP induces activation of ERKs and neuronal differentiation via a Rap-dependent, but Ras-independent pathway

We show that cAMP's activation of ERKs, as well as the induction of neurite outgrowth by both cAMP and RapV12, is largely independent of Ras, but requires Rap1. However, cells expressing both RapV12 and RasN17 showed decreased neurite length compared with RapV12 alone (Figure 3.7D) suggesting that complete differentiation by Rap may require additional Ras-dependent pathways (Frech et al., 1990).

The ability of cAMP, like that of NGF, to induce differentiation in these cells may require sustained activation of ERKs (Cowley et al., 1994; Young et al., 1994). Agents like EGF, that activate ERKs transiently, are unable to differentiate PC12 cells (Cowley et al., 1994). EGF's activation of ERKs is terminated, in part, via the direct phosphorylation of the Ras guanine nucleotide exchange factor SOS by the ERKs themselves (Porfiri and McCormick, 1996). We show that cAMP's ability to differentiate PC12 cells requires Rap1 but not Ras. cAMP's ability to activate Rap1 and ERKs via a Ras-independent mechanism may bypass this negative feedback loop regulating Ras-dependent signaling, to permit cAMP to induce sustained activation of ERKs associated with differentiation in these cells.

Rap1 is a selective activator of B-Raf

The specificity of cAMP for B-Raf is reflected in the actions of Rap1 on B-Raf. We demonstrate that Rap1 is a selective activator of B-Raf and that B-Raf can convert Rap1 and cAMP into activators of ERKs and Elk-1. In contrast, Ras can activate all known Raf isoforms. We show here that Rap1 activates B-Raf and inhibits Raf-1. Therefore, despite its initial characterization as an antagonist of Ras, Rap1 may activate MAP kinase in B-Raf-

expressing cells. This may explain the mitogenic effects of Rap1 in Swiss3T3 cells (Faure and Bourne, 1995; Yoshida et al., 1992). The ability of B-Raf to convert inhibitory PKA signals to stimulatory ones may identify a novel role for B-Raf during development of hormonally responsive tissues.

We show that the actions of cAMP on B-Raf are mediated by PKA's activation of Rap1. Both PKA activation and GTP loading of Rap1 induce the association of B-Raf with Rap1. It is possible B-Raf may be activated by recruitment to membranes (Figure 3.6C) to form a complex with Rap1 in a manner similar to that shown for Raf-1 activation by Ras (Hallberg et al., 1994; Leevers et al., 1994; Marshall, 1995). In some cells, the ability to detect an association between endogenous Raf-1 and Ras proteins is limited, suggesting that the interaction between Raf-1 and Ras is transient (Leevers et al., 1994). Differences in the activation of B-Raf and Raf-1 by Ras have been proposed (Jaiswal et al., 1996; Okada et al., 1991; Yamamori et al., 1995). Therefore, the differences in the ability to detect the association of endogenous B-Raf with Rap1 and Ras may reflect biochemical differences between the Raf isoforms, as well as differences between Ras and Rap1 (Kuroda et al., 1996).

The ability of RapN17 to inhibit cAMP's activation of B-Raf, ERKs, and differentiation in PC12 cells suggests that a Rap-GEF may be required for each of these effects of cAMP (Stacy et al., 1991). PKA increases the GTP loading of Rap1 and phosphorylates Rap at a single site (Altschuler and Lapetina, 1993; Altschuler et al., 1995). A possible relationship between these two actions is suggested by the constitutive activation of ERKs by RapD179, a mutant that mimics this phosphorylation in Rap1. It has been previously shown that phosphorylation at this site may potentiate the actions of a Rap-specific GEF, Smg-GDS *in vitro* (Hata et al., 1991). The requirement for this phosphorylation in Rap1 activation *in vivo* has not been demonstrated making it possible that phosphorylation may

lead to activation of Rap through other mechanisms and that PKA may have additional substrates to account for cAMP's activation of Rap1 in PC12 cells.

Other activators of Rap1 may stimulate B-Raf and ERKs as well. Recently, an association between the SH2-containing adapter protein Crk and a Rap1 activator, C3G, was identified (Gotoh et al., 1995). However, the expression of C3G does not potentiate cAMP's activation of ERKs (data not shown), suggesting that C3G may respond to other factors, including Crk itself. In addition, the calcium and calmodulin-dependent protein kinase (CaM kinase) IV can phosphorylate Rap1 *in vitro* (Sahyoun et al., 1991) at the same site that has been shown to be phosphorylated by PKA (Altschuler and Lapetina, 1993). Therefore, in B-Raf-expressing cells, Rap1 has the potential to integrate the actions of diverse signals to dictate cellular responses governing growth and differentiation.

Acknowledgments

We thank Drs. A. Shaw, N. Ahn, R. Maurer, R. Goodman, N. Nathanson, K. -L. Guan, U. Rapp, M. Cobb, C. Marshall, R. Davis, and G. Johnson for providing reagents. We appreciate Drs. A. Shaw, A. Misra-Press, M. Forte, B. Druker, H. Bourne, L. Feig, I. Macara, A. Wolfman, and R. Goodman for the scientific discussions.

Figure Legends

Figure 3.1 **ERK-dependent and Ras-independent stimulation of neurite outgrowth in PC12 cells by PKA.** (A) cPKA (10 μg) and RSV-β-gal (3 μg) were co-transfected into PC12 cells using lipofectamine (GIBCO/BRL). Cells transfected with cPKA appear blue in this assay and develop neuritic processes. Cells transfected with vector and RSV-ß-gal alone remain undifferentiated (data not shown). Cells transfected

with dnERKs (10 μg) (B), or MKP-2 (10 μg) (C) do not extend neurites in the presence of 8-CPT, while untransfected cells treated with 8-CPT display neuritic processes. 8-CPT still stimulates neurite extension in cells transfected with RasN17 (10 μg) (D). In contrast RasN17 inhibits neurite outgrowth in cells treated with NGF (E). The percentage of neurites in ß-gal-positive cells is indicated in the lower right corner of each panel. The bar represents 10 μm.

Figure 3.2 **Activation of ERKs and Elk-1 in PC12 cells by cAMP.** (A) Activation of ERK1 and ERK2 by cAMP. PC12 cells were treated with forskolin, NGF, EGF, alone or in combination for 10 minutes and lysates immunoprecipitated using ERK1 and ERK2 specific antibodies. Kinase assays were performed as described. The position of the ERK substrate, myelin basic protein (MBP), is shown. (B) Lack of stimulation of JNK activity by cAMP. PC12 cells were treated with vehicle, forskolin or 8-CPT for 10 minutes and lysed, or ultraviolet light (6J/m2 UVB) and lysed 60 minutes later, as indicated. JNK assays were performed as described. The position of the JNK substrate, c-jun is indicated. (C) Activation of ERK2 by the cAMP analogue, 8-CPT, the hormone VIP and the β-adrenergic agonist isoproterenol in PC12 cells. Cells were starved and pretreated with H-89 for 15 minutes or left untreated, then treated with VIP, isoproterenol or 8-CPT for ten minutes, as indicated. ERK2 kinase assays were performed as described. The position of MBP is shown. (D) Ras-independence of cAMP's stimulation of ERKs. PC12 cells were transfected with myc-ERK2 (5 μg), and either pcDNA3 (10 μg); top panel, RasN17 (10 μg); middle panel, or MEK-K97R (10 μg): bottom panel. Cells were treated with EGF, 8-CPT, or left untreated, as indicated. Lysates containing 500 μg total protein were immunoprecipitated with the anti-myc 9E10 antibody and ERK assays performed as above. Relative kinase activities are shown (untreated cells transfected with myc-ERK2 is given the value of 1). Equal levels of myc-ERK2 expression in each transfectant was confirmed by Western blotting, using 9E10 antibody (data not shown).

This assay was repeated three times with similar results. (E) Activation of ERK-dependent gene transcription by cAMP. PC12 cells were transfected with 10 μg each of cDNAs encoding vector alone, dominant negative JNK (dnJNK), dnERKs, PKI, MEK K97R, or RasN17, and treated with forskolin, or left untreated, as indicated. In addition, all cells received 3 μg of Elk-1/Gal4, 3 μg of 5xGal4-E1b luciferase and 3 μg of CMV-CAT. Activation of Elk-1 is reflected by luciferase activity and was reported as fold activation above basal normalized for CAT activity. Standard error is indicated (n=3). Right Panel: Requirement of Ras in NGF-mediated activation of Elk-1. Cells were transfected with vector or cDNA encoding RasN17 and treated with NGF. Luciferase assays of Elk-1 activity were performed as above. This serves as a control for the biological activity of the RasN17 cDNA.

Figure 3.3 **cAMP dependent activation of Elk-1 is specifically potentiated by B-Raf.** (A) Activation of B-Raf by 8-CPT and EGF in PC12 cells. Cells were treated with 8-CPT, or EGF as indicated. B-Raf was immunoprecipitated and its activity measured *in vitro* using MEK-1 as a substrate. MEK-1 phosphorylation is indicated. The upper band corresponds to autophosphorylation of B-Raf. This result is representative of three independent experiments. (B) B-Raf and Raf-1 have opposing actions on forskolin-stimulated Elk-1 activity in PC12 cells. PC12 cells were transfected with plasmids encoding either B-Raf (10 μg) or Raf-1 (10 μg) and Elk-1/Gal-4 (3 μg) and 5xGal4-E1b/luciferase (3 μg) plasmids and subsequently treated with forskolin, as indicated. Elk-1 activation was monitored by luciferase activity. Standard error is as shown (n=3). (C) B-Raf expression in PC12 cells and NIH3T3 cells. B-Raf and Raf-1 protein levels were determined by Western blot. 100 μg protein from whole cell lysates was analyzed by immunoblotting with B-Raf (left gel) and Raf-1 (right gel) antisera. Note that B-Raf was not detected in NIH3T3 cells and the 95kD form of B-Raf was the predominant form detected in PC12 cells. Raf-1 was expressed in both PC12 and NIH3T3 cells. (D)

Activation of Elk-1 in NIH3T3 cells by cAMP following transfection of B-Raf. NIH3T3 cells were transfected with plasmids encoding B-Raf (8 μg) and/or cPKA (4 μg), as indicated along with Elk-1/Gal-4 (5 μg) and 5xGal4-E1b/luciferase (5 μg) plasmids. Elk-1 activation was monitored by luciferase activity as in Figure 3.2E (n=3). (E) Opposing actions of B-Raf and Raf-1 on cAMP's regulation of Elk-1. NIH 3T3 cells were transfected with Elk-1/Gal4 and 5xGal4-E1B luciferase as in D. Cells were co-transfected with increasing amounts of B-Raf (left graph) or Raf-1 (right graph) and treated with forskolin or left untreated as indicated.

Figure 3.4 **Activation of ERKs in PC12 cells by constitutively active Rap1.** (A) Stimulation of ERK2 activity by RapV12 and RapD179. Cells were transfected with myc-ERK2 (5 μg) and RapV12 (10 μg), RasN17 (10 μg), MEK-K97R (10 μg), or RapD179 (10 μg) as indicated. Cells were lysed and transfected myc-ERK2 was immunoprecipitated with 9E10 antibody. Assays of myc-ERK activity were performed as described in Figure 3.2. Equal levels of myc-ERK expression were confirmed by Western blot using antibody 9E10 (data not shown). The results of a representative experiment are shown with relative kinase activities provided above. (B) Requirement of MEK but not Ras for activation of Elk-1 by RapV12. PC12 cells were transfected with cDNAs encoding Elk-1/Gal-4 and 5xGal4-E1b/ luciferase with 10 μg of either vector, RapV12, RasN17, or MEK-K97R alone or in combination as indicated. Luciferase activity was assayed as described in Figure 3.2E. (n=3). (C) Requirement of Rap for cAMP dependent activation of Elk-1. PC12 cells were transfected with 3 μg each of Elk-1/Gal4 and 5xGal4-E1b/luciferase and 5, 10, or 15 μg of RapN17 as indicated. Cells were treated with forskolin (n=6) or EGF (n=3) as indicated and luciferase assays performed as above. (D) Activation of Elk-1 by Rap1, cAMP, and B-Raf. PC12 cells were transfected with plasmids encoding either 10 μg B-Raf, Rap1, or RapV12, and 3 μg of Elk-1/Gal-4 and

5xGal4-E1b/luciferase plasmids, and subsequently treated with forskolin (1 μM), as indicated. Elk-1 activation was monitored by luciferase activity (n=3).

Figure 3.5 **Selective stimulation of B-Raf by Rap1.** (A) Kinase activity of B-Raf is potentiated by Rap1. COS-7 cells were transfected with cDNAs encoding B-Raf (16 μg), cPKA (4 μg), or Rap1 (16 μg), and treated with forskolin or vehicle, as indicated. B-Raf activity was detected by immune complex assay, using recombinant MEK-1 as a substrate as in Figure 3.3A (upper left gel). Control assays performed in the absence of MEK-1 showed no kinase activity (data not shown). Immunoprecipitated B-Raf levels following each transfection were identical, as judged by Western blot, using B-Raf antisera (lower left gel). The transfection of Rap1 increased the kinase activity of transfected B-Raf in untreated cells and cells stimulated with either forskolin or cotransfected with cPKA. Control assays were performed using kinase-dead MEK (MKK8E) or the phosphorylation site MEK mutant (MKK36A) as substrates (right gels). (B) RapN17 selectively blocks activation of B-Raf by PKA, but not by EGF. COS-7 cells were transfected with cDNAs encoding B-Raf (8 μg), cPKA (4 μg), RapN17 (24 μg), or RasN17 (24 μg), and treated with or without EGF, as indicated. Following immunoprecipitation with B-Raf antisera, B-Raf kinase assays were performed as described in Figure 3.3A. The expression of equal levels of transfected B-Raf was confirmed by Western blotting (data not shown). (C) RapV12 selectively activates B-Raf in COS-7 cells. COS-7 cells were either transfected with B-Raf (16 μg) and treated with 8-CPT, or were co-transfected with RapV12. In parallel, COS-7 cells were transfected with Raf-1 (16 μg) in the presence or absence of RapV12 and treated with EGF or were co-transfected with RasV12 (16 μg), as indicated. The kinase activities of B-Raf (left lanes) or Raf-1 (right lanes) were measured as described, and the position of phosphorylated MEK-1 was indicated. The expression of equal levels of transfected Raf isoforms was confirmed by Western blotting (data not

shown). (D) Potentiation of RapV12 action by exogenous B-Raf and inhibition of Raf-1 in NIH3T3 cells. Cells were transfected with 16 μg of plasmids encoding B-Raf or Raf-1 and 4 μg RapV12 as indicated in the presence of 5 μg each of Elk-1/Gal-4 and 5xGal4-E1b/luciferase. Luciferase assays were performed as previously described (n=3).

Figure 3.6 **Phosphorylation, GTP loading and coprecipitation of Rap1 proteins.** (A) Phosphorylation of Rap1 in forskolin treated PC12 cells. Cells were metabolically labeled with [^{32}P]-orthophosphate and treated with forskolin (50 μM) and IBMX (10μM). Lysates were prepared as described, and Rap1 was immunoprecipitated with or without antibody as indicated. Immunoprecipitated proteins were resolved using 12% SDS-PAGE and visualized by autoradiography. (B) Rap1 activation by 8-CPT. GTP loading was assayed in PC12 cells following transfection of His-Rap (30 μg). PC12 cells were left untreated (U) or were treated with EGF or 8-CPT for 5 min., as indicated. His-Rap was precipitated with Ni-NTA Agarose and eluates were analyzed for GTP and GDP content by thin-layer chromatography. The GTP fraction of total guanine nucleotide is given above each lane. (C) 8-CPT, but not EGF, induces the association of Rap1 with B-Raf within membranes. Membranes and cytosolic fractions were prepared from PC12 cells treated with 8-CPT, EGF, or left untreated (-), as indicated. Lysates were immunoprecipitated with Rap antisera and probed by western blot using B-Raf antisera. The migration of B-Raf in PC12 lysates is shown in the right lane. The band corresponding to the IgG heavy chain is indicated. (D) GTP dependence of the association of B-Raf kinase activity with Rap1. COS-7 cells were transfected with 10 μg of His-Rap, 12 μg of B-Raf , 5 μg of PKA, or 10 μg of His-RapV12, as indicated. His-Rap proteins were precipitated with Ni-NTA Agarose and associated B-Raf kinase activity was immunoprecipitated with B-Raf antisera and assayed for kinase activity as previously

described (upper gel). The position of MEK-1 is shown. Associated B-Raf was also detected by western blotting. The position of B-Raf is shown (lower gel).

Figure 3.7 **Stimulation of neurite outgrowth in PC12 cells by RapV12.** RapV12 (5 μg) or RasV12 (5 μg) and of RSV-ß-gal (3 μg) were co-transfected into PC12 cells using lipofectamine (GIBCO/BRL). Transfected cells appear blue in this assay. Cells transfected with vector and RSV-ß-gal alone (A) remain undifferentiated, while cells receiving RasV12 (B) or RapV12 (C) develop processes. Cells were transfected with RapV12, RSV-ß-gal and either RasN17 (D), MEK K97R (E), or dnERKs (F). RapV12-induced neurites were blocked by MEK K97R and dnERKs but not by RasN17. Cells were transfected with RapN17 and RSV- ß-gal and treated with 8-CPT (G), or NGF (H), or transfected with RSV-ß-gal in the presence of vector control and treated with 8-CPT (data not shown). Differentiation by 8-CPT (G), but not NGF (H), was blocked by RapN17. Transfection of RSV- ß-gal and vector alone had no effect on the percentage of 8-CPT induced neurites (data not shown). The percentages of ß-gal-positive cells with neurites are indicated.

Fig. 3.1. ERK-dependent and Ras-independent stimulation of neurite outgrowth in PC12 cells by PKA

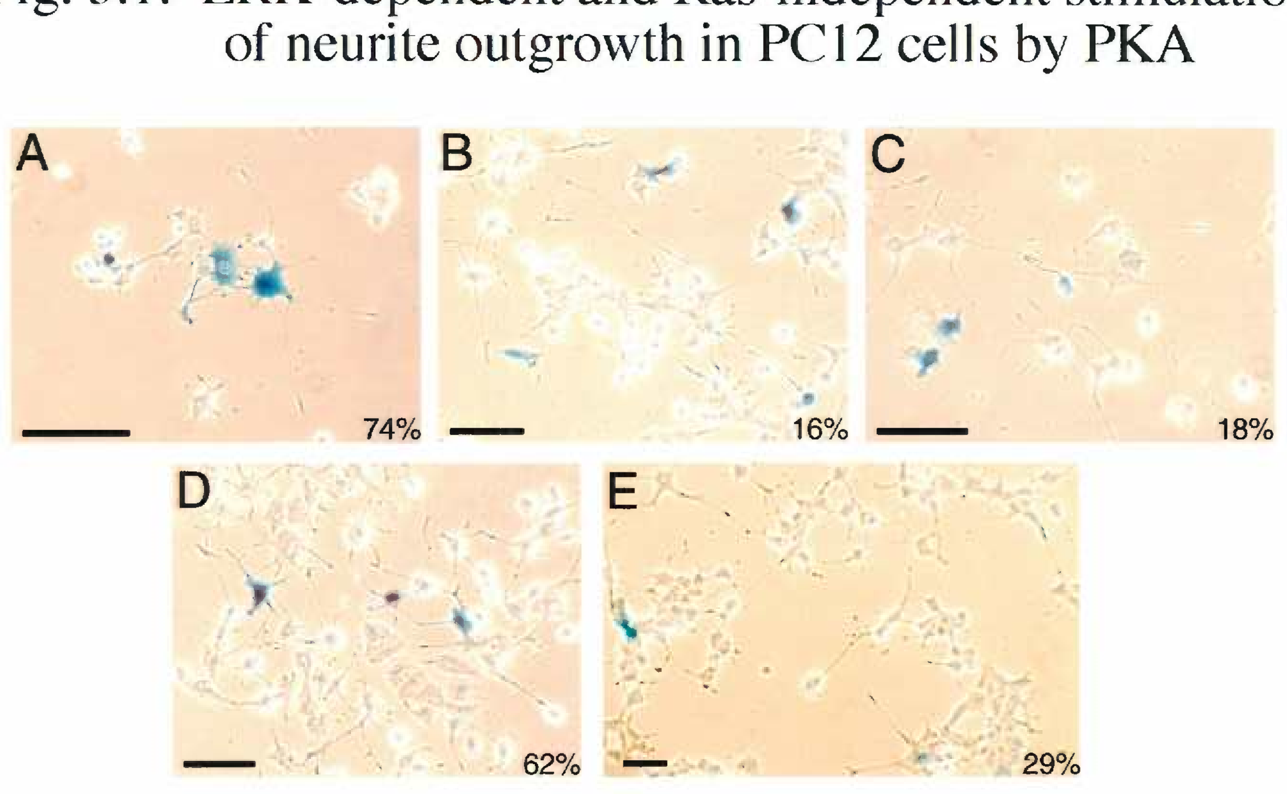
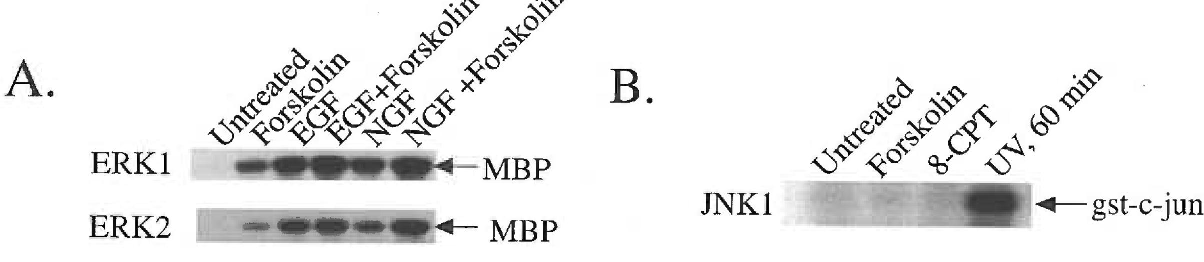
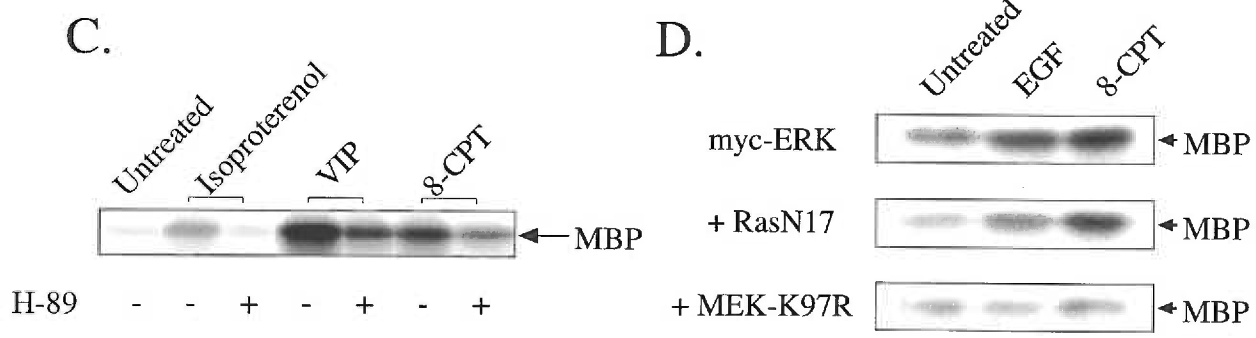


Fig 3.2. Activation of ERKs and Elk-1 in PC12 cells by cAMP





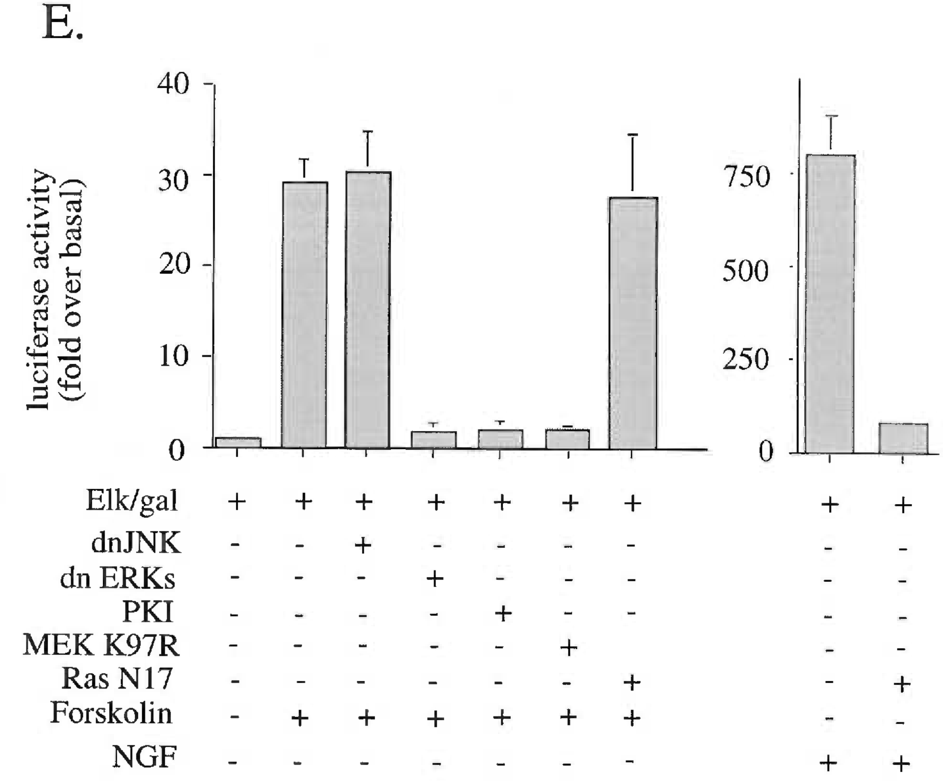


Fig. 3.3. cAMP-dependent activation of Elk-1 is specifically potentiated by B-Raf

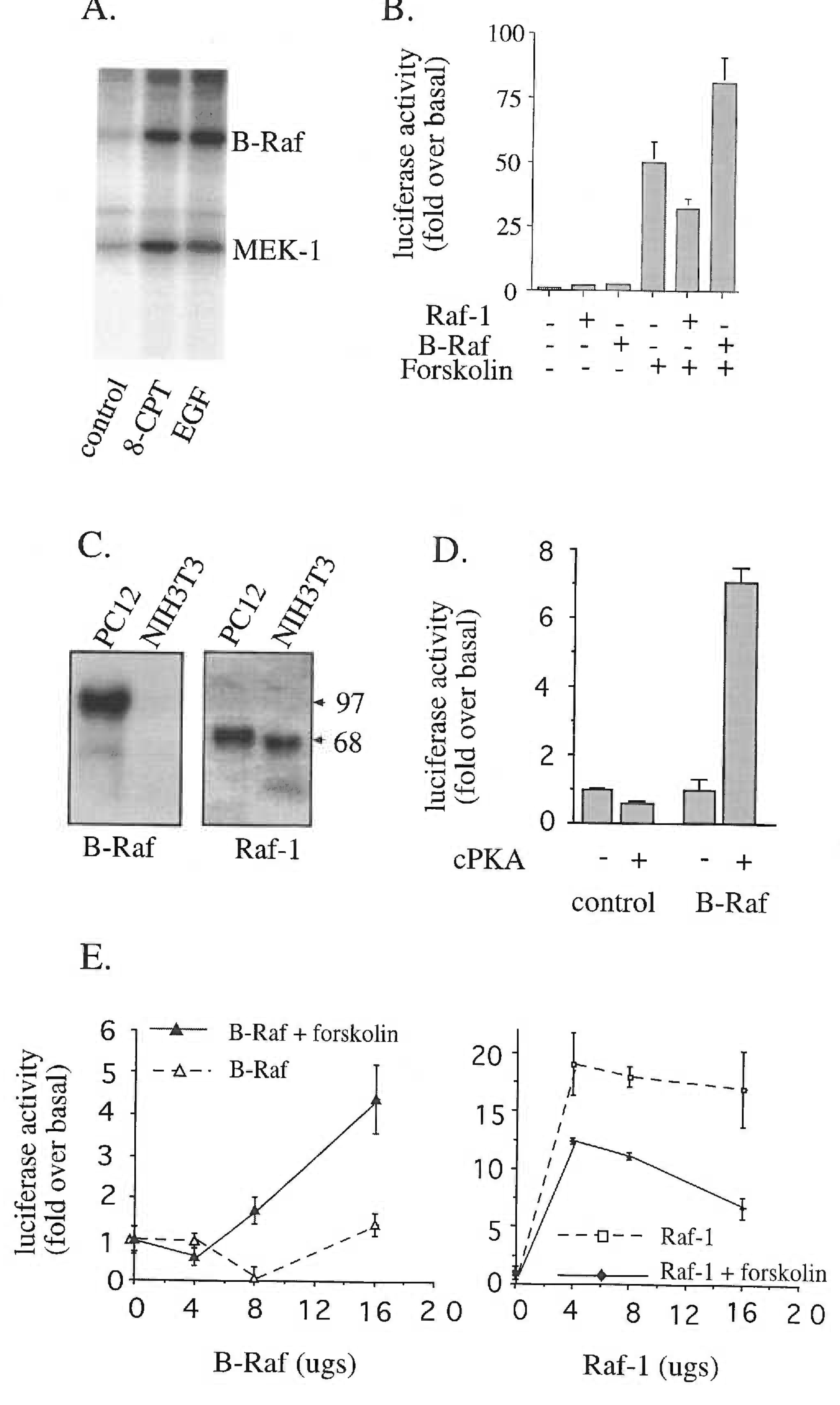


Fig. 3.4. Activation of ERKs in PC12 cells by constitutively active Rap1

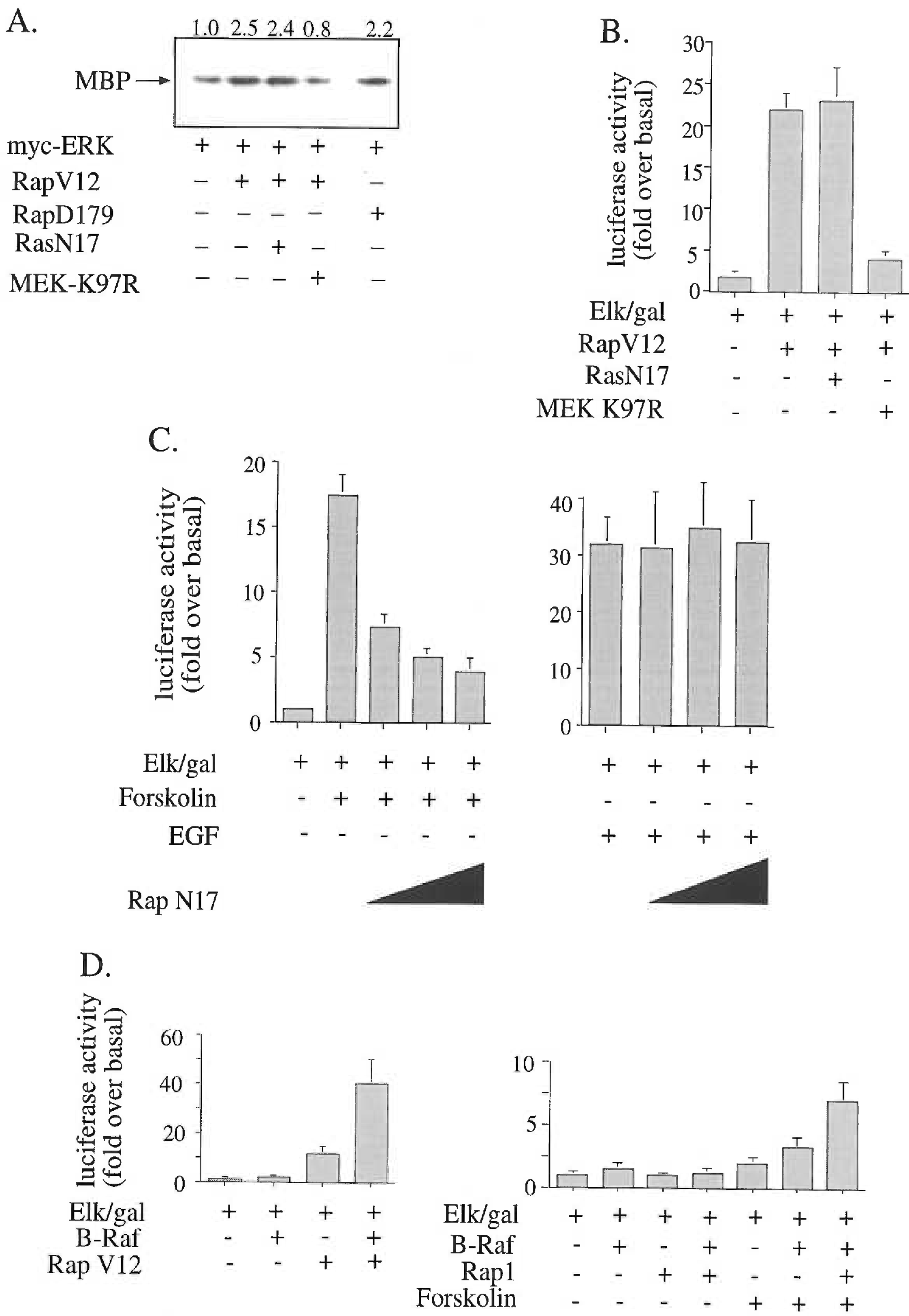


Fig. 3.5. Selective stimulation of B-Raf by Rap1

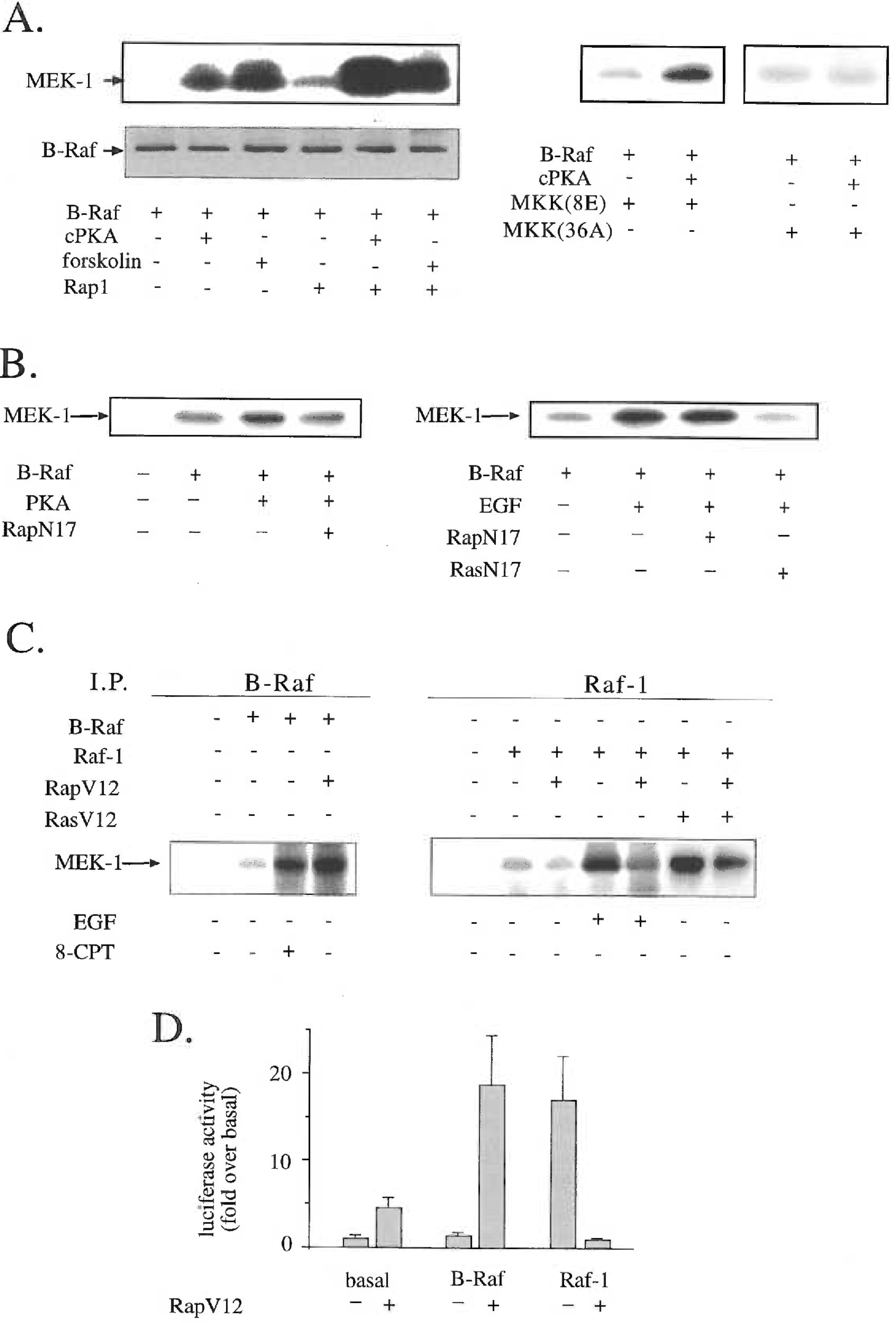


Fig. 3.6. Phosphorylation, GTP loading, and coprecipitation of Rap protein

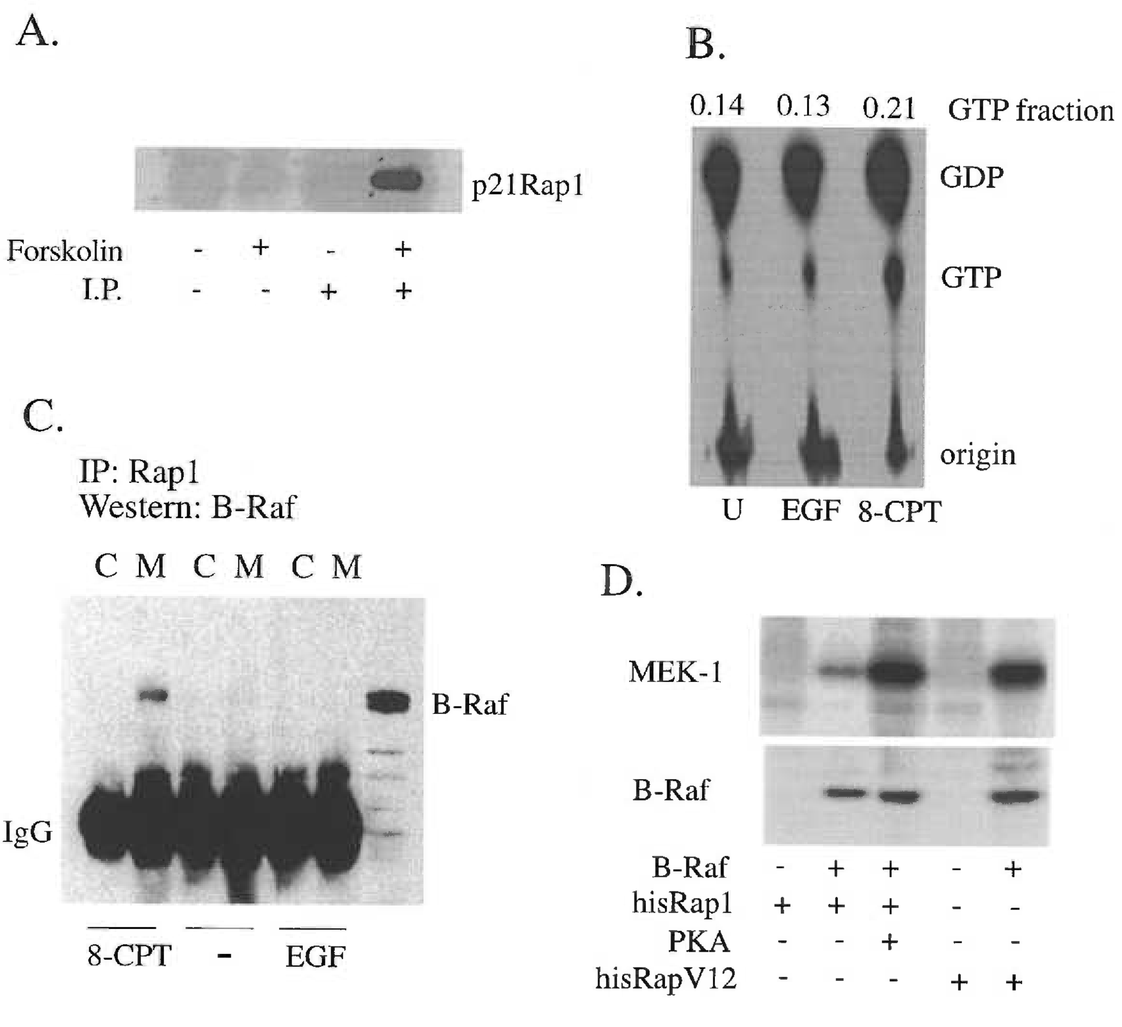
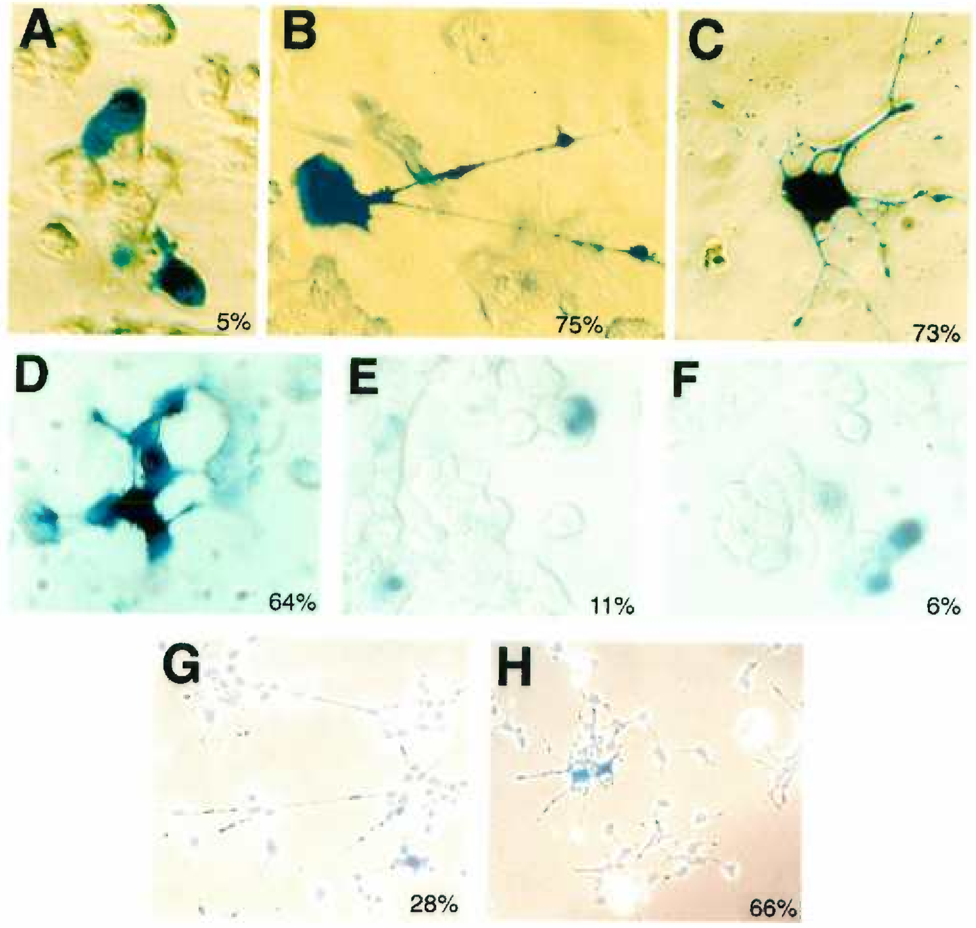


Fig. 3.7. Stimulation of neurite outgrowth in PC12 cells by RapV12



Abstract

Induction of neuronal differentiation of the rat pheochromocytoma cell line, PC12 cells by NGF requires activation of the MAP kinase, ERK (extra cellular signal regulated kinase). cAMP dependent protein kinase PKA also can induce differentiation of these cells. Like NGF, PKA's ability to differentiate PC12 cells is associated with a sustained activation of ERKs. Here we show that maximal sustained activation of ERK1 by NGF requires PKA. Inhibitors of PKA partially blocked activation of ERK1 by NGF but had no effect on activation of ERK1 by EGF. Inhibition of PKA also reduced the ability of NGF and cAMP, but not EGF, to activate the transcription factor Elk-1, reduced the induction of both immediate early and late genes following NGF treatment and blocked the nuclear translocation of ERK1 induced by NGF. We propose that PKA is an important contributor to the activation of ERK1 by NGF and is required for maximal induction of gene expression by NGF.

Introduction

Nerve growth factor (NGF) promotes the differentiation of sympathetic and sensory neurons that is characterized by morphological features of neuronal differentiation (neurite formation) and changes in gene expression including the late gene transin (Levi-Montalcini, 1987; Levi-Montalcini and Angeletti, 1968; Shackleford et al., 1993). This differentiation has been examined extensively in the rat pheochromocytoma cell line, PC12 cells, a well studied model of growth factor actions (Greene and Tischier, 1982; Greene and Tischler, 1976). In PC12 cells, neuronal differentiation by NGF requires activation of the mitogen-activated protein (MAP) kinases (also called extracellular signal regulated protein kinases, or ERKs) (Cowley et al., 1994). The mechanisms by which NGF activates ERKs have been the subject of many studies. Upon NGF binding, activation of the NGF receptor, TrkA, triggers the assembly of a multimeric protein complex that includes the small monomeric G-protein Ras (Qiu and Green, 1991). Ras activation triggers a cascade of phosphorylations on protein kinases that lie upstream of the ERKs (Ohmichi et al., 1992). Epidermal growth factor EGF also induces ERK activation via Ras. Unlike that of NGF, EGF's activation of Ras and ERK triggers a mitogenic program within PC12 cells. The ability of NGF to trigger neuronal differentiation instead of proliferation is thought to depend, in part, on its ability to activate ERKs for long, sustained periods. Sustained activation of ERKs may be required for the translocation of ERKs into the nucleus where they induce a distinct set of gene expression (Marshall, 1995). In contrast, ERK activation following EGF stimulation is transient. This is a consequence of the rapid termination of signals to ERK via a short feedback loop involving an ERK-dependent phosphorylation of the Ras activator SOS (Buday et al., 1995). This loop uncouples Ras-dependent activation of ERKs from upstream activators (Porfiri and McCormick, 1996). The activity of this feedback loop is reflected in the transient activation of Ras by EGF. Interestingly, although NGF induces a sustained activation of ERKs, Ras activation following NGF treatment of PC12 cells is terminated rapidly (Qiu and Green, 1991). Since NGF activation of ERK is

sustained despite the rapid inactivation of Ras, NGF may utilize Ras-independent pathways that are not inactivated rapidly to allow the sustained activation of ERKs. Here we test this hypothesis by examining the requirement of PKA, cAMP dependent protein kinase, for NGF's activation of ERK1.

We have recently identified a novel Ras-independent pathway by which cAMP induces sustained activation of ERKs in PC12 cells (Vossler et al., 1997). This pathway involves the Ras related small G protein Rap1 as well as the cAMP dependent protein kinase, PKA. In this study, we examine the possibility that PKA also participates in NGF signaling to the MAP kinase cascade by providing NGF a Ras-independent pathway to ERKs. We show that inhibition of PKA can inhibit signaling of NGF to ERK, to the transcription factor Elk-1 and to specific marker gene of differentiation and can block the nuclear translocation of ERK1 induced by NGF. In addition, we demonstrate that NGF, as well as PKA, can activate the small G protein Rap1 and that this activation is blocked by the PKA inhibitor PKI. Therefore, we propose that PKA participates in NGF signaling to ERKs, in part, via the activation of Rap1 and that this pathway contributes to the sustained activation of ERKs that characterizes NGF signaling.

MATERIALS AND METHODS

Materials--PC12-GR5 cells were kindly provided by Rae Nishi (Oregon Health Sciences University, Portland, Oregon). A126-1B2 cells, PKA deficient PC12 cells, and stromelysin-1 (transin) cDNA were provided by Gary Ciment (Oregon Health Sciences University, Portland, Oregon). Plasmids encoding Elk-1/Gal-4, 5xGal4-E1b/luciferase, Protein Kinase Inhibitor (cPKI) and loss-of-function mutant of PKI, $cPKI^{mut}$, were gifts of Richard Maurer (Oregon Health Sciences University, Portland, Oregon). Agarose-conjugated ERK1 (c-16) used in immunoprecipitations was purchased from Santa Cruz

Biotechnology Inc. NGF was from Boehringer Mannheim. EGF was from Sigma. Forskolin, H89, and 8-(4-chlorophenylthio)-cyclic AMP (8-CPT-cAMP), were purchased from CalBiochem.

Cell culture--PC12 cells and A126-1B2 cells were maintained in DMEM (Dulbecco-Modified Eagle Medium) plus 10% horse serum and 5% fetal calf serum on 100 mm plates to 50-60% confluence at 37° C in 5% CO_2 prior to harvesting. For immune complex assays and Northern blotting, cells were deprived of serum and maintained in DMEM for 16 hours at 37° C in 5% CO_2 prior to treatment with various reagents. 10 µM H89 was added to plates 15 minutes prior to treatment with NGF (50 ng/ml) or forskolin (10 µM). Lipid modified PKI peptide (sPKI) was added at 5µM, 10 minutes prior to treatment with NGF.

Transient transfections and luciferase assay--60-80% confluent PC12 cells were co-transfected with the indicated cDNAs using a calcium phosphate transfection kit (Gibco BRL) 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. Four hours following transfection, cells were glycerol shocked and allowed to recover in serum containing media overnight. Cells were then starved overnight in supplemented serum free media (N2) which contained DMEM with 5 µg/ml insulin, 100 µg/ml apo-transferrin, 30 µM sodium selenite, 100 µM putrescine, and 20 nM progesterone (Yao et al., 1995). Following serum deprivation cells were treated with the indicated reagents for 6 hours prior to harvesting. Luciferase assay was performed as previously described (Vossler et al., 1997). Briefly, cells were washed twice in phosphate buffered saline (PBS), scraped in PBS, spun at low speed to collect cells, and lysed by freeze-thawing three times in 100 µM K_2PO_4 (pH 7.8). For determination of Elk-1 activity, cells were transfected with 5 µg of Elk-1/Gal4 and 5 µg of 5XGal4-E1B-luciferase and other

plasmids as indicated. Luciferase activity was assayed using a luminometer (AutoLumat LB953), as previously described (Vossler et al., 1997). Cells transfected with pcDNA3 in the absence of reporter plasmids provided a baseline value that was subtracted from all subsequent measurements. Luciferase activity was reported as the fold increase above the basal levels reached in untreated cells that were transfected with the reporter plasmids alone.

Histological detection of β-galactosidase--The expression of β-galactosidase was used to identify transfectants. PC12 cells were maintained in DMEM with 10% horse serum and 5% fetal calf serum on collagen-coated plates. Plasmids were transfected along with RSV-ß-gal (3 μg/plate) using lipofectamine (GIBCO/BRL) in serum free media. After four hours 10% serum was reintroduced. Sixteen hours later the cells were washed and placed in serum free N2 media. The transfected cells were exposed to NGF (50 ng/ml) or 8-CPT-cAMP (175 μM) for 2 days prior to fixation. PC12 cells were fixed in 4% paraformaldehyde and 0.2% glutaraldehyde for 5 minutes after which cells were washed in PBS and subjected to a β-galactosidase assay. Cells were incubated in PBS containing 2 μM $MgCl_2$, 5 μM Ferric cyanide, 5 μM Ferrous cyanide, and 0.1% X-gal in overnight at 37 °C. Transfected cells were identified as those staining blue and were then counted to determine the percent of blue cells with neurites in each set of transfections. Each set of transfections was done in duplicate and at least 200 cells were counted for each experimental condition.

RNA Isolation, Riboprobe synthesis, and Northern blot analysis--RNA was isolated using RNAzol B (TEL-TEST, Inc. Friendswood, Texas) per the instructions of the manufacturer. Stromelysin (transin) riboprobes used to detect transin mRNA were synthesized following linearizing pGEM-TR1 with Hind III by using T7 RNA polymerase to make antisense RNA transcripts. Northern blotting using transin riboprobe has been

previously described (Yao et al., 1995). MKP2 riboprobe synthesis and Northern blotting using this cRNA probe were done as previously described (Misra-Press et al., 1995). All filters were scanned and quantitated using a Molecular Dynamics PhosphorImager 445SI.

ERK immune complex assay--Treated or untreated cells were lysed in a lysis buffer containing 10% Sucrose, 1% NP-40, 20 μM Tris-HCl (pH 8.0), 137 μM NaCl, 10% glycerol, 2 μM EDTA, 1 μM PMSF, 1 μg/ml leupeptin, 1 μM sodium vanadate, and 10 μM sodium fluoride. The lysates were spun at low speed to remove debris and the supernatant was assayed for ERK1 activity. One hundred μg of protein (as determined by Bradford Assay) from the supernatant was immunoprecipitated with an agarose-coupled antibody to ERK-1(C-16) (Santa Cruz Biotechnology Inc.) overnight at 4 °C. The immunoprecipitates were washed 3 times in lysis buffer and assayed for kinase activity by incubating with 25 μg myelin basic protein (MBP) and 10 μCi γ-^{32}P-ATP in 60 μl of buffer containing 40 μM Hepes (pH7.4), 40 μM $MgCl_2$, 0.1 μM ATP, 4 μM sodium vanadate, and 10 μM sodium fluoride for 30 minutes at 30° C. Reactions were terminated by the addition of 60 μl of 2X Laemmli sample buffer and analyzed by SDS-PAGE. Quantitations were performed by scanning the gel using a Molecular Dynamics PhosphorImager 445SI.

PKA Assay--Following treatment with the indicated reagents, PC12 cells were harvested and soluble PKA was measured as previously described (Carr et al., 1993). Kinase reactions were incubated for two minutes at 30° C in the presence or absence of 10 μM cAMP.

GTP loading--For GTP loading studies, PC12 cells were transfected with 15μg of poly-histidine-tagged Rap1b (6X-His-Rap), together with or without cPKI, using calcium phosphate transfection kit (GIBCO/BRL). Following transfection, cells were labeled with [^{32}P]-orthophosphate as described (Vossler et al., 1997). Rap1 was precipitated with Ni-NTA Agarose and GTP loading was assayed as described (Urano et al., 1996) with the

addition of a preclearing step using activated charcoal. Nucleotide samples were spotted on a PEI cellulose chromatography plate along with GTP and GDP standards (Sigma, St. Louis), resolved in 1M KH_2PO_4 (pH3.4), at room temperature, and analyzed using a Molecular Dynamics PhosphorImager 445SI. The GTP fraction was calculated as follows: (GTP counts/3)/[(GTP counts/3) +(GDP counts/2)].

Immunofluorescence--PC12 cells were seeded onto Permanox chamber slides (Nunc, Inc.) at 30-40% confluency, serum starved for 16 hours and treated with or without NGF (50 ng/ml) for 90 minutes. H89 (10 μM) was added 15 minutes prior to NGF, as indicated. Cells were fixed in 70% ethanol, 50 μM glycine, pH 2.0 at -20°C for 20 minutes. The cells were incubated with ERK1 antisera (1:400 dilution) or normal rabbit sera (1:400 dilution) in PBS containing 0.5% goat serum for 1 hour at room temperature. After five washes with PBS, cells were incubated with rhodamine-conjugated anti-rabbit IgG (1:800 dilution) for 1 hour at room temperature and were visualized using a Leitz DMRB microscope (Leica, Inc.).

RESULTS

Activation of ERK1 by cAMP and NGF is reduced by the inhibitor of PKA, H89-- Both NGF and agents that activate PKA, including forskolin and the cAMP analog 8-(4-chlorophenylthio)-cyclic AMP (8-CPT-cAMP), induce sustained activation of ERK1 in PC12 cells (Fig. 4.1). ERK1 activation by forskolin peaked by 20 minutes and remained elevated for at least one hour. 8-CPT-cAMP induced a similar induction of ERK1 (Fig. 4.1A). The activation of ERK1 by forskolin was completely blocked by an inhibitor of PKA, H89 (Chijiwa et al., 1990), (Fig. 4.1A), suggesting that cAMP's actions on ERK1 require PKA. NGF's activation of ERK1 was inhibited at multiple time points (20, 40, and 60 minutes of stimulation) in the presence of the PKA inhibitor H89 (Fig. 4.1B, C).

In contrast, EGF's activation of ERKs was not blocked by H89 at 5 or 20 minutes (and only minimally blocked at 10 minutes) in the data presented in Fig. 4.1D, suggesting that H89 was preferentially acting on kinases downstream of NGF at this concentration.

NGF activation of ERK1 is reduced by the protein kinase inhibitor PKI--PKA can be inhibited by the protein kinase inhibitor (PKI), a physiological inhibitor of PKA (Day et al., 1989; Patten et al., 1992). To test whether this specific inhibitor of PKA could alter NGF activation of ERK1, we treated wild type PC12 cells with a peptide corresponding to PKI sequences from amino acid 5 to 22 that has been shown to be a specific inhibitor of PKA (Day et al., 1989; Patten et al., 1992). This peptide was modified by the addition of a stearyl group at the amino terminus (sPKI) to allow penetration into the cell (Vijayaraghavan et al., 1997). *In vivo*, sPKI inhibited NGF stimulation of ERK1 minimally at early time points, but showed significant inhibitory effects at later time points (20 and 40 minutes) compared with NGF-treated cells not receiving peptide (Fig. 4.2A). Addition of sPKI *in vitro* completely inhibited 8-CPT-cAMP stimulated PKA activity (Fig. 4.2A, insert), demonstrating that the addition of the stearyl group did not interfere with the ability of PKI to inhibit PKA catalytic activity. Unrelated peptides that contained the stearyl modification did not alter NGF's ability to activate ERK1 at the time points examined[1], suggesting that the inhibition by sPKI was not due to a toxic effect of peptide. sPKI completely inhibited the activation of ERK1 by forskolin at 20 minutes (Fig. 4.2A).

NGF induction of sustained activation of ERKs is reduced in PKA-deficient PC12 cells --The requirement of PKA in NGF's activation of ERKs was also examined in a PC12 line which is deficient in cAMP responses, A126-1B2 cells (VanBuskirk et al., 1985). A126-1B2 cells contain near wild type levels of type I PKA, but have greatly reduced levels of type II PKA. Furthermore, these mutant cells display altered PKA type I and type II regulatory subunits, as judged by ion-exchange chromatography. These alterations, as well as alterations in specific PKA anchoring proteins (Cassano et al.,

1996), greatly reduce the cells' response to cAMP (VanBuskirk et al., 1985). In these cells, NGF's activation of ERK1 was reduced at 20, 40, and 80 minutes, compared to wild type cells, but appeared unchanged at early time points (2, 5, 10 minutes) (Fig. 4.2B, upper and middle panels). The activation of ERK1 by 8-CPT-cAMP in these cells was blunted in these cells (Fig. 4.2B, lower panel). Western blotting demonstrated that the decrease in ERK1 activation following NGF stimulation in A126-1B2 cells was not due to the altered expression of ERK1[1]. These results, together with the previous data, demonstrate that PKA is required for maximal activation of ERK1 by NGF, particularly at later time points.

Maximal activation of Elk-1 by NGF requires PKA--The transcriptional effects of ERKs are mediated, in part, by its activation of Elk-1, a member of the Ets family of transcription factors and a component of the serum response factor (SRF) (Gille et al., 1995; Janknecht et al., 1993; Marais et al., 1993). To determine whether PKA was required for activation of Elk-1 by NGF, we examined the activation of Elk-1 directly in a mammalian two hybrid system, that measured the transactivation of a 5xGal4-E1b/luciferase gene by an Elk-1/Gal4 fusion protein (Roberson et al., 1995; Vossler et al., 1997). These plasmids were co-transfected with or without cDNA encoding the PKA inhibitor, PKI (cPKI), or an inactive mutant of PKI ($cPKI^{mut}$) (Day et al., 1989). The maximal activation of Elk-1 by both NGF and 8-CPT-cAMP was blocked by PKI, but not blocked by the expression of $cPKI^{mut}$ (Fig. 4.3A). In contrast, the activation of Elk-1 by EGF was not blocked by either wild type or mutant PKI. Therefore, PKI specifically interferes with the activation of Elk-1 by NGF and 8-CPT-cAMP, but not EGF, demonstrating that NGF activation of Elk-1 is mediated, in part, by PKA.

Maximal activation of the "immediate-early" gene MKP-2 by NGF requires PKA--To examine further the requirement of PKA for NGF regulated gene expression, we chose to

study the immediate early gene MKP-2. It has been previously shown that MKP2 expression is rapidly increased in response to NGF in PC12 cells (Misra-Press et al., 1995). Its gene product, MAP kinase phosphatase-2, dephosphorylates and inactivates multiple members of the MAP kinase family including JNKs (cJun N-terminus Kinase) and p38 *in vitro* and *in vivo* (Enslen et al., 1996; Hirsch and Stork, 1997; Misra-Press et al., 1995). As JNKs and p38 trigger cell death in PC12 cells (Xia et al., 1995), these actions of MKP-2 have been proposed to mediate the neurotrophic action of these agents (Hirsch and Stork, 1997). MKP-2 expression in PC12 cells is also induced by a variety of other neurotrophic agents that activate ERKs, including fibroblast growth factor, insulin and cAMP[2]. Since the induction of MKP-2 mRNA by NGF may require ERK activation, we examined the role of PKA in this process. The level of MKP-2 mRNA following NGF stimulation of wild type PC12 cells was reduced significantly by H89 (Fig. 4.3B, left panel). In addition, in the PKA-deficient cell line A126-1B2, MKP-2 induction by NGF was also reduced compared to wild type PC12 cells (Fig.3B, right panel).

Maximal induction of the "late" gene transin by NGF requires PKA--The expression of the metalloprotease transin (stromelysin) is stimulated by NGF, but not EGF, and has been used as a marker for neuronal differentiation of PC12 cells(Machida et al., 1991; Nordstrom et al., 1994; Shackleford et al., 1993). Its induction by NGF is dependent on Ras, the MAP kinase kinase kinase Raf, and ERKs (D'Arcangelo and Halegoua, 1993a; Yao et al., 1995). It has been shown that this induction requires multiple transcription factors, including those of the Ets family (deSouza et al., 1995; Kirstein et al., 1996). NGF induces low levels of transin mRNA in PC12 cells within 4 hours (Nordstrom et al., 1994) and by 24 hours of stimulation, expression levels could be easily detected by Northern Blot (Fig. 4.3C). 8-CPT-cAMP could not stimulate the expression of transin in the absence of additional agents (Nordstrom et al., 1994) (Fig. 4.3C). However, 8-CPT-cAMP dramatically enhanced NGF's ability to stimulate transin (data not shown),

suggesting that NGF and PKA are synergistic in their induction of transin gene expression. The synergistic action of NGF and 8-CPT-cAMP on transin expression reflects the action of these agents on neurite outgrowth as well (Connolly et al., 1984; Gunning et al., 1981; Heidemann et al., 1985). The participation of PKA-signaling in NGF's induction of transin mRNA was examined using H89. Preincubation with H89 blocked the induction of transin mRNA by NGF, suggesting that PKA activity was required for NGF's induction of this gene (Fig. 4.3C). Neither treatment with 8-CPT-cAMP or H-89 alone stimulated transin mRNA to detectable levels (Fig. 4.3C). These data demonstrate that maximal induction of specific immediate early and late genes by NGF may require PKA.

NGF does not stimulate detectable PKA kinase activity in PC12 cells--Since we observed the involvement of PKA in NGF's signaling, we determined whether NGF could stimulate total cellular PKA activity directly. Based on the kinetics studies shown in Fig. 4.2, we treated cells with NGF over a time course extending from 5 to 40 minutes and examined total PKA activity retained within lysates prepared from those cells. We could not detect increases in PKA activity following NGF treatment at any of the time points examined (Fig. 4.4A). Similar results were seen following EGF treatment (Fig. 4.4B).. In contrast, 8-CPT-cAMP induced a five-fold increase in PKA activity at the time points examined (Fig. 4.4B). The activity within all lysates could be stimulated *in vitro* by cAMP except cells pretreated with H89, demonstrating the presence of PKA and action of H89 on PKA in this experiment (Fig. 4.4B). These results are consistent with previous reports showing that adenylyl cyclase and PKA activities are not significantly increased following NGF stimulation of these cells (Balbi and Allen, 1994; Race and Wagner, 1985). Taken together, these data raise the possibility that a fraction of the total cellular pool of PKA is activated by NGF. Alternatively, basal activity of PKA may play a permissive role in NGF regulation of ERKs.

NGF activation of the B-Raf activator Rap1 requires PKA--PKA's activation of ERK in PC12 cells requires Rap1, a small GTP binding protein in the Ras superfamily. Rap1 is a selective activator of one of isoforms of MAP kinase kinase kinase, B-Raf, and the expression of B-Raf is required for Rap1 to activate ERKs (Vossler et al., 1997). We show data that suggest that NGF utilizes PKA to activate ERK. It is possible that PKA participates in NGF's stimulation of ERK via its actions on Rap1. To test this hypothesis, we examined the GTP loading of transfected histidine tagged Rap1 (6X-His-Rap) following transfection in PC12 cells. Both 8-CPT-cAMP and NGF increased GTP loading of Rap1 in this assay (Fig. 4.5), raising the possibility that Rap1 participates in NGF signaling. Activation of Rap1 by both NGF and 8-CPT-cAMP was reduced to basal levels by the co-transfection of cPKI, suggesting that PKA contributes to the activation of Rap1 by both NGF and 8-CPT-cAMP. Since Rap1 mediates PKA's ability to activate ERKs in PC12 cells (Vossler et al., 1997), the activation of Rap1 by PKA may contribute to NGF's activation of ERKs in these cells.

PKI can block the induction of neurites induced by 8-CPT-cAMP but not NGF--We have previously shown that Rap1 activation is not required for the elaboration of neurites seen following NGF treatment of PC12 cells (Vossler et al., 1997). However, it is possible that PKA may have other actions in NGF-treated cells that are required for the differentiation phenotype. To determine whether neuronal differentiation by NGF was dependent on PKA, we transfected cPKI into wild type PC12 cells along with the marker RSV-ßgal (ß-gal). The detection of ß-gal expression permitted the identification of transfected cells (stained blue) using histochemical methods. Cells were treated with 8-CPT-cAMP or NGF and assayed for neurite outgrowth and ß-galactosidase expression as described (Vossler et al., 1997; Yao et al., 1995). Both NGF and 8-CPT-cAMP induced neurites in cells that did not express ß-gal (unstained) (74% and 65%, respectively). The same percentages of neurite outgrowth were seen in NGF- or 8-CPT-cAMP-treated cells

that had been transfected with RSV-ßgal in the absence of additional cDNAs[1] (Vossler et al., 1997; Yao et al., 1995). The expression of PKI reduced the percentage of neurites following 8-CPT-cAMP treatment (65% to 10%), while the expression of PKI minimally reduced the percentage of neurite in NGF treated cells (74% to 63%) (Fig. 4.6). This is consistent with previous reports demonstrating that NGF was able to differentiate the PKA-deficient A126-1B2 cells (Ginty et al., 1991). Therefore, although PKA contributes to the sustained activation of ERK1 by NGF in PC12 cells, it is not required for neurite outgrowth induced by NGF in these cells.

H89 blocks nuclear translocation of ERK1 by NGF--Nuclear translocation of ERKs in PC12 cells has also been associated with their sustained activation and can be detected by immunofluorescence following NGF, but not EGF treatment of these cells (Cowley et al., 1994). Nuclear localization of ERK1 was seen 90 minutes following NGF treatment (Fig. 4.7C). In untreated cells, ERK1 was mainly in cytoplasm (Fig. 4.7A). The nuclear staining seen following NGF treatment was blocked by pretreatment with H89 (Fig. 4.7E), suggesting that PKA was required for this action of NGF. Parallel samples, prepared and incubated with normal rabbit serum, showed no non-specific staining (Fig. 4.7B,D,F).

DISCUSSION

NGF's ability to stimulate differentiation of PC12 cells has been associated with a sustained activation of ERKs. In contrast, EGF's activation of ERKs is rapidly terminated (Marshall, 1995). The signaling pathways that are selectively activated by NGF to permit sustained activation of ERKs have not been fully elucidated. PKA also stimulates neuronal differentiation of PC12 cells and induces a sustained activation of ERKs (Young et al., 1994). In this study we examined the possibility that PKA contributes to the sustained activation of ERKs seen following NGF treatment. We show that maximal activation of ERKs by NGF was diminished in PC12 cells in three independent assays, using two

inhibitors of PKA, PKI and H89, and in a clonal isolate of PC12 cells that is deficient in cAMP signaling.

PKA has been previously proposed to be required for a subset of NGF's actions in PC12 cells (Damon et al., 1990). Inhibiting PKA blocks selected actions of NGF at transcriptional (43, 44) as well as post-transcriptional levels (Cremins et al., 1986; Kalman et al., 1990). NGF can augment cAMP production in some neuronal cells (Berg et al., 1995), and has been reported to stimulate the production of cAMP in PC12 membranes(Knipper et al., 1993; Schubert et al., 1978). However, neither the direct demonstration of an increase in adenylyl cyclase nor in PKA activity by NGF in PC12 cells has not been established (Balbi and Allen, 1994; Race and Wagner, 1985). We were also not able to detect PKA activation by NGF in PC12 cells (at 5, 10, 15, 20, and 40 minutes), raising the possibility that only a fraction of the total cellular PKA might be activated by the NGF signaling complex. This may be achieved by the activation of a distinct subcellular pool of PKA, either directly or indirectly, through the regulation of specific adenylyl cyclases or phosphodiesterases (Houslay and Milligan, 1997). Alternatively, PKA might be recruited to specific subcellular compartments in response to NGF to form a signal complex with activators of downstream effectors of NGF, such as Rap1. Subcellular localization of PKA with specific signaling complexes may be achieved via specific targeting proteins. For example, PKA may be anchored to multienzyme complexes via a diverse family of PKA anchoring proteins, or AKAPs (Dell'Acqua and Scott, 1997; Faux and Scott, 1996; Vijayaraghavan et al., 1997). In addition, the adapter molecule Grb2 has been implicated in targeting PKA to growth factor receptors (Tortora et al., 1997). Other kinases are also targeted to components of the NGF receptor signaling complex (Canossa et al., 1996; Volonté et al., 1993). One of these kinases, protein kinase N (PKN), has been reported to activate PKA directly (Volonté and Greene, 1995; Volonté et al., 1993). Therefore, it is possible that PKA may participate in a signaling complex through a direct

interaction with these proteins that associate with the NGF receptor/tyrosine kinase cascade.

In PC12 cells, NGF induces a sustained activation of ERKs that is associated with neuronal differentiation (Marshall, 1995). The inhibition of PKA by H89 or PKI in these cells or through the use of PC12-derived PKA-deficient cells blocked the sustained activation of ERKs by NGF significantly more than it blocked the rapid, initial portion of ERK activation. However, neither method of PKA inhibition interfered with NGF's ability to induce differentiation (Fig. 4.6) (Ginty et al., 1991; Richter-Landsberg and Jarstoff, 1986; Rydel and Greene, 1988; Yao and Cooper, 1995). Therefore, PKA does not appear to be required for NGF's induction of neurites in PC12 cells. Furthermore, while sustained activation of ERK is sufficient for triggering differentiation, it is not necessary (Peng et al., 1995; Wu and Bradshaw, 1996). Other signals than ERK activation might also be required for triggering this process, as suggested by others (Montminy et al., 1986).

The ability of PKA to augment NGF signaling may be most important in the regulation of gene expression during differentiation. Previous reports have suggested that the induction of selected genes and proteins by NGF requires PKA (Balbi and Allen, 1994; Brady et al., 1990; Cremins et al., 1986; Damon et al., 1990; Kalman et al., 1990; Machida et al., 1991). Much of PKA's transcriptional actions in neuronal cells are mediated by specific sites present in the promoter of many cAMP-responsive genes, called cAMP responsive elements (CREs) (Montminy et al., 1986). Transcriptional activation by cAMP can occur via phosphorylation of a PKA-responsive site within the trans-activation domain of the CRE binding protein, CREB (Gonzales et al., 1989). Another transcription factor, Elk-1, can also be activated by PKA in neuronal cells via PKA's activation of the MAP kinase cascade (Vossler et al., 1997). Elk-1 is a member of the Ets family of transcription factors

and is activated by ERKs by direct phosphorylation (Gille et al., 1992; Janknecht et al., 1993; Marais et al., 1993). Stimulation of ERK and Elk-1 is required for full activation of the serum response element (SRE) within the fos promoter (Cavigelli et al., 1995; Hill and Treisman, 1995; Hipskind et al., 1994) and other immediate-early genes that are activated by NGF (Greenberg et al., 1985; Millbrandt, 1986). We show here that maximal activation of Elk-1 by NGF, but not EGF, requires PKA. Therefore, PKA may contribute to the specificity of NGF's transcriptional effects via its activation of Elk-1.

PKA modulates NGF's induction of representative "early" and "late" genes, as well. We show here that one immediate early gene, MKP-2, also requires PKA for its maximal induction by NGF. The induction of "late" genes by NGF may also be regulated by PKA. One example is transin, whose expression is a marker for neuronal differentiation (Fillmore et al., 1992; Machida et al., 1991; Shackleford et al., 1993). This may be mediated, in part, by NGF's activation of ERKs since the activation of the ERK substrate, Ets, has been implicated in transin activation (Kirstein et al., 1996; Wasylyk et al., 1991; Yao et al., 1995).

One mechanism by which PKA might influence NGF signals to the nucleus is via regulation of nuclear translocation of ERKs. We have previously demonstrated that forskolin, and activator of adenylyl cyclase could promote the nuclear translocation of ERKs in EGF-treated PC12 cells (Yao et al., 1995). Here, we show data that suggest that PKA may be required for the translocation of ERKs by cells treated with NGF alone. Since nuclear translocation of ERKs correlates with their sustained activation, PKA's involvement in NGF signaling may be a consequence of its ability to augment the sustained activation of ERKs by NGF. It is also possible that PKA may have additional protein targets that help regulate the subcellular localization of ERKs in a manner that is independent of its action of ERK activity. In either case, the ability of PKA to increase the

nuclear concentration of ERK proteins following NGF treatment may account, in part, for the PKA-dependent activation of Elk-1 by NGF, as well as NGF's induction of the genes encoding transin and MKP-2.

Although Ras is required for the actions of both NGF and EGF on ERKs in PC12 cells, the mechanisms that distinguish NGF and EGF signaling to ERKs are not known. In particular, it is not known how NGF can activate ERK for extended periods. The activation of Ras by both EGF and NGF is rapidly terminated, suggesting that sustained activation of ERKs by NGF involves pathways that are downstream or independent of Ras. We propose that the activation of PKA by NGF allows NGF to activate Ras-independent pathways that are not rapidly terminated. One potential Ras-independent pathway that is activated by PKA involves the small G protein Rap1. Rap1 has been recently shown to be required for the sustained activation of ERKs by PKA in PC12 cells (Vossler et al., 1997). We show here that Rap1 is activated following NGF treatment and that this activation requires PKA. The ability of NGF to activate Rap1 distinguishes it from EGF (Vossler et al., 1997). Rap1 activation stimulates ERKs in PC12 cells via its direct activation of the MAP kinase kinase kinase B-Raf, the only known effector of Rap1 (Fig. 4.6) (Vossler et al., 1997). We suggest that the activation of Rap1 by NGF may account for the high level of B-Raf activity seen following NGF treatment of PC12 cells (Jaiswal et al., 1994; Wixler et al., 1996). We propose that NGF's activation of ERK may be enhanced by its activation of Rap1, via the action of PKA.

Acknowledgments--We are grateful to Dr. Richard Maurer, Dr. Gary Ciment for cDNAs, to Dr. Richard Maurer and Dr. John Scott for reading of the manuscript and to Heidi Conklin and Caroline Rim for excellent technical assistance.

FOOTNOTES

[1] H. Yao and P. J. S. Stork, unpublished observations.

[2] A. Misra-Press, H. Yao and P. J. S. Stork, unpublished observations

[3] R. H. York, H. Yao and P. J. S. Stork, manuscript in preparation (See York et al., 1998)..

FIGURE LEGENDS

FIG. 4.1. **Activation of ERK1 by cAMP and NGF and inhibition by PKA inhibitors.** A, Time course of forskolin's and 8-CPT-cAMP's activation of ERK1 and blockade by the PKA inhibitor H89. PC12 cells were treated with 8-CPT-cAMP (175 μM) (white squares), forskolin (10μM) (black diamonds), or forskolin and H89 (10μM) (gray squares) for the times indicated. Immune complex kinase assays were performed using myelin basic protein (MBP) as a substrate following ERK1 immunoprecipitation, as described in Materials and Methods. After resolution with SDS-PAGE, the MBP bands were analyzed by a PhosphorImager (Molecular Dynamics). The results are reported as fold increase over basal. Standard error is indicated (n=4). B, Time course of NGF's activation of ERK1 and blockade by the PKA inhibitor H89. PC12 cells were treated with NGF (50ng/ml) (white squares; n=6) or NGF and H89 (10μM) (black diamonds; n=4) for the time indicated (top panel). ERK1 kinase assay and quantitation were performed as described in A. The results are reported as fold increase over basal. Standard error is indicated. C, An example of a single experiment shows the phosphorylation of MBP by ERK1 following stimulation of PC12 cells by NGF, in the absence of H89 (top panel; NGF) or the presence of H89 (bottom panel; NGF + H89). D, Autoradiograms showing the phosphorylation of MBP by ERK1 following stimulation of PC12 cells by EGF. Top panel: the time course of EGF's actions on ERK1 in the absence of H89 (EGF). Bottom panel: the time course of EGF's actions on ERK1 in the presence of H89 (EGF + H89). The position of the phosphorylated MBP is indicated.

FIG. 4.2. **ERK1 activation by NGF is blunted in A126-1B2 cells and inhibited by PKI peptide (sPKI) in PC12 cells.** A, Inhibition of NGF-stimulated ERK activation by PKI peptide. PC12 cells were pretreated with or without sPKI peptide (20 μM) for 20 minutes and then treated with NGF (50 ng/ml) or forskolin (10 μM) for the

time indicated. Cells were lysed at the indicated times and ERK1 kinase assays performed as described in Fig. 4.1A. Insert: The inhibition of PKA *in vitro* by purified PKI protein and stearyl modified PKI (sPKI) peptide is shown to control for the function of sPKI. H89 was utilized as a positive control for PKA inhibition. B, ERK1 activation in PKA-deficient cells. A126-1B2 cells were treated with NGF in the same way as with PC12 cells. Equal amounts of protein was assayed for ERK1 activity. The time course of ERK1 activation by NGF in these cells was compared with wild type PC12 cells. Note that NGF induced ERK1 activity at later time points was reduced in A126-1B2 cells. 8-CPT-cAMP's inability to activate ERKs in these cells is shown as a control.

FIG. 4.3. **Activation of transcription by NGF requires PKA.** A, Maximal activation of Elk-1 by NGF in PC12 cells requires PKA. NGF activation of Elk is blocked by transfection of a cPKI but not by PKI mutant ($cPKI^{mut}$). PC12 cells were transfected with plasmids encoding either cPKI (10 μg), $cPKI^{mut}$ (10 μg), or vector (10 μg) and Elk-1/Gal-4 (3 μg) and 5xGal4-E1b/luciferase (3 μg) plasmids and subsequently treated with NGF (50 ng/ml), EGF (100 ng/ml) or 8-CPT-cAMP (175 μM), as indicated. Elk-1 activation was monitored by luciferase activity. Standard error is shown (n=3). B, Expression of MKP-2 mRNA by NGF is blocked by H89 and blunted in PKA-deficient cells. Both PC12 cells and A126-1B2 were starved overnight prior to NGF treatment. They were pretreated with or without 10 μM H89 for 15 minutes before NGF treatment. Cells were then incubated with 50 ng/ml NGF for the indicated time period. RNA was prepared as described in Materials and Methods. 15 μg of RNA was loaded on the gel and subsequently subjected to Northern Blotting. Note that MKP2 mRNA induced by NGF was blocked at 1.5 and 2 hours by H89. MKP2 expression following NGF treatment was also blunted in A126-1B2 cells at every time points examined. C, Expression of transin mRNA by NGF is blocked by H-89. PC 12 cells were treated and RNA was prepared as in B. RNA samples from each treatment were Northern blotted with transin probe. 10 μM

H89 was added onto plates 15 minutes prior to NGF or 8-CPT-cAMP treatment in samples indicated.

FIG. 4.4. **NGF does not stimulate PKA kinase activity in PC12 cells.** PC12 cells were treated *in vivo* with 8-CPT-cAMP (175 μM), NGF (50 ng/ml), EGF (100 ng/ml) or H89 (10 μM) as indicated. All treatments refer to 10 minute incubations *in vivo*. In addition, each condition was either untreated (light bars) or treated with cAMP (dark bars) *in vitro*, to identify cAMP dependent protein kinase (PKA) activities within each lysate. The data are representative of two separate experiments. A, Time course of NGF treatment, at 0, 5 10, 15, 20, and 40 minutes. B, Lysates from cells left untreated or treated with EGF, 8-CPT-cAMP, or H89 for 5 or 15 minutes. Note that neither NGF nor EGF increased PKA activity from PC12 cells and 8-CPT-cAMP induced a five-fold increase in PKA activity. The data are presented as the percent of maximal stimulation of PKA activity measured from the untreated control (in the presence of cAMP *in vitro*).

FIG. 4.5. **GTP loading of Rap1 by cAMP and NGF is blocked by PKI.** GTP loading was assayed in PC12 cells following transfection of 6X His-Rap (15 μg), in the presence or absence of cPKI (10 μg). Cells were metabolically labeled with [^{32}P]-orthophosphate and treated with NGF or 8-CPT-cAMP for 10 or 30 minutes as indicated. Lysates were prepared as described in Materials and Method. His-Rap was precipitated with Ni-NTA Agarose and GTP and GDP content in the elutes were analyzed by thin layer chromatography. The GTP fraction of total guanine nucleotide is shown above each lane.

FIG. 4.6. **Inhibition of PKA does not interfere with neurite outgrowth.** A, PC12 cells were transfected with RSV-ß-gal and cPKI and treated with 8-CPT-cAMP or NGF for two days as indicated. Cells were then stained with X-gal as described in

Materials and Method. ß-gal was used as marker for transfected cells, therefore, blue cells represent transfected cells. Cells with neurites were counted from both blue and white cells. PKI and ß-gal-transfected cells were treated with 8-CPT-cAMP (left panel) or NGF (right panel). The percentage of neurite-bearing cells is included in the text.

FIG. 4.7. **Inhibition of PKA blocks nuclear translocalization of ERK1 by NGF.** PC12 cells were left untreated (A, B) or treated with NGF (50 ng/ml) for 90 minutes (C, D, E, F). Cells were treated with NGF alone (C, D) or following a 15 minute pretreatment with H89 (10 μM) (E, F). Following fixation, cells were incubated with ERK1 antisera (A, C, E) or normal rabbit serum (B, D, F), and visualized by fluorescent microscopy.

Fig. 4.1. Activation of ERK1 by cAMP and NGF and inhibition by PKA inhibitor

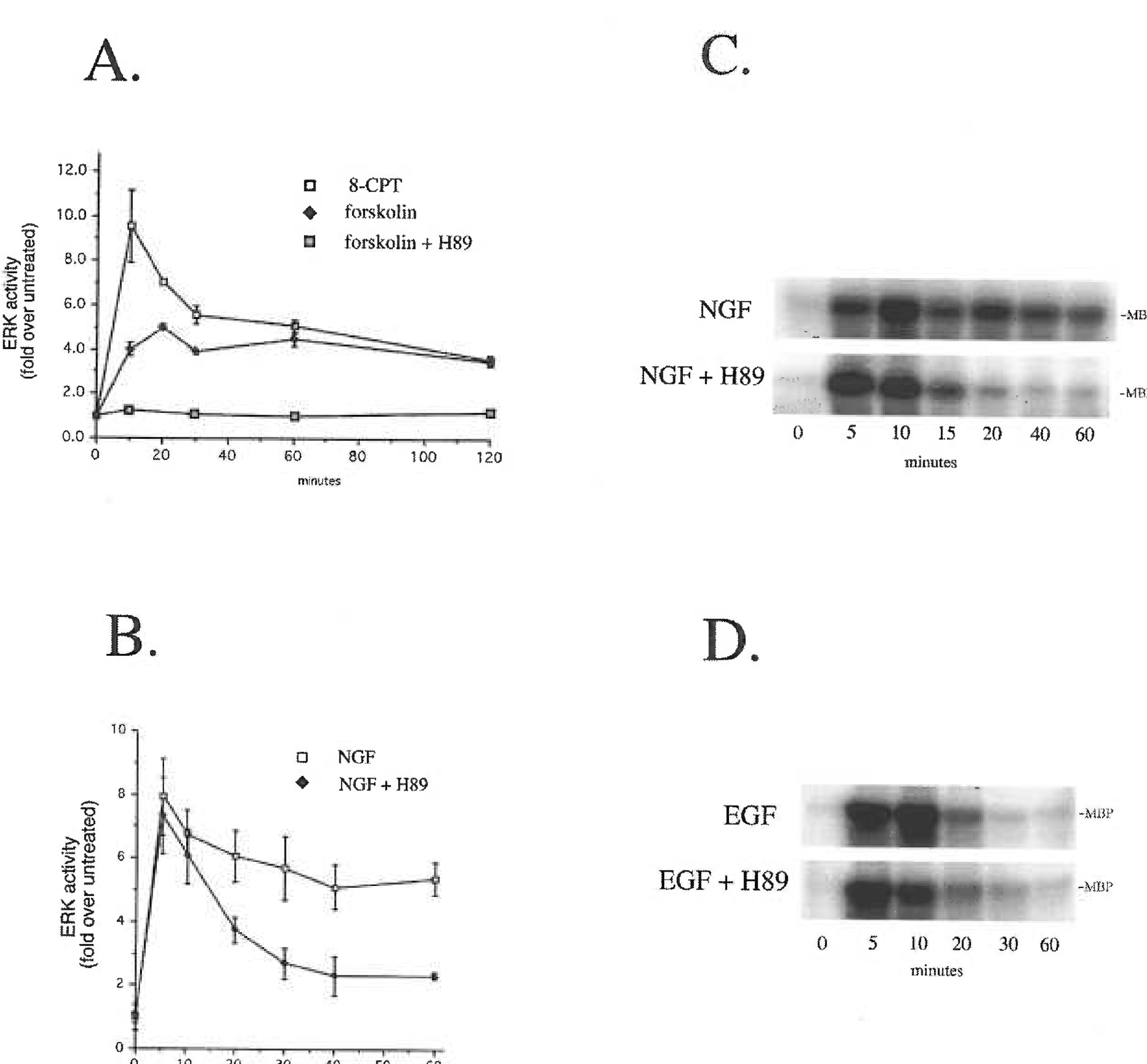
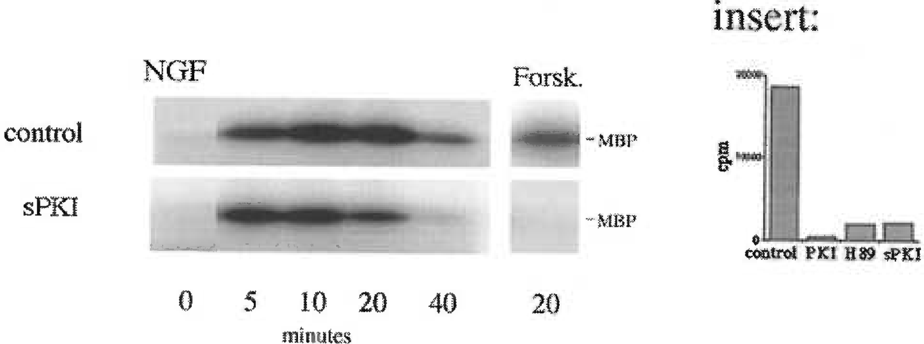


Fig. 4.2. ERK1 activation by NGF is blunted in A126-1B2 cells and inhibited by PKI peptide (sPKI) in PC12 cells



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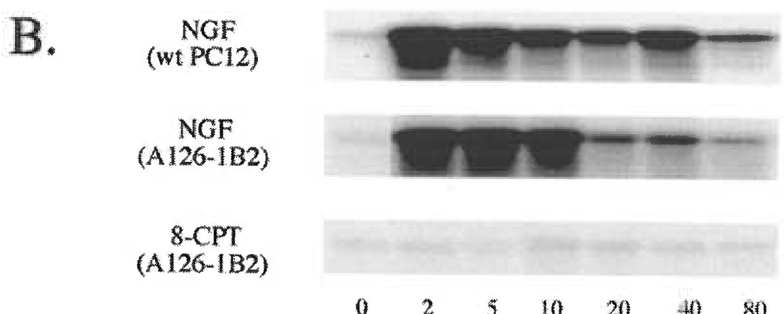
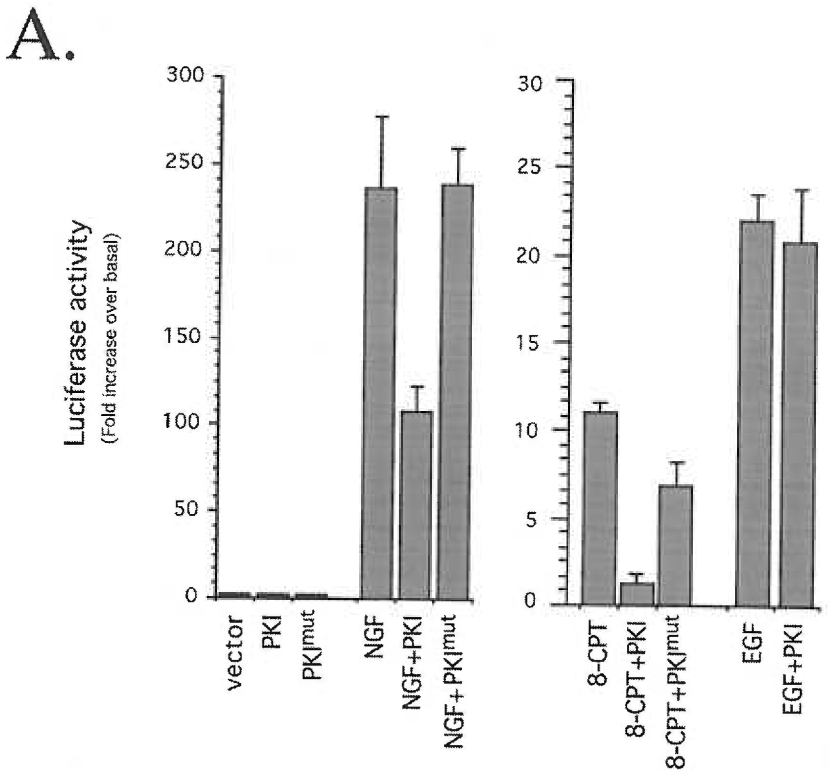


Fig. 4.3. Transciptional activation by NGF requires PKA

A.



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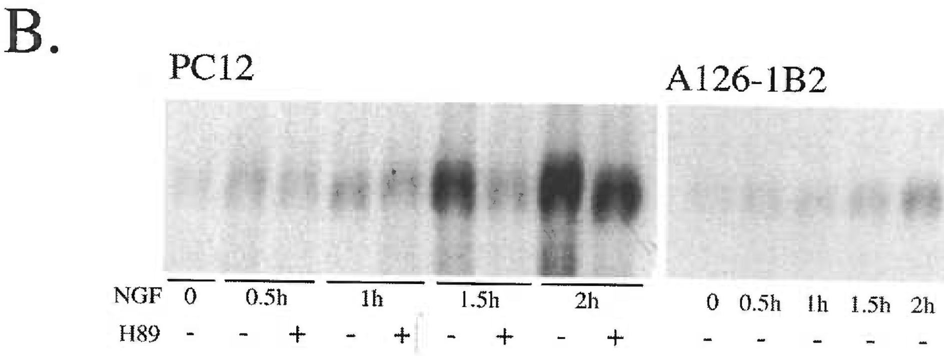

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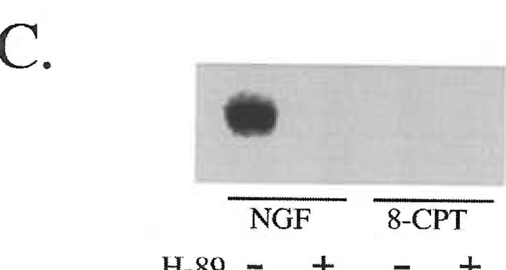
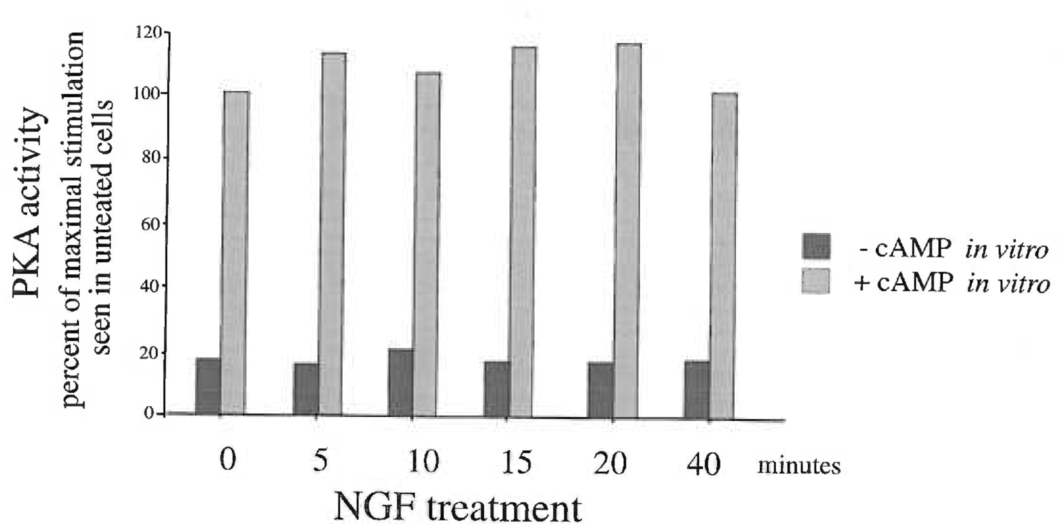


Fig. 4.4. NGF does not stimulate PKA activity in PC12 cells



B.

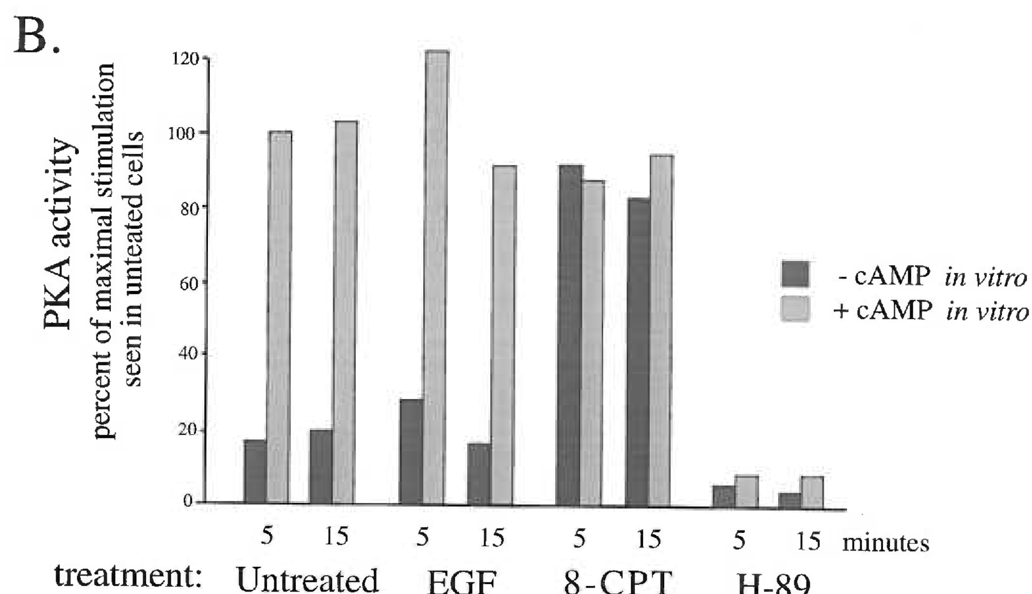


Fig. 4.5. GTP loading of Rap1 by cAMP and NGF is blocked by PKI

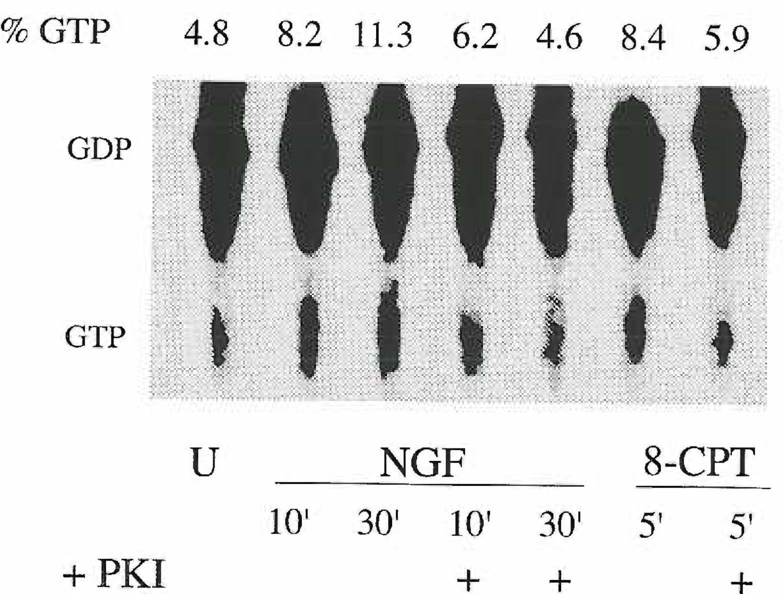


Fig. 4.6. Inhibition of PKA does not interfere with neurite outgrowth induced by NGF

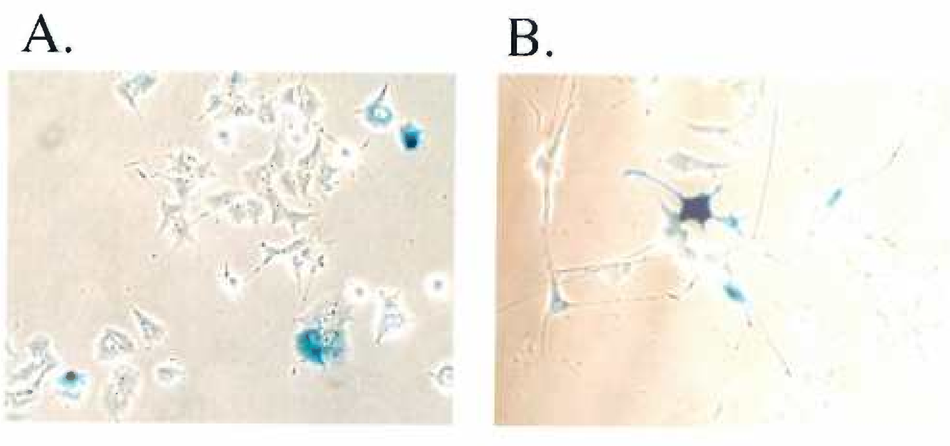
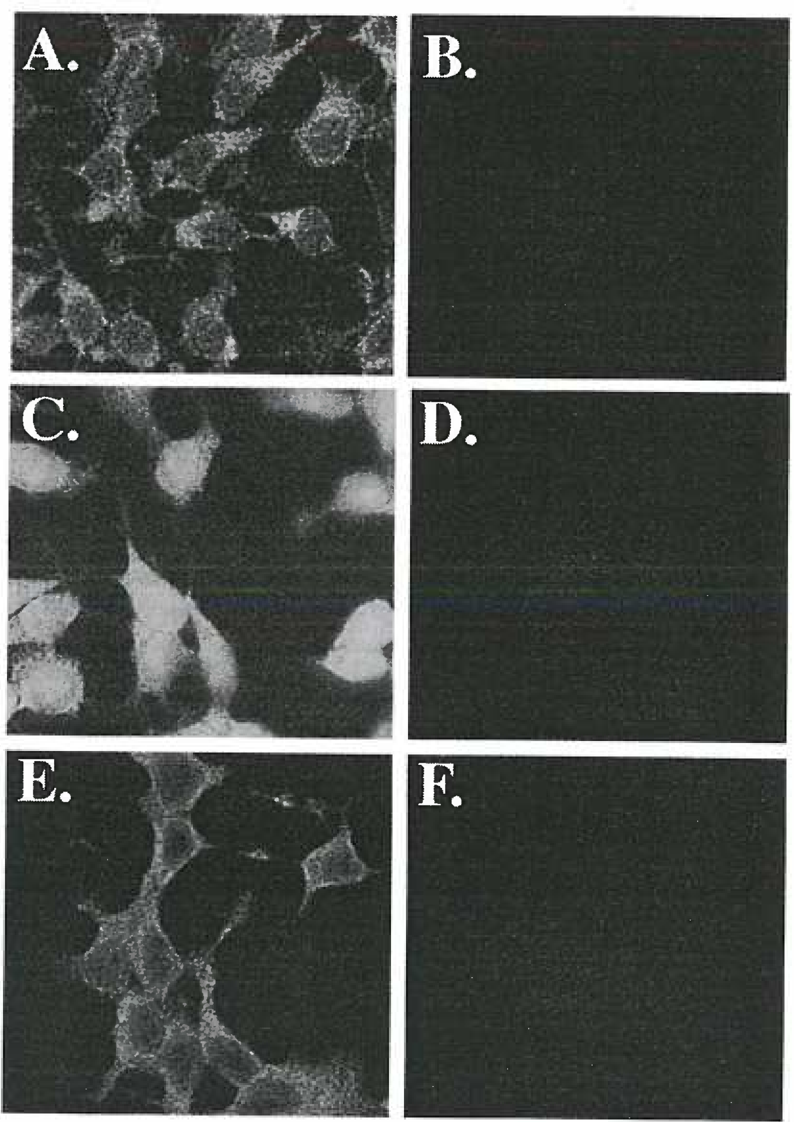


Fig. 4.7. Inhibition of PKA blocks nuclear translocation of ERK1 by NGF



Chapter 5

Summary and Discussion

5.1 cAMP can activate MAP kinase in a cell-type specific manner

The adenylyl cyclase activator forskolin and cAMP analogue 8-CPT can both activate cAMP dependent protein kinase PKA. In this study, I have shown that forskolin (Chapter 2) and 8-CPT (Chapter 3) can activate MAP kinases via PKA in PC12 cells. In addition, in these cells, these agents synergize with growth factors EGF and NGF to stimulate MAP kinase. This synergism is also reflected in the activation of MAP kinase-dependent transin gene expression (Chapter 2). These results are consistent with the ability of PKA to activate MAP kinase in other cell types following stimulation by the hormone which acts through Gs coupled receptors to increase intracellular cAMP concentrations (Englaro et al., 1995; Hazlerigg et al., 1996; Vossler et al., 1998). However, these actions of cAMP contrast to those seen in fibroblasts where cAMP antagonizes growth factor's activation of MAP kinases. Therefore, whether cAMP activates or inhibits MAP kinase is dependent on the cell type. In addition, the consequences of ERK activation *in vivo* are likely to be cell-specific. For example, MAP kinase activation in PC12 cells can lead to proliferation or differentiation. One of the parameters that dictates the physiological response to MAP kinase activation is the duration of that activation. Therefore, the time-course of ERK activation also needs to be considered when examining the consequence of MAP kinase activation in any given cell type. It is possible that ERK activity may need to attain a certain threshold in either magnitude or duration to stimulate a downstream response.

The physiological relevance of cAMP activation of MAP kinase is that cAMP is the intracellular messenger of a family of hormones that activate adenylyl cyclase. Hormonal activation of MAP kinase was also examined in PC12 cells by using the

hormone VIP (vaso-intestinal hormone) or the hormone analog isoproterenol. VIP and isoproterenol are well known activators of adenylyl cyclase through binding to VIP receptor and β-adrenergic receptors, respectively. Both of these agents are able to stimulate MAP kinase (Fig.3.2C). The ERK activation by these agents are blocked by PKA inhibitor H89, suggesting this stimulation is dependent on PKA. This involvement of PKA further suggests a role of Gsα in hormonal activation of ERK and is supported by the studies using microinjection of constitutively active Gsα mutants. The expression of constitutively active Gsα mutants in COS cells induces ERK activation (Faure et al., 1994). These cells, unlike other fibroblasts, express moderate level of B-Raf (Yao and Stork's unpublished data). Recently, however, it was reported that isoproterenol activates ERKs via β-adrenergic receptors through a Ras-dependent pathway in HEK293 cells utilizing βγ subunits (Crespo et al., 1995). Although these results may reflect a β-adrenergic action that is independent of cAMP, they do not address the mechanism by which cAMP activates MAP kinase. We propose that Gsα can couple to MAP kinase via PKA in a subset of cells through the MAP kinase kinase kinase, B-Raf, and the small G protein Rap1. This is further discussed below.

5.2 PKA activates MAP kinase via a novel pathway

In the middle section of this dissertation, I examined the mechanism by which cAMP activates MAP kinase in PC12 cells. Initial studies demonstrated that MAP kinase activation and neurite outgrowth by cAMP were independent of Ras and we identified a role for a related small G protein, Rap1, a known substrate of PKA in cAMP's actions. By using constitutively activated mutant of Rap1, RapV12, we demonstrated that Rap is sufficient for MAP kinase activation in PC12 cells. We also generated an the interfering mutant of Rap1, RapN17, which, like RasN17, has reduced binding affinity to guanine nucleotides (Berghe et al., 1997). Our results showed that RapN17 blocked 8-CPT-stimulated ERK activity, Elk-1 activation and neurite outgrowth in PC12 cells,

demonstrating that Rap1 is necessary for MAP kinase activation and neuronal differentiation by cAMP. This conclusion is supported by data showing that cAMP can activate Rap1 by stimulating its GTP loading (Chapter 3). PKA phosphorylates Rap1 (Chapter 3) on a single site, serine 179, near the carboxyl-terminus *In vivo* and *in vitro* (Altschuler and Lapetina, 1993; Lerosey et al., 1991; Quilliam et al., 1991). Replacing this serine residue with aspartic acid (which mimics phosphoserine) results in a mutant Rap1 which is able to activate MAP kinase in the absence of cAMP (Chapter 3). These data suggest that phosphorylation of Ser179 by PKA plays a part in Rap1 activation. This PKA phosphorylation site is adjacent to the site of geranyl-geranyl methyl ester modification that enables Rap1 to bind to the membrane and is neither part of the GTPase domain nor the effector domain. Replacing the cysteine at the lipid modification site prevents membrane localization and activation of Rap, demonstrating that like that of Ras, membrane attachment of Rap is required for activation by GTP loading. However, this soluble mutant is still capable of being phosphorylated by PKA *In vivo*. Therefore, PKA phosphorylation is not sufficient for Rap1 activation, unless it is membrane-bound. One possible model to explain why PKA can activate Rap only when it is membrane-associated is that an additional membrane protein is required for PKA's effects. Additional studies by our laboratory suggest that additional proteins might be involved in Rap1 activation by PKA (York et al. paper in press). Two potential candidates for this protein are the Rap-specific GEFs (Rap-GEFs), Smg-GDS (Kaibushi et al., 1991) and C3G (Gotoh et al., 1995) and Rap GAP. Rap GAP is a substrate of PKA *in vitro* (Polakis et al., 1992). It has been shown that C3G greatly and specifically enhances the GTP-loading of Rap1 (Berghe et al., 1997). PKA phosphorylation has been shown to sensitize Rap1 to the actions of the Rap-GEF Smg-GDS *in vitro* (Hata et al., 1991) and this mechanism has been proposed as an explanation for the increased GTP loading of Rap1 seen following PKA treatment of platelets and fibroblasts (Altschuler et al., 1995; Lerosey et al., 1991). On the other

hand, recent data in our laboratory suggest that PKA phosphorylation of Rap1 may not be necessary for PKA's ability to activate Rap1. Serine 179 and 180 can be replaced with alanines to generate a Rap1 mutant that is incapable of being phosphorylated by PKA. This mutant does not significantly block cAMP activation of MAP kinase nor does it block cAMP's stimulation of Rap1 GTP-loading and its association with B-Raf (York et al. paper in press). These results suggest that direct phosphorylation of serine 179 is not the only mechanism by which cAMP activates Rap1. Further studies will be required to determine all of the PKA targets that mediate Rap1 activation.

After the identification of the mechanism by which cAMP activates MAP kinase in PC12 cells, studies were carried out by other groups that demonstrated similar MAP kinase activation by hormones that utilize G-coupled receptors in other cell types. M. H. Verheijen and his colleague showed that parathyroid hormone (PTH) also has cell type specific action on MAP kinase via PKA (Verheijen and Defize, 1997; Verheijen and Defize, 1995). In osteosarcoma cells, this hormone inhibits MAP kinase (Verheijen and Defize, 1995). In contrast, in Chinese hamster ovary (CHO) R15 cells stably expressing the rat PTH/PTH-related peptide receptor, PTH stimulates MAP kinase also via PKA. This stimulation of MAP kinase by PTH was independent of Ras, Gi, phorbol ester-sensitive PKC, elevated intracellular calcium levels, or release of Gβγ subunits. Their findings further support our results of a Ras-independent action of cAMP.

5.3 A model for the cell type-specificity of Rap1 and cAMP action

cAMP has cell type specific effects on cell proliferation and transformation that correlate with its effects on MAP kinase. Rap1's actions correlate with those of cAMP. Like cAMP, Rap1 is present in all cells. This suggest that the cell-type specificity of these agents is generated by a downstream effectors. In this dissertation, I have presented

data demonstrating that the distinct B-Raf expression pattern in different cell types dictates cAMP and Rap1's ability to activate MAP kinase. B-Raf expression is restricted to certain tissues and cell types, such as brain, neuronal cells and endocrine cells. The tissue expression pattern of B-Raf correlates with the tissue specific actions of both cAMP and Rap1. In this study, pheochromocytoma PC12 cells and rat fibroblast NIH3T3 cells were used as models of B-Raf positive and negative cell lines, respectively. In PC12 cells, B-Raf is expressed to high levels as is Raf-1. However, in NIH3T3 cells, B-Raf is not detectable by western blotting. In PC12 cells, cAMP/PKA inhibits Raf-1 but stimulates B-Raf. This action of PKA is mediated by Rap1. Activation of Rap1 by either PKA or mutation of Gly12 of Rap1 to Val12 (RapV12) inhibits Raf-1 but stimulates B-Raf. In NIH3T3 cells, cAMP and Rap1's inhibitory effects on MAP kinase can be reversed by the introduction of exogenous B-Raf. Furthermore, a dominant negative mutant of Rap1, RapN17, inhibits B-Raf activation by PKA. These results demonstrate that Rap1 is necessary for B-Raf activation and B-Raf is sufficient for cAMP and Rap1's activation of ERKs. Another group has recently published a paper in which they demonstrated recombinant RapV12 can activate B-Raf *in vitro* (Kuroda et al., 1996; Ohtsuka et al., 1996). This result supports our finding *In vivo* that B-Raf is sufficient for Rap1 activation of MAP kinase. The strong evidence showing that B-Raf can convert both Rap1 and cAMP into activators of MAP kinase demonstrate that B-Raf is sufficient for this action of cAMP/Rap1. However, we have not yet established the necessity of B-Raf in these actions. Although interfering mutants of Raf-1 interrupted cAMP and Rap1 signaling to ERKs (Rim and Stork's unpublished data), we are not able to definitively demonstrate that B-Raf is required for cAMP's or Rap1's activation of MAP kinase, largely because available Raf interfering mutants are not specific for B-Raf. Further biochemistry studies on the B-Raf structure and functional domains need to be done to produce a specific B-Raf interfering mutant.

We believe that Rap1 may mediate, in part, the inhibition of Raf1 and MAP kinase cascade by PKA in B-Raf negative cells. It is possible that Rap1 may mediate, in part, the inhibition of Raf-1 and MAP kinase cascade by PKA in B-Raf negative cells. However, other models to explain PKA inhibition of MAP kinase signaling have been proposed. For example, Sturgill has proposed a model that PKA inhibits MAP kinase by direct phosphorylation of Ser43 in Raf-1 which he proposed uncouples Raf-1 from Ras. The strongest evidence that this model proposed by Sturgill is incomplete is that the replacement of Ser43 with Ala does not reduce PKA's inhibitory actions (Lee Graves, personal communication). At the present time, we are not able to determine whether the inhibition of Raf-1 by Rap is direct through its sequestration of Raf 1 from Ras or indirect through its competition for SOS. We propose a model that cAMP can activate MAP kinase in B-Raf expressing cells and Rap1 mediates cAMP's activation of MAP kinase. The PKA/Rap/B-Raf pathway represents a novel pathway leading to MAP kinase activation independently of Ras. We have demonstrated the existence of a signaling pathway involving the ubiquitous small G protein Rap1 and the tissue-specific Raf isoform, B-Raf. cAMP's actions on B-Raf are mediated, at least in part, through the action of PKA on Rap1. We predict that cells where cAMP activates MAP kinase will express B-Raf, and that Rap1 will activate, rather than inhibit, MAP kinase cascade, by activating B-Raf in these cells. In another words, the tissue specific expression of B-Raf dictates whether cAMP and Rap1 activate or inhibit MAP kinase. Therefore, differential B-Raf expression adds another level of specificity to signal transduction pathway. Moreover, since Rap1 antagonizes Ras in B-Raf-negative cells, the temporal and spatial regulation of B-Raf expression may provide a powerful developmental switch that converts negative proliferative signals into positive ones.

There are at least three implications that follows from our studies of Rap1/B-Raf signaling. First, we have shown that the physical association of Rap1 and B-Raf is not sufficient for full activation of B-Raf by Rap1 suggesting two components to B-Raf activation. Therefore, it is possible that this is a common mechanism for the activation of other Raf isoforms including the activation of Raf-1 by Ras. For both Raf-1 and B-Raf, it will be important to identify other potential proteins or factors involved in their full activation by small G proteins. Second, because B-Raf expression can change cAMP's specificity, the differential expression of B-Raf during development might dictate differential proliferative responses to external signals that activate Rap1. The same signals could have the opposite effect in B-Raf deficient-cells. Through this mechanism, Rap/B-Raf might provide a spatial and temporal control of organogenesis. Third, in addition to cAMP, other signals can also utilize Rap/B-Raf pathway. For example, we have recently demonstrated that NGF can also activate Rap1/B-Raf. In addition, it has also been shown that CaM kinase IV phosphorylates Rap1 at the same site as PKA *in vitro* (Sahyoun et al., 1991). It is likely that these stimuli can activate Rap1/B-Raf pathways to regulate specific physiological functions *In vivo.*

5.4 Growth factor specificity

We have recently examined the possibility that NGF utilizes PKA and Rap1 to induce sustained activation of MAP kinase. This idea is based on two observations: 1) both NGF and cAMP both activate MAP kinase for sustained periods; 2) both agents preferentially activate B-Raf (Chapter 3; Jaiswal et al., 1994; Jaiswal et al., 1996). We have addition experimental evidence suggesting that PKA is important for NGF signaling to MAP kinase. We show that PKA inhibitors PKI and H89 inhibited MAP kinase activation by NGF primarily at later time points. Sustained activation of MAP kinase by NGF was also blunted in PKA-deficient PC12 cells, A126-1B2.

The possibility that Rap1 was activated by PKA in response to NGF was also tested. We also observed that NGF increased Rap GTP-loading and this increase was by PKI. This result suggests that PKA may be required for NGF's activation of Rap1. Rap1 in turn may be important for NGF signaling to MAP kinase. Like cAMP, NGF promoted the association of Rap1 and B-Raf. Both PKA inhibitors (Chapter 3) and RapN17 (York et al., 1998) diminished sustained activation of MAP kinase by NGF, and NGF induced transin gene expression. These findings are consistent with other reports that blocking PKA activity decreases NGF induced gene expression such as sodium channel and tyrosine hydroxylase. Therefore, PKA is required for multiple aspects of NGF's signaling. These findings thus reveal a novel mechanism for regulating the time course of activation of ERK pathway by NGF and cAMP. Via the activation of Rap1, PKA may contribute to the growth factor specificity. We propose that the ability of NGF to activate PKA (and Rap1) is a major distinguishing feature between NGF and EGF in PC12 cells. These results further suggest that the balance between Ras and Rap1 activation, the expression of their targets and perhaps the compartmentalization of these signaling molecules all play important roles in regulating the level and time course of ERK activation.

At this moment, we are not able to establish how NGF or its receptor TrkA activates PKA. However, our studies (York et al., 1998) have shown that the activation of Rap1 and MAP kinase by NGF involves SH2 and SH3 domain containing adapter molecule Crk and the Crk SH3-binding guanine nucleotide releasing-protein, C3G. C3G is specific guanine nucleotide releasing factor for Rap1(Gotoh et al., 1995) and was originally identified by its ability to interact with members of the family of Crk proteins (Tanaka et al., 1994; Matsuda et al., 1994). *In vivo*, C3G binds specifically to the N-terminal SH3-domain of Crk proteins, but not to other SH3-containing proteins, including Grb2. Co-transfection of exogenous Crk L or Crk L and C3G potently augment the activity of Rap1, ERK and ERK substrate Elk-1.

Rap1 and Ras are activated by C3G and Sos, respectively (Gotoh et al., 1995). Although C3G primarily interacts with Crk proteins (Ichiba et al., 1997), Sos can couple to both Grb2 and Crk proteins (Feller et al., 1995; Tanaka et al., 1993). NGF's activation of Sos may be mediated by Crk adaptors rather than Crb2, since Ras activation by NGF can be inhibited by the expression of interfering mutants of Crk (Matsuda et al., 1994). However, these interfering mutants of Crk have no effect on EGF-stimulated ERK activity (Tanaka et al., 1995), suggesting that the ability of Crk proteins to mediate ERK activation is specific for NGF. To test this possibility, we utilized a truncated mutant of C3G, CBR, containing the Crk-binding region, but lacking the catalytic domain to test the possibility that the ability of Crk proteins to mediate ERK activation is specific for NGF but not for EGF. The results of our experiments demonstrated that in PC12 cells CBR selectively blocked NGF's activation of ERK, whereas EGF signaling to ERK was not affected by this mutant at any time point. These results indicate that this CBR interferes selectively with Crk pathways, and confirm that EGF does not signal to ERK via Crk proteins. Therefore, we conclude that Crk proteins are required to activate early (Ras-dependent) and late (Rap1-dependent) actions of NGF on ERKs.

5.5 PC12 cells and Neuronal Differentiation

Growth factors, such as basic FGF (bFGF) and NGF, that induce PC12 cell differentiation also stimulate prolonged activation and nuclear localization of MAP kinase. This observation, together with studies involving constitutive activation or inhibition of MAP kinase, led to the hypothesis that sustained activation of MAP kinase is necessary and sufficient for neuronal differentiation (Cowley et al., 1994; Marshall, 1995; Pang et al., 1995). Two of our findings described above: first, forskolin synergizes with EGF to induce sustained activation of MAP kinase; second, PKA inhibitors and RapN17 convert NGF's sustained activation of MAP kinase into

transient activation; allow us to test the model that proposes sustained activation of MAP kinase is necessary and sufficient for neuronal differentiation more carefully. Our data demonstrated that costimulation with forskolin and EGF is able to stimulate at least two aspects of differentiation; neurite outgrowth and "late" gene transin expression. These two phenotypes are also associated with MAP kinase nuclear translocation. However, inhibitors of PKA, PKI (Chapter 4) and H89 (Chapter 2), failed to inhibit NGF induced neurite processes but H89 was able to inhibit nuclear localization of ERKs (Chapter 4). Inhibition of Rap1 also blocks sustained activation of MAP kinases by NGF but not neurite outgrowth (York et al., 1998). Therefore, the role of PKA/Rap1/B-Raf pathway in NGF signaling can be best appreciated as being required for some aspects of neuronal differentiation but only sufficient for others. Taken together, these data and previous studies suggest that: 1) MAP kinase activation is necessary and sufficient for morphological changes such as neurite outgrowth (Chapter 2 and Yao and Stork's unpublished data); 2) the sustained activation of MAP kinase is sufficient but is not necessary for neurite outgrowth; 3) the ability of H89 to block nuclear localization but not neurite outgrowth demonstrates that nuclear localization of MAP kinase is not required for neurite outgrowth as well; 4) sustained activation of MAP kinase is required for other aspects of differentiation such as electrical excitability and neuronal marker gene transin expression. Other downstream targets of NGF receptor TrkA, such as PLC (Stephens et al., 1994) and the protein tyrosine phosphatase SHPTP2 (Wright et al., 1997) appear to have an undefined role. The role of low affinity NGF receptor p75 in neuronal differentiation also has not been identified. Therefore, since different neuronal differentiation events require distinct signals, it is important to examine every aspects of differentiation when studying neuronal differentiation. It will also be important to determine the generality of these observations in PC12 cells by investigation neuronal cell culture system other than PC12.

5.6 Potential physiological functions of PKA/Rap-1/B-Raf pathway

Although all the studies presented here have utilized transformed cell lines, it is important to speculate on the significance of these studies for organism as a whole. Two potential roles will be discussed below.

1) Physiological relevance: cAMP in long term facilitation.

The best characterized models of synaptic plasticity is long-term facilitation (LTF) of the connections between sensory and motor neurons that control the gill-withdrawal reflex in Aplysia (Bailey and Kandel, 1993). Previous studies have revealed that one of the intracellular signal transduction pathways that is critical for the initiation of LTF utilizes the second messenger cAMP. The effects of cAMP on LTF are mediated at least in part through PKA (Bailey et al., 1996; Carew, 1996; Goelet et al., 1986). Kandel and colleagues provide compelling evidence that MAP kinase plays a critical role in LTF. MAP kinase activation is both associated and necessary for the establishment of long-term, but not short term, facilitation in Aplysia. Stimulation that induces LTF lead to the translocation of MAP kinase into the nucleus of the presynaptic cell (sensory neurons). Exposure of presynaptic cells to agents that elevate cAMP level also caused MAP kinase activation and translocation of MAP kinase, raising the possibility that PKA may activate MAP kinase in these cells. Interestingly, within the sensory-motor cocultures, the activation of MAP kinase was specific to the presynaptic cells; addition of activators of cAMP did not trigger MAP kinase translocation in the postsynaptic neurons (Kelsey et al., 1997). Our model may help explain the cell type specific actions of cAMP on MAP kinase in sensory and motor neurons and therefore, provides one of the molecular mechanisms for cAMP and MAP kinase in the regulation of LTF. Our laboratory is currently investigating the role of Rap1 and B-Raf in the activation and translocation of MAP kinase in primary neuronal cultures.

2) Therapeutic significance: Reversing T cell anergy

Recently, the role of Rap1 in dictating the anergic response in T lymphocytes has been demonstrated. Anergy, or the lack of responsiveness of T cells to antigens presented to them, is a physiological response that restricts T-cell responses to foreign antigens and a pathophysiological response that limits tumor immunity during carcinogenesis. The development of anergy in T cell models is the result of constitutive activation of Rap1 which serves to limit Ras-dependent signaling via the T cell receptor (Boussiotis et al., 1997). The mechanism of this antagonism of Ras-dependent signaling by activated Rap1 is identical to that seen in fibroblasts following the expression of RapV12 (Cook et al., 1993) or following cAMP treatment (Cook and McCormick, 1993) and may occur only in the absence of B-Raf expression. Since lymphocytes express abundant Rap1 but no B-Raf, the antagonism of Rap1 may mediate cAMP's antagonism of T cell response as well (Tamir and Isakov, 1994). The discovery of B-Raf as a positive effector of Rap1, that can activate MAP kinase, opens up new therapeutic routes to the reversal of anergy, via the ectopic expression of B-Raf within B-Raf-negative lymphocytes. These avenues are currently being explored in our laboratory.

5.7 Conclusion

In conclusion, the studies presented in this dissertation demonstrate the following:

1) cAMP can activate MAP kinase in PC12 cells and this activation is required for cAMP's synergistic actions with EGF and NGF. This is in contrast to what was shown in fibroblasts where cAMP has inhibitory effects on MAP kinase. In PC12 cells, one of the consequences of MAP kinase activation is neuronal differentiation. Costimulation with EGF and forskolin stimulates potent sustained activation of MAP

kinase which is associated with MAP kinase nuclear localization and PC12 cell morphological differentiation and transin gene expression.

2) cAMP activation of MAP kinase is mediated by Rap1 and B-Raf. This pathway is independent of Ras but requires MEK. Rap1 and B-Raf also mediate cAMP induction of MAP kinase-dependent transcription factor Elk-1 and PC12 morphological differentiation. We propose a model that the restricted tissue expression of B-Raf dictates cAMP's tissue specific effects on MAP kinase.

3) PKA/Rap1/B-Raf pathway might represent a common mechanism of sustained activation of MAP kinase. NGF induces sustained activation of MAP kinase in part via PKA/Rap pathway. Inhibition of PKA selectively diminishes the late period of MAP kinase activation by NGF. NGF stimulates neuronal differentiation in PC12 cells and primary neuronal cells. By blocking NGF's signal to MAP kinase with PKA inhibitors, it was demonstrated that sustained activation is not necessary for PC12 neurite outgrowth.

Fig. 5.1. Mulitple pathways to ERK activation: EGF, NGF, and cAMP

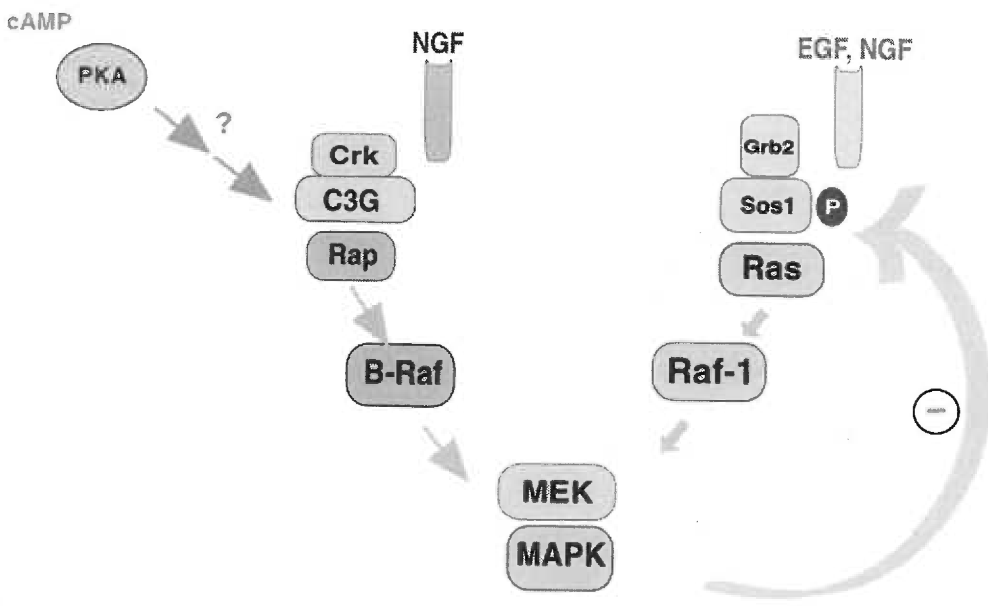


Fig. 5.1. Mechanisms of activation of MAP kinase through Rap1 and Ras. The growth factors NGF and EGF can activate MAP kinase via similar and distinct pathways. Both factors activateMAP kinase via Ras-dependent pathways that are terminated by the phosphorylation of SOS (see arrow). Only NGF can activate MAP kinase via Rap1. This requires the Rap exchanger C3G. cAMP, via PKA, can also activate MAP kinase in a Rap1-dependent pathway. The specific targets of PKA include Rap1 itself and possibly additonal proteins upstream of Rap1.

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